The Regulation and Function of Fizz1 and Ym1 in Th2 Inflammation

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By

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Abstract

Macrophages are key effector cells in not only the immune system but also in physiological processes such as wound healing and tissue repair. Macrophage activation pathways stimulated by the innate recognition of bacterial products and the T helper 1 (Th1) cytokine IFN-γ which lead to a proinflammatory or "classically activated" macrophage phenotype have been well studied. However macrophages can also be activated by Th2 cytokines such as IL-4 and IL-13, by Fc receptor ligation and by hormonal influences such as glucocortioids. These latter pathways are less well delineated and the functional macrophage phenotypes they generate in vivo are less well understood than classical activation. Macrophage exposure to IL-4 and / or IL-13 leads to "alternative activation" and alternatively activated macrophages have been implicated in the response against helminth parasites and in wound healing.

We have developed a model system for generating alternatively activated macrophages in vivo. Implant of the filarial nematode *Brugia malayi* into the peritoneal cavity of mice gives rise to a cellular response dominated by macrophages with a profoundly Th2 dependent phenotype. These nematode elicited macrophages (NeMΦ) are potent suppressors of cellular proliferation and express the enzyme Arginase1, a marker of alternative activation as well as two further markers of alternative macrophage activation, Ym1 and Fizz1.

We investigated the kinetics of the NeMΦ phenotype and found that whilst late expression of Arginase1, Fizz1 and Ym1 was a driven by an adaptive Th2 response these genes were also expressed at the earliest timepoints after implant indicating that they had a role in the response to the surgical procedure used to implant *B. malayi*. The early, injury associated expression of Arginase1 mRNA was found to be dependent on IL-4 whilst Fizz1 and Ym1 mRNA expression in this context was dependent on IL-13 signalling through the IL-4Rα.

We also found that Fizz1 and Ym1 expression were confined to the lung stage of infection with the gastrointestinal nematode *Nippostrongylus brasiliensis* where these genes may play a role in the regenerative response after the parasite migration through this tissue. Finally an examination of the regulation the regulation of these genes in a trematode model and found that, surprisingly, Fizz1 and Ym1 expression may also be controlled by IFN-γ perhaps through modulation of IL-13 activity.

Abbreviations

AAMΦ	Alternatively Activated Macrophage
AMCase	Acidic Mammalian Chitinase
aMΦ	Alveolar Macrophage
APC	Antigen Presenting Cell
Arg1	Arginase1
AU	Arbitrary Units
B.M.	Bone Marrow
b.p.	Base Pair
BALF	Broncho-alveolar Lavage Fluid
BSA	Bovine Serum Albumen
CAMΦ	Classically Activated Macrophage
CPM	Counts per Minute
CRGP	Calcitonin Related Gene Peptide
DC	Dendritic Cell
ECM	Extracellular Matrix
ELISA	Enzyme Linked Immunosorbant Assay
Eos	Eosinophil
ES	Excretory / Secretory
Fizz	Found in Inflammatory Zone
GAG	Glycosaminoglycan
GalN	Galactosamine
GlcN	Glucosamine
GM-CSF	Granulocyte-Monocyte Colony Stimulating Factor
i.p.	Intraperitoneal
IFN-γ	Interferon-gamma
IFN-R	Interferon Receptor
Ig	Immunoglobulin
IL	Interleukin
IL-13R	Interleukin 13 Receptor

IL-4R	Interleukin 4 Receptor
iNOS	Inducible Nitric Oxide Synthase
IPTG	Isopropyl-β-D-thiogalactopyranoside
iv	Intravenous
KLH	Keyhole Limpet Haemocyanin
LPS	Lipo-polysaccharide
mf	Microfilaria
MHC	Major Histocompatibility Complex
MIF	Macrophage Inhibitory Factor
MΦ	Macrophage
NeMΦ	Nematode Elicited Macrophage
NES	Nippostrongylus Excretory / Secretory
NGF	Nerve Growth Factor
NKT	Natural Killer Cell
NO	Nitric Oxide
OAT	Ornithine Transaminse
ODC	Ornithine Decarboxylase
PAMP	Pathogen Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PEC	Peritoneal Exudate Cells
PFA	Parafomaldehyde
PMN	Polymorphonuclear
PRR	Pattern Recognition Receptor
RA	Radiation Attenuated
RAG	Recombinase Activating Gene
RNA	Ribonucleic Acid
RT-PCT	Reverse Transcriptase Polymerase Chain Reaction
s.c.	Subcutaneous
SEA	Schistosome Egg Antigen
s.e.m.	Standard Error of the Mean

STAT	Signal Transducer and Activator of Transcription
TBS	Tris Buffered Saline
TGF	Transforming Growth Factor
Th	T helper Cell
TLR	Toll Like Receptor
TNF	Tumour Necrosis Factor
WT	Wild Type

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Chapter 1 - Introduction

The evolutionary and social history of humans is thoroughly entangled with that of our helminth parasites. From early migratory waves out of Africa, to the domestication of animals and the rise of large settlements and civilizations we have been exposed to new parasites and doubtless carried the old ones with us (Cox, 2002). In recent years the rise of HIV has had an impact on the biology and infection patterns of various parasitic infections (Borkow & Bentwich, 2004), (Capron & Dessaint, 1992), (Harms & Feldmeier, 2005). Additionally it has been suggested that the adoption of urban habitats by the definitive hosts of some helminths could raise the risk of human infection (Deplazes *et al.*, 2004). Thus the evolutionary pressures faced by both man and his helminth parasites continues. Currently some 2 billion people worldwide are thought to suffer some kind of helminth infection (Colley *et al.*, 2001), (World Health Organization, 2005). The symptoms of helminth infection are rarely severe but the large number of people infected together with the higher infection rates in areas of severe poverty make the helminth diseases a large public health problem on a worldwide scale (World Health Organisation, 2002), (World Health Organization, 2005). The advent of effective public hygiene and control measures has almost entirely removed the threat of these diseases in the developed nations. However a breakdown in these mechanisms can result in rapid and widespread infection returning as a recent study of trichinellosis in Serbia shows (Djordjevic *et al.*, 2003). Helminths do not just infect humans. In both the developed and third world helminth infection of livestock has considerable economic impact. Indeed in the western world the increasing interest in organic farming could see a resurgence in livestock infection rates (Waller & Thamsborg, 2004). In the third world the economic impact of helminth disease on livestock contributes in turn to poverty and perpetuates conditions which make control of these diseases difficult (World Health Organisation, 2002).

1.1. Helminths – the nature of disease

Most of the helminth diseases are caused by only two phyla; Nematoda (including *Ascaris lumbricoides* and *Brugia malayi*) and Platyhelminths (including *Schistosoma* spp. and *Fasciola hepatica*) (MacDonald *et al.*, 2002). Different parasites have specific niches within a host and so pathology manifests in different ways. The liver, gut and bladder pathology that occur in chronic schistosomiasis reflects the dwelling place of these organisms. *S. mansoni* prefers to live in the veins of the portal circulation and *S. haematobium* in the urinary bladder. Thus the symptoms of chronic *S. mansoni* infection include hepato-splenomegaly as a result of granuloma formation in the liver and gut

whereas *S. haematobium* infection leads to haematuria and can potentiate bladder cancer (Khurana *et al.*, 2005). Likewise the different diseases caused by the filarial nematodes *Wucheria bancrofti* and *Onchocerca volvulus* arise from the tissue distribution of these organisms. *W. bancrofti* migrates to major lymphatic ducts and the pathology associated with this organism is classical elephantiasis with dependent lymphoedema. *O. volvulus* however can cause skin disfigurement or river blindness as dying microfilariae, trapped in the tissues or eyes, cause allergic inflammation and tissue damage (Janovy & Roberts, 1996).

The range of symptoms shown by infected individuals in areas where filarial disease is endemic varies widely; the effects caused by the filarial nematodes *B. malayi* and *W. bancrofti* can range from none at all and an ability to completely clear infection to sub-clinical lymphatic changes but no overt disease to severe lymphodema and pronounced morbidity. Interestingly the individuals most debilitated usually have the most robust anti-filarial immune responses. These differences suggest genetic, environmental or immune mediated influences as well as parasite localisation within the host all affect the outcome of disease (Lammie *et al.*, 2002).

1.2. The Th2 Immune response

The immune system is broadly capable of two different responses, the proinflammatory Th1 response which is considered anti-bacterial, protective against intracellular infections (Seder & Paul, 1994) and is involved in pathologies such as autoimmune diseases (Skurkovich & Skurkovich, 2003) and the Th2 response which is seen in response to extracellular infections such as helminth parasites and in the pathology of asthma and allergy (Negrao-Correa & Teixeira, 2006). These responses can be temporally divided into an innate early response and a later, more specific adaptive response.

The primary function of the immune system is the recognition and removal of invading organisms. The mechanisms by which microbial stimuli elicit an early immune response were revealed when Charles Janeway proposed the concept of an innate immune system adapted to recognize evolutionarily conserved molecular patterns on pathogens and termed these pathogen associated molecular patterns (PAMPs); the innate receptors for PAMPs he termed pathogen recognition receptors (PRRs) (Janeway, 1989), (Medzhitov *et al.*, 1997). This model has proven remarkably robust and has provided a framework for the investigation not only of the innate system but for how the innate and adaptive systems are bridged. Central to the success of the model was the discovery of the receptors for a wide range of PAMPs. These receptors were identified on the basis of their similarity to the Drosophila protein Toll, which is important in dorso-ventral patterning and insect innate immunity, and were

termed Toll-like receptors (TLRs) (Medzhitov *et al.*, 1997). A family of TLRs was soon discovered (Rock *et al.*, 1998) and there are currently some 13 known members of the mammalian TLR family (Hopkins & Sriskandan, 2005).

The type 2 immune response is shaped by the production of the cytokine interleukin (IL-) 4 (Kopf *et al.*, 1993). Exposure of naïve CD4+ve T cells to IL-4 results in the T helper (Th) 2 T cell phenotype characterised by the production of other Th2 cytokines such as IL-5, IL-9 and IL-13 (Kopf *et al.*, 1993), (Seder & Paul, 1994). In addition IL-4 promotes an Ig class switch in B cells to IgE and upregulates the mannose receptor and the enzyme Arginase1 in macrophages (Stein *et al.*, 1992), (Corraliza *et al.*, 1995). IL-4 also drives the mobilisation of eosinophils through the tissue expression of the chemotactic factor eotaxin (Mochizuki *et al.*, 1998) and the T cell derived cytokine IL-5 (Kopf *et al.*, 1993). The Th2 immune response has profound effects on the expression of the Th1 immune response. IL-4 actively suppresses the production of the key Th1 cytokine IFN-γ (Wagner *et al.*, 1989), (Vercelli *et al.*, 1990), (Munder *et al.*, 1998) thus regulating the adaptive arm of immunity. In macrophages IL-4 stimulation leads to Arginase1 upregulation, and this enzyme is a key marker of macrophage activation by Th2 cytokines. Arginase1 inhibits the action of the enzyme inducible nitric oxide synthase (iNOS), the key macrophage marker of Th1 activation, by substrate competition (Munder *et al.*, 1998). Thus the Th1 and Th2 immune responses are to some extent functionally opposed.

1.2.1. The innate type 2 response

The innate response is required to set up the later adaptive response and the correct arm of the immune system must be activated at an early stage. Whilst the innate type 1 response has been extensively investigated the early events which shape a type 2 response were, until recently, comparatively ignored. The Th2 adaptive response is seen in many, if not all, helminth infections. Several cell types rapidly infiltrate tissues after helminth infection. These include mast cells, basophils and eosinophils (Else & Finkelman, 1998), (Min *et al.*, 2004), (Klion & Nutman, 2004), (Voehringer *et al.*, 2004). All three cell types derive from CD34+ve precursors in the bone marrow. Mast cells are large granulocytic cells, are released from the bone marrow and undergo a final maturation step when they reach the epidermal and sub-mucosal tissues (Austen & Boyce, 2001) where they release histamine in response to stimulation. Eosinophils are also granulocytes but are released from the bone marrow as mature cells. They are capable of releasing many different effector molecules and pro-inflammatory mediators from their cytoplasmic granules (Klion & Nutman, 2004). Eosinophil development is dependent on the cytokine IL-5 which functions in the later stages of differentiation and maturation of eosinophils in the bone marrow (Clutterbuck *et al.*, 1987), (Sanderson, 1988). IL-5 is essential for the mobilisation of

eosinophils from the bone marrow in response to helminths (Ovington *et al.*, 1998), (MacDonald *et al.*, 2003) although a small IL-5 independent population of eosinophils also exists (Nishinakamura *et al.*, 1996). Basophils are not found in healthy tissue but infiltrate tissues rapidly during inflammation (Falcone *et al.*, 2001b), (Voehringer *et al.*, 2004).

1.2.2. Mast cells

Mast cells are associated with the immune response against parasitic intestinal helminths and the expulsion of these organisms from the gut. Mast cell degranulation can be triggered by the cross linking of the high affinity IgE receptor, FcεRI, by IgE bound to multivalent antigen or in an IgE independent manner (Else & Finkelman, 1998). Degranulation leads to histamine, prostaglandin and TNF-α release and a local inflammatory reaction with a resultant influx of other inflammatory mediators, cells and antibody (Janeway *et al.*, 2001). Mast cell responses are particularly important in the expulsion of gastrointestinal parasites and changes in gut wall motility and increases in intestinal mucus production can be triggered by mast cells (Gay *et al.*, 2000), (McDermott *et al.*, 2003). The importance of mast cells in the expulsion of intestinal helminths is variable however with these cells being required for successful expulsion of some nematodes but not others (Else & Finkelman, 1998).

1.2.3. Eosinophils

Whilst mast cells are associated with the expulsion of parasites from their preferred niche eosinophils have been implicated in a more direct attack. Eosinophilia in the blood and tissues is a long standing observation in parasite infections (Taliaferro & Sarles, 1939). In the late 1970s and early 1980s it was noted that eosinophils could mediate the killing of parasites in vitro in the presence of antibody and / or complement (Kazura & Grove, 1978), (Haque *et al.*, 1981), (David *et al.*, 1980) and it was thought that the role of these cells was to deposit the toxic contents of their granules directly onto the surface of the worm and effect killing (Behm & Ovington, 2000). Additional epidemiological evidence of a role for eosinophils in control of parasite infections comes from the findings that raised antibody levels and eosinophilia provide some protection against re-infection with *S. mansoni* and *S. haematobium* after curative treatment (Sturrock *et al.*, 1983), (Hagan *et al.*, 1985).

Animal models of helminth infection have, however, given somewhat contradictory results. In *Litomosoides sigmodontis* infection IL-5 overexpressing mice have lower worm burdens than WT mice indicating that an abundance of eosinophils can provide a measure of protection (Martin *et al.*, 2000). Further evidence of intimate eosinophil involvement in the killing of helminths comes from

experiments in which the larvae of *Strongyloides stercoralis* in diffusion chambers were implanted under the skin of mice. In these experiments eosinophils were the only cells which accumulated in the chamber during parasite killing and the addition of anti-IL-5 antibody prevented killing from taking place illustrating a role for eosinophils (Rotman *et al.*, 1996). However migrating *S. stercoralis* L3 were still killed in the absence of eosinophils in secondary infection indicating that while eosinophils may play a role in the response to tissue migratory stages they are not indispensable (Rotman *et al.*, 1996). The removal of IL-5 activity also increased the worm recovery in mice given a secondary infection of *Strongyloides venzuelensis* but in this case only the tissue migratory stage was affected (Korenaga *et al.*, 1991). These data illustrate that whilst eosinophils may be required for the killing of the migratory stages of some helminth infections in a secondary response they are not indispensable. Indeed the inhibition of IL-5 using monoclonal antibody does not change the susceptibility of infection in several models of intestinal helminth infection and IL-5 overexpressing mice that develop a pronounced basal eosinophilia also show no changes in susceptibility to many helminth infections (Klion & Nutman, 2004). Thus despite the evidence that eosinophils are markedly upregulated in parasite infection and that the the products released by eosinophil degranulation can effect helminth killing the evidence for a direct role in vivo remains contradictory.

1.2.4. Basophils

Basophils have long been associated with immunity to ectoparasites like ticks. Recently however their involvement in immunity against helminths has become more apparent. Basophils, along with eosinophils are recruited into the lung in mice infected with the helminth parasite *N. brasiliensis* and produce large quantities of IL-4 (Voehringer *et al.*, 2004). Like mast cells, basophils express the high affinity IgE receptor, FcεRI and release IL-4 after crosslinking of this receptor. The involvement of IgE implies an adaptive response but basophils can degranulate in an antibody-dependent but antigen-independent manner and rapidly release IL-4 (Gibbs *et al.*, 1996), (Aumuller *et al.*, 2004). Thus the presence of IgE, bound non-specifically to helminth constituents can drive an early IL-4 release.

1.2.5. Adaptive Th2 response

The adaptive Th2 response is seen primarily in response to macroscopic parasites like helminths and in atopic conditions such as asthma and eczema. In atopy it is thought that the immune system is reacting inappropriately to innocuous antigen. Whereas the Th1 response is characterized by the cytokine IFN-γ, the Th2 response is characterised by a wider variety of cytokines, including IL-4 and IL-13. The Th2

cytokines IL-4 and IL-13 display significant functional overlap despite sharing only 25% amino acid similarity. For example IL-13 can mediate the upregulation of MHC class II on monocytes and B cells as well as promoting a class switch to IgE in the latter. It can also inhibit proinflammatory cytokine production (Wynn, 2003) and is thought to be important in the granulomatous reactions to some parasites (Chiaramonte *et al.*, 1999), (Corry, 1999), (Wynn, 2003). The overlap between these two cytokines can be attributed to the fact that they share a common receptor chain. There are two receptors for IL-4; the type I receptor consists of the IL-4Rα chain and the common cytokine receptor gamma chain (cγ) and binds only IL-4 (Nelms *et al.*, 1999). The type II receptor consists of the IL-4Rα chain and the IL-13Rα1 chain and binds both IL-4 and IL-13 (Mueller *et al.*, 2002), (Hershey, 2003). Binding of IL-4 or IL-13 to the type II receptor leads to activation of members of the janus family of tyrosine kinases (JAKs), a phosphorylation cascade and phosphorylation and dimerisation of the signal transducer and activator of transcription 6 (STAT 6) which migrates to the nucleus and activates transcription of IL-4 and IL-13 responsive genes (Kaplan *et al.*, 1996).

Another cytokine considered important in the type 2 response is IL-10. IL-10 is secreted by T regulatory cells (Fehervari & Sakaguchi, 2004), Th2 CD4 +ve cells (Fiorentino *et al.*, 1989) and macrophages (Redpath *et al.*, 2001). Originally characterised as broadly suppressive it is now recognised that IL-10 can also promote various aspects of the Th2 immune response. One critical function of IL-10 is to check the magnitude of immune responses so that neither the type 1 nor type 2 responses overreach their functional capacity and cause pathology (Pestka *et al.*, 2004). The ability of IL-10 to suppress responses across the immunological spectrum is seen in IL-10 deficient mice which develop not only spontaneous enterocolitis (type 1 response) (Kuhn *et al.*, 1993) but also have exacerbated asthmatic / allergic responses (type 2 response) (Tournoy *et al.*, 2000).

One of the characteristic adaptive responses seen in the Th1 context is the production of antibody for specific antigen by terminally differentiated plasma cells. In that response the presentation of antigen by B cells to differentiated Th1 cells acts as a stimulus for the B cell to switch antibody isotypes from IgM to mainly IgG. Specifically the Th1 cytokine IFN-γ induces a switch to IgG2a and IgG3 in the mouse (Janeway *et al.*, 2001). Similarly in the Th2 response IL-4 induces an isotype switch to IgG1 and IgE in the mouse, and IgG4 and IgE in humans. IL-13 can also promote this isotype switch (Punnonen *et al.*, 1993). In humans there is some evidence that the IgG4 subclass plays a protective role against the pathology of filarial infection at the cost of circulating microfilaria by inhibiting the function of IgE (Kurniawan *et al.*, 1993). Human IgG4 is not able to bind the complement component C1q and set complement cleavage in motion or mediate antibody dependent cellular toxicity (Bruggemann *et al.*, 1987), (Bindon *et al.*, 1988). Thus cross recognition of antigen by IgG4 versus IgE

can lead to a blocking of IgE effector mechanisms (Ndhlovu *et al.*, 1996) and indeed IgG4 has also been shown to block the killing of schistosomula mediated by other IgG subclasses and eosinophils in vitro (Khalife *et al.*, 1989).

Unlike the human situation where the IgG subclass does not seem to play an active role in the the anti-helminthic response, IgG1 in the mouse has been implicated in the successful vaccination of mice against helminth infection. Inoculation with a 24kDa antigen from *Ascaris suum* induces IgG1 but no IgE and provides partial protection upon challenge infection (Islam *et al.*, 2005). An early elevation of IgG1 has also been observed in a mouse strain resistant to *Litomosoides sigmodontis*, the only patent murine filarial experimental system (Marechal *et al.*, 1997), whilst an elevated IgE response occurs in a susceptible strain (Le Goff *et al.*, 2002) although IgG1 was also elevated in this strain later in the infection. Thus in the filarial systems an important caveat to note is that IgG4 in humans seems to play a role in protecting against IgE mediated pathology but have little to do with parasite control, whilst in murine systems IgG1 seems to play a role in the active removal of the parasite from the system.

1.2.6. *Bridging the innate / adaptive gap in the Th2 response*

Like the Th1 response the Th2 response requires some initiating signal akin to the TLR2 or TLR4 activation which occurs in bacterial infection (Hopkins & Sriskandan, 2005). The re-use of a functional leitmotif is a feature of much biology and evidence is emerging that TLRs also play a role in the induction of the innate type 2 response through interaction with helminth PAMPs. Schistosome egg antigen (SEA), a soluble extract from Schistosome eggs can drive a dendritic cell phenotype that pushes naïve T cells to initiate a Th2 response (MacDonald *et al.*, 2001). The glycan lacto-N-fucopentaose III (LNFPIII) when glyco-conjugated can drive a very similar DC phenotype through TLR4 (Thomas *et al.*, 2003). Differential signalling is involved in the push to a Th2 biasing DC phenotype rather than a Th1 biasing DC phenotype after engagement of TLR4. The crucial difference appears to be a transient translocation of NFκB to the nucleus rather than the sustained NFκB activity seen in response to LPS / TLR4 engagement (Thomas *et al.*, 2005). Evidence of a role for basophils in the early induction of the Th2 response comes from their expression of TLRs. Indeed basophils not only express TLR2 and TLR4 but selectively express IL-4 and IL-13 in response to peptidoglycan signaling through TLR2 whereas they do not respond to LPS (TLR4 signalling) (Bieneman *et al.*, 2005).

1.3. Resistance to helminths

The helminth infections that seem most amenable to rapid resolution by the Th2 response are those involving gastrointestinal nematodes. The commonly used mouse models are *Trichuris muris* (model of human whipworm infection), *Nippostrongylus brasiliensis* (model of hookworm infection) and *Heligmosomoides polygyrus* (also a model of hookworm infection) and infection with each of these results in a Th2 immune response (Finkelman *et al.*, 1997), (Else & Finkelman, 1998). The immune effector mechanisms leading to the removal of these infections is CD4 +ve T cell dependent and in each infection there is a stereotypical response involving Th2 cytokines, mastocytosis and changes to gut physiology (Khan & Collins, 2004). However the immediate mechanism of parasite expulsion in each case differs, perhaps as a result of the niche the organisms inhabit.

Infection with tissue dwelling helminths is somewhat complicated by the fact that the immune responses invoked can lead to profound pathology and their role in parasite clearance remains uncertain. The pathology accompanying filarial infection with e.g. *B. malayi* includes inflammatory damage to lymphatic vessels which can lead to sub-clinical changes in lymph flow or to debilitating lymphoedema (elephantiasis). Granulomatous responses around dead and dying filarial worms include macrophages and eosinophils (Vickery *et al.*, 1991), two cell types capable of releasing substances toxic to lymphatic tissue. Another complicating factor is the presence of the intracellular bacteria *Wolbachia* inside *B. malayi* and the release of *Wolbachia* from dead or dying worms can play a role in inflammatory pathology (Taylor, 2002).

1.3.1. A successful response – N. brasiliensis

Infection with *N. brasiliensis* in the mouse results in the expulsion of the parasite from the gut some 6-10 days after L3 larvae are introduced through the skin. The effector response takes the form of a mastocytosis and increase in both gut motility and mucus production and absolutely requires the Th2 cytokine IL-13 (Urban *et al.*, 1998) and signalling through IL-4Rα (Barner *et al.*, 1998). In this case the actions of IL-13 seem to be concentrated on the cells of the gut wall (Urban *et al.*, 2001) and IL-13 has been shown to increase gut motility, mucus production (Zhao *et al.*, 2003) and gut epithelial cell turnover (Cliffe *et al.*, 2005). Indeed in IL-13 deficient mice there is a failure in the goblet cell hyperplasia related to expulsion and this leads to susceptibility to *N. brasiliensis* indicating that the goblet cell response is required and is IL-13 dependent (McKenzie *et al.*, 1998). This is supported by the finding that the expression of IL-4Rα on non-bone marrow derived cells is required for the expulsion of *N. brasiliensis* (Urban *et al.*, 2001). The mastocytosis that arises as a result of *N.*

brasiliensis infection does not seem to be important in worm expulsion (Madden *et al.*, 1991) although mast cells may play a role in damage to the tissues of the intestine (Gay *et al.*, 2000). Further supporting the relative redundancy of the immune effector mechanism was the finding that immune mediated damage was not required for the expulsion of *N. brasiliensis* in mice (Ishiwata *et al.*, 2002). The finding that many of the immune responses in *N. brasiliensis* infection are not required (e.g. IL-4 upregulation, mastocytosis) illustrates the stereotypical nature of the response as well as the redundancy in that response.

1.3.2. A pathological response – *Schistosoma mansoni*

In contrast to the successful expulsion of *N. brasiliensis* mediated by IL-13, the same cytokine has a prominent role in the pathology associated with murine models of schistosomiasis. Infection with *S. mansoni*, like infection with *N. brasiliensis* begins in the skin when cercariae enter the body. After a transformation to schistosomula they enter the vasculature and travel to the portal circulatory system, mature into adult worms and proceed to spend the rest of their lives in copula with the female shedding eggs. These eggs attach to and pass through the walls of the blood vessels and then the wall of the gut from where they are eventually shed in the faeces thus restarting the lifecycle. There are two pathological processes associated with *S. mansoni* infection. The early, debilitating but usually self limiting fever (called Katayama fever) is believed to be associated with a Th1 response. Indeed investigation using IL-4 deficient mice has revealed that an inability to repress the early Th1 response with a Th2 response leads to cachexia and death (Brunet *et al.*, 1997). Thus, in mice, the later Th2 response is required to modulate an early, potentially fatal Th1 response.

The second pathology associated with *S. mansoni* infection is the cirrhosis, resultant portal hypertension, ascites and varices which arise from hepatic fibrosis. As the eggs leave the female many of them are swept by the flow of blood back towards the liver where they become lodged. Glycoproteins from these eggs are hepato-toxic (Dunne *et al.*, 1981), (Dunne *et al.*, 1991) and the response to this is the generation of a CD4+ve T cell dependent granuloma around the egg isolating it from the surrounding liver tissue. The eggs thus encased eventually die and the granuloma resolves leaving a fibrotic plaque (Wynn *et al.*, 2004b). This fibrotic process is dependent on IL-13 (Chiaramonte *et al.*, 1999), (Fallon *et al.*, 2000). The control of fibrosis seems to depend to some extent on the IL-10, IL-13/STAT-6 driven expression of the putative IL-13 decoy receptor IL-13Rα2 which limits the fibrotic response (Chiaramonte *et al.*, 2003). Thus whilst the Th2 driven granuloma are required to prevent hepatic damage the potent Th2 cytokine IL-13 can lead to a tissue destructive response.

1.4. Worms fight back

It is well recognised that helminth infection, as well as driving a Th2 response leads to a general suppression of the immune system. This is particularly evident in both filarial and schistosome infections (Yazdanbakhsh, 1999). Suppressive effects even fall outside specific immune interactions such that alternatively activated macrophages (AAMΦ) from *B. malayi* implanted mice suppress the proliferation of many different cell lines (MacDonald *et al.*, 1998). It is now recognised that helminth parasites secrete various factors that can polarise or modulate the immune system. The excretory / secretary products from *N. brasiliensis* (NES) are profound Th2 adjuvants (Holland *et al.*, 2000), *B. malayi* ES products lead to cellular suppression of T cells but a Th2 pattern of cyokine production (Allen & MacDonald, 1998), ES 62, an immunomodulatory molecule from the filarial nematode *Acanthocheilonema viteae* has similar properties (Marshall *et al.*, 2005) and ES products from schistosome cercariae can modulate the early immune response (Jenkins *et al.*, 2005) and this presumably alters the later responses in schistosome infection. These are only a few examples of multiple immune modulatory factors that are secreted by parasites (Maizels & Yazdanbakhsh, 2003), (Maizels *et al.*, 2004) indicating that this is a general strategy employed by helminths to avoid the effector mechanisms of the immune system.

Since the subsequent immune response is determined early in infection the interactions which lead to biased responses would also be evident at the earliest timepoints. The innate type1 response results in a strongly pro-inflammatory phenotype and is triggered by, for example, bacterial components binding to TLRs on dendritic cells (DC). This is paradigmatically represented by the well studied response to lipopolysaccharide (LPS) found on the surface of Gram negative bacteria (Palsson-McDermott & O'Neill, 2004). As discussed above the DC response to helminth PAMPs also informs the subsequent immune response leading to a Th2 bias and this is seen in nematodes (Balic *et al.*, 2004) as well as trematodes (MacDonald *et al.*, 2001).

The question that helminth immunomodulation raises is what advantage, if any, does the parasite gain from the Th2 response? In the context of macrophages one of the key markers of activation driven by Th2 cytokines is the upregulation of Arginase1. The biochemical pathways set in motion by Arginase1 lead to the production of polyamines – molecules essential for cell proliferation and repair (Mills, 2001). There is evidence that obligate helminth parasites lack the biochemical machinery to make lower order polyamines (Sharma *et al.*, 1991) but can utilise those made in the host (Tekwani *et al.*, 1995). Thus one advantage may be a simple parasitism adaptation to steal the hosts resources. Indeed in *S. mansoni* infection the levels of circulating polyamines are elevated and this may be to the

parasites advantage (Abdallahi *et al.*, 2001).

The other potential reason for manipulation of the host response would be to avoid the effector functions of the immune system. One of the exciting developments in immunology recently has been the unravelling of the mechanisms by which so called regulatory T cells down modulate immune responses (Maloy & Powrie, 2001). The gut nematode *H. polygyrus*, known to be profoundly immunomodulatory (Maizels *et al.*, 2004), is capable of inducing regulatory T cells and the regulatory response extends well beyond the immediate vicinity of the parasite to include immune reactions distant from the site of infection (Wilson *et al.*, 2005). In schistosomiasis there is also evidence of a parasite induced IL-10 from both T cell and non T cell sources (Hesse *et al.*, 2004) and this may benefit both the worm and the host (Mangan *et al.*, 2004).

Thus the immunological mechanisms deployed by both mouse and man are often insufficient to clear helminth infection because these processes are subverted. The modulation of the immune system no doubt underlies the longevity of many helminth infections and the usually peaceful coexistence between these parasites and their hosts.

1.5. Macrophage activation

Macrophages are functionally diverse effector cells derived from blood borne monocytes (Gordon, 1999), (Gordon & Taylor, 2005). In some tissues such as the lung, liver, skin, gut and nervous system there are systems of resident macrophages. Indeed the gut is thought to have the largest resident macrophage population in the body (Smith *et al.*, 2005). Macrophages are part of the professional antigen presenting cell (APC) repertoire of the body and as such are in a unique position of being effector cells as well as informing the innate to adaptive immune transition. The signals generated during innate and adaptive immunity have profound activation effects on these cells.

1.5.1. Classical macrophage activation

The best delineated macrophage activation pathway is that induced by the Th1 cytokine IFN-γ and a second signal such as LPS binding to TLR4 or danger signals (e.g. heat shock proteins) (Taylor *et al.*, 2005), (Gordon & Taylor, 2005). Macrophages activated in this way are termed classically activated (CAMΦ). Classical activation leads to an effector cell with a strong proinflammatory bent, capable of generating and releasing reactive oxygen and nitrogen products, actively phagocytic and specialised in the destruction intracellular parasites such as leishmania as well as extracellular bacteria (Goerdt *et al.*, 1999). A key marker of classical activation is inducible nitric oxide synthase (iNOS or NOS2) which

generates nitric oxide from arginine. CAMΦ also secrete proinflammatory cytokines and chemokines (e.g. IL-12, IL-8, TNF-α, MIP-1α), can stimulate a proinflammatory response and can push naïve T cells into the Th1 phenotype. Thus CAMΦ not only respond to the Th1 cytokine response but actively promote it.

1.5.2. *Tc2 macrophage activation*

The immune response can be extremely powerful and CAMΦ must be tightly controlled if the impact of inflammation on tissues is to be minimised. Consequently mechanisms exist to dampen the proinflammatory activity of CAMΦ. This response can be downregulated through ligation of the Fcγ receptors (FcγR) on both IFN-γ primed macrophages and on unprimed macrophages (Anderson & Mosser, 2002b). Like the CAMΦ phenotype this so called type-II macrophage phenotype requires a second signal in the form of a macrophage stimulatory signal (e.g. LPS) (Anderson & Mosser, 2002b), (Mosser, 2003). FcγR ligation alongside TLR4 activation by LPS leads to a loss of IL-12 expression in macrophages and the secretion of large amounts of the down regulatory cytokine IL-10 and protection of mice against lethal LPS doses (Anderson & Mosser, 2002a). Despite their anti-inflammatory properties type-II macrophages continue to express Th1 cytokines such as TNF, IL-1 and IL-6. However these type-II macrophages were capable of biasing T cells to a Th2 phenotype and IL-4 production (Anderson & Mosser, 2002b).

1.5.3. *Alternative macrophage activation*

Exposure of macrophages to IL-4 or IL-13 results in a distinct phenotype with upregulation of Arginase1, the mannose receptor and MHC class II (Stein *et al.*, 1992), (Doyle *et al.*, 1994), (Corraliza *et al.*, 1995). Cells activated in this way have been termed "alternatively activated" macrophages (AAMΦ) and have a proposed role in anti-parasite and allergic responses, wound healing and post inflammatory repair processes (Gordon, 2003), (Gordon & Taylor, 2005). One mechanism by which the Th1 and Th2 cytokines induce opposing phenotypes in macrophages is through the upregulation of either iNOS or Arginase1. These enzymes compete for a common substrate, the amino acid arginine. Whereas iNOS catalyses the generation of nitric oxide and thence other nitrogen radicals (e.g. peroxynitrate) from arginine, Arginase1 catalyses the generation of ornithine used in the formation of polyamines (essential for cell proliferation and produced via ornithine decarboxylase) and proline (used in collagen manufacture and produced via ornithine amino transferase) (Mills, 2001).

The action of type-II activated macrophages on naïve T cells (i.e. the driving of a Th2 response) and

the alternatively activated phenotype that IL-4Rα signalling confers on naïve macrophages suggests that there may be a continuum of macrophage activation states in a properly controlled inflammatory episode to initiate inflammation (CAMΦ), reduce that inflammation (type-II MΦ) and then clear up debris and promote healing (AAMΦ). This continuum requires that all stages of macrophage activation function properly and it is likely that there is a degree of redundancy and overlap in the functional responses of the distinct macrophage phenotypes. The picture is complicated by the heterogeneity of macrophage activation states with a recent review by Gordon listing no less than 5 different activation pathways for these cells (Gordon, 2003).

1.6. Previous work in the laboratory

In order to examine the regulation and roles of individual macrophage phenotypes it is useful to be able to generate these cells in vivo. Our group have generated alternatively activated macrophages (AAMΦ) in vivo through the implantation of the filarial nematode *B. malayi* into the peritoneal cavity of mice (MacDonald *et al.*, 1999). We have termed AAMΦ generated in this way Nematode elicited macrophages (NeMΦ - pronounced "nee macs"). The phenotype of these cells was largely dependent on host IL-4 (Loke *et al.*, 2002) but will also be influenced by other in vivo factors such as other cytokines and parasite derived products. The cellular population in the peritoneal exudate of these mice is stable over a period of many weeks. This model system allowed us to study the phenotype and function of AAMΦ at sites of chronic infection with cells derived in vivo.

NeMΦ generated using the *B. malayi* implant system were found to be potent suppressors of cellular proliferation, suppressing the proliferation of a number of tumour cell lines as well as cell lines derived from immune cells (Loke *et al.*, 2000a). Interestingly the suppression of T cell growth did not affect the ability of T cells to make cytokine responses after stimulation with cognate antigen indicating that suppression did not result from a lack of antigen presentation (Allen *et al.*, 1996). The suppressive phenotype required IL-4, cell-to-cell contact and halted progression through the cell cycle at two points – the G0/G1 and G2/M phases (MacDonald *et al.*, 1999), (Loke *et al.*, 2000a). Proliferative suppression was not dependent on IL-10, IL-5, NO, prostaglandins or IFN-γ (Allen *et al.*, 1996), (MacDonald *et al.*, 1999). The proliferative block was reversible and after removal of NeMΦ from cultures of naïve T cells the T cells were found to make Th2 cytokine responses to antigen (Loke *et al.*, 2000b). Thus although suppressive, NeMΦ primed naïve T cells to differentiate into Th2 cells.

The gene expression pattern of NeMΦ was examined using EST analysis, subtractive hybridisation and expression array analysis revealing that NeMΦ had a distinctive gene expression profile (Loke *et al.*,

2002). The most abundantly expressed, IL-4 dependent gene, representing 10% of the total mRNA, was Ym1. Ym1 is a protein with similarity to chitinases but no apparent chitinase activity (Jin *et al.*, 1998) lacking a glutamate (Glu 140) residue thought to be required for chitinase activity (Sun *et al.*, 2001). Biochemical analysis demonstrated that Ym1 was a lectin which bound to saccharides with free amine groups such as glucosamine (GlcN) and galactosamine (GalN) (Chang *et al.*, 2001). Lectins are capable of crosslinking cell surface carbohydrates and Ym1 may act as an effector molecule by binding to the surface of an invading parasite and acting as a signal or binding molecule for host effector mechanisms. Ym1 also has a proposed interaction with extracellular matrix materials and is implicated in healing of trauma caused by physical and chemical agents (Hung *et al.*, 2002). This indicated that it could have a role in the sequestration of the parasite from the rest of the body through granuloma formation. Ym1 was also elevated in AAMΦ generated in response to infection with *Trypanosoma brucei* (Raes *et al.*, 2002) and thus it appears to be a general marker for AAMΦ. Interestingly Ym1 can also form crystals and these have been implicated in pathogenic situations and in aged mice of some strains (Harbord *et al.*, 2002).

The second most abundant IL-4 dependent gene expressed by NeMΦ from *B. malayi* implanted mice was Fizz1 (Loke *et al.*, 2002). Fizz1 was first described in a mouse model of asthma and was produced by Clara cells and type II pneumocytes in that model. Fizz1 is one of a three member family which includes Fizz2 and resistin (Fizz3). These three family members have different tissue expression patterns (Holcomb *et al.*, 2000). Fizz1 was found to have neuro-modulatory properties such that it could inhibit nerve growth factor mediated cell survival (Holcomb *et al.*, 2000). Since the initial description Fizz1 has also been found to inhibit pre-adipocyte differentiation (Blagoev *et al.*, 2002), promote the differentiation of fibroblasts to myofibroblasts (Liu *et al.*, 2004b) and is highly expressed in the lung after damage (Liu *et al.*, 2004a). The expression of Ym1 and Fizz1 can be driven in macrophages in vitro by culturing them in the presence of IL-4 or IL-13 (Nair *et al.*, 2003).

1.7. Thesis aims

The use of the *B. malayi* implant model has uncovered a macrophage phenotype which was previously unknown. The NeMΦ's we work with manufacture and secrete large quantities of both Fizz1 and Ym1. NeMΦs represent an excellent tool for examining the role of macrophages in chronic helminth infection. In this thesis I wish to elucidate AAMΦ function in vivo by asking is the NeMΦ phenotype confined to the peritoneum or are Fizz1, Ym1 and other Th2 associated proteins also expressed in acute settings? In addition the quantitative gene expression, especially Ym1, indicates that this protein plays a major role in the response to *B. malayi* implant and so it may have a role in physiological

settings associated with Th2 cytokines. Bearing this in mind I address the following broad questions in this thesis:

- How are the proteins expressed by NeMΦ regulated?
- Is this MΦ phenotype confined to the peritoneal cavity?
- Are these AAMΦ strictly dependent on helminth infection?

Chapter 2 - Materials & Methods

2.1. Mice

For all experiments mice used were 6-12 weeks old at the start of the experiment. C57BL/6, C57BL/6 IL-4 deficient (IL-4 -/-), BALB/c , BALB/c IL-4 deficient and BALB/c IL-4 Rα deficient mice were bred in house in the Ann Walker Facility, University of Edinburgh. BALB/c IL-4 deficient mice (Noben-Trauth *et al.*, 1996) were originally a kind gift of Eileen Devaney (University of Glasgow). BALB/c IL-4 Rα deficient mice (Barner *et al.*, 1998) were originally a kind gift from Frank Brombacher (University of Cape Town, South Africa). 129 wild type (WT) and IFN-γ receptor deficient (IFN-γ R-/-) mice were maintained in the animal facility at the University of York. For some experiments C57BL/6 and BALB/c animals were purchased from Harlan-UK (Bicester, UK).

2.2. Parasite Infections

2.2.1. *Brugia malayi* & Surgical Implant

Adult *B. malayi* parasites were obtained from infected jirds (*Meriones unguiculatus*) purchased from TRS Laboratories (Athens, GA) or maintained in house. Adult worms were removed from the peritoneal cavity of jirds that had been euthanised by cardiac puncture under anaesthesia, and washed 5 times in warm media (RPMI, Invitrogen). Mice were surgically implanted intra-peritoneally (i.p.) with 5 live adult female and 1 live adult male *B. malayi*. Three to four weeks later the mice were euthanised by cardiac or brachial artery puncture under anaesthesia followed by asphyxiation with CO_2 to ensure death. Generally naive mice or mice injected i.p. with 0.8 ml of 4% thioglycollate medium (Brewer modified); (Becton Dickinson) for three days were used as controls. For the time course experiment control mice were subjected to the surgical procedure for implant without any implantation of worms. The peritoneal exudate cells (PEC) were harvested by thorough washing of the peritoneal cavity with 15 ml of ice-cold RPMI media.

2.2.2. *Nippostrongylus brasiliensis*

The *N. brasiliensis* life cycle was maintained in house. Mice were infected (s.c.) with 200-400 L3 larvae. After 6-7 days, the mice were sacrificed and the lung tissue, small intestine, mesenteric lymph nodes, thoracic lymph nodes and spleen were removed for RNA extraction, proliferation studies or the

examination of cytokine production. Airway cells were recovered by lavage of the airways with incomplete RPMI. The first aliquot of lavage fluid was used for subsequent ELISAs and western blots. The day prior to sacrifice food was withdrawn to ensure the digestive tract was clear.

2.2.3. *Schistosoma mansoni*

The *S. mansoni* experiments were performed at the University of York. A Peurto Rican isolate of *S. mansoni* was maintained by routine passage through LACA strain mice and albino *Biomphalaria glabrata* snails. Cercariae were exposed to 20 kilorads of γ-radiation using a ^{60}Co source (Department of Radiology, Cookridge Hospital, Leeds). Mice were anaesthetised and given a single vaccination of 500 radiation attenuated (RA) cercariae by application to an area of shaved skin on the right flank of the mouse. At 28 days post vaccination mice were euthanised by severing the aorta under anaesthetic and various tissues taken for further examination. Broncho-alveolar lavage was carried out by instilling 500ul of 3% lignocaine in PBS into the lungs of the mice. The first aliquot of broncho-alveoar fluid (BALF) was used for subsequent ELISA and western blotting. The lungs were then further lavaged with a total of 5mls of fluid. Cells were collected from both aliquots.

2.3. Cell Culture & Techniques

2.3.1. *Purification of macrophages by adherence*

Peritoneal or lung lavage cells were plated in 12 well plates at $1x10^6$ cells/ml. Following 2-3 hours adherence at 37°C, the non-adherent cells were removed, leaving a cell population highly enriched for macrophages. Macrophage purity was previously assessed by FACS staining with the F4/80 antibody for macrophage identification. Purity was routinely greater than 85% (M. Nair - unpublished)

2.3.2. *Generation of bone marrow derived macrophages*

Bone marrow derived macrophages were prepared by harvesting the bone marrow from the femur and tibia of C57BL/6 mice. Following the lysis of the erythrocytes using red blood cell lysis buffer (Sigma), the cells were washed and plated in 24-well plates at $2.5x10^5$ cells/well in Dulbecco's Modified Media (DMEM), supplemented with 25% Fetal Calf Serum (FCS) (Gibco), 25% L929 supernatant (ATCC n° CCL1), 2mM L-glutamine, 0.25U/ml penicillin and 100μg/ml streptomycin (Dransefield *et al.*, 1996).

2.3.3. Lymph node and Spleen Preparation

A single suspension of lymph nodes or splenocytes was prepared by mashing the tissue through a cell strainer (BD Falcon) and then passing the cells through a 25-gauge needle. Lymph node cells were plated at $1x10^6$ / ml for ELISA or proliferation studies. Following red blood cell lysis the splenocytes were also plated at $1x10^6$ cells/well for ELISA or proliferation studies.

2.3.4. Cultures and cell lines

The murine lymphoma cell line EL-4 (ATCC No. TIB-39) was cultured in RPMI 1640 medium (Gibco) supplemented with 2mM glutamine, 0.25 units/ml penicillin, 100 μg/ml streptomycin and 10% FCS (complete medium) at 37°C in 5% CO_2.

2.3.5. In vitro activation of cells

For activation by Th2 cytokines cells were treated overnight with IL-4 (10 or 20ng/ml) and/or IL-13 (10ng/ml) (Pharmingen). For classical activation, macrophages were treated overnight with 100μg/ml LPS (Sigma) and 10U/ml recombinant IFN-γ (Pharmingen).

2.4. Microscopy & Photography

2.4.1. Cytocentrifuge preparations and immunostaining

Cells from the broncho-alveolar lavage (BAL) were resuspended at $2x10^6$ / ml. $4x10^5$ cells were centrifuged onto polylysine slides, allowed to air dry overnight then fixed and stained with Diff-Quik reagent (Dade-Behring) according to the manufacturers directions for differential cell counting. For immunostaining cells were fixed after cytospin using 4% paraformaldehyde (PFA) and air dried overnight. The cells were then re-hydrated in PBS and staining carried out using the α-Ym1 antibody (1:500) and the VectaStain Rabbit Ig kit (Vector Laboratories) or the DakoCytomation LSAB-2 system (K0673, DakoCytomation) according to the manufacturers directions. For lung section the lungs were removed whole from animals and fixed by direct instillation via the trachea of 4% PFA. After embedding in paraffin wax 5μm thick sections were cut and stained using the ABC technique with DAB reagent (Vector Laboratories) according to the manufacturers directions. Photographs were taken under x40 or x100 objective lens using a Sony Cybershot F707 digital camera attached directly to the eyepiece of the microscope by M99 adapters supplied by the Martin Microscope Company (South

Carolina). Post image capture processing (white balance etc) was carried out using Adobe Photoshop version 7 (Adobe Software) or the Gnu Image Manipulation Program (www.gimp.org).

2.5. Cell suppression / proliferation assays

2.5.1. Suppression Assay

To assess their suppressive capacity, macrophages from the peritoneal lavage were purified by adherence, as described above and co-cultured in 96-well flat-bottomed plates with EL-4 cells ($1x10^4$ cells/well) for 48 hours. 1μCi of [^{3}H]TdR in 10μl complete medium was then added to each well. Plates were incubated overnight before harvesting and counting using a liquid scintillation counter (Microbeta 1450, Trilux). Triplicate or quadruplicate measurements per sample were performed. Results were plotted as counts per minute (CPM).

2.5.2. Proliferation Assay

Lymphocytes or splenocytes were resuspended at $1x10^6$ cells/ml and incubated at 37°C with antigen, the mitogen Concanavalin A (ConA) or media alone as a negative control. After 6 hours the supernatant was removed and 1μCi of [^{3}H]TdR in 10μl was added to each well. After a further 18 hours incubation cell proliferation was measured using a liquid scintillation counter (Microbeta 1450, Trilux).

2.6. ELISA

2.6.1. Parasite antigen preparation

Adult *N. brasiliensis* antigen was prepared by homogenisation of mixed sex worms in PBS on ice followed by centrifugation at 10,000 x g for 20 minutes. The resultant supernatant was passed through a 0.2μm filter. Protein concentration was assessed by Bradford assay. 190μl Coomassie Plus reagent (Pierce) was added to 10μl antigen. Plates were read at 570nm. A standard curve of diluted BSA (Pierce) was used to quantify protein in mg/ml.

2.6.2. Cell cultures

Single cell suspensions of lymph nodes or splenocytes were prepared as described above. The cells

were treated with medium alone, *N. brasiliensis* antigen (Nb) (5μg/ml), *N. brasiliensis* excretory / secretory products (NES) (5μg/ml) or Concanavalin A (Con A) (1μg/ml). After 48 hours culture 100μl supernatant was removed for cytokine assays.

2.6.3. Cytokine detection

Maxisorp (Nunc) 96 well plates were coated with α-IL-4, α-IL-5, α-IL13 or α-IFN-γ in 0.06M carbonate buffer and incubated overnight at 4°C. After washing the plates were blocked with 5% BSA (Sigma) in carbonate buffer for IL-4, IL5 and IFN-γ or 1% BSA / 5% sucrose (Sigma) in PBS for IL-13 for 2 hours at 37°C. After blocking 50μl of sample (culture supernatant or BAL fluid depending on the experiment) was added to each well. Serial 2 fold dilutions of each recombinant mouse cytokine in PBS and 0.05% Tween (PBST) were used to make up a standard curve. Ranges for the standard curves were; IL-4 8ng / ml – 1.9pg / ml; IL13 16ng / ml 3.9pg / ml; IFN-γ 50ng / ml – 12pg / ml; IL5 100ng / ml – 40 ng / ml. All cytokines were purchased from Pharmingen. Plates were incubated overnight at 4°C.

After washing with TBS / 0.1% Tween-20 (Sigma) (TBST), biotinylated anti-cytokine antibodies diluted in 5% FCS (Gibco) / TBST were added to the plates as follows; IL-4 (5ng/ml), IFN-γ (5ng/ml), IL-5 (5ng/ml) (all from Pharmingen) and IL-13 (500ng/ml) (R & D Systems). After a 1 hour incubation at 37°C ExtrAvidin (Sigma) at 75μl/ml was added to the IL-4, IL-5 and IFN-γ plates. For IL-13 horse radish peroxidase conjugated streptavidin (Amersham Biosciences) was added at 5ng / ml in 5% FCS / TBST. *p*-Nitrophenyl Phosphate (pnpp) substrate (Sigma) was prepared according to the manufacturers instructions and added to the plates at 100μl/ml. The plates were monitored for colour change and read at 405nm.

The IL-13 Rα2 ELISA was carried out according to the method of Mentink-Kane et al (Mentink-Kane *et al.*, 2004). Maxisorp plates were coated with recombinant mouse IL-13 (R&D Systems) in PBS and incubated overnight. After washing with PBS and 0.05% Tween-20 (PBST) the plates were blocked with 5% milk in PBST. A standard curve of IL-13 Rα2 (R&D Systems) was prepared in PBST. After washing with TBST, biotinylated goat anti-mouse IL-13 Rα2 at a concentration of 156ng / ml in TBST was added to each well. Following a 2 hour incubation at 37°C horse radish peroxidase conjugated streptavidin (Amersham Biosciences) was added at 5ng / ml and the plates incubated for a further hour at 37°C. Following washes in TBST and dH_2O 100μl of the ABTS substrate (KPL). Plates were monitored for colour change and read at 450nm.

2.7. Antibodies

The antibodies used in the western blotting and immunostaining were anti-peptide antibodies raised in rabbits against peptides from both Fizz1 and Ym1 by Genosphere Biotechnologies (France). Peptides were conjugated to KLH and the sequences were residues 32 - 46 (ENKVKELLANPANYP) for Fizz1 (Entrez Accession number: AAG02143) and residues 154 - 168 (IPRLLLTSTGAGIID) for Ym1 (Entrez Accession number: 1VF8_A). Polyclonal serum against Ym1 was originally a kind gift of Alan Wilson and Pat Coulson (University of York).

2.8. Western Blotting.

20µl of peritoneal lavage or BAL fluid samples were run out at 150V for 45 minutes on 4%-12% NuPage gels (Invitrogen). The samples were then blotted onto nitrocellulose membrane (BioRad) at 30V for 1 hour. The membranes were blocked for 30 minutes using Starting Block TBS Blocking Buffer (Pierce). After washing with TBS / 0.2% Triton-X / 0.05% Tween-20 (TBST-T) anti-Ym1 (1:5000) or anti-Fizz1 (1:2000 in Blocking Buffer) were added. After 1 hour at room temperature or 4°C overnight incubation the membrane was washed and HRP conjugated goat anti-rabbit-Ig antibody (1:2000 in Blocking Buffer) was added. After a further hour incubation and washing the ECL reagent (Amersham Biosciences) was applied according to the manufacturers instructions.

2.9. Molecular Biology

2.9.1. RNA extraction

For the recovery of RNA from tissue samples tissues were removed from the animal and stored in RNALater (Ambion) at -80°C. After homogenisation of tissues in Trizol (Invitrogen), RNA was recovered according to the manufacturers instructions with the addition of 5µg glycogen (Invitrogen) to promote RNA precipitation. 1 – $2x10^6$ cells from the airways, culture or cell suspensions were placed into Trizol and then RNA extracted as detailed for tissues. The quality of the RNA was assessed by agarose gel electrophoresis (1% agarose) and ethidium bromide staining and the RNA was DNAse (Promega) treated (if required) and quantified by spectrophotometry using a GeneMeter (ABGene) at A260 (1 A260 unit = 40µg RNA/ml). The purity was determined by the A260/A280 ratio. RNA was stored at -80°C in RNA storage buffer (Ambion).

2.9.2. Reverse Transcription

1µg of RNA was used for the synthesis of first strand cDNA. Reaction volumes were 20µl each containing 1mM of each dNTP (Stratagene), 0.5µg oligo dT (Promega), 1U RNAse inhibitor (Promega) and 50U MMLV reverse transcriptase (Stratagene) in 1x reaction buffer (Stratagene). The conditions used for reverse transcription were 20°C for 10 minutes, 37°C for 1 hour and 99°C for 5 minutes.

2.9.3. Real time RT-PCR

Real time PCR of β-*actin*, *Fizz1*, *Ym1*, *Arginase1*, *AMCase* and *IL13Rα2* cDNA was carried out using the LightCycler (Roche Molecular Biochemicals). Five 2-fold serial dilutions of a positive sample of cDNA were used as a standard curve in each reaction. The LightCycler software was used to calculate the expression level for each gene from the appropriate standard curve. The PCR amplifications were carried out in 10µl reactions containing 1µl cDNA, 4mM $MgCl_2$, 0.3M primers, 1µl of LightCycler-DNA SYBR Green mix and 6.2µl dH_2O. The PCR conditions for *b-actin*, *Fizz1*, *Arginase1* were as follows; 95^{o}C for 30s denaturation, 55^{o}C for 5s annealing, 72^{o}C for 12s elongation and acquisition of SYBR Green fluorescence at 85^{o}C. For *AMCase* the acquisition temperature was 82^{o}C; for *IL13Rα2* the acquisition temperature was 78^{o}C. For *Ym1* the annealing temperature was 67^{o}C and the acquisition temperature was 80^{o}C. The expression level of each gene was then expressed in relation to the expression level of β-*actin* to control for differing levels of cDNA in each sample. The highest level of expression for a given gene in a given sample was arbitrarily set to 100 to allow for a common scale in graphical presentation. The primers for each gene are detailed in table 1.

2.9.4. Standard PCR

Standard PCR reactions were generally carried out with 2.5U Taq polymerase (Roche), 0.25mM each dNTP, 0.2µM primers and 1x PCR buffer (Roche) in a total volume of 25µl. The conditions were as follows - denaturation at 94°C for 5 minutes, then 35 cycles of 20 sec 94°C, 30 sec 55°C, and 90 sec 72°C, followed by 10 minutes at 72°C. The PCR products were visualised by gel electrophoresis (1% agarose) and ethidium bromide staining.

Gene	Forward Primer	Reverse Primer
β-actin	*TGGAATCCTGTGGCATCCATGAAAC*	*TAAAACGCAGCTCAGTAACAGTCCG*
Fizz1	*GGTCCCAGTGCATATGGATGAGACCAT AGA*	*CACCTCTTCACTCGAGGGACAGTTG GCAGC*
Arginase1	*CAGAAGAATGGAAGAGTCAG*	*CAGATATGCAGG GAGTCACC*
AMCase	*GTCTGGCTCTTCTGCTGAATGC*	*TCCATCAAACCCATACTGACGC*
Ym1	*TCACAGGTCTGGCAATTCTTCTG*	*TTTGTCCTTAGGAGGGCTTCCTCG*
IL-13Rα2	*CGCAAATGTTGATAGCGACAG*	*CCAAGCCCTCATACCAGAAAAAC*
iNOS	*GCATTTGGGAATGTAGACTG*	*GTTGCATTGGAAGTGAAGCGTTT*
IL-10	*AGCCGGGAAGACAATAACTG*	*CATTTCCGATAAGGCTTGG*
IL-6	*TTCCATCCAGTTGCCTTCTTGG*	*TATCTCTCTGAAGGA TCTGGC*
IL-4	*GAATGTACCAGGAGCCATATC*	*CTCAGTACTACGAGTAATCCA*

Table 1: Primers used throughout this project.

2.10. Statistical Analyses

Graphs were prepared using Prism (version 3.0; GraphPad Software, Berkeley, CA) or the R (www.r-project.org) environment for statistical computing (R Development Core Team, 2006). Statistical significance between groups was assessed using the Kruskal-Wallis test and if significance was seen Dunn's post test or pairwise Wilcox tests with Holm's p-value correction for multiple tests (Holm, 1979) was used to pinpoint differences. Unless otherwise stated all dotplots show individual data points with a bar representing the mean. Barplots show the mean with error bars representing +/- 1 standard error of the mean (s.e.m.). Timecourse data is shown as points joined by a line with error bars representing the s.e.m.

Chapter 3 - Kinetics of the NeMΦ Phenotype

3.1. Introduction

Exposure of macrophages to the cytokine IL-4 results in a distinct phenotype with upregulation of Arginase1, the mannose receptor and MHC class II. Cells activated in this way have been termed "alternatively activated" macrophages (AAMΦ) (Stein *et al.*, 1992) to differentiate them from macrophages activated by the pro-inflammatory Th1 cytokine IFN-γ (Goerdt & Orfanos, 1999). Subsequent studies revealed that the cytokine IL-13 acted similarly to IL-4 in the induction of the AAMΦ phenotype (Doyle *et al.*, 1994). AAMΦ have a proposed role in anti-parasite and allergic responses, wound healing and post inflammatory repair processes (Gordon, 2003).

In order to examine the regulation and roles of AAMΦ in vivo we utilised a model system whereby large numbers of MΦ are generated through the implantation of the parasitic nematode *B. malayi* into the peritoneal cavity of mice (Allen *et al.*, 1996). In C57BL/6 mice the phenotype of these NeMΦs is largely dependent on host IL-4 (MacDonald *et al.*, 1998) but will be influenced by other in vivo factors including parasite derived products, host IL-10 and glucocorticoids. The cellular population in the peritoneal exudate of *B. malayi* implanted mice is stable over a period of many weeks. We have previously characterised various aspects of the NeMΦ phenotype including the ability to block cellular proliferation (MacDonald *et al.*, 1999), (Loke *et al.*, 2000a), the requirement for IL-4 in the production of Arginase1 and the proteins Fizz1 and Ym1 (Loke *et al.*, 2002), the ability of NeMΦs to induce Th2 differentiation in naïve T cells (Loke *et al.*, 2000a) and the requirement for T cells in NeMΦ recruitment (MacDonald *et al.*, 2003). Because of the dependence on IL-4 for many aspects of the NeMΦ phenotype, we consider these cells to represent a form of AAMΦ in vivo.

NeMΦ and related AAMΦ phenotypes are increasingly being recognised as important effector cells in a variety of Th2 mediated biological processes (Allen & Loke, 2001), (Smit *et al.*, 2004), (Peters-Golden, 2004). We wanted to further understand the development and recruitment of these cells in nematode infection and therefore undertook a timecourse analysis of the response to *B. malayi* implantation into the peritoneal cavity. This gave us insight into the kinetics of NeMΦ development as well as the context in which these cells arise. We have examined cell recruitment into the peritoneal cavity and the kinetics of key markers of the NeMΦ phenotype including suppression of proliferation and the expression of Fizz1 and Ym1 as well as the cytokine environment in which these markers arise.

3.2. Results

3.2.1. Cellular Kinetics

In order to investigate the in vivo kinetics of the NeMΦ phenotype we undertook a timecourse study of C57BL/6 mice implanted with *B. malayi* adult nematodes (implant group). As a control for the effects of surgery we also undertook an analysis of mice subjected to the surgical procedure required for *B. malayi* implant but without the implant of nematodes (sham surgery group). The implantation of *B. malayi* into the peritoneal cavity caused a sustained influx of cells (Figure 1). The kinetics of cellular recruitment in both groups was similar until day 3. After this point the *B. malayi* implanted mice had a consistently higher number of cells in the peritoneal cavity. By day 21 the number of cells in the peritoneal cavity of the implanted mice had increased some 75 fold over the naïve level and was still rising. In contrast cell numbers in the sham surgery group plateaued at day 10 and remained considerably lower than that of the implanted mice until the experiment terminated.

Previously we had shown that the cellular population of *B. malayi* implanted mice at day 21 was approximately 75% macrophages with eosinophils and lymphocytes making up the remainder (MacDonald *et al.*, 1999). The data presented here for day 21 is broadly similar to that previous study with the exception that we find fewer eosinophils than lymphocytes at day 21 while the original study found the reverse (Table 2). The biggest early difference between the implanted and sham surgery groups in the composition of the cellular infiltrate was the rapid increase in eosinophil numbers in the implanted group. Eosinophil numbers were significantly higher in the implanted group from day 1 and this was consistent throughout the experiment.

There is a pronounced early neutrophilia in both groups of mice and although the difference was not significant the number of neutrophils recruited into the peritoneal cavity of the implanted mice was higher. Neutrophilia has been described before in response to i.p *Brugia* sp infection with both adult worms (Falcone *et al.*, 2001a) and infective larvae (Ramalingam *et al.*, 2003). In our model system this response is partly due to the surgical implantation technique we use, as demonstrated by the rise in neutrophils in the sham surgery mice. Given the role of neutrophils in innate defence their presence early after implant is not surprising especially as the innate defences could be triggered by the presence of the endosymbiont *Wolbachia* in *B. malayi* (Taylor *et al.*, 2000) as well as by surgical trauma.

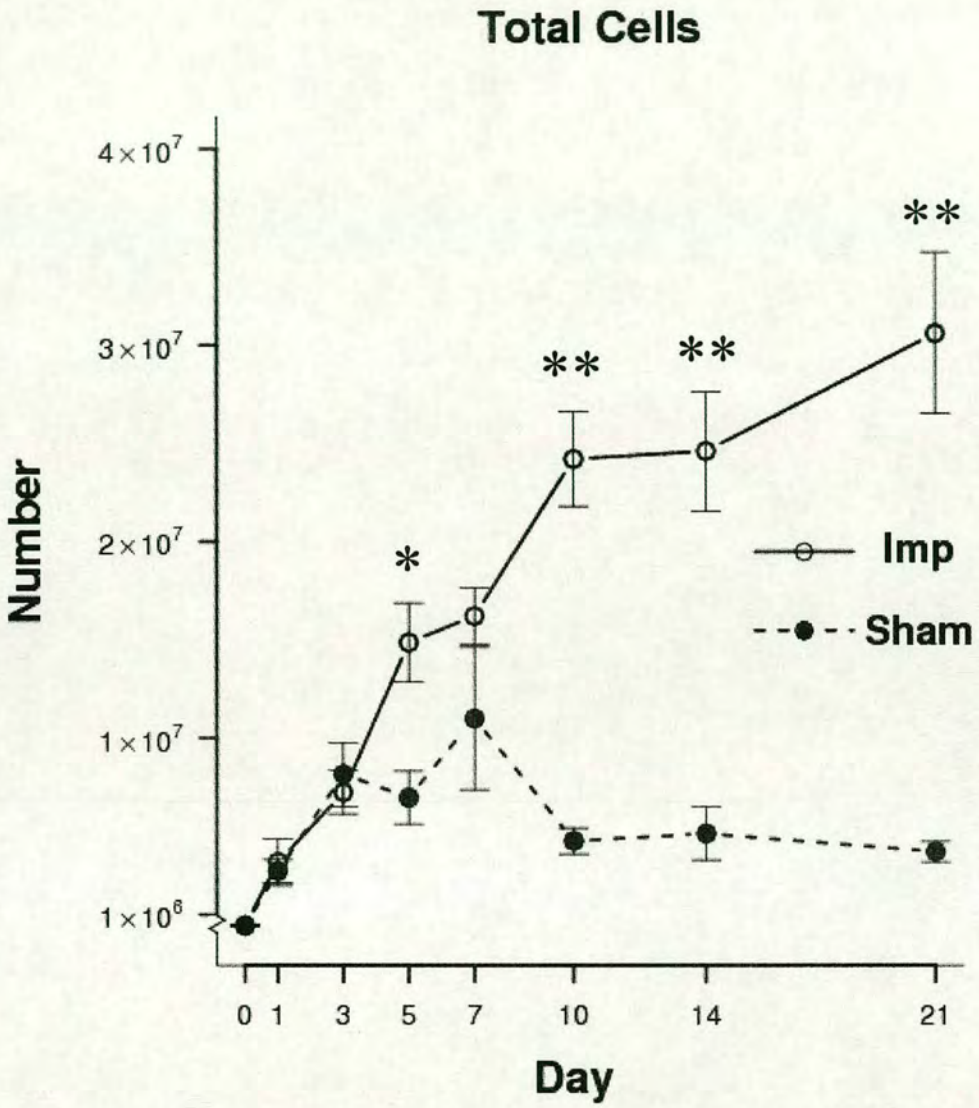

Figure 1: Implant of B. malayi into the peritoneal cavity of C57BL/6 mice causes a sustained cell recruitment. n= 5 mice per group for each day. * = $p < 0.05$, ** = $p < 0.01$.

Monocyte numbers increased immediately in both groups though there were no significant differences until day 14 when monocytes disappeared from the peritoneal cavity of the sham surgery mice; by day 21 neither group had an appreciable monocyte population in the peritoneal cavity. In both groups there was an early gradual rise in lymphocyte numbers. Between days 7 and 10 the number of lymphocytes in the implanted mice increased and was then maintained until day 21. In contrast lymphocyte numbers in the sham surgery group began to fall at day 7. Macrophage numbers at days 1 and 3 were very similar between the two groups. At day 5 however the number of macrophages in the peritoneal cavity

of implanted mice increased and continued rising throughout the duration of the experiment. By day 21 macrophages were the dominant cell type in the peritoneal cavity of the implanted mice with some $2.22x10^7$ (+/- $9.93x10^6$) cells present, representing ~ 75% of the PEC. In the sham surgery group although macrophages represented 53% of cells present, there were far less of them in terms of total cell number (Table 3).

Implant	Day 0	Day 1	Day 3	Day 5	Day 7	Day 10	Day 14	Day 21
Eos	*0.01*	*0.67 +/- 0.3*	*0.63 +/- 0.2*	*1.6 +/- 0.7*	*1.98 +/- 0.9*	*2.56 +/- 1.16*	*1.39 +/- 0.62*	*1.95 +/- 0.87*
Monos	*0*	*1.82 +/- 0.9*	*2.42 +/- 1.0*	*3.03 +/- 1.3*	*0.17 +/- 0.07*	*0.4 +/- 0.18*	*0.02 +/- 0.001*	*0*
Macs	*0.26 +/- 0.1*	*0.23 +/- 0.1*	*1.59 +/- 0.7*	*5.8 +/- 2.6*	*10.7 +/- 4.7*	*13.9 +/- 6.2*	*17.5 +/- 7.8*	*22.2 +/- 9.9*
Neutros	*0*	*1.27 +/- 0.6*	*0.86 +/- 0.4*	*0.68 +/- 0.3*	*0.57 +/- 0.25*	*0.4 +/- 0.18*	*0.22 +/- 0.009*	*0.16 +/- 0.07*
Lymphos	*0.16 +/- 0.007*	*0.4 +/- 0.2*	*1.7 +/- 0.8*	*3.79 +/- 1.7*	*2.79 +/- 1.2*	*6.92 +/- 3.7*	*5.5 +/- 2.5*	*6.28 +/- 2.8*

Table 2: Implant cell numbers. Numbers are shown as $x10^6$ +/- sem.

Sham	Day 0	Day 1	Day 3	Day 5	Day 7	Day 10	Day 14	Day 21
Eos	*0.02*	*0.07 +/- 0.03*	*0.29 +/- 0.14*	*0.16 +/- 0.08*	*0.1 +/- 0.05*	*0.1 +/- 0.05*	*0.002 +/- 0.001*	*0.001 +/- 0.0007*
Monos	*0*	*1.41 +/- 0.2*	*2.2 +/- 1.1*	*1.6 +/- 0.81*	*1.6 +/- 0.8*	*0.01 +/- 0.005*	*0*	*0*
Macs	*0.38 +/- 0.07*	*0.46 +/- 0.18*	*2.4 +/- 1.2*	*2.3 +/- 1.4*	*3.6 +/- 1.8*	*2.0 +/- 1.0*	*1.7 +/- 0.9*	*1.3 +/- 0.7*
Neutros	*0*	*0.75 +/- 0.26*	*1.5 +/- 0.74*	*0.63 +/- 0.3*	*0.12 +/- 0.06*	*0.001 +/- 0.0007*	*0.008 +/- 0.004*	*0.0003 +/- 0.0001*
Lymphos	*0.06 +/- 0.001*	*0.53 +/- 0.12*	*1.6 +/- 0.83*	*2.2 +/- 1.1*	*4.3 +/- 2.1*	*1.3 +/- 0.7*	*2.39 +/- 1.2*	*1.1 +/- 0.6*

Table 3: Sham cell numbers. Numbers are shown as $x10^6$ +/- sem.

3.2.2. Kinetics of proliferative suppression

Macrophages recruited into the peritoneal cavity in response to *B. malayi* are able to suppress cell proliferation (Allen *et al.*, 1996) and this suppressive ability is IL-4 dependent (Loke *et al.*, 2000a). We therefore examined the ability of the peritoneal cells from both implanted and sham surgery mice to suppress the EL4 murine lymphoma cell line in co-culture. Figure 2 shows that the cells in the implanted group displayed a rapid and profound ability to suppress EL4 cell proliferation from day 3 onwards. This suppressive ability was maintained until day 21 in the implant group. In the early stages of the timecourse cells from the sham surgery mice were also suppressive but by day 10 they had lost this ability.

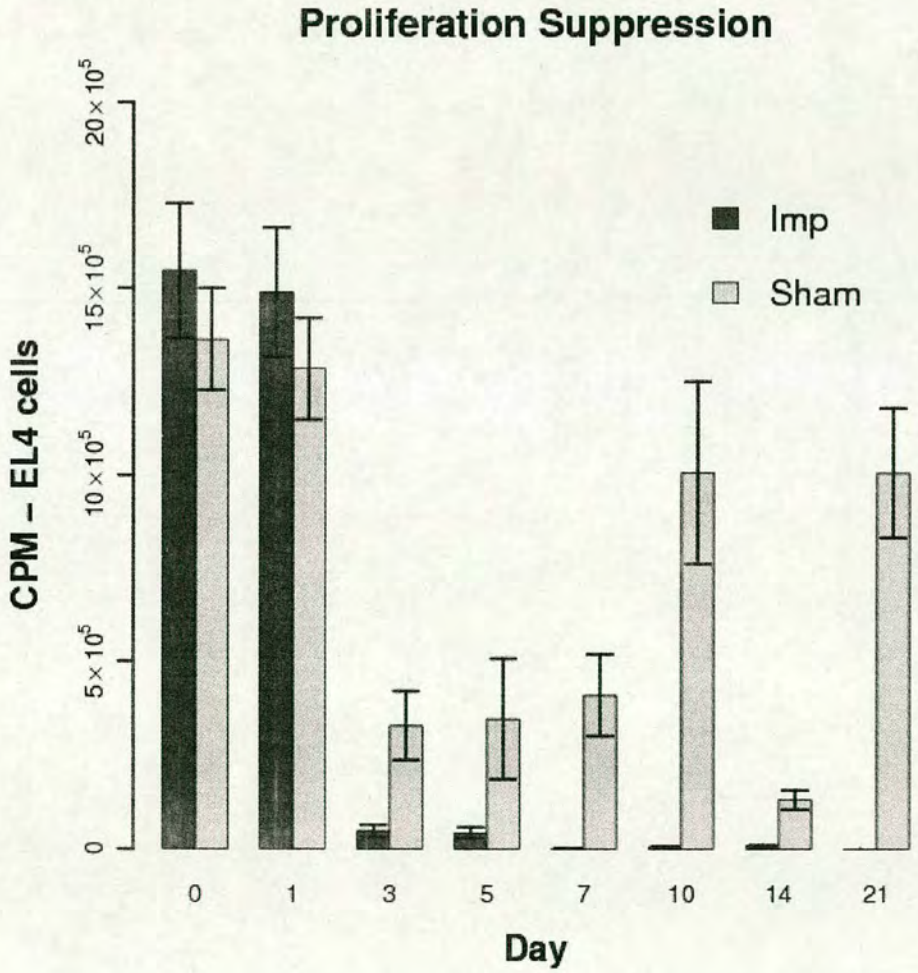

Figure 2: Peritoneal exudate cells (PEC) from implanted or sham surgery mice were cultured with the EL4 lymphoma cell line. Proliferation of EL4 cells was monitored by the uptake of ^{3}H labelled thymidine. The suppression generated by implanted cells is more profound and longer lasting than that generated by sham PEC. CPM = counts per minute. Error bars represent sem, n= 5 mice per group per day.

3.2.3. Fizz1, Ym1, Arginase1 and iNOS Kinetics

The genes Fizz1, Ym1 and Arginase1 are upregulated in response to the implantation of *B. malayi* into the peritoneal cavity and are characteristic of the NeMΦ phenotype (Loke *et al.*, 2002). Previously we had only assessed expression of these genes 2 to 3 weeks after worm implant. In order to monitor the development of the NeMΦ phenotype we performed real time RT-PCR on PEC and measured the expression of these genes over time. In addition we examined the expression of inducible Nitric Oxide Synthase (iNOS). This is a marker of classical macrophage activation (Satriano, 2004) and by means of substrate competition is directly antagonistic to Arginase1 (Corraliza *et al.*, 1995), (Munder *et al.*, 1998). The Fizz1 and Ym1 data shown in figures 3 and 4 and discussed below has been published (Nair *et al.*, 2005).

In the implanted mice there was an immediate rise in the level of message for all genes examined (Figure 3). The expression level of these genes continued to increase throughout the experiment. In the sham surgery group there was a spike of Ym1 expression at day 1 (Figure 3B) which was equivalent to the expression level in the implanted mice. Similarly Fizz1 and Arginase1 expression (Figure 3A & 3C) was also elevated at day 3 in the sham surgery mice. At day 5 the expression of all three genes had fallen to naive levels in the sham surgery group and remained at these levels until the end of the experiment.

At all time points from day 5 onwards there was significantly higher expression of Fizz1, Ym1 and Arginase1 in the implanted mice. Throughout the early timecourse there was also an increase in iNOS expression in both groups (Figure 3D). However only at the later timepoints did the expression in the implanted mice become significantly higher than that seen in the sham surgery mice. Indeed whereas the level of iNOS expression in the sham mice began to decrease from day 5 onwards in the implanted mice iNOS expression did not change from this timepoint.

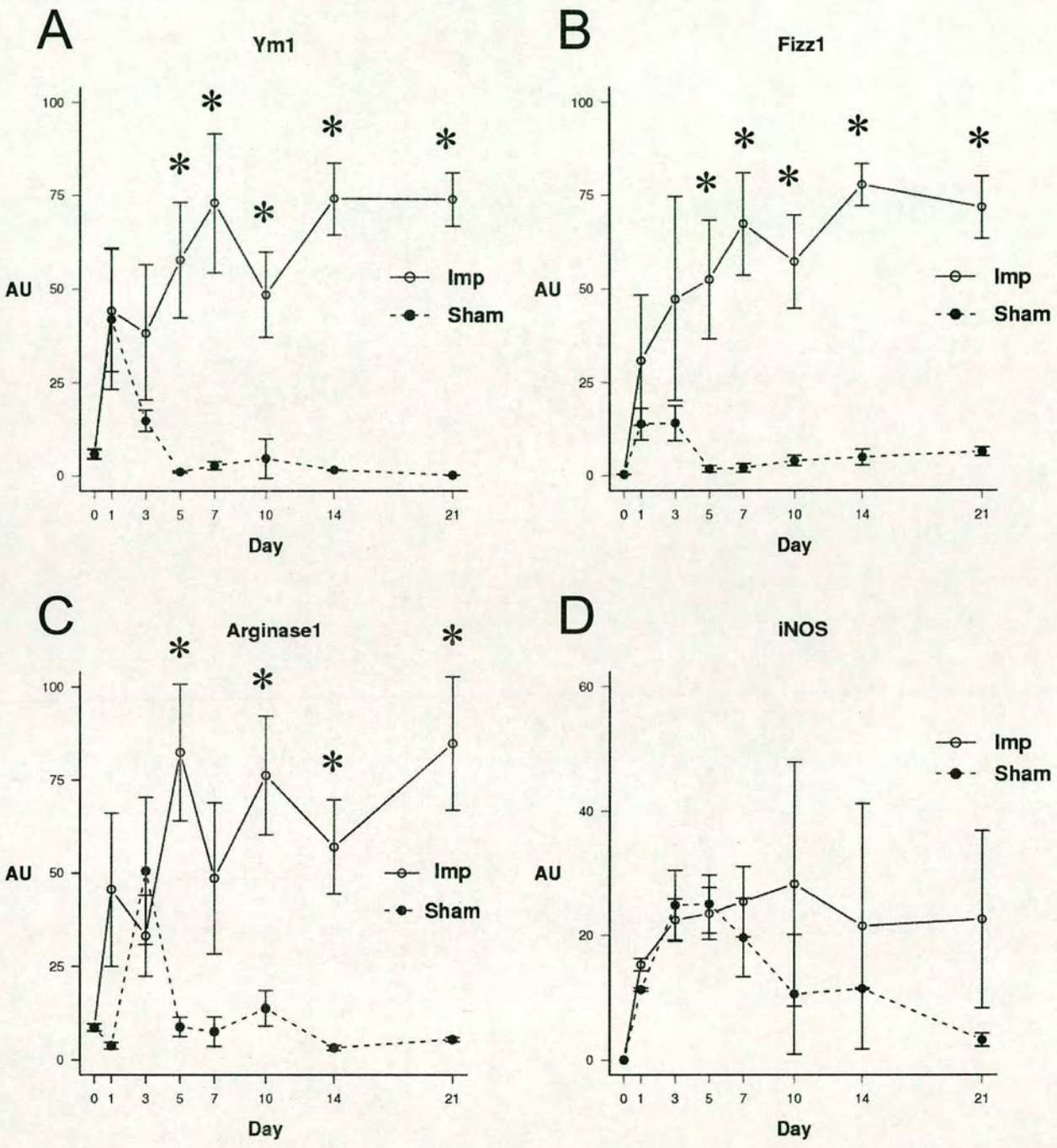

Figure 3: Using real time RT-PCR we examined the expression of Ym1 (A), Fizz1 (B) and Arginase1 (C). We also examined the expression of iNOS (D) a marker of classical macrophage activation. Implant n = 5. Sham n = 4 or 5. Error bars represent s.e.m. $*$ = $p < 0.05$.

3.2.4. Fizz1 and Ym1 are secreted proteins

In order to examine whether the mRNA expression pattern of Fizz1 and Ym1 was matched by protein secretion we undertook western blotting of the peritoneal lavage fluid at each timepoint using polyclonal rabbit anti-peptide antibodies. Figure 5 shows that the secretion of Ym1 protein into the peritoneal lavage fluid follows the mRNA expression pattern in the implanted mice. There is an initial low level of Ym1 present and then at day 7 the protein becomes much more apparent. Fizz1 was not detectable by western blot in the first 5 days of the timecourse in the implanted mice but at day 7 there is evidence of protein in the peritoneal lavage fluid. From this point onwards Fizz1 protein is detectable in implanted mice. Ym1 was detectable in the peritoneal lavage of the sham surgery group until day 7 but was undetectable after that point. We could not detect Fizz1 by western blot at any time in the sham operated group reflecting the lower level of mRNA expression in these mice compared to the implanted mice. Immunohistochemical staining using the ABC technique and the antibody raised against a Ym1 peptide (Figure 5) reveals that NeMΦ stain intensely for Ym1 (red staining Figure 5A). There is also some evidence of small areas of discrete staining on microfilaria washed from the peritoneal cavity of implanted mice. Staining with an irrelevant antibody reveals no non-specific binding (Figure 5B) on NeMΦ or on microfilaria. Likewise there is no positive staining seen on thioglycollate recruited macrophages (Figure 5C).

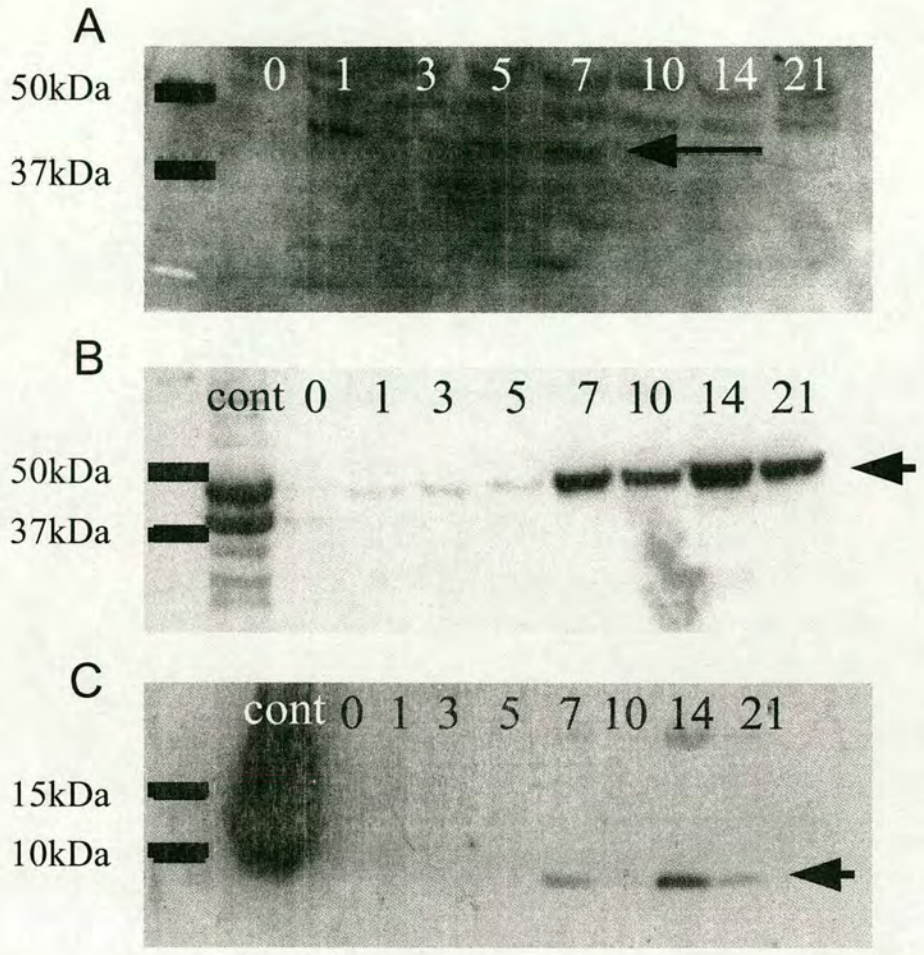

Figure 4: Western blot of Ym1 in sham surgery mice (4A) and implanted mice (4B), and Fizz1 (4C) in implanted mice illustrates that secretion of these proteins into the peritoneal cavity follows mRNA expression. In the implanted mice Ym1 (4B) is detectable throughout the timecourse and Fizz1 (4C) from day 7 onwards. In sham surgery mice Ym1 (4A) is detected from day 1 until day 7. Fizz1 was undetectable in the sham surgery mice. cont = recombinant protein. Arrows indicate position of native protein.

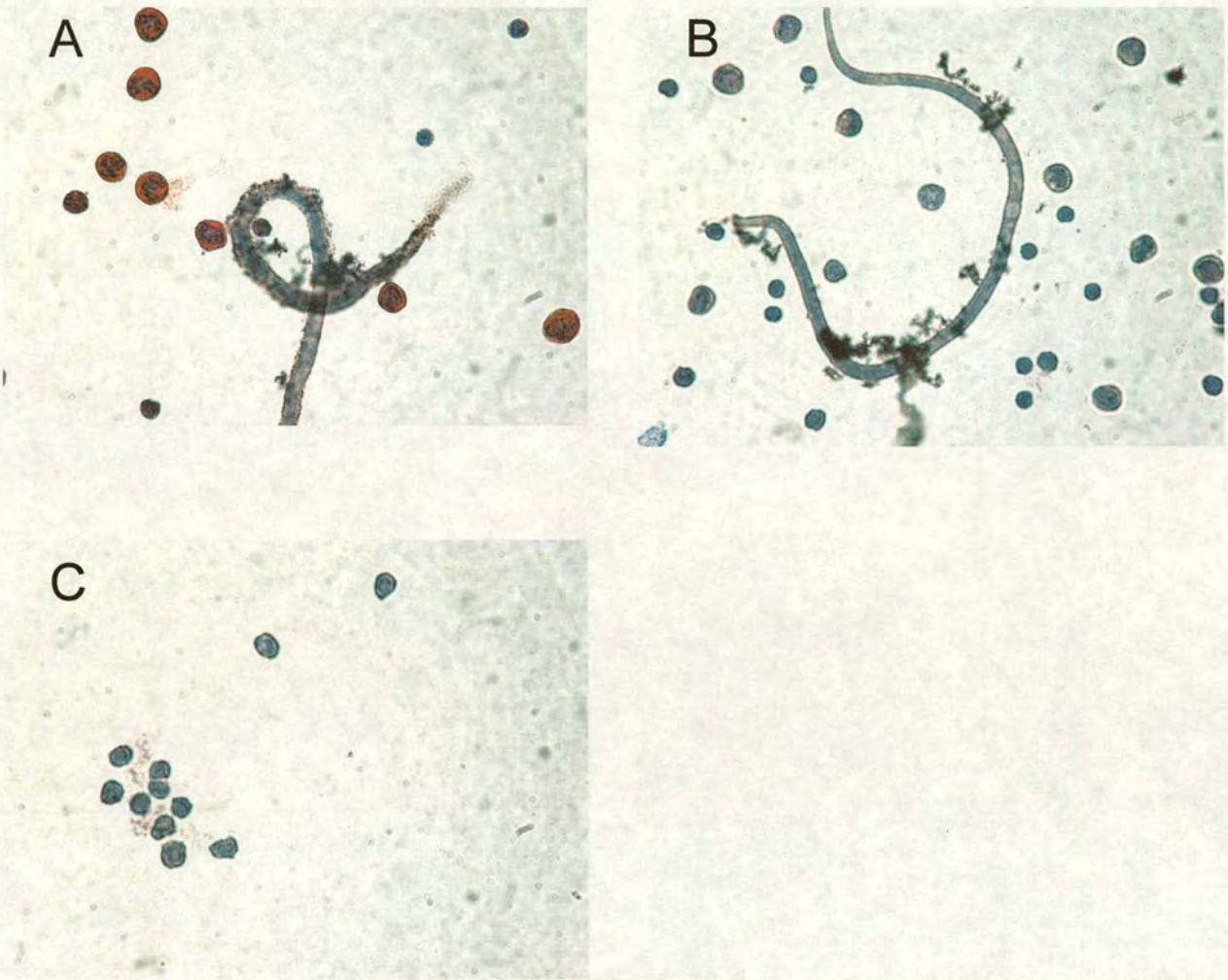

Figure 5: Immunohistochemical staining of Ym1 in NeMΦ. Ym1 is clearly visible (red stain) in NeMΦ (5A) there is also slight staining of *B. malayi* microfilaria (5A). NeMΦ and microfilaria stained with irrelevant antibody (5B) and thioglycollate recruited macrophages stained with anti-Ym1 (5C) show no evidence of positive staining. Magnification x1000.

3.2.5. Ym1 has a role in B. malayi encapsulation

The association between Ym1 and wound healing suggested that Ym1 may be involved in an immune response against *B. malayi* involving the encapsulation of the parasite and thus its sequestration away from nutrients. At day 21 after implant we occasionally recovered adult worms ensheathed in a granulomatous substance (Figure 6A). Western blotting of homogenates made from encapsulated worms revealed a positive signal for Ym1 (Figure 6B Lane Enc) whereas homogenates made from free worms did not (Figure 6B Lane UEnc). There was also no reactivity in the culture supernatant used to

culture free worms indicating that the reactivity we were seeing was not due to a secreted *B. malayi* chitinase (Figure 6B Lane UES). Furthermore immunostaining of both encapsulated and free worms retrieved from the peritoneal cavity at day 21 showed that the material around the encapsulated worms was indeed strongly positive for Ym1 (Figure 6C) whereas unencapsulated worms do not stain for this protein (Figure 6D).

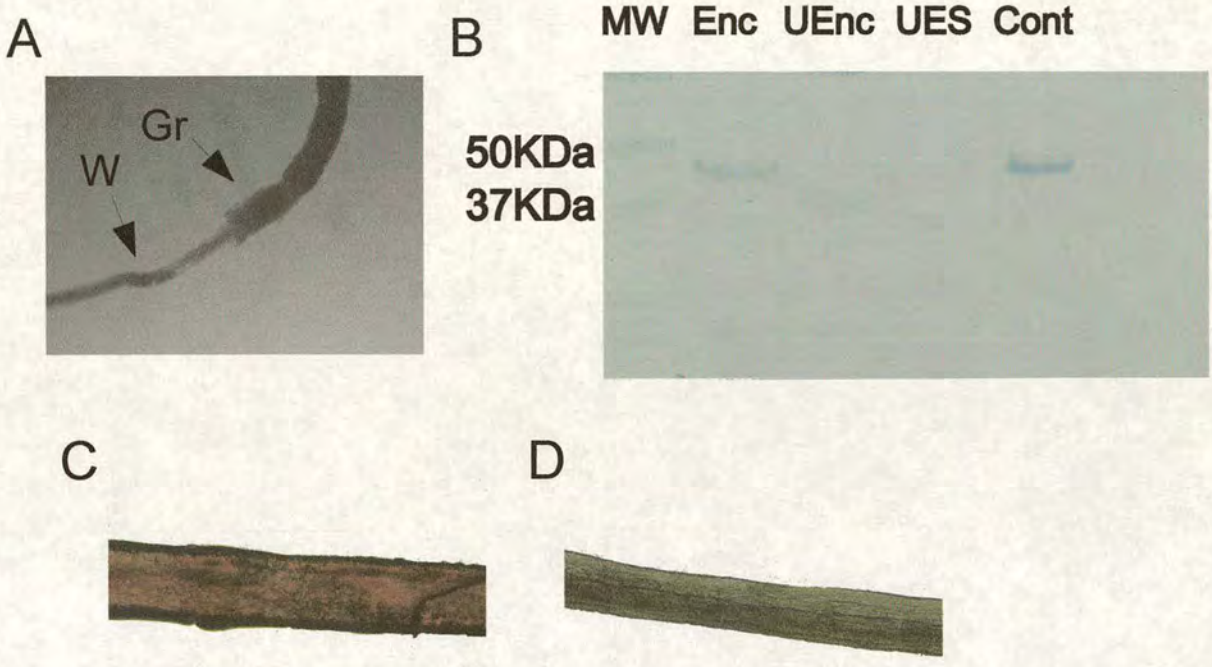

Figure 6: At day 21 after *B. malayi* implant some adult worms (W) are encapsulated in a granulomatous material (Gr) (A). Western blotting reveals that homogenate made from encapsulated worms stains positively for Ym1 (Lane marked Enc in B) whereas homgenate made from free worms does not (Lane marked UEnc in B). There is no Ym1 cross reactivity in the culture supernatant from free worm (Lane marked UES in B). The granulomatous material around encapsulated worms stains positively for Ym1 (C) whilst the surface of free worms does not (D). Cont = positive control.

3.2.6. NeMΦ Morphology

Our previously published data suggested that the structural phenotype and appearance of NeMΦ was different from that of thioglycollate recruited macrophages (Nair *et al.*, 2003). In order to examine this more closely we undertook electron microscopic examination of NeMΦ from both WT and IL-4 deficient mice as well as examining thioglycollate recruited cells. Confirming our light microscopy findings (Nair *et al.*, 2003) electron microscopy reveals that NeMΦ from both WT and IL-4 deficient

mice have a rounded, ball-like appearence (Figures 7A and 7B respectively). The thioglycollate recruited cells have a flattened, fibroblastic like appearance (Figure 7C). The rounded phenotype of NeMΦ is not dependent on IL-4 as it is seen in the cells from IL-4 deficient mice (7B) as well as those from WT mice (7A).

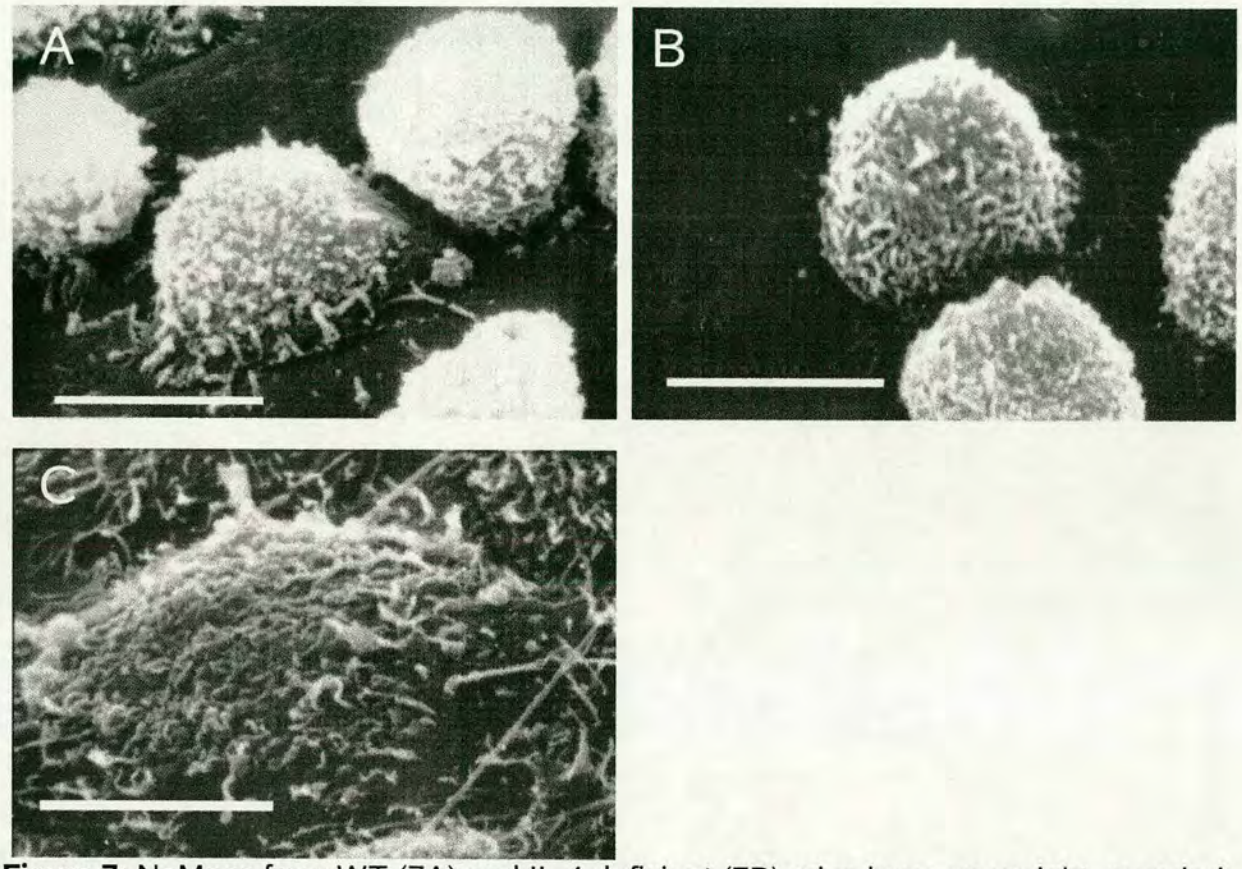

Figure 7: NeMacs from WT (7A) and IL-4 deficient (7B) mice have an upright, rounded shape in culture. In contrast thioglycollate recruited macrophages (7C) assume a more flattened and fibroblastic like shape. Magnification x 4000. Bar = 10 μm.

3.2.7. The Cytokine Response

The unexpected early expression of Fizz1, Ym1 and Arginase1 suggests that surgical intervention induces a similar response to nematode implant, albeit transiently. Thus, we chose to examine by real time RT-PCR the expression of a range of cytokines and inflammatory markers to examine how the kinetics of expression could shape the cellular response.

In general, the cytokine responses were similar in the early stages of the timecourse with differences becoming noticeable after day 5 (Figure 8). The NeMΦ phenotype is dependent on IL-4 and so in order

to investigate the temporal relationship between this cytokine and Fizz1 / Ym1 expression, key markers of NeMΦ, we examined the mRNA expression of IL-4 in PECs from both the implanted and sham surgery mice. There was no expression of IL-4 mRNA in PECs from untreated mice (Figure 8A Day 0). In both groups there was a rise in message at day 1 although this rise was larger in the implanted group where the levels of IL-4 mRNA rose rapidly between days 5 and 7 and then reached a plateau which was maintained until the end of the experiment. In the sham surgery mice there was a more subtle rise in IL-4 mRNA which peaked at day 5 and fell to baseline between days 10 and 14. At no point was the level in the sham surgery group greater than that in the implanted group. IL-4 expression was thus closely correlated with Fizz1 and Ym1 expression in the implanted mice. In the sham surgery group however IL-4 expression was more sustained than the expression of either Fizz1 or Ym1. This may relate to the relative importance of IL-13 in this innate response (see Chapter 4).

IL-6 has recently been shown to be intimately involved in the early differentiation of naive CD4+ T cells to Th2 cells (Diehl & Rincon, 2002). The dependence of the NeMΦ phenotype on IL-4 led us to investigate the kinetics of IL-6 expression in our system (Figure 8B). IL-6 showed a small rise in mRNA expression in the sham surgery group and by day 5 levels had returned to baseline. In contrast in the implanted group where we see a pronounced Th2 response there was a steady rise in IL-6 mRNA levels until day 10; from this point on they remained steady until the end of the experiment. We have subsequently shown that NeMΦ produce very high levels of IL-6 (unpublished observation) and so the IL-6 we have detected in the implanted mice is likely from this source rather than T cells.

IL-10 was originally characterised as a Th1 suppressive cytokine on the basis of inhibition of Th1 cytokine expression (Fiorentino *et al.*, 1989), (Fiorentino *et al.*, 1991). This classification was further supported by the finding that lack of IL-10 leads to enterocolitis in mice (Khun *et al.*, 1993). IL-10 can drive a rise in Arginase1 activity in macrophages independently of IL-4 or IL-13 whilst suppressing iNOS expression (Munder *et al.*, 1998) supporting a role for this cytokine in the generation of the AAMΦ phenotype. However IL-10 has also been implicated in the suppression of the Th2 response (Tournoy *et al.*, 2000). The concurrent expression of both iNOS and Arginase1 led us to examine IL-10 expression. There was a detectable level of IL-10 mRNA in the PEC of naïve mice (day 0) which increased rapidly in both groups in the early stages of the experiment (Figure 8C). There was a large spike of IL-10 mRNA expression at day 3 in the sham surgery group. The level of IL-10 then began to fall in this group although it did not reach baseline levels until day 21. At day 5 differences between the groups became apparent; the fall in IL-10 expression in the sham surgery group was paralleled by a rise in the implanted group which peaked at day 10 and was then maintained until the end of the experiment. AAMΦ are a major source of IL-10 and we have also observed IL-10 in our NeMΦ

(unpublished observation). Therefore along with IL-6 the IL-10 expression we have documented here is likely to be derived predominantly, at least in the later stages from NeMΦ.

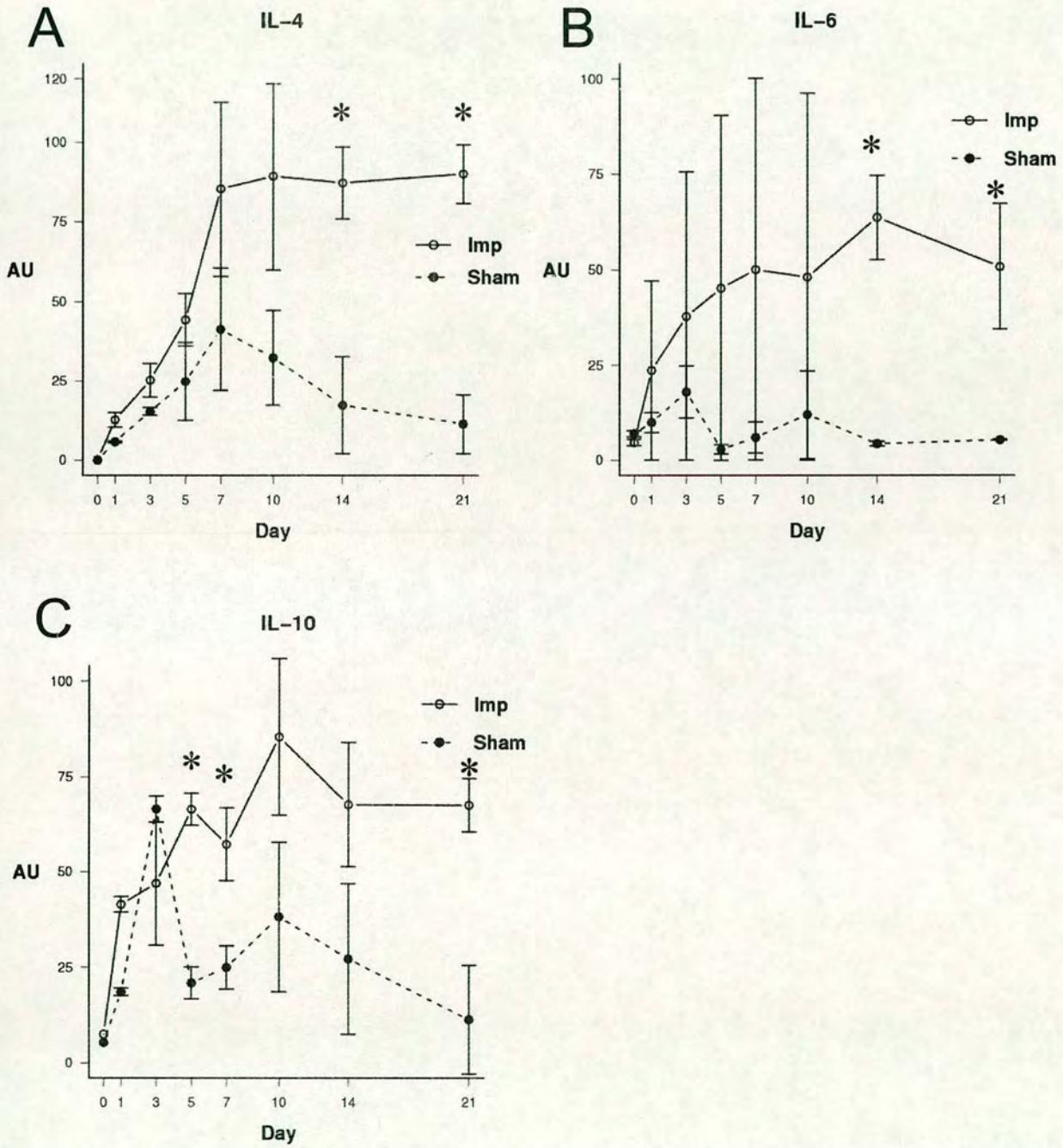

Figure 8: Real time RT-PCR was used to examine the expression of the cytokines IL-4 (A), IL-6 (B) and IL-10 (C) in the PEC of implanted and sham surgery mice. In each case the presence of *B. malayi* drove a sustained expression of these genes. There are also early IL-4 and IL-10 responses in the sham surgery mice. Error bars represent the s.e.m. n=5 mice per groups per day. $*$ = $p < 0.05$.

3.3. Discussion

The data presented in this chapter has expanded our understanding of the early events that shape the NeMΦ phenotype. We have illuminated the kinetics of this phenotype and the immunological context in which NeMΦs arise as well as gained information about the dynamics of the markers of alternative macrophage activation Fizz1, Ym1 and Arginase1. In addition we have made some surprising discoveries with regard to early innate responses in this setting.

3.3.1. Differences in eosinophil recruitment between implanted and wounded mice are immediately apparent

In both the sham surgery and implanted mice there is a rapid influx of cells into the peritoneal cavity. Although the absolute number of cells recruited in the first 3 days in both groups is similar there is then a rapid divergence in the response. Total cell number in the sham mice is more or less maintained from day 3 onwards whereas in the implanted mice cell numbers continue to increase. The composition of the early cellular infiltrate is different between groups and the most obvious early difference is in the number of eosinophils recruited. The implanted mice demonstrate an immediate rise in eosinophil numbers whereas in the sham mice the number of eosinophils does not rise until day 3. The higher level of eosinophils in the implanted mice is easily explainable by the presence of the parasite which is known to drive an early eosinohpil recruitment (Ramalingam *et al.*, 2003) partly through excretory / secretory products (Ramirez *et al.*, 2006).

However the lower numbers of eosinophils in the sham surgery mice may be explained by migration of these cells into the wound. We did not count the cells in and around the wound and so may have underestimated the number of eosinophils recruited in the sham surgery group mice. Eosinophils migrate into cutaneous wounds where they express and secrete members of the transforming growth factor (TGF) family (Elovic *et al.*, 1998). Whilst this migration would also have occurred in the implanted mice the presence of the parasite may have meant that a higher number of eosinophils were retained in the peritoneal cavity.

Ym1 was initially identified as an eosinophil chemotactic protein upregulated in a model of schistosomiasis (Owhashi *et al.*, 2000) and thus may play a role in the eosinophil response to *B. malayi* implant. We have previously published data detailing the relationship between the *B. malayi* macrophage inhibitory factor (Bm-MIF), Ym1 expression and eosinophil recruitment (Falcone *et al.*, 2001a) and shown that sustained exposure to Bm-MIF can drive Ym1 expression and eosinophil recruitment. Consistent with a role for Ym1 in eosinophil recruitement Welch *et al*, using a model of

allergic peritonitis saw eosinophil recruitment in the absence of eotaxin and CCL5 message along with an increase in Ym1 expression firstly by neutrophils and then macrophages (Welch *et al.*, 2002).

However, a role for Ym1 in eosinophil recruitment remains controversial. In a model of allergic asthma Webb *et al* saw little evidence for an eosinophil chemotactic role for Ym1 in vitro or in vivo (Webb *et al.*, 2001). Our present data show that eosinophil numbers increase rapidly in response to *B. malayi* implant. The difference in eosinophil recruitment between the groups despite equivalent initial Ym1 expression indicates that although Ym1 may have an eosinophil chemotactic role it is not the primary determinant of eosinophil levels in response to *B. malayi* implant.

3.3.2. *Early Ym1 & Fizz1 expression is an innate response to injury.*

Within the first 24 hours of the timecourse there was an equivalent rise in Ym1 gene expression in both the implanted and sham surgery groups which was reflected in the level of Ym1 protein detected in the peritoneal cavity. Fizz1 gene expression also rose in both groups although this was more pronounced in the implanted mice. The increasing expression of both these genes after injury alone suggests that this is an innate response to tissue damage and may be quite different in character from the later adaptive Th2 response we see in the implanted mice. The rapid rise in Ym1 expression precedes the arrival of large numbers of macrophages in the peritoneal cavity but expression does coincide with the rise in neutrophil numbers. Neutrophils have previously been characterised as a source of Ym1 (Harbord *et al.*, 2002) and early expression of Ym1 in a mouse model of allergic peritonitis was attributed to neutrophils (Welch *et al.*, 2002). In addition the loss of Ym1 expression in the sham surgery mice occurs when neutrophil numbers drop in the peritoneal cavity (see Table 3). Thus the early expression of Ym1 in the sham surgery group may come from neutrophils rather than macrophages. The source of early Fizz1 expression in both groups remains elusive. Although the number of macrophages in the peritoneal cavity of a naïve mouse is small they are the dominant population (Tables 2 and 3). These resident macrophages may express Fizz1 in response to trauma and then be replaced from day 3 onwards by a new population. Functionally both Fizz1 and Ym1 remain ill-defined but expression has previously been seen in response to tissue injury (Hung *et al.*, 2002), (Li *et al.*, 2005); roles for Ym1 and Fizz1 in this context will be discussed more fully in Chapter 4.

The finding that the early Fizz1 response is higher in the implanted animals indicates that *B. malayi* can drive immediate Fizz1 gene expression. Fizz1 belongs to a family of proteins involved in the metabolism of fats and is expressed in adipose stromal tissue (Steppan *et al.*, 2001). The manipulation of local Fizz1 levels may affect lipid metabolism and lead to the release of fats usable by the parasite.

Fizz1 mRNA is at its highest natural level in the gonadal fat pads of mice which are within the peritoneal cavity (Bing *et al.*, 2002) and thus Fizz1 could be involved in the modulation of lipid levels in this space. These lipids could also be used as fuel by the immune cells recruited into the peritoneal cavity by the infection. However expression levels of Fizz1 decrease during lactation one of the most metabolically demanding processes in the body (Bing *et al.*, 2002) indicating that lipid metabolism may decrease Fizz1 expression. Given the variety of functions ascribed to Fizz1 (Holcomb *et al.*, 2000), (Teng *et al.*, 2003b), (Liu *et al.*, 2004b) it is likely that the role this protein plays in the immune and growth responses is context dependent.

3.3.3. *Early iNOS and Arginase1 responses*

Arginase1 and iNOS are considered counter regulatory macrophage markers of the Th1 and Th2 responses respectively. Specifically the upregulation of Arginase1 driven by the Th2 cytokines IL-4 and IL-13 leads to lower iNOS activity by competition for their common substrate L-arginine (Rutschman *et al.*, 2001). In our surgical trauma system the early expression of mRNA for these enzymes is innate as it occurs before adaptive responses have had time to arise. Previous investigations into the metabolism of L-arginine in wounds have found that the early response is dominated by the expression of iNOS and the generation of NO and citrulline (Albina *et al.*, 1990) and the source of iNOS expression was neutrophils (Frank *et al.*, 1998). IFN-γ has also been shown to regulate the proinflammatory phenotype of neutrophils (Ellis & Beaman, 2004) and upregulate the oxidative burst and the expression of iNOS (McCall *et al.*, 1991), (Yamashita *et al.*, 1997). The microfilariae of filarial nematodes are known to drive a type 1 response with the upregulation of IFN-γ (Lawrence *et al.*, 1994). This in turn drives iNOS expression. The presence of microfilaria in the peritoneal cavity may therefore be responsible for the prolonged iNOS expression in the PECs. We have never detected iNOS mRNA expression in NeMΦ or nitric oxide production by these cells and thus, in the later stages of the timecourse some other cell type must be responsible for iNOS expression. Exposure to IL-4 can drive iNOS expression in eosinophils (Paoliello-Paschoalato *et al.*, 2005) and the persistence of eosinophils as well as the presence of IL-4 indicates that these cells may be responsible for sustained iNOS expression in the implanted group. iNOS mRNA levels are equivalent between the two groups at day 1 suggesting that the early response is driven by surgery and that there is no great contribution by *B. malayi.* Thus early iNOS expression is likely a bacteriostatic / bacteriocidal response to surgical trauma whilst the later expression results either from the presence of IFN-γ inducing microfilaria, the action of IL-4 on eosinophils or both. We did not measure NO generation and thus it is possible that the high level of Arginase1 expression we observe would prevent NO generation through competition

for the common substrate arginine.

Like early Fizz1 expression, early Arginase1 expression is driven by *B. malayi*. The early upregulation of Arginase1 may be to the advantage of the parasite. This enzyme catalyses the hydrolysis of L-arginine to ornithine and urea. Ornithine can then be converted to polyamines essential for cell proliferation and proline essential for collagen synthesis (Witte & Barbul, 2003). There is evidence that obligate filarial parasites lack the biochemical mechanisms to manufacture polyamines (Wittich *et al.*, 1987), (Sharma *et al.*, 1991) but possess mechanisms to absorb them from the host (Tekwani *et al.*, 1995). The upregulation of Arginase1 may therefore feed the ornithine decarboxylase (ODC) pathway and provide polyamines for growth and the production of *B. malayi* young. The question of whether early innate and the later Th2 dependent Arginase1 expression is beneficial or detrimental to the parasite merits investigation. The pattern of iNOS and Arginase1 expression in the sham surgery mice reflects previous findings on the activity of these enzymes in healing wounds (Albina *et al.*, 1990). The role of Arginase1 in wound healing will be discussed in Chapter 4.

3.3.4. *The adaptive response drives increased macrophage numbers.*

The late cellular response to *B. malayi* implant is dominated by macrophages. However this dominance only begins to become apparent around day 7 after implant. After this timepoint the macrophage numbers in the implanted mice increased rapidly whilst in the sham surgery mice macrophage numbers remained constant until the end of the experiment. The early equivalency of macrophage numbers between the two groups suggests that up until day 7 the response to surgery drives the macrophage response and after that point sustained recruitment is driven by the presence of *B. malayi*. The timing of the rise in macrophage numbers indicates that the adaptive response drives this later increase. In view of this hypothesis, it is interesting to note that whilst lymphocyte numbers are not different between the groups and do not change between days 5 and 7, the expression of markers of alternative macrophage activation (Arginase1, Ym1, Fizz1) and the expression of IL-4 all peak around this time in the implanted mice.

We have previously noted that one of the hallmark properties of NeMΦ is contact dependent suppressive ability (Allen *et al.*, 1996), (Loke *et al.*, 2000a) and that this depends upon host IL-4. Also, the timing of the rise in suppressive ability suggested a requirement for the adaptive response (MacDonald *et al.*, 1998). In the present study we examined the suppressive ability of the whole peritoneal cell population and found significant suppression from day 3 onwards. Some of this is due to the surgical intervention as the sham surgery group also displayed a significant suppressive ability at

day 3. However by day 10 the sham surgery cells were no longer able to suppress EL4 cell proliferation while the PEC from implanted mice continued to suppress EL4 proliferation until day 21. We did not investigate the IL-4 dependence of the early suppression and it is possible that the mechanisms underlying this are different from the later IL-4 dependent NeMΦ suppressive ability. Nitric oxide (NO) is known to be suppressive and as seen in figure 3 message for iNOS is upregulated early in the response in the sham surgery mice and then falls. Thus NO could be responsible for the early cellular suppression we see in the sham surgery mice.

Thus, the large increase in macrophage numbers together with increases in Fizz1 and Ym1 secretion, Arginase1 and IL-4 expression and loss of suppressive ability in the sham surgery mice indicate that a qualitative change in the response takes place around day 7. This would be consistent with a change from mainly innate immune mechanisms to an adaptive response.

3.3.5. *Ym1, Fizz1 and Arginase1 in the late response.*

As discussed above the immediate Fizz1, Ym1 and Arginase1 responses may be related wholly to wound healing. Ym1 is upregulated in many different parasite infection systems (Raes *et al.*, 2002), (Loke *et al.*, 2002), (Donnelly *et al.*, 2005) and we have documented expression in NeMΦ and other APCs in response to helminth parasites (Nair *et al.*, 2005). The putative role of Ym1 in wound healing suggests it may play a part in attempts by the host to control the parasite by encapsulation. A useful coping mechanism for the host would be to isolate the parasite in a collagenous network of fibres and thus prevent movement, feeding and reproduction. This has been seen in other parasite models and macrophages are central in this process (Shuhua *et al.*, 1998). Some of the worms in the *B. malayi* implant system become surrounded by a granulomatous material which is indeed rich in Ym1 (Figure 6) This mechanism may also involve Arginase1. The conversion of arginine to ornithine catalysed by Arginase1 can lead to increased proline levels via the ornithine amino transferase (OAT) pathway. Proline is essential for the production of collagen which is a constituent of filarial granulomatous lesions (Jeffers *et al.*, 1987).

Alternatively, Ym1, Fizz1 and Arginase1 may be required to repair parasite induced tissue damage. Ym1 expression has been seen in response to traumatic injury (Chang *et al.*, 2001) (and see Chapter 4) and Arginase1 has a documented role the physiology of wound healing (Albina *et al.*, 1988), (Albina *et al.*, 1990) while Fizz1 expression has been seen in models of pulmonary tissue remodelling (Holcomb *et al.*, 2000), (Li *et al.*, 2005), (Teng *et al.*, 2003a). Interestingly, a study by Donnelly et al using a *Fasciola hepatica* infection model showed that whilst Ym1, Fizz1 and Arginase1 were upregulated by

parasite infection only Fizz1 and Arginase1 were upregulated by the excretory / secretory (E/S) products of the parasite (Donnelly *et al.*, 2005). Infection with this organism involves damage to the intestinal wall and thus natural infection may require Ym1 expression as part of the repair process whilst injection of E/S products does not. The initial expression of these genes may be an innate event related to tissue damage but their prolonged expression in chronic injury may require IL-4.

Fizz1 could also be involved in altering the sensitivity of immune cells to other signalling molecules. Fizz1 can modulate the responses to nerve growth factor (NGF) (Holcomb *et al.*, 2000) and since NGF and the NGF receptor are expressed by a wide variety of immune cells including mast cells, eosinophils and lymphocytes (Bonini *et al.*, 2003) Fizz1 may function to modulate the behaviour of immune cells as the type 2 response develops in parasite infection.

3.3.6. The Cytokine Response

IL-4 is considered the paradigmatic Th2 cytokine and is upregulated in response to most helminth infections. IL-4 expression reaches a maximum at day 7 coinciding with the appearance of the NeMΦ phenotype and underlining the reliance of this phenotype on IL-4. Interestingly, the expression of IL-4 peaks before the peak in lymphocyte numbers. This may reflect the previously described ability of NeMΦs to present antigen and drive cytokine expression by T cells while preventing cell proliferation (Allen *et al.*, 1996).

The IL-6 response to *B. malayi* implant has not been previously described. The data we present shows that there is basal expression of this cytokine in the peritoneal cavity of naïve mice and it is upregulated early in the implanted mice. IL-6 is able to stimulate the Th2 response and bias naïve CD4 positive T cells towards the Th2 phenotype and IL-4 secretion while suppressing the Th1 response (Diehl & Rincon, 2002). The early rise in IL-6 expression in the implanted mice indicates that the swaying of the immune response towards Th2 begins immediately. IL-6 has been characterised as an acute phase cytokine and is expressed by a wide variety of cell types including those associated with tissue injury (i.e. fibroblasts, keratinocytes and endothelial cells) (Diehl & Rincon, 2002) thus it is surprising that we see little upregulation at any point in the sham surgery group although many of these cell types would be expected to remain in and around the wound and may not be found in the peritoneal exudate population.

Whilst IL-10 is upregulated in both groups the expression of this cytokine is initially higher in implanted mice indicating that *B. malayi* drives rapid IL-10 expression. IL-10 is thought of as a Th2 and generally suppressive cytokine; two properties encompassed in the finding that IL-10 deficient

mice develop a Th1 driven gastroenteritis (Kuhn *et al.*, 1993). Data from our laboratory indicates that NeMΦs produce IL-10 and although this cytokine plays no role in NeMΦ suppressive ability (MacDonald *et al.*, 1998), IL-10 is known to induce an anti-inflammatory phenotype in macrophages (Stumpo *et al.*, 1999). IL-10 can also upregulate Arginase1 through synergy with IL-4 and / or IL-13 (Munder *et al.*, 1998) and as discussed above this may be beneficial for the parasite.

3.3.7. *NeMΦ appearance in the peritoneal cavity*

The electron microscopy studies we undertook were based upon previous observations in the laboratory that the shape of NeMΦs and thioglycollate recruited macrophages were different when viewed under the light microscope (Nair *et al.*, 2003). We hoped to illuminate differences between these cell types from closer examination of these cells. Thioglycollate recruits macrophages partly through the generation of oxygen radicals and the creation of an inflammatory environment dominated by Th1 cytokines and partly through the engagement of the macrophage scavenger receptor by advanced glycosylation endproducts (AGEs) in aged thioglycollate (Li *et al.*, 1997). AGEs produce superoxide when they interact with receptors on cell surfaces (Loske *et al.*, 1998) and thus contribute to tissue damage which signals further macrophage recruitment. Thioglycollate has also been shown to be chemotactic and mitogenic for macrophages (Kirstein *et al.*, 1990), (Yui *et al.*, 1994). We found that thioglycollate recruited cells have a markedly different gross appearance with a fibroblastic shape compared to the rounded shape of NeMΦs. Bosetti et al indicate the presence of oxidised stimuli causes an extended, fibroblastic shape similar to that seen in our thioglycollate recruited cells (Bosetti *et al.*, 2003).

In contrast to the thioglycollate recruited cells NeMΦs from both wild type and IL-4 deficient *B. malayi* implanted C57BL/6 mice have a rounded appearance; this shape is not, therefore IL-4 dependent. The rounded appearance of macrophages has been associated with both resident macrophages and macrophages involved in the destruction of helminth L3 larvae (Bosetti *et al.*, 2003), (Shuhua *et al.*, 1998), where rounded macrophages adhere to and encase helminth larvae. We do not see macrophage adherence to *B. malayi* microfilaria or adult worms in our implant model although some worms are found encased in a granulomatous material which is rich in Ym1 (Figure 6).

3.3.8. Conclusions

- Eosinophil recruitment is a rapid response to parasite infection.
- Fizz1, Ym1 and Arginase1 are all upregulated in response to tissue trauma.
- Sustained macrophage numbers and late Fizz1, Ym1 and Arginase1 expression required the presence of the parasite and presumable the adaptive immune response. after *B. malayi* implant.
- Expression of these genes may be an effector response or beneficial to the parasite.

Chapter 4 - Regulation of Fizz1 & Ym1 in Surgical Trauma

4.1. Introduction

The data in Chapter 3 strongly implicated Fizz1 and Ym1 in the innate response to trauma. The hypothesis that these genes play a role in early resolution to injury was supported by the observation that message for Arginase1, an important component of the early wound healing response (Albina *et al.*, 1988), (Albina *et al.*, 1990), (Witte *et al.*, 2002) was also upregulated. We knew from previous work that Arginase1, Fizz1 and Ym1 expression in NeMΦ required IL-4 (Loke *et al.*, 2002) and the presence of CD4 +ve T cells (P. Loke - Unpublished observation). Thus the late response of these genes is entirely dependent on the Th2 response. We wanted to examine early expression of these genes, characterised by the response to surgical trauma, more closely and specifically ask: 1) is this a truly innate response or does it require input from cells of the adaptive immune system and 2) is the early induction of Fizz1 and Ym1 dependent on IL-4 and / or IL-13?

Tissue damage leads to a stereotypical response which can be divided into three overlapping phases; inflammation, proliferation and resolution (Park & Barbul, 2004). Initially there is the formation of a platelet plug with early extracellular matrix (ECM) formation provided by blood coagulation. These processes allow an influx of neutrophils and macrophages into the affected area to clean up cellular debris and invading bacteria. The release of growth factors as this first stage ends results in the migration of keratinocytes across the area to provide an epithelial barrier and the recruitment of fibroblasts which generate ECM proteins and and lay down granulation tissue. Myofibroblasts then provide contracting forces within this granulation tissue to bring the wound edges together as endothelial cells re-vascularise the area. This process leaves a collagen based scar tissue which undergoes further remodelling over a series of months (Midwood *et al.*, 2004).

The early immune response to tissue damage is reported to be a type 1-like response. Neutrophils recruited into the wound area produce high quantities of reactive oxygen species and release damaging products from their granules resulting in the death of not only bacteria but also healthy cells in and beyond the wounded area (Martin & Leibovich, 2005). The arrival of macrophages heralds the removal of debris and dead cells from the area. Initially macrophages are involved in the inflammatory phase with the generation of reactive molecules and phagocytosis being their main functions (Park & Barbul, 2004). As this stage proceeds however the activation state of macrophages changes and they secrete various cytokines and growth factors to promote the proliferative phase of healing (Park & Barbul,

2004). The final role for macrophages in the wound healing process seems to be the degranulation of the wound to make way for the laying down of granulation tissue by fibroblasts (Martin & Leibovich, 2005). So although the immune environment of the wound begins with a very pro-inflammatory bias, after 3 to 5 days this response is downregulated and repair proper begins to take place.

The data shown in Chapter 3 (Figures 3 & 4) suggested that there might be a novel requirement for type 2 cytokines in the acute wound setting. We thus examined the type 2 dependence of Fizz1 and Ym1 in this setting. We carried out further timecourse experiments in IL-4 and IL-4Rα deficient mice utilising the surgical model as detailed in Chapters 2 and 3. Using real time RT-PCR we assessed the expression of Fizz1 and Ym1 mRNA before surgery and over the 5 days after surgery. We also examined the secretion of Fizz1 and Ym1 by western blotting. In addition we used RAG1 deficient mice to examine any potential requirement for natural killer T (NKT) cells which may produce early Th2 cytokines (Kronenberg, 2005) or γ / δ T cells which can produce Th2 cytokines (Wesch *et al.*, 2001), and have a role in promoting keratinocyte responses after injury (Jameson *et al.*, 2004), (Jameson *et al.*, 2004) and promote neutrophil migration after injury (Toth *et al.*, 2004).

4.2. Results

4.2.1. Surgical trauma causes cell recruitment into the peritoneal cavity

As detailed in Chapter 3 surgical trauma to the abdomen causes an influx of cells into the peritoneal cavity of WT mice. In this set of experiments we examined the response of IL-4, IL-4Rα and RAG1 deficient mice to a surgical wound. Our chief aim was to detail the requirement for IL-4, IL-4Rα signalling and the adaptive immune system in the regulation of early Fizz1 and Ym1 expression. As a first step we examined the number and type of cells recruited into the peritoneal cavity in response to surgery. In all three experiments there was an increase in the total number of cells recruited into the peritoneal cavity over the 5 days following trauma in both the WT and gene knockout mice (Figure 9). The temporal pattern of the response was also similar in each experiment with an influx of cells into the peritoneal cavity on days 1 and 3 followed by a fall in numbers at day 5. In the IL-4 (Figure 9A) and RAG1 deficient (Figure 9C) mice there were significantly fewer cells in the peritoneal cavity at day 5 than were seen in the WT mice ($p<0.05$) but this difference was not seen in the IL-4Rα deficient mice (Figure 9B). Thus, there does not seem to be a deficiency in total cell recruitment in IL-4, IL-4Rα or RAG1 knockout mice within the first 3 days after surgical trauma.

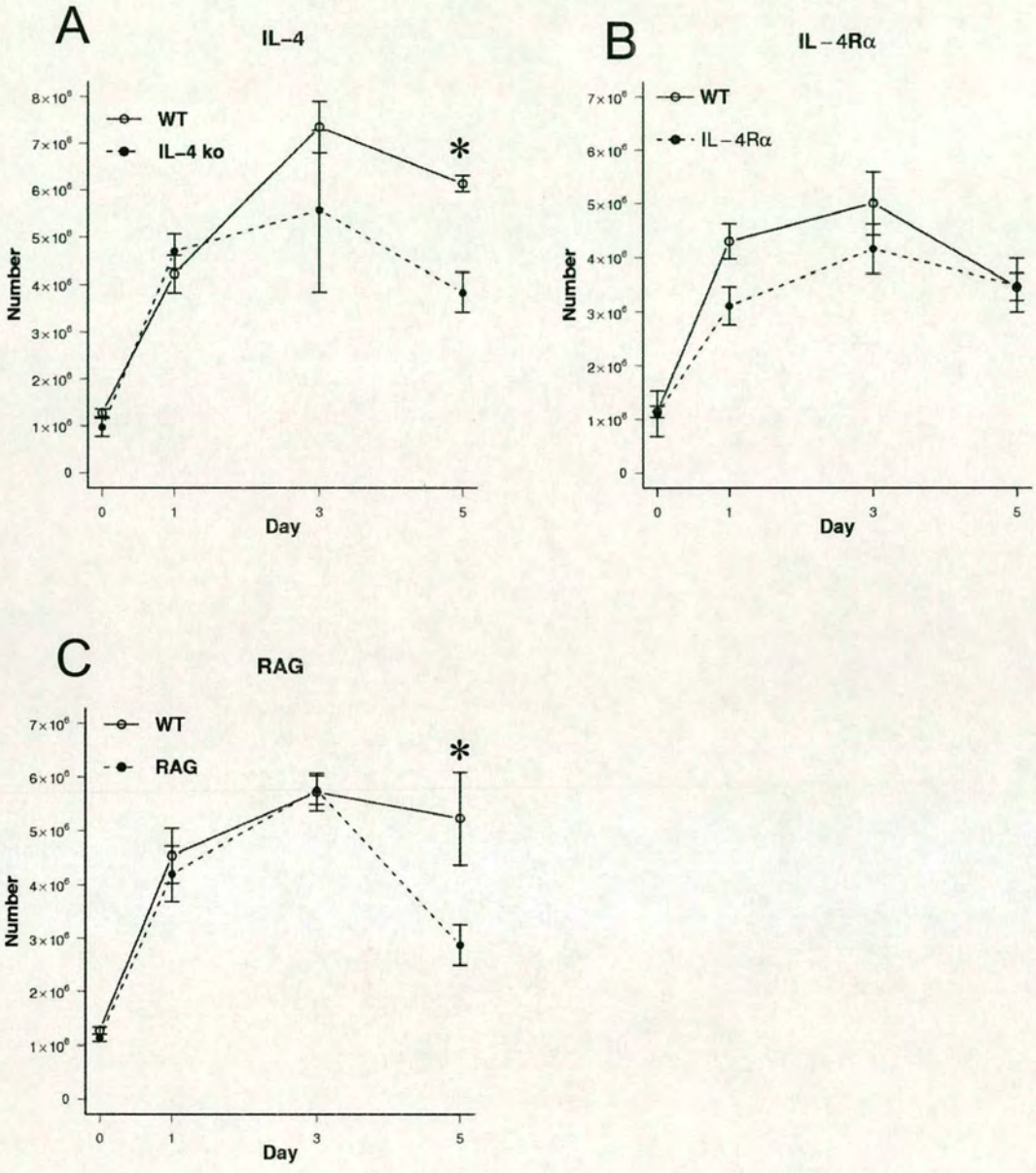

Figure 9: Cell recruitment into the peritoneal cavity in IL-4 deficient (A), IL-4Rα deficient (B) and RAG1 deficient (C) mice after surgical trauma. In each case there were 4 or 5 WT mice and 5 gene knockout mice per timepoint. Peritoneal lavage was carried out on each mouse and the total number of cells counted using the Casy Counter (Scharfe Systems).

* = $p < 0.05$.

4.2.2. Cell recruitment patterns in IL-4 deficient, IL-4Rα deficient and RAG1 deficient mice after surgical trauma

Whilst the dynamics of total cell recruitment into the peritoneal cavity in response to surgical trauma were similar in the different groups of mice examined, there are differences in the composition of the cellular influx (Figure 10). There were fewer eosinophils in the peritoneal lavage fluid of the IL-4 deficient mice than their WT counterparts over the course of the experiment (Figure 10A) although this difference was only significant at day 3 ($p = 0.02$). Neutrophil numbers were also significantly reduced at day 3 in the IL-4 deficient mice ($p = 0.02$). The IL-4Rα deficient mice (Figure 10B) had a lower recruitment of neutrophils and eosinophils at all timepoints compared to their WT counterparts. Whilst this could represent a faster consumption of dead or damaged granulocytes by macrophages in these mice there was no significant difference in the macrophage numbers between the groups at any time. Reduced eosinophil recruitment is also seen in IL-4 deficient mice implanted with *B. malayi* (MacDonald *et al.*, 2003) suggesting a role for Th2 cytokines in the eosinophil response. The most obvious difference in the RAG1 deficient mice (Figure 10C) was the complete lack of lymphocytes, as expected. The only other significant difference in the RAG1 deficient mice compared to their WT counterparts was an elevation in the neutrophil numbers at day 3 ($p = 0.01$). Taken together the recruitment data suggest that some aspects of the granulocyte response are dependent on Th2 cytokines but that NKT cells or lymphocytes are not the source of early Th2 cytokine release since granulocyte numbers in the RAG1 deficient mice were no different than the WT mice.

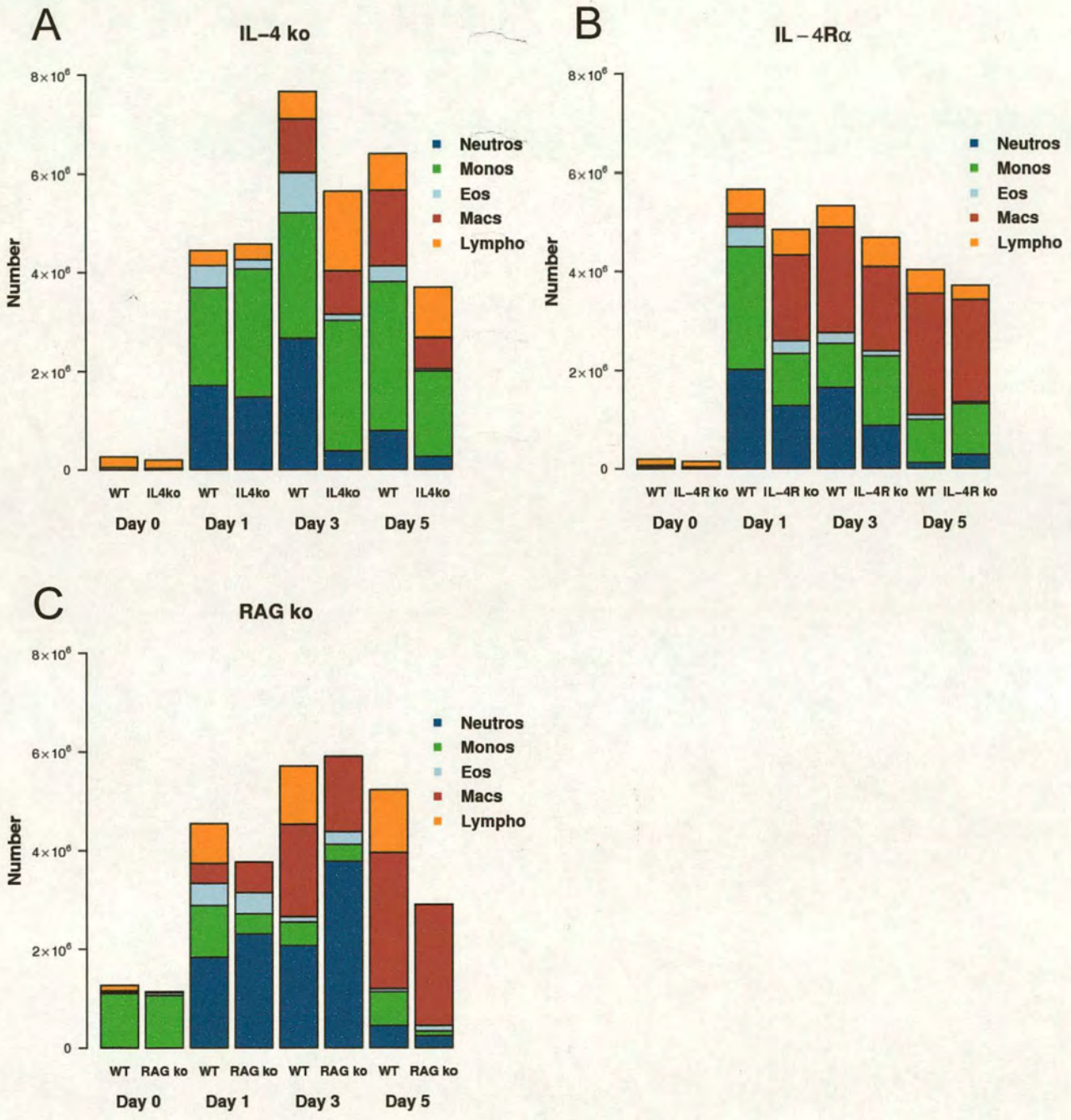

Figure 10: Differential cell counts from the peritoneal lavage fluid of IL-4 deficient (A), IL-4Rα deficient (B) and RAG1 deficient (C) mice after surgical trauma. Peritoneal lavage fluid was subjected to cytospin and individual cell counts types were counted by routine staining. The percentage of each cell type was calculated form these counts and the total cell count (Figure 9) and these percentages were used to estimate the total number of each cell type present. N= 4 or 5 WT mice and 5 gene knockout mice per timepoint and two fields of at least 150 cells were counted per mouse.

4.2.3. IL-4 is required for the expression of Arginase1 but not Fizz1 and Ym1 after surgical trauma

The previous section detailing the requirement for innate Th2 cytokine activity in the generation of the full cellular response suggests that innate Th2 activity might also be important in the Ym1 and Fizz1 responses after surgical trauma. In order to assess the requirement for Th2 cytokines in the expression of Fizz1 and Ym1 after surgical trauma we used real time RT-PCR to examine the mRNA expression of these genes in the PEC population described in the previous section by real time RT-PCR. We also used western blot to detect the presence of Fizz1 and Ym1 protein in the peritoneal lavage fluid after surgery. Figure 11 shows that despite the lack of IL-4 the expression of Ym1 (Figure 11A) is unaffected with responses being identical in both the WT and IL-4 deficient mice. Fizz1 (Figure 11B) shows an intermediate response with expression being somewhat lower on day 3 and significantly lower on day 5 in IL-4 deficient mice compared to WT mice. Arginase1 (Figure 11C) shows a profound dependence of IL-4 with expression heavily compromised in the IL-4 deficient mice. These data support a role for the innate production of IL-4 in the full expression of Fizz1 and Arginase1.

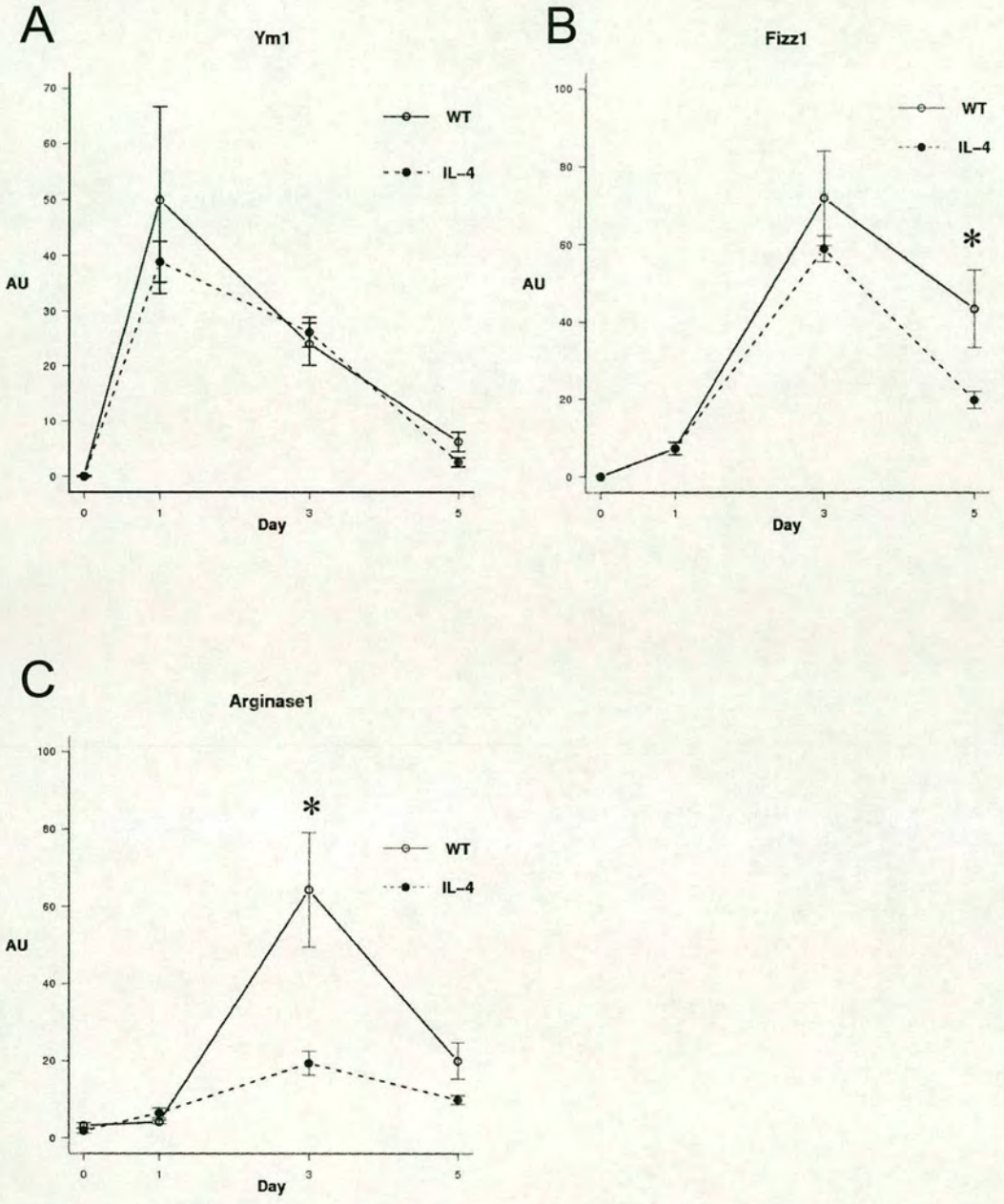

Figure 11: Real time RT-PCR reveals the mRNA expression pattern of Ym1 (A), Fizz1 (B) and Arginase1 (C) in peritoneal cells of WT and IL-4 deficient C57BL/6 mice after surgery. N = 4 or 5 WT mice and 5 gene knockout mice per timepoint. * = $p < 0.05$.

Figure 12 confirms that Ym1 protein is secreted into the peritoneal cavity after surgical trauma in both WT and IL-4 deficient mice. In both groups of mice Ym1 is undetectable in the peritoneal lavage fluid

of an uninjured mouse. At day 1 however presence of the Ym1 protein is marked and persists until day 3 before falling at day 5. This reflects the expression of Ym1 mRNA seen in figure 11. We could not detect Fizz1 expression in the peritoneal lavage fluid at any timepoint from either group.

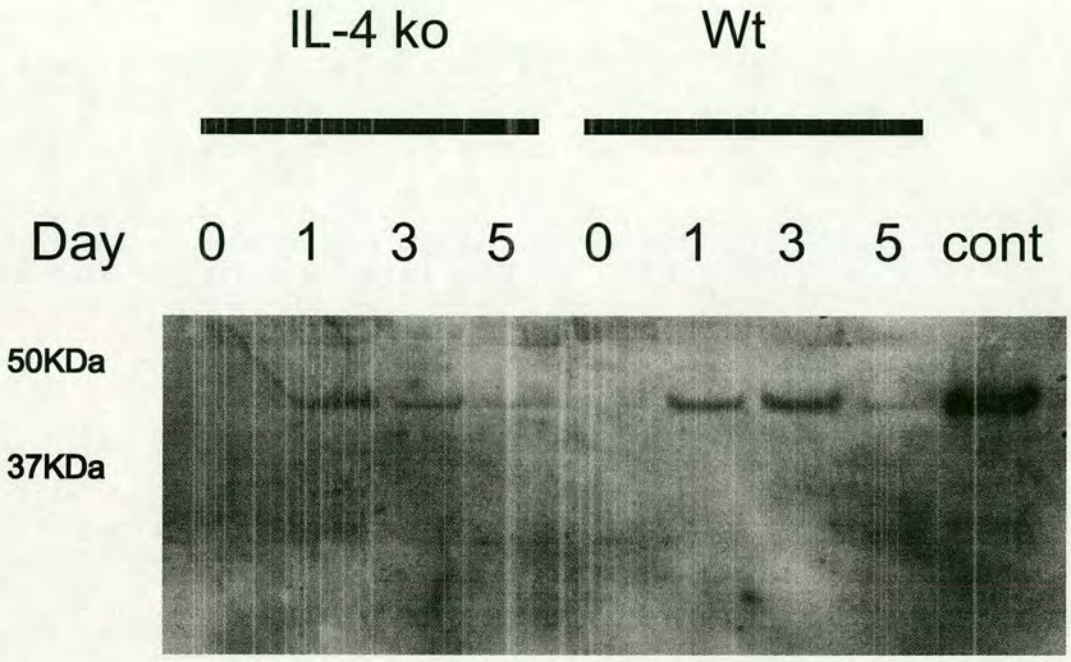

Figure 12: Western blot confirms that there is secretion of Ym1 protein into the peritoneal cavity in both WT and IL-4 deficient mice after surgery.

4.2.4. Type 2 cytokines are required for Fizz1 and Ym1 expression but not for secretion of Ym1 into the peritoneal cavity

The expression of Fizz1, Ym1 and Arginase1 is regulated by signalling through IL-4 receptor α (IL-4R α) (Stutz *et al.*, 2003), (Welch *et al.*, 2002). The finding that lack of IL-4 had different effects on the expression of Fizz1, Ym1 and Arginase1 with a profound effect on Arginase1 expression and no effect on Ym1 expression led us to examine the effect of a lack of responsiveness to both the main type 2 cytokines IL-4 and IL-13. We therefore undertook a second timecourse experiment in IL-4Rα deficient mice. This receptor is shared between IL-4 and IL-13 (Hershey, 2003) and accounts for much of the functional overlap of these cytokines. The IL-4Rα deficient mice were only available on the BALB/c background and so the control mice in this experiment are BALB/c and not C57BL/6.

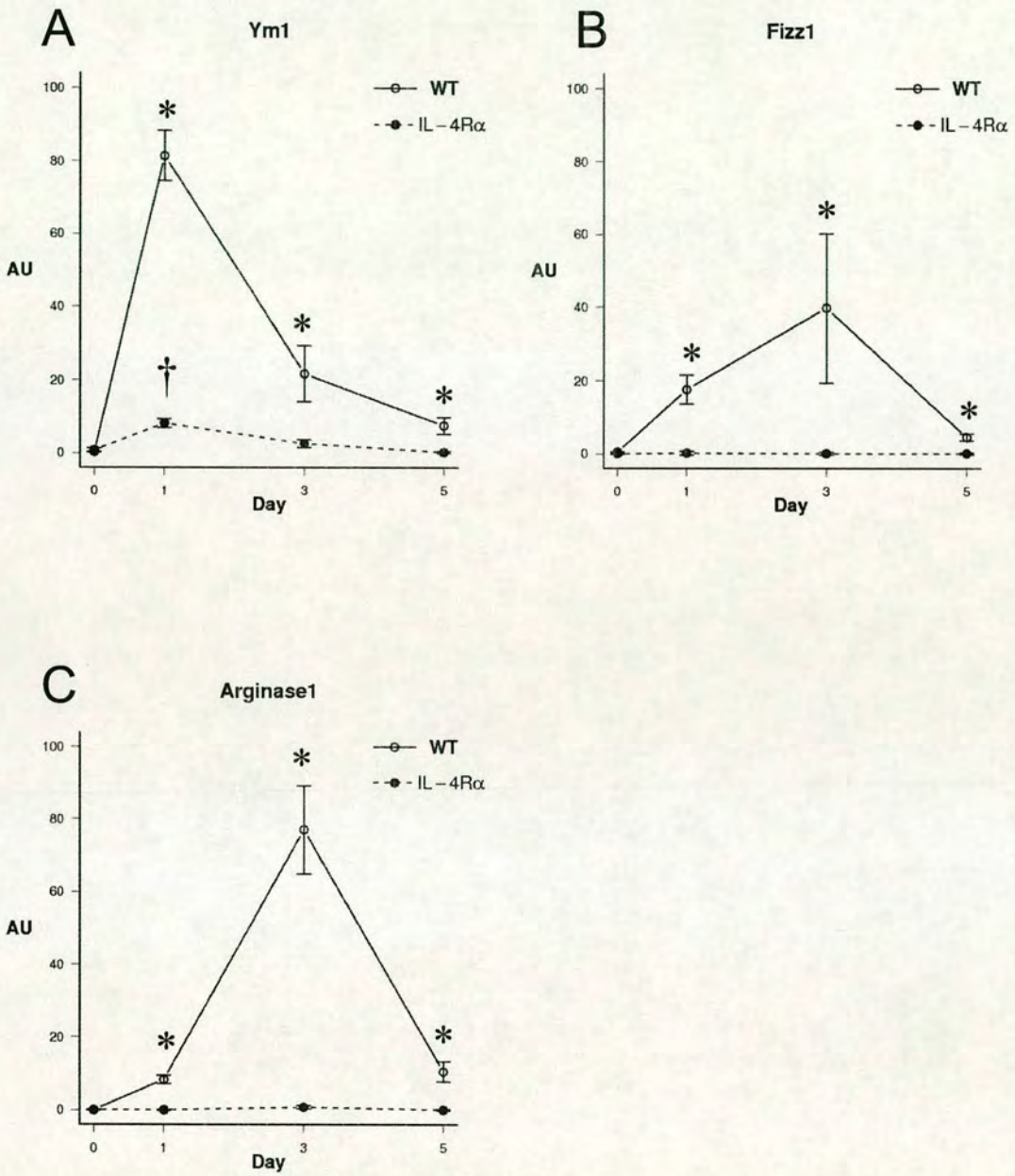

Figure 13: Real time RT-PCR reveals the mRNA expression pattern of Ym1 (A), Fizz1 (B) and Arginase1 (C) in the PECs of WT and IL-4 Rα deficient BALB/c mice after surgery. N = 4 or 5 WT mice and 5 gene knockout mice per timepoint. $*$ = $p < 0.05$ compared to IL-4Rα deficient group. † = $p < 0.05$ compared to day 0.

As shown in figure 13A a lack of signaling through IL-4Rα results in near ablation of Ym1 mRNA expression after surgery. There is a small but significant rise ($p < 0.05$) in Ym1 expression after surgery in the IL-4Rα deficient mice at day 1 but this is some ten times lower than that seen in WT mice (Figure 13A). The expression of both Fizz1 and Arginase1 is completely ablated in the IL-4Rα deficient mice (Figures 13B and 13C). Rather surprisingly when we carried out the western blotting procedure to assess the secretion of Ym1 protein into the peritoneal cavity we found that there was a

signal in the IL-4Rα deficient mice comparable to that seen in the WT mice (Figure 14).

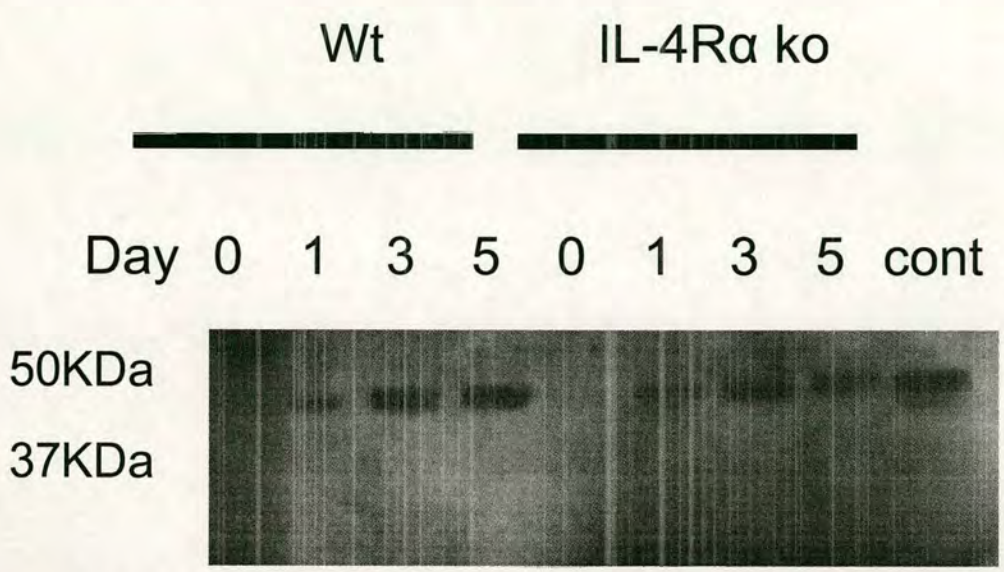

Figure 14: Western blotting reveals that IL-4Rα deficient mice secrete Ym1 into the peritoneal cavity despite a lack of mRNA expression by peritoneal exudate cells.

These data suggest that whilst innate IL-4 is required for the full expression of Arginase1 mRNA and to some extent for Fizz1 mRNA expression (Figure 11), IL-13 is more important in the regulation of the early expression of both these genes and Ym1. However the western blot data suggest that there is a source of preformed Ym1 and this protein is released after surgical trauma. We did not examine cells from around the wound which may have expressed Ym1 mRNA or protein in the absence of IL-4Rα signalling and these could be responsible for Ym1 protein release into the peritoneal cavity.

4.2.5. Fizz1 and Ym1 do not depend on NKT cells as the early source of type 2 cytokines

The early expression of Fizz1 and Ym1 in response to trauma suggests that this is an innate response rather than dependent on adaptive immunity. We have shown that this early response requires signalling through IL-4Rα and thus the activity of the Th2 cytokines IL-4 and IL-13 . Amongst the candidate cells for early production of IL-4 and IL-13 are the natural killer T (NKT) cells. These cells, characterized by expression of a Vα14-Jα18 TCR rearrangement in the mouse, although incompletely understood, have wide ranging functions in the immune system (Kronenberg, 2005). One of the defining features of NKT cells is the ability to secrete large amounts of cytokines when stimulated

including IFN-γ, IL-4, IL-13, IL-12, TNF and GM-CSF. Given the early Fizz1 and Ym1 response in the context of surgical trauma we examined the response of these proteins in RAG1-deficient mice which lack NKT cells, γ/δ T cells and conventional lymphocytes.

RAG1 deficient mice displayed mRNA expression patterns of Ym1 (15A), Fizz1 (15B) and Arginase1 (15C) comparable to those seen in WT mice. Although Ym1 expression (15A) at days 1, 3 and 5 was reduced in the RAG1-deficient animals compared to the WT animals this did not reach statistical significance at any point. Fizz1 mRNA expression (15B) in the RAG1-deficient animals was identical to that seen in the WT animals until day 5 when there was a slight non-significant reduction. The expression of Arginase1 message was also not significantly different between the groups at any of the timepoints assessed. These data therefore suggest that the early source of IL-4 and IL-13 required for the full expression of Ym1, Fizz1 and Arginase1 after surgical trauma is not NK or γ/δ T cells and that this response is a form of innate immunity which does not require cells of the adaptive immune system.

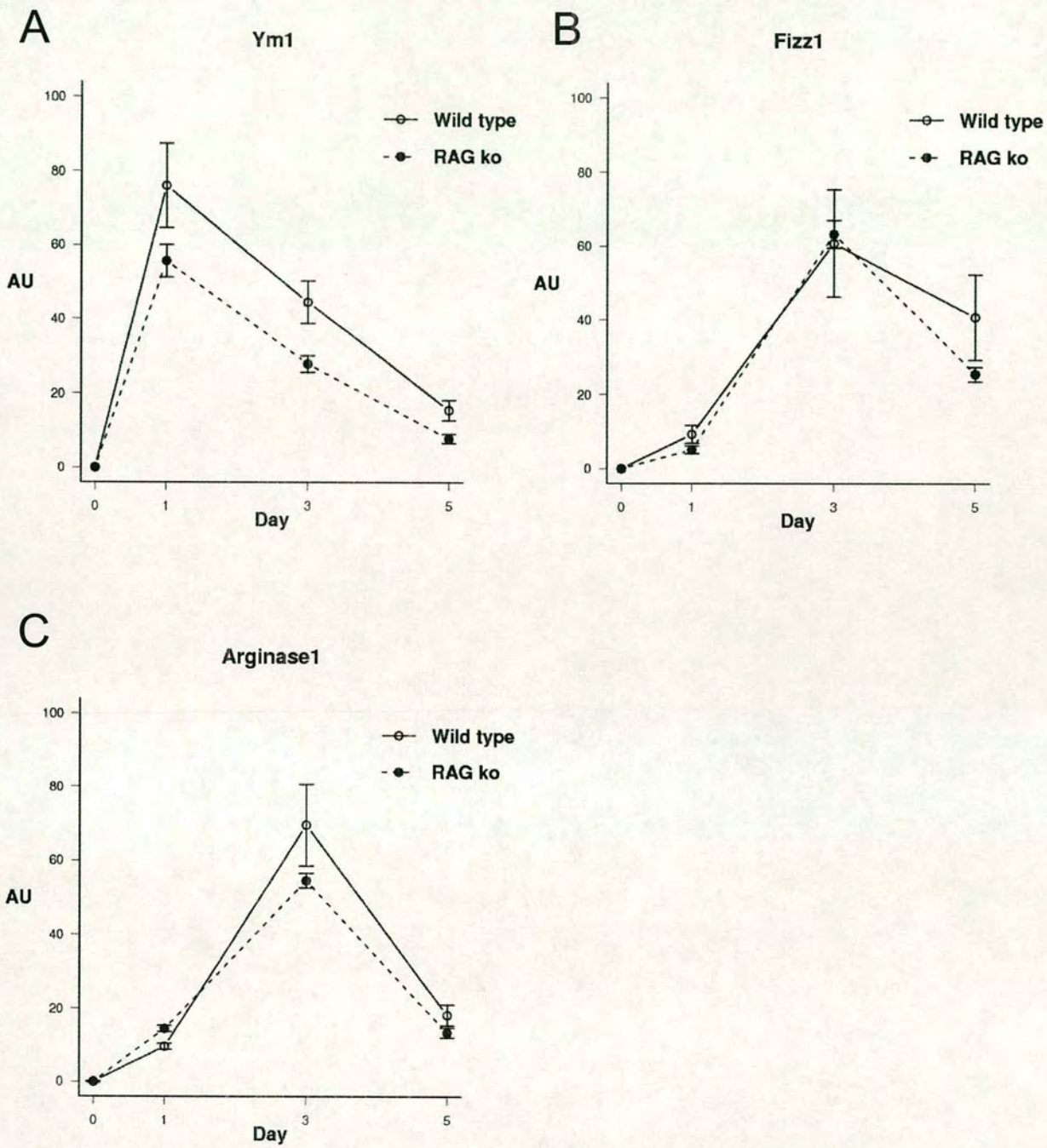

Figure 15: Real time RT-PCR reveals the mRNA expression pattern of Ym1 (A), Fizz1 (B) and Arginase1 (C) expression in the PECs of WT and RAG1 deficient C57BL/6 mice after surgery. 4 or 5 WT mice and 5 gene knockout mice per timepoint.

4.3. Discussion

We have used IL-4, IL-4Rα and RAG1 deficient mice in these experiments to examine whether Fizz1 and Ym1 rely on the Th2 cytokines IL-4 and IL-13 or on lymphocytes for their expression immediately following wounding. Our results suggest that IL-13 is the key cytokine governing these proteins in this innate setting. The use of RAG1 deficient mice allows us to exclude NKT cells and γ/δ T cells as early sources of IL-13 but the cells responsible for expression of this cytokine remain unknown. Mast cells, basophils and eosinophils are all capable of a rapid, innate production of IL-4 and IL-13 (Gessner *et al.*, 2005). These cell types could be responsible for the early IL-13 release and type 2 stimulation required for Fizz1 and Ym1.

4.3.1. The cellular response to an incisional wound

In our examination of the Fizz1 and Ym1 response to wounding we concentrated on the first 5 days after an incisional surgical wound. During this time the inflammatory phase of wound healing is initiated and mostly completed and the proliferative phase, by day 3, is well underway. The pattern of cell recruitment we have detailed in the peritoneal cavity reflects that documented by others in the wound itself (Park & Barbul, 2004).

The purpose of the early neutrophil infiltration into the wound site is mainly wound debridement and the phagocytosis of bacteria which may have entered the body through the damaged skin and / or epithelium. The data presented above show that there is a reduction in the number of neutrophils at day 3 in the IL-4 and IL-4Rα deficient mice. The timing of this reduction coincides with the arrival of macrophages in the peritoneal cavity and may represent an increased phagocytosis of neutrophils by macrophages in these mice, one of the main functions of macrophages at this stage in wound repair (Leibovich & Ross, 1975). IL-4, but not IL-13 has been shown to delay human neutrophil apoptosis (Girard *et al.*, 1996), (Girard *et al.*, 1997) and the lack of IL-4 or IL-4 responsiveness in the murine system could therefore lead to a greater rate of neutrophil apoptosis and a faster clearance of neutrophils in the IL-4 and IL-4Rα deficient mice. Alternatively because we assessed neutrophil numbers in the peritoneal cavity and not around the wound itself the possibility exists that there is a faster neutrophil migration to the wound surface in the knockout animals than in the WT animals.

As well as engaging in wound debridement neutrophils recruited into wounds upregulate genes involved in the recruitment of other immune cells and extracellular matrix (ECM) re-modelling (Theilgaard-Monch *et al.*, 2004). Thus, the early neutrophil infiltration serves not only to clean the wound area but also to prepare the site for the arrival of cells involved in the healing processes.

Although a greater rate of neutrophil clearance might therefore be expected to delay wound closure it has been found that neutrophils are not required for the proper healing of wounds (Simpson & Ross, 1972). Indeed it has been argued that the largely pro-inflammatory nature of the neutrophil anti-bacterial response may be responsible for delaying wound healing. In a study of wound healing in neutropenic mice it was found that epidermal closure was faster in these mice compared to neutrophil sufficient mice (Dovi *et al.*, 2003) indicating that the closure of the epidermal layer may be delayed by the early neutrophil response. This latter study was carried out on antibiotic maintained mice housed in clean conditions and the likelihood of immunocompromised animals surviving even a trivial wound in the wild without infection is small. Thus, the inflammatory response, while antagonising wound healing does ensure that the animal remains infection free.

In our study there was a marked increase in the number of eosinophils in the peritoneal cavity of WT mice on day 1 after wounding compared to both the IL-4 and IL-4Rα deficient animals (Figure 10). Reduced eosinophil recruitment in IL-4 deficient mice is consistent with data we have previously published in the *B. malayi* model system (MacDonald *et al.*, 2003). There are two main mechanisms for the recruitment of eosinophils to local tissues. The chemokine eotaxin serves to mobilise the blood pool of eosinophils and promote local recruitment and the cytokine IL-5 promotes the generation of eosinophils in the bone marrow and thus maintains or increases the circulating pool of eosinophils (Collins *et al.*, 1995), (Mould *et al.*, 1997). Both these responses can be modulated by a lack of IL-4 or IL-4 responsiveness. Eotaxin, although constitutively expressed in the skin of mice (Rothenberg *et al.*, 1995), is strongly induced in dermal fibroblasts by IL-4 and TNF (Mochizuki *et al.*, 1998). In the IL-4 and IL-4Rα deficient mice the IL-4 component of this response would be missing. Likewise the IL-4 driven IL-5 response (Kopf *et al.*, 1993) would also be impaired. The reduced eosinophil recruitment seen in the IL-4 and IL-4Rα deficient mice may therefore reflect a failure to properly upregulate eotaxin in injured skin and / or IL-5 production. The consistency in eosinophil numbers between the RAG1 deficient and WT mice suggests that lymphocytes play little role in the early post injury eosinophil recruitment.

A role for eosinophils in wound healing is proposed on account of their recruitment into the wound and surrounding tissues and their sequential secretion of transforming growth factor (TGF) family members; first TGF-α then TGF-β1 (Wong *et al.*, 1993). TGF family members are pleiotropic proteins with roles in many physiological processes including wound healing (Chin *et al.*, 2004). Eosinophils are not the only source of TGF family members at wound sites. Platelets release large quantities of TGF-β1 upon degranulation (Hosgood, 1993) and keratinocytes are also capable of synthesising and releasing this cytokine (Ansel *et al.*, 1990) so the relative paucity of eosinophils in the IL-4 and IL-4Rα

deficient mice might not be expected to affect the availability of this growth factor and have any impact on TGF-β dependent healing processes.

In summary, the pattern of cellular recruitment into the peritoneal cavity following an incisional abdominal wound is similar to that seen in wounds themselves and the surrounding tissue. The rapid loss of neutrophils in IL-4 and IL-4Rα deficient mice may reflect an increased rate of neutrophil apoptosis leading to a higher rate of phagocytosis by macrophages. The finding that eosinophil recruitment in RAG1 deficient and WT mice is identical implies that lymphocytes are not important in early, injury related eosinophil recruitment. However, the lower level of eosinophil recruitment in the IL-4 and IL-4Rα deficient mice suggests that IL-4 from innate sources regulates some aspects of the eosinophil response.

4.3.2. *Ym1 in wound healing*

The data presented above illustrates that Ym1 expression in the peritoneal cavity is not limited to parasitic infection. Furthermore, whilst expression is relatively unaffected by a lack of IL-4 a lack of IL-4Rα signalling greatly reduces Ym1 mRNA expression in cells recovered from peritoneal lavage fluid. This indicates that IL-13 plays an important role in Ym1 expression in this system. The source of IL-13 in this system is not lymphocytes as Ym1 expression in RAG1 deficient mice was equivalent to that seen in WT mice. Intriguingly despite a loss of Ym1 message in PECs from IL-4Rα deficient mice, Ym1 protein remains detectable in the peritoneal lavage fluid from these mice. This may represent preformed Ym1 from an as yet unidentified source. Efforts to identify cells from the PEC population responsible for Ym1 expression by immunostaining were unsuccessful.

Ym1 expression has previously been detected in the context of a stab wound model in the CNS (Hung *et al.*, 2002) where microglial cells were identified as Ym1 positive and expression was seen 3 days after the wound had occurred. In another model of CNS damage to the olfactory neurons Ym1 was also identified although in this case macrophages were not definitively identified as the cells secreting or expressing Ym1 (Giannetti *et al.*, 2004). In this latter model Ym1 expression was associated with an ongoing but futile regenerative response (Giannetti *et al.*, 2004) indicating that Ym1 is involved in the recovery of tissue after damage. However, the function of Ym1 in wound healing remains unclear. Ym1 has been reported to bind saccharides with a free amine group such as glucosamine (GlcN) and galactosamine (GalN) as well as heparin and heparan (Chang *et al.*, 2001), to have a β-N-acetylgluosaminidase activity at low pH and to be able to cleave single glycosaminoglycan (GAG) groups (Harbord *et al.*, 2002). Tissue damage can be exacerbated in inflammation by the NO mediated

oxidation of heparin and heparan chains in the ECM (Vilar *et al.*, 1997), thus one role of Ym1 in the early stages after tissue damage could be binding to heparin / heparan groups and thus limiting the damage caused to the ECM by the release of oxidative products from inflammatory cells. This would be an especially important function given the important role these carbohydrates have in processes such as cell migration and remodelling (Tumova *et al.*, 2000). The Ym1 driven modification of GAG groups in the ECM could also contribute to signalling processes that are important in normal healing.

In the *B. malayi* model system sustained Ym1 expression is dependent on the Th2 cytokine IL-4 and CD4 +ve T cells (Loke *et al.*, 2002), (P. Loke – Unpublished data). In the wound healing model we examined the cytokine dependence of Ym1 by using IL-4 and IL-4Rα deficient mice. Whilst a lack of IL-4 did not affect the expression of Ym1 message or protein a lack of IL-4Rα signaling led to a profound failure of Ym1 mRNA expression in PECs whilst Ym1 protein secretion seemed to be unaffected. Using electron microscopy and immunostaining Harbord et al demonstrated Ym1 in the azurophilic granules of neutrophils recruited into the peritoneal cavity 18 hours after thioglycollate injection (Harbord *et al.*, 2002). The presence of pre-formed Ym1 in neutrophils could explain why Ym1 protein is detectable in the peritoneal lavage fluid, especially in the IL-4Rα deficient animals where we see a profound loss of Ym1 mRNA. The presence of Ym1 protein in the absence of mRNA in IL-4Rα deficient mice also implies that the processes leading to pre-formed Ym1 in cells do not require Th2 cytokines whilst the de novo production of Ym1 by PECs in response to injury does.

4.3.3. *Fizz1 in wound healing*

Fizz1 expression in PECs after surgery was slightly reduced by a loss of IL-4 but the loss of IL-4Rα activity resulted in a complete ablation of Fizz1 expression. As detailed in Chapter 3 we could not detect Fizz1 protein in the peritoneal lavage fluid of WT or knockout mice before or after surgical trauma. This presumably reflects the lower levels of protein produced in response to injury as compared to *B. malayi* implant (Chapter 3, Figure 4). Much of the work on Fizz1 in response to tissue damage and remodeling has been done in the lungs; indeed Fizz1 was first identified in the lungs of allergic mice (Holcomb *et al.*, 2000). Fizz1 was found to inhibit the nerve growth factor (NGF) mediated survival of embryonic rat dorsal root ganglion neurons and the NGF mediated upregulation of Calcitonin Gene Related Peptide (CGRP) in adult dorsal root ganglion neurons (Holcomb *et al.*, 2000) indicating that Fizz1 was an antagonist of NGF. NGF is not only expressed in wounded tissue but actively aids in the process of healing (Matsuda *et al.*, 1998), (Werner & Grose, 2003), (Hasan *et al.*, 2000) and so the modulation of NGF functions by Fizz1 may be a key part of the early response after wounding in the skin.

Fizz1 can potentiate the differentiation of fibroblasts to myofibroblasts, essential for laying down the collagenous network required to heal wounds (Liu *et al.*, 2004b). This process is more important in the proliferative phase of wound healing rather than the inflammatory phase and this phase is beginning at day 3 when we see the peak in Fizz1 expression. Thus early Fizz1 expression may contribute to the induction of the myofibroblast phenotype and promote the progression to the proliferative phase of healing. Since we did not examine the levels of Fizz1 expression in and around the wound we cannot be sure that local expression levels were higher than that seen in the peritoneal cavity. Thus local responses could have a profound effect on the fibroblast phenotype. Fizz1 is a potent promoter of vaso-constriction (Teng *et al.*, 2003a) and this property would be useful after the inflammatory phase to limit the recruitment of proinflammatory cells from the circulation into the wound.

4.3.4. Arginase1 in wound healing

Of the three genes we examined, Arginase1 has the best defined role in wound healing and displayed the greatest dependence on type 2 cytokines with expression being half maximal in the absence of IL-4 and completely ablated in the absence of IL-4 Rα signalling. The dependence of Arginase1 expression on IL-4 and IL-13 has been well documented (Munder *et al.*, 1998), (Louis *et al.*, 1999), (Wei *et al.*, 2000), (Chang *et al.*, 2000), (El-Gayar *et al.*, 2003). Arginase1 expression in macrophages can also be driven by catecholamines and TGF-β and this expression is potently induced when macrophages are exposed to catecholamines, TGF-β and Th2 cytokines (Barksdale *et al.*, 2004) all of which are released in abundance after trauma to tissues. The expression of Arginase1 in wound sites is thought to be restricted to macrophages (Albina *et al.*, 1988) and wound derived fibroblasts (Witte *et al.*, 2002) and the rise in Arginase1 mRNA at day 3 after surgery is consistent with the arrival of macrophages in the peritoneal cavity (Figure 10) and with data detailing a rise in Arginase1 expression in the proliferative phase of wound healing (Albina *et al.*, 1990).

The importance of Arginase1 in the wound healing response is underlined by its pivotal role in the generation of polyamines, which are essential for cell proliferation (Wallace *et al.*, 2003) and proline, an essential component of collagen (Lodish *et al.*, 2000). Arginase1 converts L-arginine to ornithine and urea (Albina *et al.*, 1990). Ornithine can be converted to proline by the enzyme ornithine amino transferase (OAT) or to the polyamine putrescine by ornithine decarboxylase (ODC). The higher order polyamines, spermidine and spermine, are then synthesised from putrescine (Wallace *et al.*, 2003). A bias in arginine metabolism in favour of ornithine and urea leads to faster wound healing and growth (Seifter *et al.*, 1978) partly through an increase in collagen deposition and an increase in wound strength (Canturk *et al.*, 2001).

4.3.5. Conclusions

- The cellular response in the peritoneal cavity to a wound in the abdominal wall reflects the reported profile of cell recruitment into wounded tissue.
- The granulocyte response shows some degree of dependence on Th2 cytokines.
- Ym1 does not show any IL-4 dependence in its mRNA expression after surgical trauma whilst Fizz1 is partially IL-4 dependent and Arginase1 is profoundly IL-4 dependent .
- In the absence of IL-4 or IL-13 signalling there was a profound loss of Ym1, Fizz1 and Arginase1 mRNA expression after surgery suggesting a profound role for innate IL-13 in this setting.
- There was no requirement for lymphocytes in the expression of Ym1, Fizz1 or Arginase1 indicating that the expression of these genes after surgery is a wholly innate response
- There may a source of pre-formed Ym1 in the response to tissue damage as, despite a lack of Ym1 mRNA in IL-4Rα deficient mice, protein was detectable in the peritoneal lavage fluid.

Chapter 5 - Regulation of Fizz1 and Ym1 in *N. brasiliensis* Infection

5.1. Introduction

The Th2 response with upregulation of the cytokines IL-4, IL-13, IL-5 and IL-9 is a characteristic feature of nematode infection. These cytokines inform responses from the immune and other systems which can lead to removal or containment of the parasite (Urban *et al.*, 1991), (Else *et al.*, 1994), (Bancroft *et al.*, 1998), (Richard *et al.*, 2000). The susceptibility of BALB/c and the resistance of C57BL/6 mice to *Leishmania* (Alexander *et al.*, 1999) helped to define immune bias in these two strains. Specifically, BALB/c mice tend towards a Th2 response whereas C57BL/6 mice tend towards a Th1 response and the availability of these strains has facilitated the study of how the prototypical Th1 and Th2 cytokines influence the outcome of infection. Infection with the gastrointestinal nematode *N. brasiliensis* causes a profound Th2 immune response (Mahida, 2003). The initial site of infection is the skin where L3 larvae enter the host; they then travel via the vasculature to the lungs and enter the airways. In the lung a moult from L3 to L4 larvae takes place and the parasite is coughed up, swallowed and once in the gastrointestinal (GI) tract becomes sexually mature and begins to lay eggs which are shed in the faeces (Taliaferro & Sarles, 1939) thus restarting the lifecyle. Within 10-14 days *N. brasiliensis* has usually been expelled from the gut in resistant hosts.

The majority of the literature dealing with *N. brasiliensis* infection has focused on the gut where the parasite reaches maturity. However, as detailed above, the migration of *N. brasiliensis* is triphasic with the skin and lung as well as the gut being involved. *N. brasiliensis* migration through the host lung results in a profound Th2 response, (Watkins *et al.*, 1996), (Matsuda *et al.*, 1998), (Voehringer *et al.*, 2004) tissue damage (Ramaswamy *et al.*, 1991), (McNeil *et al.*, 2002) and the formation of granuloma (Arizono *et al.*, 1996) around worm debris. Thus, in the lung the organism has to mount not only immune responses to try and contain the infection but also an effective healing response to repair damage to delicate and vital tissues.

The gut stage of *N. brasiliensis* infection leads to a Th2 dependent response in the gastrointestinal tract (Urban *et al.*, 1998). This response also leads to pathology although damage seems to be caused by the immune system and in particular the extensive mastocytosis that accompanies *N. brasiliensis* infection at this site (Perdue *et al.*, 1989), (D'Inca *et al.*, 1992) rather than directly by the parasite. Although Th2 cytokines are absolutely required for the expulsion of *N. brasiliensis* from the gut the protoypic Th2

cytokine IL-4 is not (Lawrence *et al.*, 1996), (Barner *et al.*, 1998). Instead the functionally related cytokine IL-13 plays a key role in this process (Urban *et al.*, 1998).

The *B. malayi* implant is a model of human chronic infection with a filarial nematode and illustrated that Fizz1 and Ym1 are expressed in this context. The data from the wound healing experiments (Chapter 3) indicated that Fizz1 and Ym1 were also expressed in response to acute trauma. The brevity of *N. brasiliensis* infection in the murine host makes it a good model for acute helminth infection. We therefore used *N. brasiliensis* infection to investigate the expression patterns of Fizz1 and Ym1 in acute helminth infection and at two separate sites in the body (lung and gut). We have previously documented the IL-4 dependence of Fizz1 and Ym1 expression in the peritoneal cavity of IL-4 deficient C57BL/6 mice after *B. malayi* implant i.e. in the absence of IL-4 C57BL/6 mice do not upregulate Fizz1 or Ym1 after implant. In BALB/c mice however, a deficiency in IL-4 leads to reduced expression of these proteins but not a total loss (Nair, 2003). In the absence of IL-4 BALB/c mice demonstrate compensation by up regulating IL-13 in the spleen. This is sufficient to drive Ym1 and Fizz1 expression as these proteins are not seen in IL-4Rα deficient mice unable to respond to IL-4 or IL-13 (Nair, 2003). We therefore investigated the cytokine regulation of Fizz1 and Ym1 in the *N. brasiliensis* infection model and confirmed the cytokine response in C57BL/6 and BALB/c mice.

The involvement of the lung in *N. brasiliensis* infection led us to also examine the expression of the recently described functional mammalian chitinase acidic mammalian chitinase (AMCase) (Boot *et al.*, 2001) in addition to Ym1. Ym1 and AMCase are 67% similar at the level of amino acid sequence and AMCase has recently been implicated in the pathogenesis of asthma (Zhu *et al.*, 2004).

5.2. Results

5.2.1. Ym1, AMCase and Arginase1 Expression are Limited to the Lung in N. brasiliensis Infection in C57BL/6 Mice.

We have previously shown that the Fizz1 and Ym1 are highly upregulated following *B. malayi* implant, a model system that reflects chronic filarial infection in humans. In Chapter 4 we showed that the expression of Fizz1 and Ym1 was also an acute and innate response to injury. In order to investigate whether the expression of these genes is limited to the *B. malayi* model or to the peritoneal cavity, we chose to examine murine infection with the intestinal nematode *N. brasiliensis*. We first analysed the expression of Fizz1, Ym1, the mammalian chitinase AMCase and Arginase1 in the lung and small intestine of infected C57BL/6 mice because this is the strain we routinely use in the *B.*

malayi implant model. Real time RT-PCR (Figure 16) revealed that Ym1 (16A), AMCase (16B) and Arginase1 (16C) were all upregulated in the lung at day 6 post infection. At this point the parasite has cleared the lung and is present in the gut. However despite the increase in markers of alternative macrophage activation in the lung no significant expression was seen in gut tissue of C57BL/6 mice. We also examined the expression of Fizz1 and Ym1 in BALB/c mice and found Ym1 expression was upregulated in the gut of BALB/c mice after *N. brasiliensis* infection (see (Nair *et al.*, 2005)).

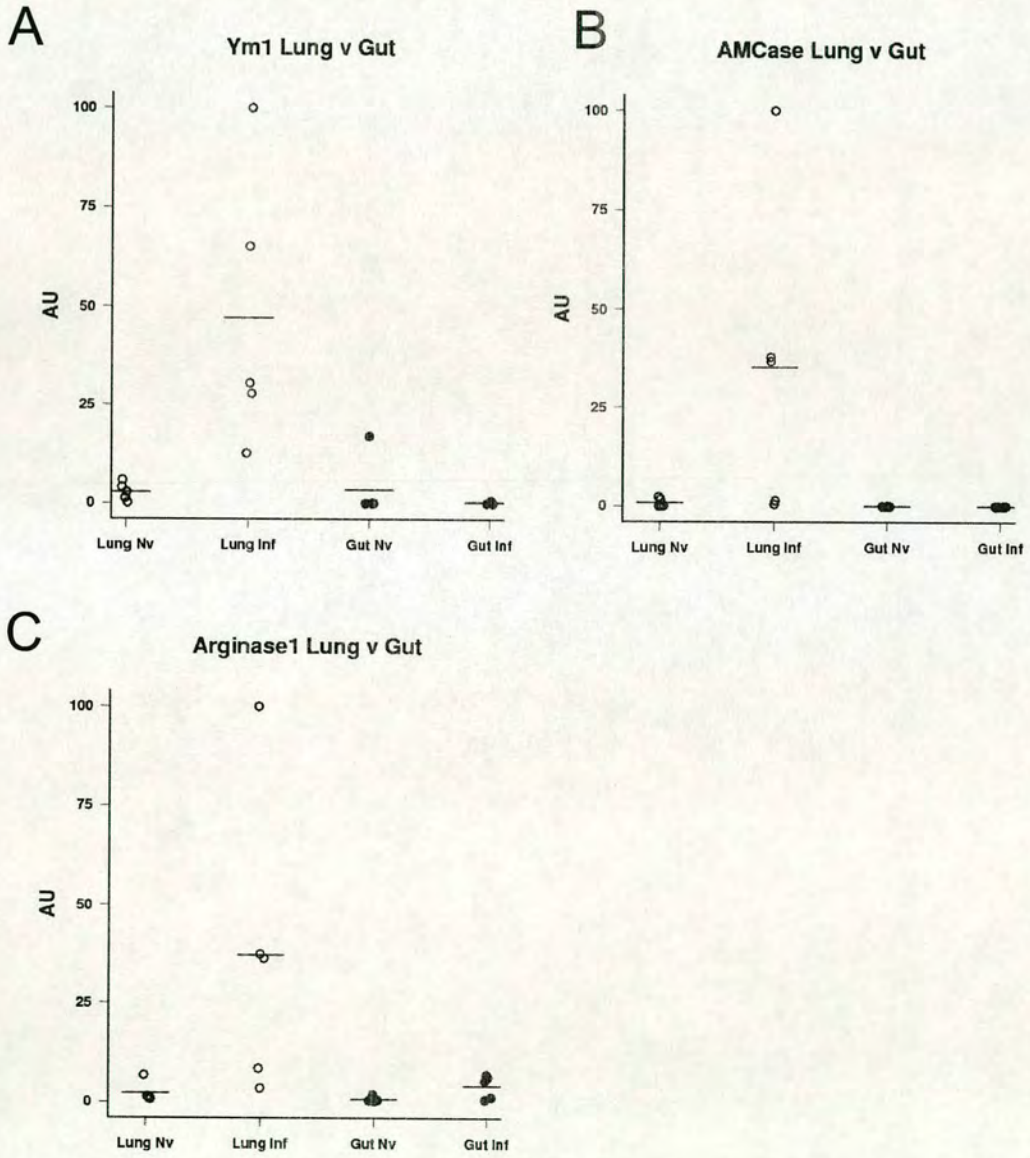

Figure 16: Tissue distribution of Ym1 (A), AMCase (B) and Arginase1 (C) expression after *N. brasiliensis* infection. C57BL/6 mice were infected subcutaneously with 400 L3 *N. brasiliensis* larvae. Real time RT-PCR was used to examine the levels of gene expression 6 days after infection. Each point represents an individual mouse and the bar indicates the mean.

5.2.2. Fizz1 and Fizz2 Have a Reciprocal Tissue Distribution

Fizz1 is one of a multigene family that includes Fizz2, Fizz3 and the hormone resistin (Holcomb *et al.*, 2000), (Gerstmayer *et al.*, 2003). Fizz2 has been identified in the gut after infection with *Trichinella muris*, *Trichuris spiralis* and *N. brasiliensis* (Artis *et al.*, 2004). These organisms are not just phylogenetically distinct but also occupy distinct compartments within the gut; *T. muris* burrows into the mucosa of the intestine, *T. spiralis* burrows into the mucosa of the caecum and appendix (Baron, 1996) and *N. brasiliensis* sits on the mucosa in the small intestine (Ogilvie, 1974). The finding that each of these organisms stimulates the production of Fizz2 in the gut indicates that this protein is an important component of the response against parasites in this tissue. The expression of Fizz1 alongside Fizz2 would suggest that these proteins play different roles in response to infection. To examine this issue we used real time RT-PCR to assess the expression pattern of Fizz1 and Fizz2 in response to *N. brasiliensis* infection in both the lung and gut. We found a reciprocal tissue distribution of Fizz1 and Fizz2 mRNA expression six days after *N. brasiliensis* infection. In the lung Fizz1 expression predominates (Figure 17A) whereas in the gut there is higher Fizz2 expression (Figure 17B). This is consistent with our data from BALB/c mice (Nair *et al.*, 2005) and that of others (Artis *et al.*, 2004) who demonstrated upregulation of Fizz2 specifically in the goblet cells of the small intestine.

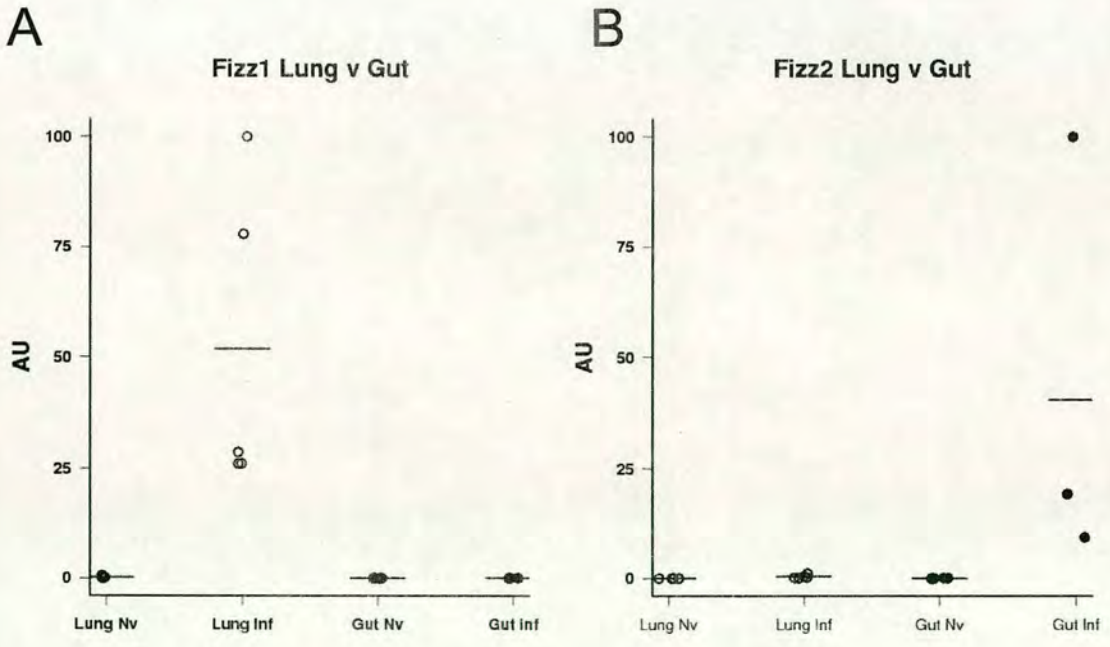

Figure 17: Real time RT-PCR reveals that Fizz1 (A) and Fizz2 (B) have a reciprocal tissue mRNA expression pattern with Fizz1 being elevated in the lung and Fizz2 elevated in the gut. Each point represents a single mouse and the bar indicates the mean.

5.2.3. IL-4 dependence of Fizz1, Ym1 and AMCase in the lung tissue of *N. brasiliensis* infected C57BL/6 mice

The migration of *N. brasiliensis* through the lung results in considerable trauma in this organ (Ramaswamy *et al.*, 1991), (Coyle *et al.*, 1998), (McNeil *et al.*, 2002). The wound healing data presented in Chapter 4 shows that early Fizz1 and Ym1 expression in response to trauma occurs in the absence of IL-4 but not IL-4Rα signalling and is an innate response. In order to examine the expression of these Th2 associated genes in an infection setting associated with trauma we investigated the levels of expression in the lungs and gut of infected WT and IL-4 deficient C57BL/6 mice. In the lung tissue Ym1 (Figure 18A), Fizz1 (Figure 18B) and AMCase (Figure 18B) all displayed IL-4 dependence with levels of expression after infection lower in IL-4 deficient mice. Fizz1 (Figure 18B) displayed the greatest dependence on IL-4 with expression ablated after infection in the knockout animals. This contrasts with the data presented in Chapter 4 where, after acute trauma, the expression of Ym1 and Fizz1 was relatively unaffected by a lack of IL-4 but did require IL-13. However the expression level in the *N. brasiliensis* model was examined 6 days after infection and at this timepoint the innate response to tissue damage could be waning and the adaptive immune response may be more important in driving the expression of these genes. Thus, the lack of IL-4, required for Th2 differentation, may have more impact on the expression of these genes at this timepoint. This would be consistent with the data presented in Chapter 3 showing a large increase in Fizz1 and Ym1 protein in the peritoneal cavity at day 7 in the *B. malayi* implant model. We were unable to detect the expression of Arginase1 mRNA in the lung tissue of infected mice and, consistent with the data from WT C57BL/6 mice (Figures 16 & 17), we were unable to detect Fizz1 and Ym1 mRNA expression in the gut of IL-4 deficient C57BL/6 mice (data not shown).

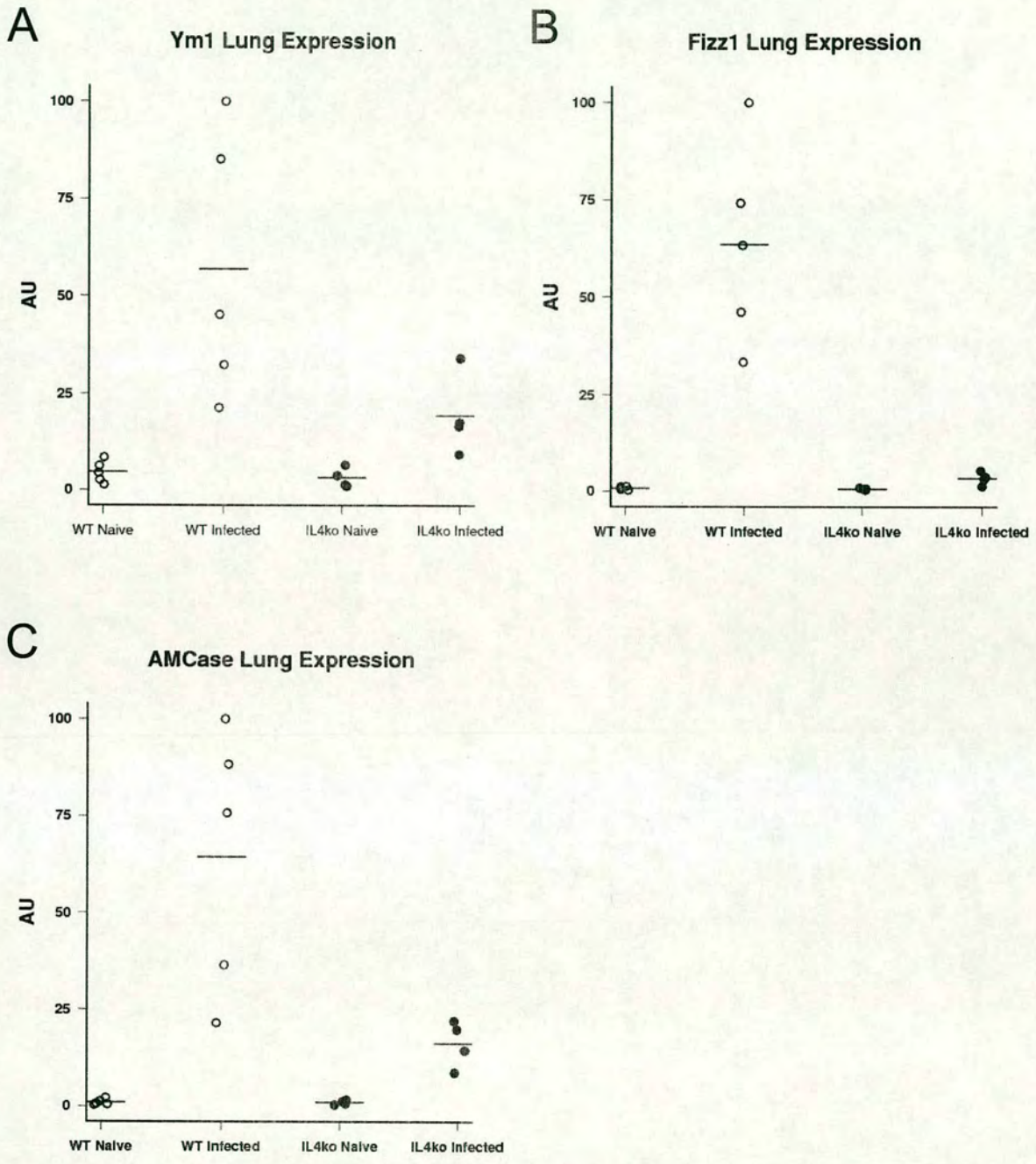

Figure 18: Real time RT-PCR shows that the expression of Ym1 (A) and AMCase (C) mRNA in whole lung tissue is partly dependent on IL-4 whereas Fizz1 mRNA expression (B) is wholly IL-4 dependent. Each point represents a single mouse and the bar indicates the mean.

5.2.4. *Fizz1 and Ym1 mRNA expression is partly IL-4 dependent and Arginase1 mRNA expression is fully IL-4 dependent in Airway Cells*

The data shown in Figure 18 was derived from homogenised whole lung tissue. We also examined the mRNA expression of Ym1 (Figure 19A), Fizz1 (Figure 19B) and Arginase1 (Figure 19C) in cells recovered from the bronchoalveloar lavage (BAL) fluid. The expression patterns we saw were largely similar to those seen in the lung tissue with clear IL-4 dependence in the expression of Ym1 and Fizz1. However while we had failed to see Arginase1 upregulated in the lung tissue it was evident that this gene was strongly upregulated in BAL cells and displayed a profound IL-4 dependence (Figure 19C). In contrast whilst we had seen robust expression of AMCase in whole lung tissue we were unable to identify expression of this gene in the cells from the BAL fluid. Fizz1 expression was much less IL-4 dependent in the BAL cells than in whole lung tissue (Figure 19B). One possible explanation for the differences in expression between lung tissue and BAL cells could be that different cells types activate different pathways in response to Th2 cytokines. Lung epithelium, clara cells, fibroblasts and myofibroblasts can respond to IL-4 and IL-13 (Kuperman *et al.*, 2005), (Whittaker *et al.*, 2002), (Ingram *et al.*, 2004), (Saito *et al.*, 2003) but little is known about the molecular events post signalling in these cell types. Thus the end result of macrophage stimulation with IL-4 / IL-13 and bronchial epithelium stimulation with the same cytokines is likely to be somewhat different.

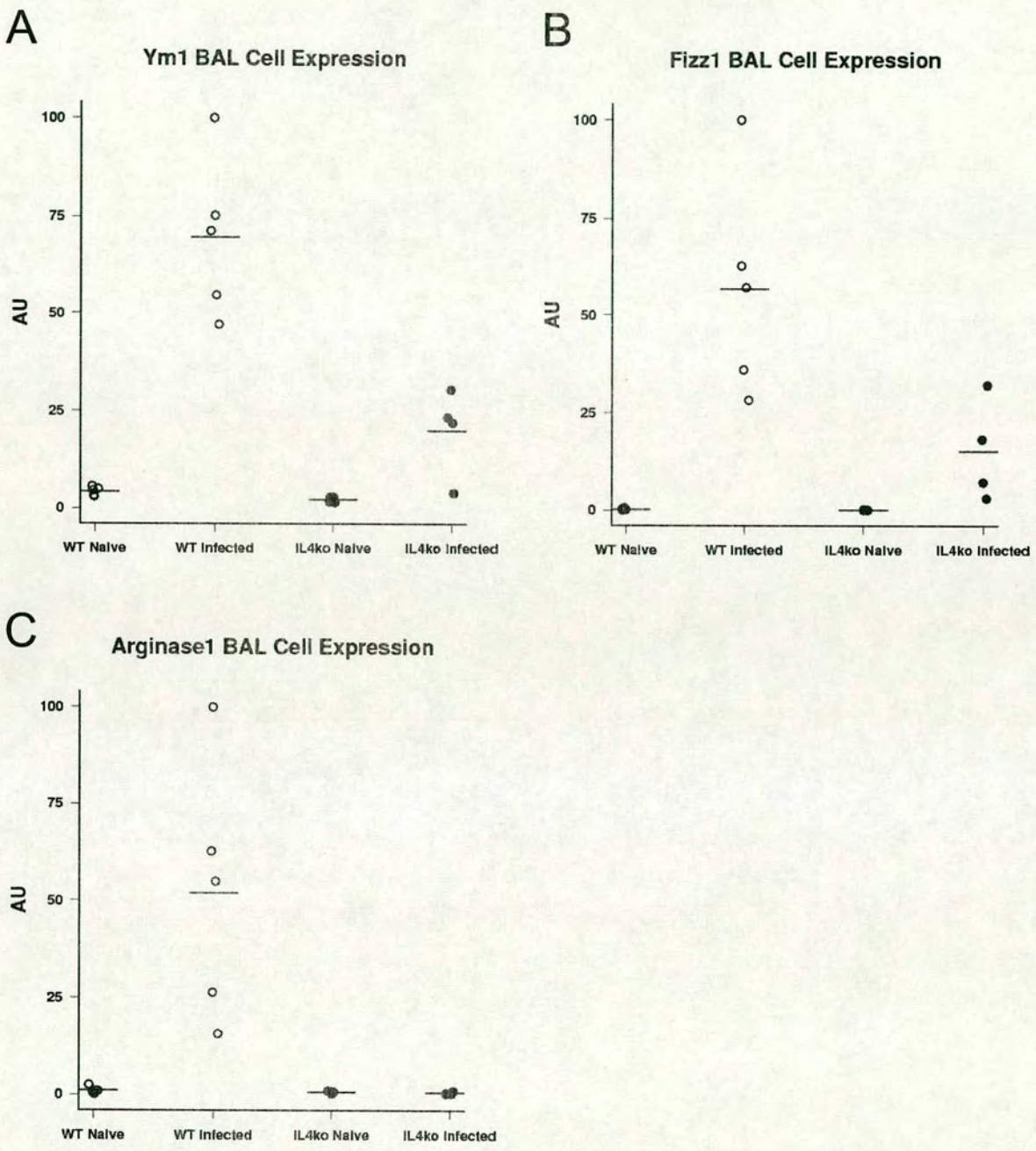

Figure 19: Real time RT-PCR reveals that the expression of Ym1 (A) and Fizz1 (B) mRNA in airway cells is partly dependent on IL-4 whilst Arginase1 mRNA expression (C) is wholly dependent on IL-4. Each point represents a single mouse and the bar indicates the mean.

5.2.5. Secretion of Fizz1 and Ym1 Proteins Reflects Expression Patterns

In order to investigate whether the lower level of message for Ym1 and Fizz1 in the IL-4 deficient mice was reflected in lower protein expression we carried out western blotting on BAL fluid from naïve and infected mice. Consistent with our data for mRNA expression in the lung and airway cells we found that the levels of both proteins were raised in the BAL fluid of infected animals (Figure 20). Ym1 protein was detectable in the airspaces of naïve WT C57BL/6 mice (20A) and protein levels rose after infection with *N. brasiliensis*. Ym1 protein was also detectable in the BAL of naïve IL-4 deficient C57BL/6 mice but this was at lower levels than seen in the naïve WT mice. As in the WT mice, Ym1 protein levels rose in the IL-4 deficient mice after infection but the signal for Ym1 protein remained lower than that seen in the naïve WT mice. These data indicate that although IL-4 is not essential it is required for full Ym1 expression in the lung environment. The western blot data for Fizz1 (20B) revealed a similar pattern of protein expression to that seen for Ym1 except that there was no detectable Fizz1 protein in the BAL fluid of naïve animals. Although there was a rise in Fizz1 protein expression in both WT and IL-4 deficient C57BL/6 mice after infection the rise was lower in the IL-4 deficient animals than that seen in the WT mice.

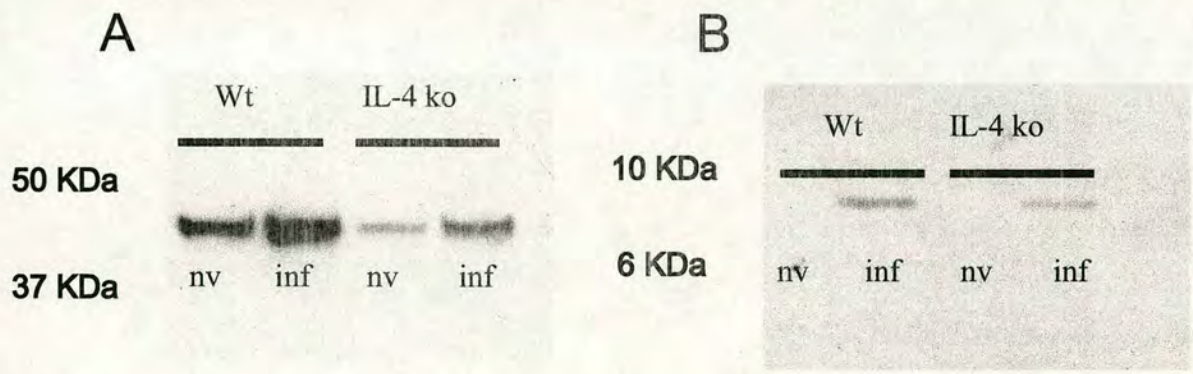

Figure 20: Western blotting of BAL fluid from naive and infected animals confirms that both Ym1 (20A) and Fizz1 (20B) are secreted and that their secretion pattern reflects the mRNA expression patterns with lower levels of protein detectable in IL-4 deficient animals after infection compared to WT animals.

5.2.6. *Immunohistochemistry reveals the pattern of Fizz1 and Ym1 staining in the Lung*

Ym1 protein has previously been localised to the bronchial epithelium as well as alveolar macrophages in both normal and pathological lungs (Ward *et al.*, 2001). In their original characterisation of Fizz1 Holcomb et al identified expression in bronchial epithelium and type II pneumocytes in the lungs of allergic mice (Holcomb *et al.*, 2000). They did not find any Fizz1 reactivity in alveolar macrophages. However we found a rise in Fizz1 mRNA expression in the airway cells in response to *N. brasiliensis* infection. Macrophages are the predominant cells in the airways of both naïve and infected animals (unpublished data) and we were intrigued to find Fizz1 expression in the airway cells of these animals, presumably from macrophages. We therefore undertook an immunohistochemical examination of the lungs of infected mice to localise the expression of both Ym1 and Fizz1.

Using immunohistochemistry we identified positive staining for Ym1 in the lungs of both naïve WT and IL-4 deficient mice infected with *N. brasiliensis* (Figure 21A & Figure 21C). Quantitatively this data is in agreement with the western blot data presented in figure 20. In naïve WT mice there was positive staining indicating Ym1 expression in the lungs of uninfected animals. The positive staining in the lungs of both naïve and infected IL-4 deficient mice was less intense and less widespread than that seen in WT mice. Indeed the staining in infected IL-4 deficient mice (21D) was comparable to that seen in naive WT mice (21A). The strongest staining in naive WT mice was generally seen in the alveolar macrophages (21A, bottom arrow). In addition we identified positive staining in platelets within blood vessels in the lung in both naïve (21A, top arrow) and infected (21B, bottom arrow) WT mice. Intravascular clotting was seen in both WT and IL-4 deficient mice and was not related to infection status. It is likely that this clotting was a post mortem event. The identification of positive Ym1 staining in platelets has not previously been described. Platelets either carry preformed Ym1 or bind Ym1 from another source since they are unable to synthesize proteins de novo. The presence of Ym1 in or on platelets potentially points to a role for this protein in clotting or early tissue repair.

In the infected WT mice there was intense Ym1 staining in bronchial epithelial cells (21B, top arrow), alveolar macrophages, extravasating macrophages (21B, middle arrow) and throughout the inflammatory infiltrate. In addition we identified large areas of positively staining proteinaceous material in the airways of some of the WT mice (21B). There were large numbers of cells, possibly macrophages infiltrating this material. Characteristically the formation of granuloma in the lungs of rodents infected with *N. brasiliensis* has involved mutlinucleated giant cells and mononuclear cells infiltrating the granuloma material (Arizono *et al.*, 1996). Thus the strongly Ym1 positive material may

represent the granuloma surrounding either a trapped parasite or moult material from the L3 to L4 moult. In the infected IL-4 deficient mice there was a similar Ym1 staining pattern to that seen in infected WT mice though less intense. Positive staining of the bronchiolar epithelium (21D, top arrow) was less intense than that seen in the WT mice as was the staining in the alveolar macrophages (21C, bottom arrow).

The pattern of Fizz1 reactivity in the lungs of naïve and infected mice was quite different (Figure 22). In agreement with the western blot data presented in figure 20 there was no Fizz1 in detectable in the lungs of either naïve WT (22A) or IL-4 deficient (22B) mice. In the infected WT mice staining was seen throughout the epithelial border of many of the airways (22B). Positive staining was notably absent from eosinophils (22B, top arrow) and macrophages (22B, bottom arrow). In infected IL-4 deficient animals there was patchy staining of the bronchiolar epithelium. This was both less widespread and less intense than the staining seen in infected WT mice. A similar pattern of Fizz1 reactivity was seen in compensatory lung growth after pneumonectomy although in that study Fizz1 was also detected in the endothelial cells of vessels and type II pneumocytes (Li *et al.*, 2005). In the developing lung Fizz1 expression has been detected in bronchial epithelial cells and type II pneumocytes (Wagner *et al.*, 2004). Thus in the context of lung growth Fizz1 may have a more widespread expression than that seen in *N. brasiliensis* infection.

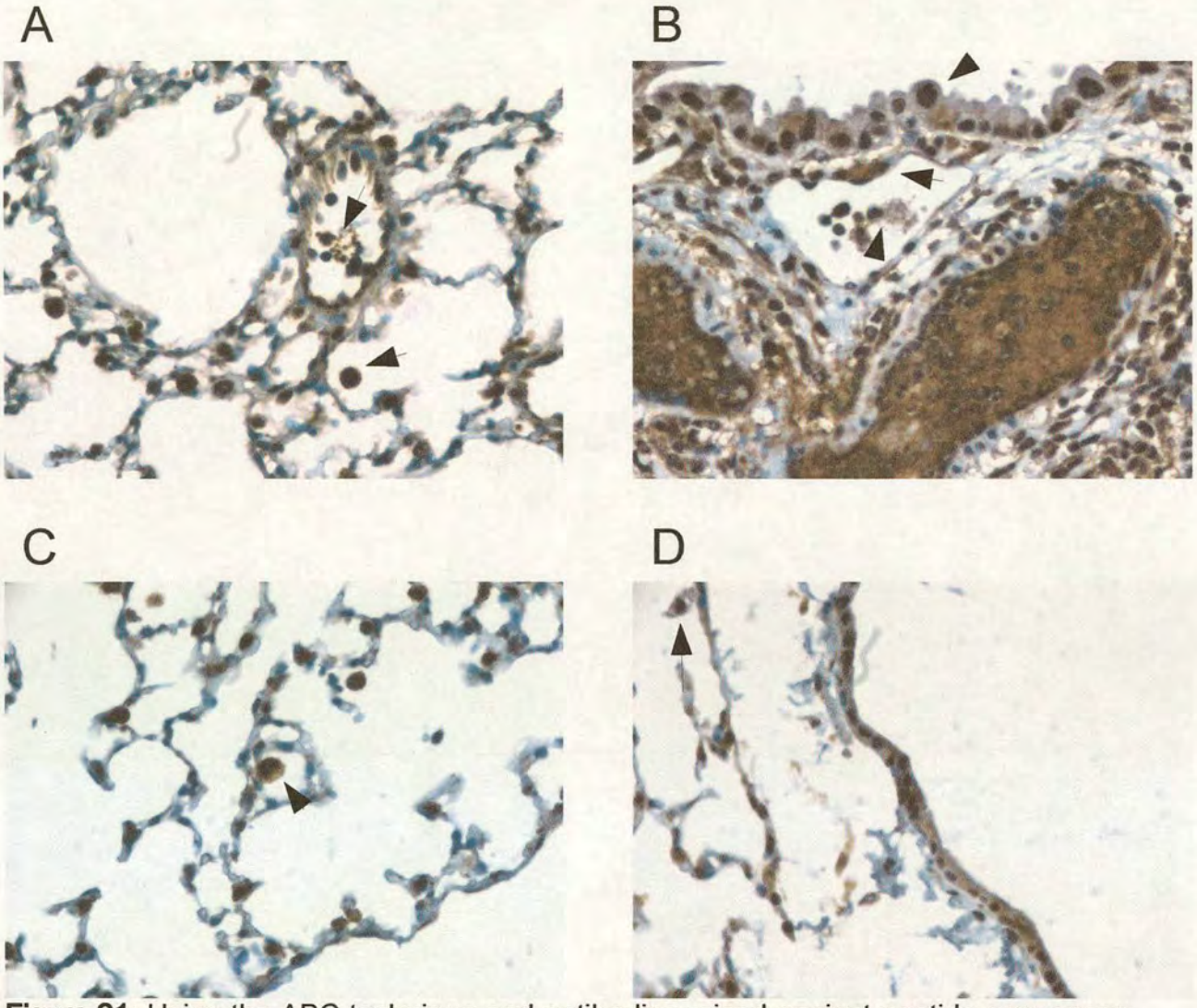

Figure 21: Using the ABC technique and antibodies raised against peptide sequences from Ym1 we investigated the distribution of this protein in the lung after *N. brasiliensis* infection in WT C57BL/6 (21A, 21B) and IL-4 deficient mice (21C, 21D). Ym1 was identified by brown DAB staining and lungs were counterstained with haematoxylin. In naive WT mice (21A) Ym1 protein is present in alveolar macrophages, platelets (arrowed) and epithelial tissue. In *N. brasiliensis* infected WT mice (21B) the expression pattern is similar but much stronger and there are areas of high immunoreactivity around extravasating and migrating macrophages (arrowed) and in the larger airways. Naive IL-4 deficient mice (21C) express lower levels of Ym1 than WT mice and expression is more concentrated in alveolar macrophages (arrowed) than in epithelium. There is an upregulation of expression in infected IL-4 deficient mice (21D) with marked expression in bronchial epithelium and alveolar macrophages (arrowed). Magnification x400. Day 6 post infection.

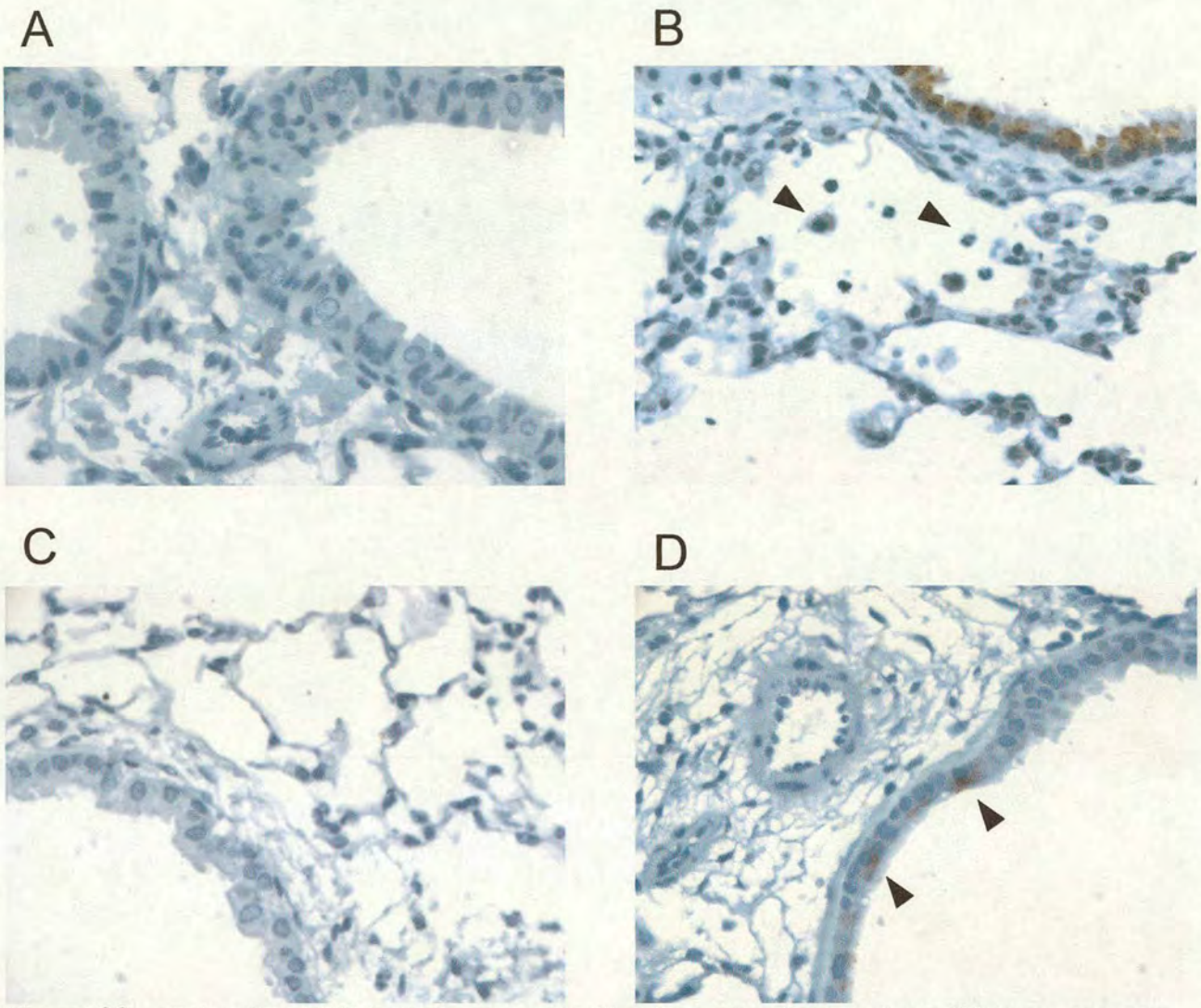

Figure 22: . Using the ABC technique and antibodies raised against peptide sequences from Fizz1 we investigated the distribution of this protein in the lung after *N. brasiliensis* infection in WT C57BL/6 (22A, 22B) and IL-4 deficient mice (22C, 22D). Fizz1 was identified by brown DAB staining and lungs were counterstained with haematoxylin. There is no Fizz1 staining in either WT (22A) or IL-4 deficient (22C) naive mice. In infected WT mice Fizz1 reactivity is strong in the bronchiolar epithelium but is notably absent from both alveolar macrophages and eosinophils (arrowed). The staining pattern in infected IL-4 deficient animals (22D) is similar to that seen in infected WT animals but is less intense and less confluent. Magnification x400. Day 6 post infection.

5.2.7. Local IL-13 expression is important for the Th2 response in IL-4 deficient C57BL/6 mice

The data presented in figures 19, 20, 21 and 22 suggests that IL-4 deficient C57BL/6 mice can upregulate Fizz1 and Ym1 in the lung after infection with *N. brasiliensis* whilst our data from the *B.*

malayi implant model had suggested the that these mice failed to upregulate Fizz1 and Ym1 after implant. In addition we saw Ym1 expression in the intestine of BALB/c mice (Nair *et al.*, 2005) but not C57BL/6 mice (Figure 16 and data not shown). These findings led us to examine the cytokine response in the spleen and draining lymph nodes of C57BL/6 and BALB/c mice.

Splenocytes from BALB/c and C57BL/6 mice were restimulated with media alone or *N. brasiliensis* excretory / secretory products (NES). After infection both WT C57BL/6 and BALB/c mice were able to mount a Th2 response in the spleen as demonstrated by the upregulation of IL-4 (Figure 23A & C) upon antigen stimulation. There was also an IL-4 response in naïve animals in this experiment. NES has been shown to promote a potent type 2 cytokine response in naïve mice (Holland *et al.*, 2000) which is dependent on MHC class II (Holland *et al.*, 2005). Thus the IL-4 production we see in naïve animals reflects the potency of NES as a type 2 cytokine adjuvant. The specificity of the response is however demonstrated by the difference in IL-13 production seen in IL-4 deficient C57BL/6 and IL-4 deficient BALB/c mice. In the spleens of IL-4 deficient C57BL/6 mice there is no production of IL-13 in response to NES co-culture despite a robust response in the WT mice (Figure 23D). In contrast there is a strong compensatory IL-13 response in IL-4 deficient BALB/c mice (Figure 23B). These data indicate that IL-4 deficient C57BL/6 mice do not compensate for the lack of IL-4 with a systemic IL-13 response when a type 2 immune response is required. In contrast IL-4 deficient BALB/c mice are able to upregulate splenic IL-13 production and do so robustly. This is in broad agreement with our previous work in the *B. malayi* model showing that IL-4 deficient C57BL/6 mice failed to upregulate IL-13 in the draining lymph nodes or the spleen and did not express Fizz1 and Ym1 in peritoneal exudate cells (PEC) but IL-4 deficient BALB/c mice upregulated splenic IL-13 leading to Fizz1 and Ym1 expression in PECs (Nair, 2003).

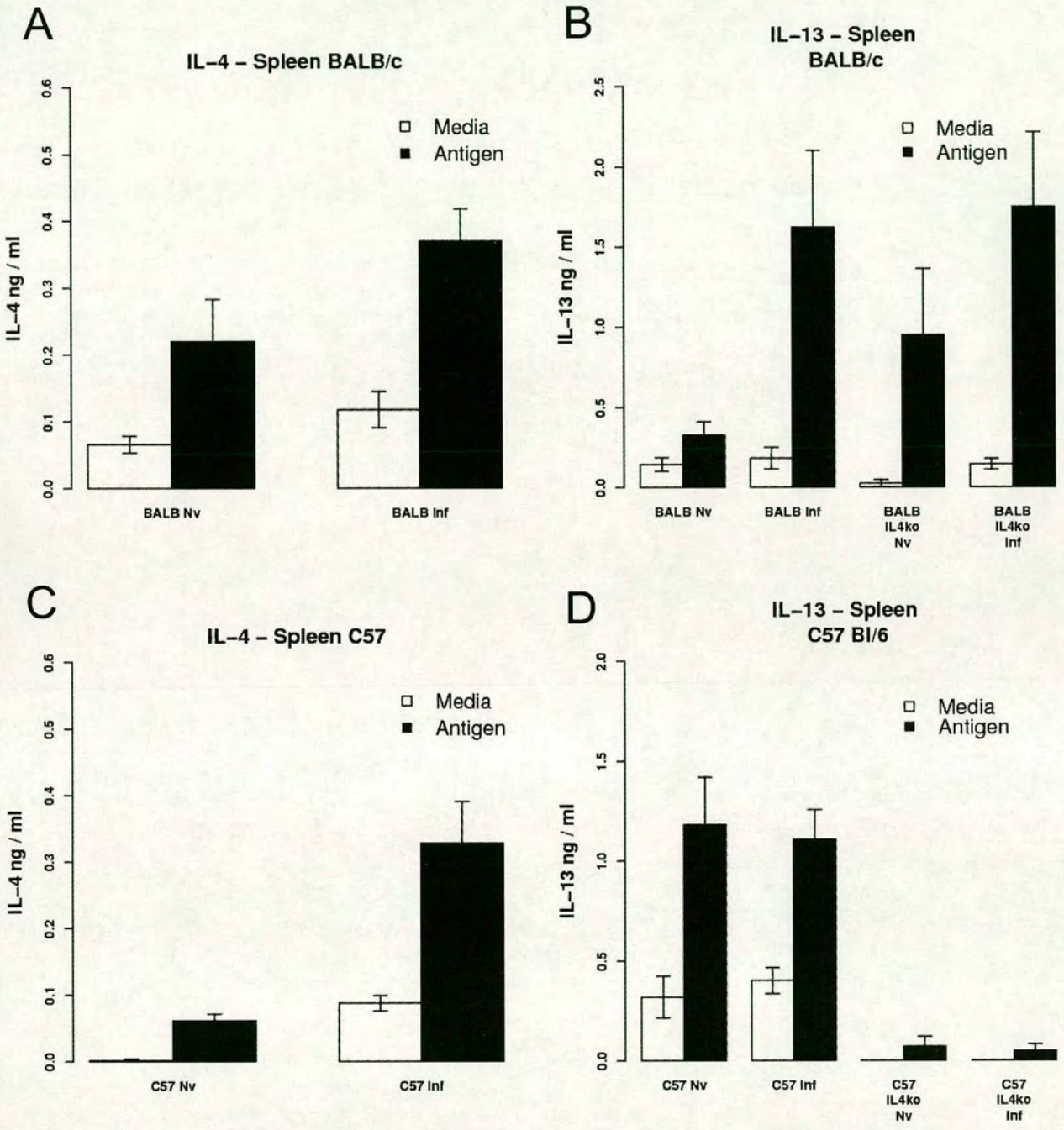

Figure 23: The Th2 response in the spleen of BALB/c (A & B) and C57BL/6 (C & D) mice infected with *N. brasiliensis*. Splenocytes were homogenised and restimulated with NES antigen and ELISA was used to examine the levels of IL-4 (A & C) and IL-13 (B & D) in the culture supernatant.

Although in the spleen and peritoneal draining lymph nodes C57BL/6 IL-4 deficient mice fail to make IL-13, the situation in the lymph nodes of the gut was strikingly different. Here, the response in the mesenteric lymph nodes of WT and IL-4 deficient C57BL/6 mice (Figure 24) illustrated that in the absence of IL-4 C57BL/6 mice can compensate with local IL-13. Cells from infected WT mice of both strains produced significantly more IL-4 than naïve mice (24A & C) although the potent Th2 adjuvant effect of NES also resulted in IL-4 production from the cells of naïve mice (24A & C). Cells from infected WT and IL-4 deficient BALB/c mice also produced significantly more IL-13 than naïve mice although there was again a background level of IL-13 production (24B). The profile of IL-13 production from the mesenteric lymph node cells of C57BL/6 mice was different (24D). Mesenteric lymph node cells from infected WT mice produced no IL-13 whilst cells from IL-4 deficient C57BL/6 mice produced abundant IL-13 (Figure 24D). These data may directly explain the presence of Ym1 in the intestine of BALB/c (Nair *et al.*, 2005) mice but not C57BL/6 mice. In BALB/c mice IL-13 alone is sufficient for the expression of Ym1 whilst in C57BL/6 mice both IL-4 and IL-13 are required but paradoxically in the presence of IL-4 we do not see IL-13 expression.

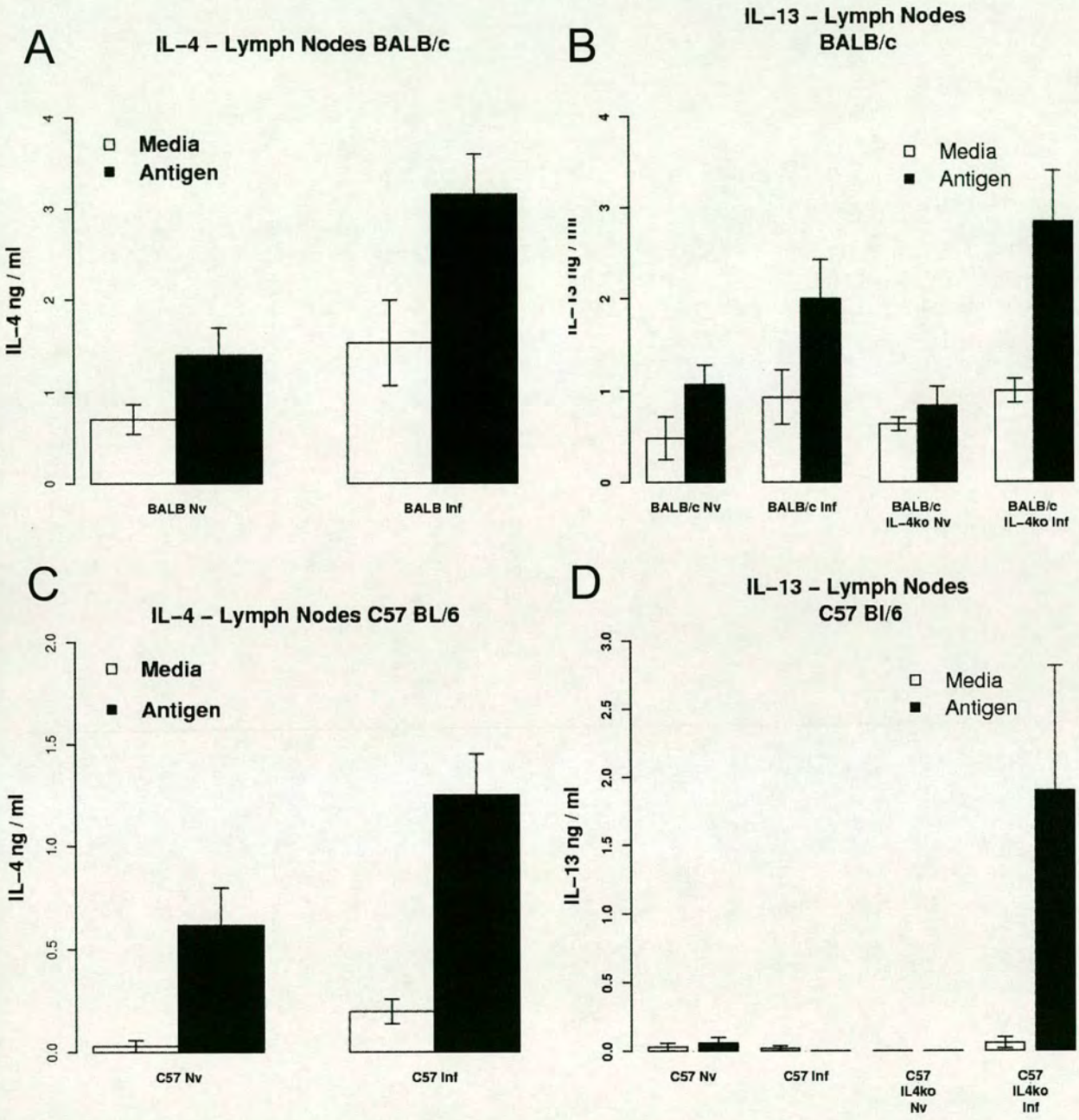

Figure 24: The Th2 cytokine response in the lymph nodes of BALB/c (A & B) and C57BL/6 (C & D) mice infected with *N. brasiliensis*. Mesenteric lymph nodes were homogenised and the cells restimulated with media alone or NES antigen. ELISA was used to examine the levels of IL-4 (A & C) and IL-13 (B & D) in the culture supernatant. N= 5 mice per group.

5.3. Discussion

We and others have previously reported the upregulation of Fizz, Ym1, Arginase1 and AMCase in response to parasite infection (Loke *et al.*, 2002), (Nair *et al.*, 2005), (Raes *et al.*, 2002), (Donnelly *et al.*, 2005) suggesting an important role for these proteins in this context. This chapter shows that Fizz1, Ym1, Arginase1 and AMCase are upregulated in an acute nematode infection and that there is differential expression of the closely related Fizz1 and Fizz2 proteins in the lung and gut. Furthermore we have visualised the expression of Fizz1 and Ym1 in the lungs of infected mice providing useful information on both the pattern of expression and the potential functions of these proteins, demonstrating for the first time the presence of Ym1 on platelets. Finally we have also shown that despite a strict dependence on IL-4 in C57BL/6 mice for the expression of these proteins after *B. malayi* implantation, IL-4 deficient C57BL/6 mice infected with *N. brasiliensis* are able to upregulate Fizz1 and Ym1 and compensate for a lack of IL-4 with localised IL-13 production.

5.3.1. The cellular response to N. brasiliensis in the lung

The migration of *N. brasiliensis* through the lung in a primary infection is a quiet process, immunologically speaking. Despite the damage the parasite does there is little in the way of inflammatory reaction whilst the parasite is present in the lungs during a primary infection (Ramaswamy *et al.*, 1991), (Daly *et al.*, 1999), (McNeil *et al.*, 2002). The number of immune cells in the alveoli does not begin to increase until 60 to 72 hours post infection, after most of the larvae have left the lung (Daly *et al.*, 1999), (McNeil *et al.*, 2002). The lack of inflammatory response may be partly accounted for by the finding that *N. brasiliensis* excretory / secretory (NES) products inhibit the migration of neutrophils into the lung in response to LPS (Keir *et al.*, 2004). It has previously been shown that LPS instillation into the lungs causes a rise in both PMN number and macrophage number in the BAL fluid (Lopez & Yong, 1986). In the study by Keir et al macrophage numbers were unchanged indicating that the population of alveolar macrophages remained constant and there was no migration of monocytes / macrophages in response to LPS (Keir *et al.*, 2004). However this study only addressed the cellular population in the BAL 6 hours after LPS instillation and this may be too soon to see an appreciable macrophage response. Thus, NES could postpone both neutrophil and macrophage migration into the lung whilst *N. brasiliensis* completes the lung stage of its migration.

Alveloar macrophages might be expected to play an effector role against the lung stage of *N. brasiliensis* and macrophages certainly play a role in the attrition of the migratory stage of *N. brasiliensis* as demonstrated by the finding that i.v. injection of carbon particles, which adversely

affects macrophage function (Stuart *et al.*, 1978), leads to higher worm load (Wibawa *et al.*, 2002). Whether this attrition occurs during the skin or lung stages is, however, unclear. In the lungs of *N. brasiliensis* infected rats macrophage numbers do not increase until at least 8 days after infection and were actually decreased at 72 hours (McNeil *et al.*, 2002). Eosinophil, basophil and lymphocyte responses in the lung also peak after the parasite has left the lung. All three cell types express IL-4 and so promote a Th2 response (Dent *et al.*, 1999), (Min *et al.*, 2004), (Voehringer *et al.*, 2004) and thus may contribute to the increased expression of Ym1, Fizz1, Arginase1 and AMCase we have found.

5.3.2. *Fizz1, Ym1, Arginase1 and AMCase play repair roles in the lung after N. brasiliensis infection*

We examined both the BAL cells and lung tissue of *N. brasiliensis* infected mice 6 days after infection by which time the pulmonary stage of parasite migration is complete. At this time the lung is clear of parasites and the post injury response is underway (McNeil *et al.*, 2002). The Ym1, Fizz1, Arginase1 and AMCase we found in the lung tissue and BAL cells of the infected mice may therefore play a role in repair processes rather than having an anti parasite role. The expression of Ym1 throughout the inflammatory infiltrate in the lungs of infected mice and around extravasating macrophages (Figure 21B) indicates that this protein may have a role in the movement of immune cells from the blood stream into infected or damaged tissue.

The expression of Fizz1 in the bronchiolar epithelium is similar to that published by other groups using various models of lung injury or pathology (Holcomb *et al.*, 2000), (Li *et al.*, 2005), (Liu *et al.*, 2004b). In these studies expression was detected in normal, uninfected mice and is in contrast to our data indicating that there is no expression of Fizz1 in lung tissue of naïve mice. Using real time RT-PCR and western blotting we confirmed the presence of Fizz1 protein in BAL fluid and mRNA expression in cells retrieved from the BAL fluid. The presence of Fizz1 protein in the bronchiolar epithelium accounts for the western blot data as Fizz1 is a secreted protein but we could not detect positive staining for Fizz1 in any of the leucocytes present in the airspaces. This leaves open the question of which of the cells recovered from the BAL fluid are responsible for the BAL fluid derived cell mRNA expression data we observed.

The function of Fizz1 in the lungs of infected animals is unknown. As noted above Fizz1 has been implicated in the response to lung damage in a number of pathology models. It is anti-apoptotic in the developing lung (Wagner *et al.*, 2004) and also upregulated in compensatory lung growth after peumonectomy (Li *et al.*, 2005) demonstrating a role in lung growth in the adult as well as in the

embryo. However Fizz1 has also been implicated in fibrosis as it can stimulate the differentiation of fibroblasts into myofibroblasts (Liu *et al.*, 2004b). The finding that Fizz1 can inhibit the nerve growth factor mediated survival of embryonic neurons (Holcomb *et al.*, 2000) has also implicated this protein in the hyper-responsiveness that occurs in asthma. The expression of Fizz1 after *N. brasiliensis* infection may be required for the properly controlled growth of various lung tissues after insult.

The patterns of Arginase1 and AMCase expression have not previously been reported in the context of *N. brasiliensis* infection and here we show that the expression of both these genes is limited to the lung. AMCase expression was further only detected in lung tissue. This functional chitinase has a role in the pathology of lung disease and has been specifically implicated in the pathophysiology of asthma where its effects were mediated by IL-13 (Zhu *et al.*, 2004). In that report AMCase expression was detected in epithelial cells and in macrophages. We did not detect AMCase expression in the cells recovered from the airways of mice and so it is unlikely that this gene is expressed in macrophages in *N. brasiliensis* infection. Furthermore in contrast to the study of allergy by Zhu et al we found that full AMCase expression required IL-4. AMCase may have role in defence in the lung against chitin bearing organisms (Boot *et al.*, 2001) such as fungi which are inhaled and settle in the lung. In the context of our study the rise in AMCase may be more to do with lung tissue remodelling and repair than an anti-parasite response.

Arginase1 is one of the prototypical markers of alternatively activated macrophages (Morris *et al.*, 1998) and is upregulated in NeMΦ (Loke *et al.*, 2002). In response to *N. brasiliensis* infection Arginase1 expression is only found in the cells recovered from the airways and this expression is highly IL-4 dependent. Arginase1 expression can be driven by IL-13 in vitro (Chang *et al.*, 2001) but the lack of Arginase1 expression in the airway cells of the IL-4 deficient animals suggests a failure of the alternatively activated macrophage phenotype due to a lack of IL-4 and that the upregulation of Arginase1 by IL-13 requires additional signaling by IL-4. Thus the regulation of Arginase1 expression in vivo may be different from that seen in vitro. As discussed in Chapter 4 Arginase1 has a crucial role in tissue repair and the finding that this enzyme is expressed in the context of pulmonary damage points to a role in tissue repair processes.

5.3.3. Granuloma in the the lung after N. brasiliensis infection

Granulomatous deposits have been previously been described in response to *N. brasiliensis* infection in rats (Ramaswamy *et al.*, 1991) and in mice (Daly *et al.*, 1999). The formation of these proteinaceous bodies is dependent on mast cells and they consist of histiocytic cells, fibroblasts and lymphoid cells

and produce Th2 cytokines (Arizono *et al.*, 1996), (Matsuda *et al.*, 2001). The focus of these granulomatous bodies is unknown. As *N. brasiliensis* migrates through the lung there is a moult from L3 to L4 larvae and granulomatous material may form around worm remains after the moult. However worm remains have not been observed in these bodies (Daly *et al.*, 1999), (Matsuda *et al.*, 2001). The finding of strong Ym1 staining in the granulomatous bodies seen in the lung (Figure 21) indicates that Ym1 may have a role in the binding of cells or the granulomatous material in this type of response.

This is the first report of Ym1 on platelets. This, together with the wound healing data presented in Chapter 4 illustrate a potential role for Ym1 in the clotting process. The lack of a proper cell structure in platelets implies that the Ym1 we see would either have to be a pre-formed component of platelets or be bound to the outside of these cell fragments after originating from another source. Ym1 has been implicated in haematopoesis but expression in this setting is by immature neutrophils and not megakaryoblasts or platelets (Nio *et al.*, 2004). Finding Ym1 protein associated with platelets suggests that the granulomatous material seen in the lungs of the infected mice may be due to haemorrhage. If Ym1 is involved in binding cells together then expression associated with extravascular coagulation may be expected.

5.3.4. The response to N. brasiliensis in the gut

We found that there was no expression of Ym1, Fizz1, AMCase or Arginase1 mRNA in the small intestine of C57BL/6 mice after *N. brasiliensis* infection. In contrast to the damage seen during the pulmonary migratory stage, the intestinal stage of infection produces little direct parasite induced damage as *N. brasiliensis* remains on the surface of the gut epithelium and does not penetrate the tissues (Ogilvie, 1974). The lack of Fizz1, Ym1 and Arginase1 expression in the small intestine may reflect the relative lack of trauma in this tissue as infection, then expulsion proceed.

Mastocytosis is a common response to helminth infection in the gut (Miller, 1996) and *N. brasiliensis* infection is no exception (Arizono *et al.*, 1996), (Madden *et al.*, 1991), (Rosbottom *et al.*, 2002). The ability of bone marrow derived connective tissue type mast cells to upregulate Ym1 expression in response to IL-4 has recently been described (Lee *et al.*, 2005) and thus it is somewhat surprising that we do not see an upregulation of Ym1 in the intestine after *N. brasiliensis* infection where the immune response is Th2 dominated and mast cells are numerous. One possible explanation is that the intestinal mastocytosis in response to *N. brasiliensis* is dominated by mucosal type mast cells and not connective tissue type mast cell (Bienenstock *et al.*, 1982), (Shelburne & Ryan, 2001), (Mahida, 2003), (Dehlawi & Goyal, 2003) and these mast cell subtypes may respond differently to Th2 cytokines. The role of

Ym1 in the intestine could also be redundant as an equivalent role could be played by chitinases or other chitinase-like molecules. Ym2 and AMCase protein are expressed in the stomach and detection of AMCase protein in the small intestine has been attributed to trickle down from the stomach (Nio *et al.*, 2004), (Boot *et al.*, 2001), (Suzuki *et al.*, 2002); the functional chitinase chitotriosidase is expressed in both the stomach and Paneth cells of the small intestine (Boot *et al.*, 2005) and could also be upregulated in the *N. brasiliensis* infection setting. The disruption of egg shells in *Heligmosomoides polygyrus* infection in mice has been attributed to chitinase secreting *Bacillaceae sp.* in the gastrointestinal tract (Lewis & Mathers, 1988) and it may therefore benefit the host to change the intestinal chemistry to favour the growth of chitinase secreting bacteria and interrupt the parasite lifecycle.

The differential expression of Fizz1 and Fizz2 is consistent with previously published data on the expression of these genes in response to both helminth infection (Artis *et al.*, 2004), (Nair *et al.*, 2005) and in normal tissue (Holcomb *et al.*, 2000). In *T. muris* infection Fizz2 expression is limited to the goblet cells (Artis *et al.*, 2004); we have not yet determined the cells responsible for Fizz2 expression in *N. brasiliensis* infected mice but it seems likely that goblet cells fulfil this role. Unlike Fizz1, Fizz2 has not been detected in macrophages. Mucus produced by goblet cells acts as a first line of defence against a range of bacterial, fungal and helminth parasites (Khan & Collins, 2004) and the composition and quantity of mucus production can be influenced by the immune system (Khan *et al.*, 1995), (Khan & Collins, 2004). Fizz2 is a component of intestinal mucus (He *et al.*, 2003) and increased expression is dependent on the Th2 response with an absolute requirement for STAT-6 and IL-4Rα (Vogelzang, 2004), (Artis *et al.*, 2004). A role for Fizz2 in defence against intestinal nematodes has been suggested by the finding that this protein binds to chemosensory pores on the surface of *T. muris* in the mouse intestine and may interfere with the ability of the parasite to detect changes in its environment (Artis *et al.*, 2004). Proof of principle for this mechanism was established using recombinant Fizz2 to block the chemotactic migration of *Strongyloides stercoralis* larvae (Artis *et al.*, 2004). The efficacy of this mechanism in vivo has not been tested but it could result in a lack of response to local stimuli and a failure to upregulate binding proteins which normally allow the parasite to maintain position in the intestine.

The lack of Ym1, Fizz1 and Arginase1 expression in the intestine of C57BL/6 mice indicate that there may be little role for alternatively activated macrophages in this stage of *N. brasiliensis* infection. Whilst there is a large macrophage population in the gastrointestinal tract (Lee *et al.*, 1985), (Smith *et al.*, 2005) increased numbers of macrophages are not a reported feature of the cellular response in *N. brasiliensis* infection and loss of IL-4 responsiveness in macrophages and neutrophils does not affect

the rate of *N. brasiliensis* expulsion from the gut (Herbert *et al.*, 2004). If alternatively activated macrophages are not important in the expulsion of *N. brasiliensis* from the gut then a lack of Arginase1, Fizz1 and Ym1 expression may not be surprising.

5.3.5. The lack of Ym1, Fizz1 and Arginase1 in the gut after N. brasiliensis infection may reflect strain differences

The expression of Fizz1 and Ym1 in the lung is not dependent on IL-4 although this cytokine is required for the full response (Figures 21 & 22). This contrasts with previous findings in the *B. malayi* implant model where we saw a lack of Ym1 and Fizz1 expression in IL-4 deficient C57BL/6 mice after parasite implant (Nair, 2003). Expression of both Fizz1 and Ym1 are regulated by STAT-6 activity (Stutz *et al.*, 2003), (Welch *et al.*, 2002). Interestingly in the former study Fizz2 expression was also seen in the lungs in response to allergic challenge but this expression was found to be independent of both IL-4 and STAT-6. In the *B. malayi* implant system IL-4 deficient BALB/c mice compensated for a lack of IL-4 by upregulating IL-13 production from the spleen. This compensatory response was not seen in IL-4 deficient C57BL/6 mice (Nair, 2003). In response to *N. brasiliensis* infection however we saw expression of both Fizz1 and Ym1 in IL-4 deficient C57BL/6 mice in the lung. Splenocytes from infected WT mice displayed a cytokine expression pattern similar to that seen in the *B. malayi* model system with rises in IL-4 expression from the spleen in response to infection. There was however a marked difference between the strains in the location of the IL-13 production in IL-4 deficient mice. In IL-4 deficient C57BL/6 mice there was no rise in splenic IL-13 expression but instead an increase in the expression of this cytokine from the mesenteric lymph nodes. In BALB/c mice there was expression of IL-13 in both the draining lymph nodes and the spleen. Thus the local Th2 response seems to be more important in C57BL/6 mice.

The data presented in this chapter indicate that, of the genes we have examined, only Fizz2 is expressed in the gut of C57BL/6 mice. We have previously reported the expression of both Fizz1 and Ym1 in the gut after *N. brasiliensis* infection (Nair *et al.*, 2005). These latter studies were carried out using BALB/c mice whereas the data presented in figures 16 to 21 were gathered from C57BL/6 mice. Evidence of Fizz1 expression in the small intestine of BALB/c mice comes from work carried out by Wang et al in which Fizz1 and Fizz2 expression were seen in the small intestine of BALB/c mice 10 days after *N. brasiliensis* infection; the expression of Fizz1 was localised to the colonic goblet cells (Wang *et al.*, 2005). In the WT C57BL/6 mice there is an IL-4 response but not an IL-13 response in the mesenteric lymph nodes. In IL-4 deficient C57BL/6 mice there is an unpregulation of IL-13 but

obviously no IL-4. Thus in C57BL/6 but not BALB/c mice both IL-4 and IL-13 may be required to drive Fizz1 and Ym1 expression in the gut and the contemporaneous expression of both cytokines is not seen in the draining lymph node 6 days after *N. brasiliensis* infection.

Differences in the mast cell responses between strains could also influence the expression of Ym1 in the gut after nematode infection. BALB/c mice contain a higher number of mast cells in the intestinal mucosa than C57BL/6 mice (Gurish *et al.*, 2001) and there is a strain variation in the mast cell response between BALB/c and C57 mice in response to gastrointestinal helminth infection (Tuohy *et al.*, 1990) perhaps due to C57 mice having smaller numbers of mast cell precursors in the bone marrow (Brown *et al.*, 2003). Thus the lack of mast cell derived Ym1/2 may be the result of lower numbers of mast cells in the C57BL/6 mice used in these studies or strain differences in the mast cell response between C57BL/6 and BALB/c mice.

5.3.6. Conclusions

- The expression of Fizz1, Ym1, AMCase and Arginase1 in *N. brasiliensis* infection is site specific with these proteins being important in the response to the lung stage but not the intestinal stage of infection in C57BL/6 mice.
- Fizz1 and Fizz2 are differentially expressed and the function of these proteins may be subtly different between the two sites examined.
- Pre-formed Ym1 may be important in clotting or repair processes.
- The regulation of type-2 cytokines in C57BL/6 and BALB/c mice is different with a broader IL-13 response seen in BALB/c mice in keeping with their classification as high type 2 responders.

Chapter 6 - Regulation of Fizz1 and Ym1 in a Schistosome Vaccination Model

6.1. Introduction

We have shown that Ym1 and Fizz1 are upregulated in both the early (Chapter 3) and chronic stages of the *B. malayi* implant model (Loke *et al.*, 2002), in an innate manner in response to surgical trauma (Chapter 4), and in response to acute helminth infection with *N. brasiliensis* (Chapter 5), (Nair *et al.*, 2005). Thus expression of these proteins is a feature of the Th2 response in infection and is driven by the Th2 cytokine IL-13 in the non-infection setting. Consistent with this, most in vivo studies have found that expression of Ym1 and Fizz1 is dependent on Th2 cytokines with Fizz1 having the most stringent requirement for IL-4 or IL-13 (Welch *et al.*, 2002), (Webb *et al.*, 2001), (Raes *et al.*, 2002), (Loke *et al.*, 2002) and we have been able to directly induce the expression of Ym1 and Fizz1 in vitro using bone marrow derived macrophages exposed to IL-4 or IL-13 (Nair *et al.*, 2003). The data presented in Chapter 4, our previously published data (Nair *et al.*, 2005) and the data of others (Chang *et al.*, 2001), (Li *et al.*, 2005) suggests that these proteins are involved in wound healing. Their upregulation during helminth infection may therefore reflect a requirement to repair the damage caused by parasite migration or alternatively they may play a role in the removal of intact or damaged parasites.

The vaccination of mice with radiation attenuated (RA) *Schistosoma mansoni* cercariae is the only vaccination model in which effective vaccine induced immunity to schistosomiasis can be demonstrated (Bergquist *et al.*, 2002). This system involves at least three distinct sites of interaction between the host and the invading organism. Firstly the cercariae enter the host through the skin causing an innate reaction. Secondly, during a prolonged stay in the skin *S. mansoni* antigen is presented to lymphocytes in skin draining lymph nodes. Finally the migration of the organism is halted in the vasculature of the lungs, perhaps for physical reasons. The efficacy of vaccination is largely dependent on an innate Th1 response (Wilson *et al.*, 1999) and alveolar macrophages express Ym1 after vaccination (R.A. Wilson, pers comm).

In contrast to the eosinophil rich response seen in the cutaneous stage of *N. brasiliensis* infection (Daly *et al.*, 1999) RA *S. mansoni* cercariae stimulate an innate immune reaction dominated by neutrophils but also including dendritic cells, macrophages and Langerhans cells (Mountford & Trottein, 2004) as they enter the skin. The antigen presenting cells in the skin exposed to the cercariae after vaccination

secrete large quantities of IL-12 (Hogg *et al.*, 2003) and so are primed to promote the Th1 response seen in the skin draining lymph nodes (Betts & Wilson, 1998). Thus from the earliest stages of infection there is a bias towards the Th1 response in *S. mansoni* infection. After a prolonged stay in the skin, but successful transformation to schistosomulae the attenuated parasites make their way to the lungs. Once in the lungs they are unable to migrate onward through the capillary beds and instead breakthrough through into the alveolar space perhaps due to neuromuscular problems arising as sequelae of irradiation (Mountford *et al.*, 1988), (Harrop & Wilson, 1993). This induces an influx of Th1 biased CD4+ve T cells into the lungs which are thought to act as memory cells and protect this organ in challenge infection (Coulson & Wilson, 1993). After challenge the lung is the site of highest attrition with an effector response in the form of an aggregate of immune cells (mainly lymphocytes and macrophages) around the intravascular parasites (Crabtree & Wilson, 1986). This is thought to be protective as it prevents the onward migration of schistosomulae through the lungs.

The effectiveness of vaccination is dependent on an intact Th1 response with IFN-γ being particularly important. Indeed administration of a monoclonal antibody to IFN-γ leads to an abrogation of immunity (Smythies *et al.*, 1992) and the effector foci in the lungs of IFN-γ depleted, IFN-γR deficient and IL-12p40 deficient animals are looser and more diffuse than those found in WT mice (Smythies *et al.*, 1992), (Wilson *et al.*, 1996), (Anderson *et al.*, 1998) suggesting that they may be less effective at holding the parasite in the lungs. The importance of IFN-γ in this system led us to investigate the regulation of the Th2 dependent proteins Ym1 and Fizz1 in a helminth setting unusually associated with Th1 cytokines. We used real-time RT-PCR and western blotting to examine the expression of Ym1 and Fizz1 in both WT mice and IFN-γR deficient mice vaccinated with RA *S. mansoni* cercariae. In addition we examined the cytokine profile in the lungs of vaccinated mice in order to assess the response driven by vaccination alone and the potential impact of the immune response on the expression patterns we found.

6.2. Results

6.2.1. Vaccination with irradiated S. mansoni cercariae causes an influx of cells into the lungs

We have established that Fizz1, Ym1 and Arginase1 require type 2 cytokines for full expression in nematode infection, with a particular requirement for IL-13 in the case of the response to injury. Having seen Fizz1, Ym1, Arginase1 and AMCase expression in the lung and airways in *N. brasiliensis* infection (Chapter 5) we chose to examine the expression of these genes in the schistosome radiation attenuated (RA) cercariae vaccination model because this system involves the lung but has a large type1 cytokine (IFN-γ) component. Additionally this model system gave us the opportunity to examine the response to a trematode parasite rather than a nematode parasite.

Having previously used C57BL/6 and BALB/c mice to characterize the response to nematode infection we began by assessing the response to vaccination with RA *S. mansoni* cercariae in these strains. We found that vaccination causes an influx of cells into the lung (Table 4) and that both before and 28 days after vaccination macrophages make up the majority of the cellular population recoverable by bronchial lavage. Although both the total number of cells and the numbers of macrophages, eosinophils and lymphocytes significantly increased after vaccination there were no differences in these parameters between the two strains. We did not find neutrophils in the bronchial lavage of either the C57BL/6 or BALB/c mice.

Strain	Macrophages	Eosinophils	Lymphocytes
C57BL/6 Nv	*0.6 +/- 0.104 x10^6*	*0*	*0.02 +/- 0.004 x10^6*
C57BL/6 Vacc	*1.3 +/- 0.211 x10^6*	*0.5 +/- 0.005 x10^6*	*0.2 +/- 0.026 x10^6*
BALB/c Nv	*0.5 +/- 0.113 x10^6*	*0*	*0.01 +/- 0.002 x10^6*
BALB/c Vacc	*1.3 +/- 0.119 x10^6*	*0.4 +/- 0.009 x10^6*	*0.3 +/- 0.076 x10^6*

Table 4: Differential count of cells recovered from the airways of C57BL/6 and BALB/c mice before and 28 days after vaccination with RA *S. mansoni* cercariae. Numbers represent the mean +/- s.e.m. n= 5 per group.

6.2.2. Vaccination causes a rise in Ym1, Fizz1 and Arginase1 expression in the BAL cells of mice

We next examined the expression of Ym1, Fizz1 and Arginase1 by real-time RT-PCR in the BAL cells (Figure 25). In both strains of mice Ym1 (25A) and Arginase1 (25C) mRNA was detectable in the BAL cells prior to vaccination with C57BL/6 mice displaying higher levels than BALB/c mice. Vaccination led to a rise in Ym1(25A), Fizz1 (25B) and Arginase1 (25C) expression levels by day 28 in both strains with the highest levels consistently seen in the C57BL/6 mice.

We also analysed the secretion of Ym1 and Fizz1 protein by western blot in the BAL fluid (Figure 26). This revealed that the pattern of Ym1 protein (Figure 26A) in the BAL fluid reflected the mRNA expression pattern seen using real-time RT-PCR. In particular there was a stronger signal from the BAL fluid of naïve C57BL/6 mice than that of the naïve BALB/c mice. In both strains there was a stronger Ym1 signal after vaccination. Fizz1 protein was undetectable in the BAL fluid of either strain prior to vaccination and we could not detect this protein in the BALB/c mice after vaccination either (Figure 26B); Fizz1 protein was, however detectable in the BAL fluid of the C57BL/6 mice after vaccination. This suggests that the small rise in mRNA expression seen in BALB/c mice is either not enough to enable the detection of Fizz1 protein in these mice after vaccination or that the half life of this protein in the BALB/c strain is shorter.

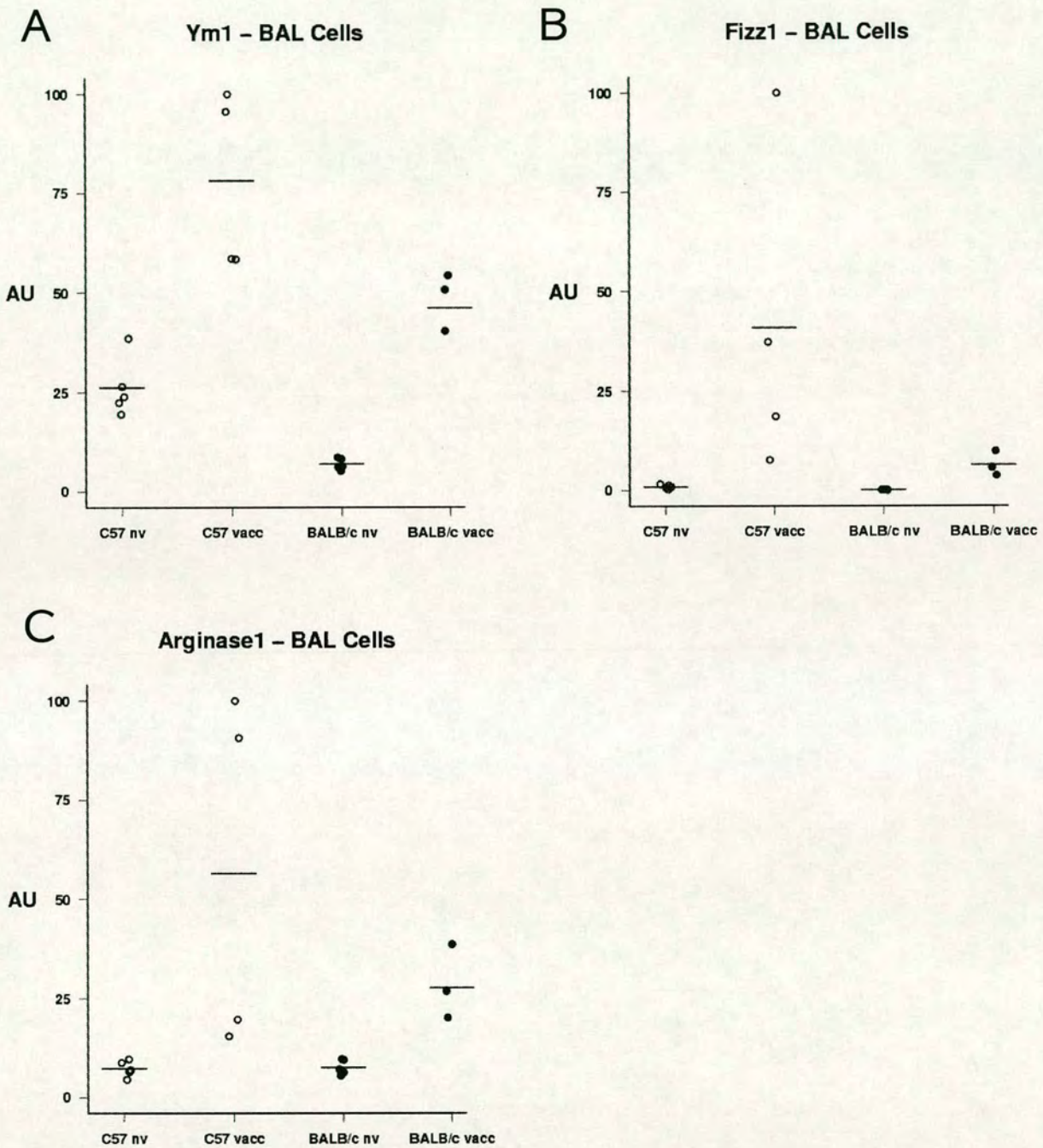

Figure 25: Ym1 (A), Fizz1 (B) and Arginase1 (C) are all upregulated in airway cells 28 days after vaccination with RA *S. mansoni* cercariae. Expression was analysed using real time RT-PCR. The highest expression was arbitrarily assigned a value of 100 and each of the other points were then plotted in relation to this. Each point represents a single mouse and the bar indicates the mean.

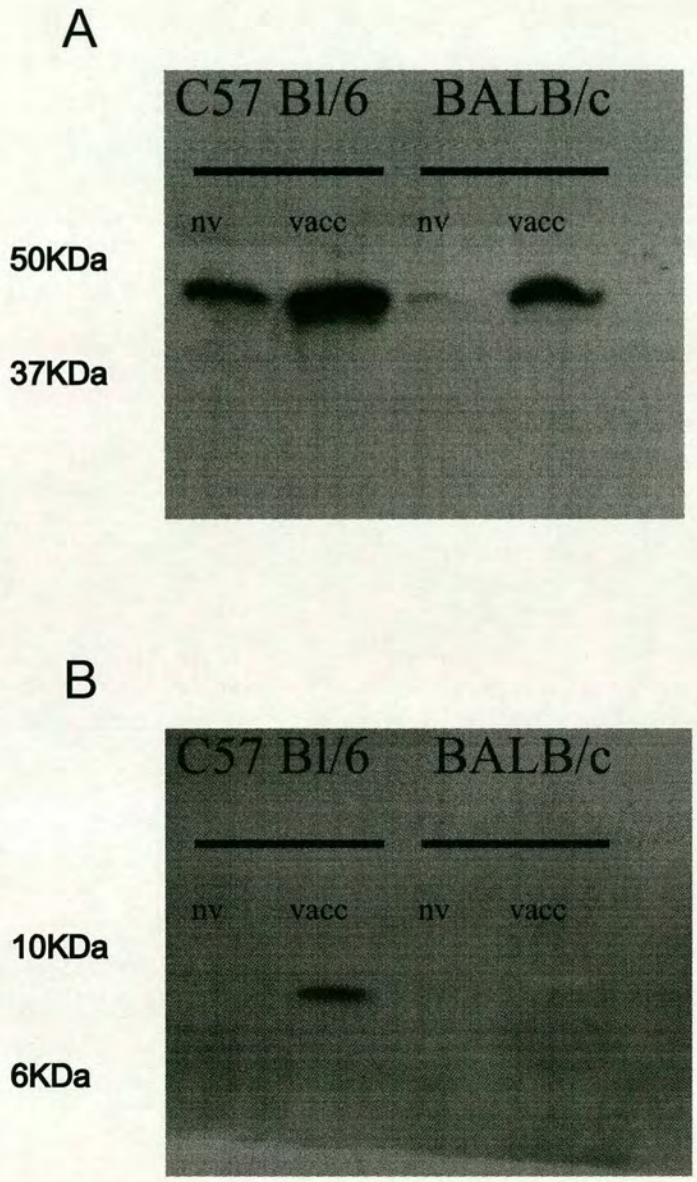

Figure 26: Western blotting reveals that there is a higher level of Ym1 protein (26A) in the BAL fluid of C57BL/6 mice both before and 28 days after vaccination. In both C57BL/6 and BALB/c mice Ym1 protein levels rose after vaccination. Fizz1 (26B) was only detectable in vaccinated C57BL/6 mice.

6.2.3. Ym1 and Fizz1 but not AMCase are upregulated in the lungs of vaccinated C57BL/6 and BALB/c mice

The pattern of Fizz1 and Ym1 expression seen in the airway cells was mirrored by the expression of these genes in the lung tissue. Expression of Ym1 mRNA was detectable in the lung tissue of both strains of mice prior to vaccination and, as seen in the BAL cells vaccination induced an increase in Ym1 mRNA levels. Interestingly, in this experiment Fizz1 mRNA was detected in the lungs of these mice prior to vaccination, a finding we had not previously recorded and the levels found in the C57BL/6 mice were elevated compared to those seen in the BALB/c mice. After vaccination Fizz1 expression (Figure 27B) rose in the lung tissue of both strains with expression levels again being higher in the C57BL/6 mice. We were able to detect AMCase mRNA expression in the lung tissue of both strains prior to vaccination (Figure 27C) although unlike Ym1 and Fizz1 there was no strain specific difference in this expression. Contrary to our observations in the *N. brasiliensis* infection system (Chapter 5) AMCase did not show any rise in mRNA expression level after vaccination with irradiated *S. mansoni* cercariae (Figure 27C).

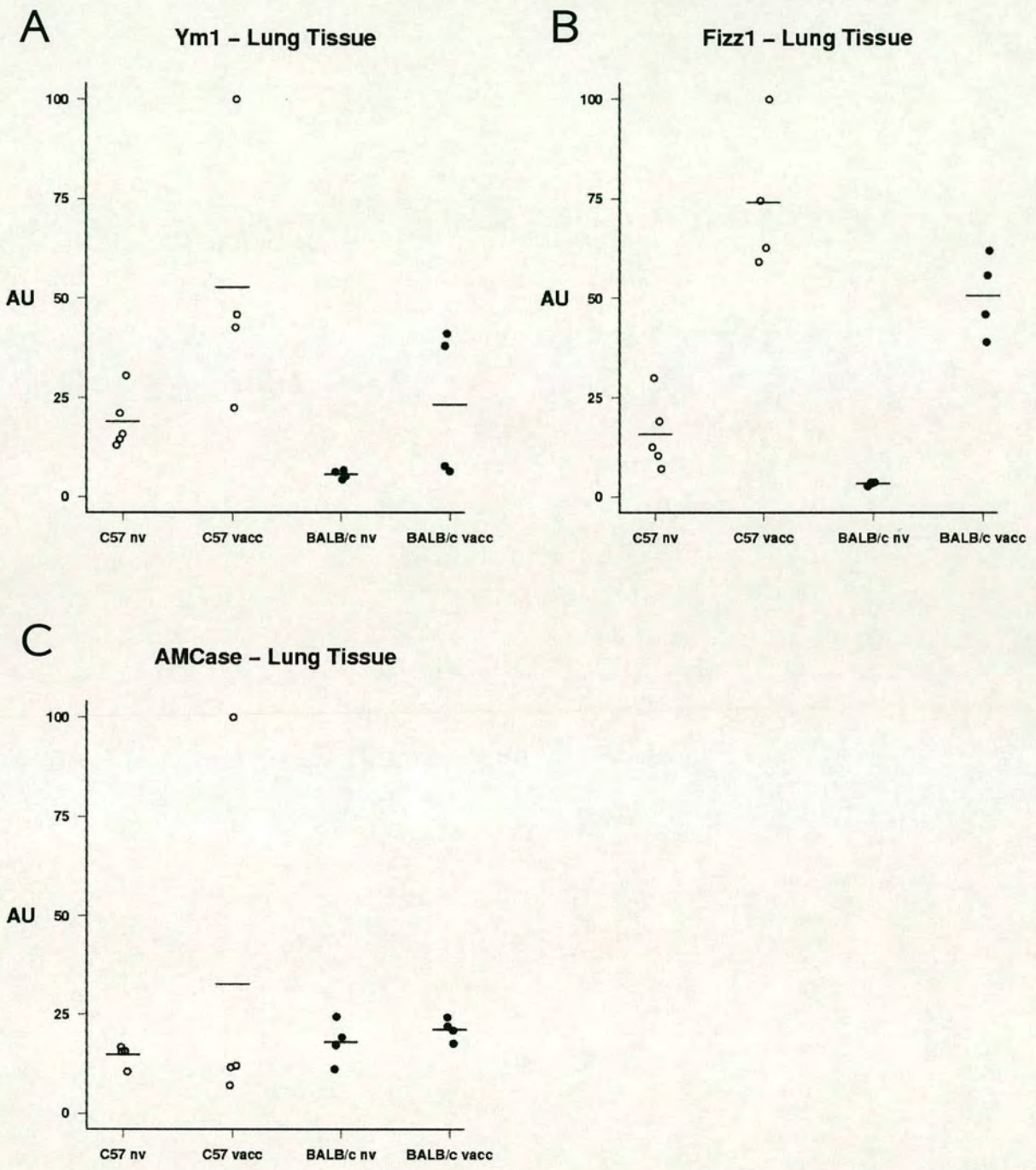

Figure 27: Ym1 (A), Fizz1 (B), and AMCase (C) are all upregulated in lung tissue of C57BL/6 and BALB/c mice 28 days after vaccination with RA *S. mansoni* cercariae. Expression was analysed using real time RT-PCR. The highest expression was arbitrarily assigned a value of 100 and each of the other points were then plotted in relation to this. Each point represents a single mouse and the bar indicates the mean.

6.2.4. The cytokine response in BALB/c mice after vaccination is more pronounced

The efficacy of the irradiated cercariae vaccination system is strongly dependent on IFN-γ (Smythies *et al.*, 1992) although in the absence of this cytokine a Th2 response can occur and provide some protection (Mountford *et al.*, 2001). Much of the work examining the polarisation of the immune response in the lungs of these animals has been undertaken after both vaccination and challenge. In order to assess the polarisation of the immune response following a single vaccination we examined the levels of the cytokines IL-4, IL-13 and IFN-γ in the BAL fluid of vaccinated only mice using ELISA. Our results, presented in figure 28, show that there is a mixed Th1 / Th2 response in both C57BL/6 and BALB/c mice but surprisingly, in view of the Ym1, Fizz1 and Arginase1 expression profiles in both strains the overall immune response is much more pronounced in BALB/c mice.

In terms of IL-4 (Figure 28A) there were also differences in the levels detectable in naïve mice. There was no IL-4 detectable in the BAL fluid of unvaccinated C57BL/6 mice. In contrast this cytokine was detectable in the BAL fluid of BALB/c mice. There was a strong response in terms of IL-4 after vaccination in both strains with significantly higher levels seen in the BALB/c mice. Thus in terms of IL-4, BALB/c mice not only have a higher level in the lungs prior to vaccination but also have a higher level post vaccination befitting their reputation as high Th2 responders. Whilst IL-13 was found in the BAL fluid of both strains prior to vaccination (Figure 28B) only the BALB/c mice displayed a rise in the level of this cytokine after vaccination (Figure 28B). IFN-γ (Figure 28C) was not detectable in the BAL fluid of either the C57BL/6 or BALB/c mice prior to vaccination. There was, however a rise in this cytokine in both strains after vaccination with the BALB/c mice displaying a significantly higher level than the C57BL/6 mice. Thus vaccination with RA *S. mansoni* cercariae leads to a mixed Th1 / Th2 response in the lungs of both C57BL/6 and BALB/c mice but the cytokine response is more pronounced in the BALB/c mice.

Taken together the real time RT-PCR, western blotting and ELISA data indicate that the vaccination of mice with irradiated *S. mansoni* cercariae leads to a rise in the levels of both Fizz1 and Ym1 in the airways and lung. C57BL/6 mice have a higher basal level of Ym1 expression and secretion and express both Fizz1 and Ym1 to a greater extent than the BALB/c mice. Given that these genes are heavily influenced by Th2 cytokines it is of interest that the BALB/c mice make a larger response in terms of both Th1 and Th2 cytokines measured in the bronchial lavage fluid than the C57BL/6 mice but have lower expression of Fizz1 and Ym1.

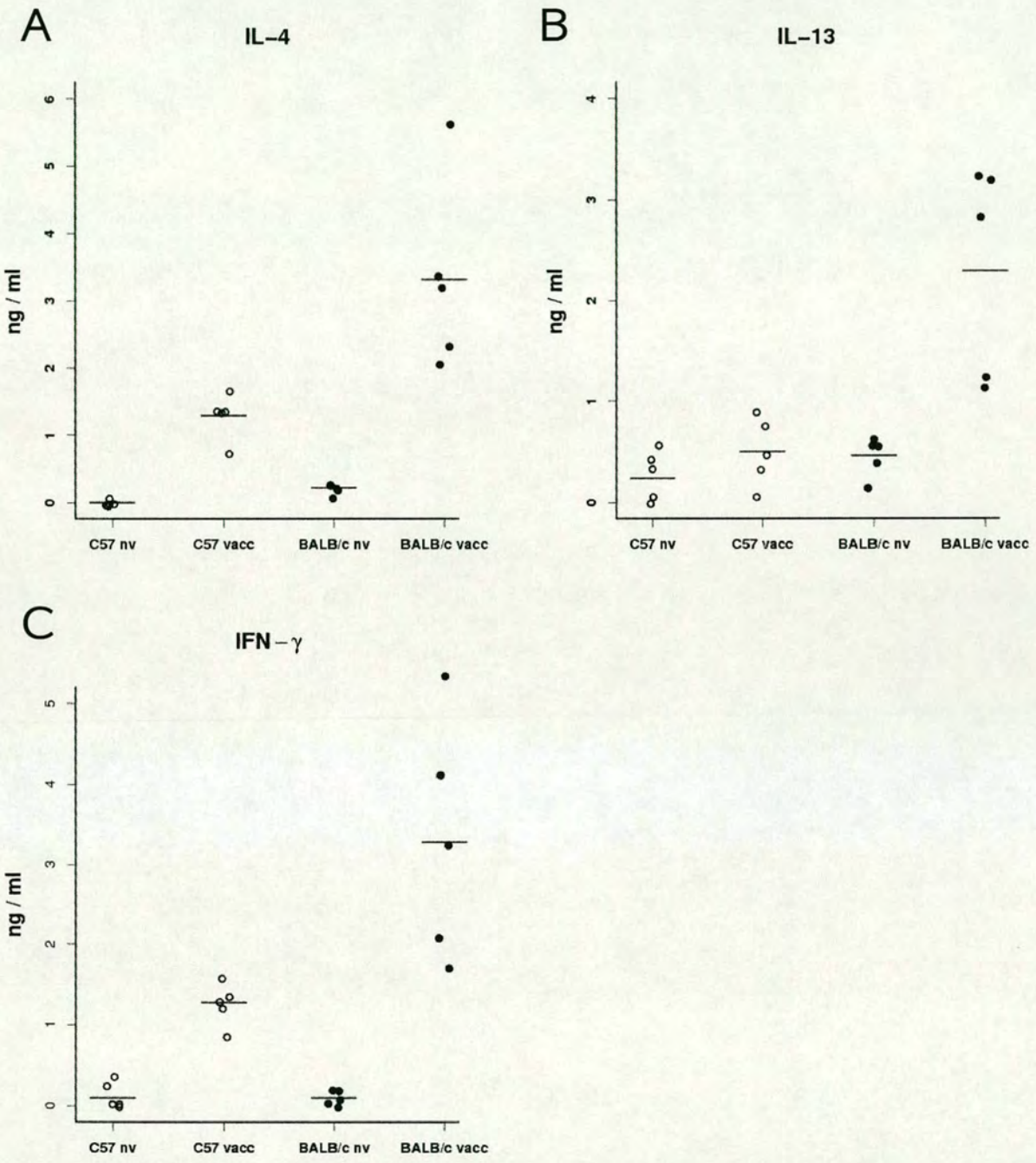

Figure 28: IL-4 (A) is detectable in the BAL fluid of BALB/c, but not C57BL/6, mice prior to vaccination. After vaccination IL-4 is seen in the BAL fluid of both C57BL/6 and BALB/c mice. There is an equivalent level of IL-13 (B) in the BAL fluid of both strains prior to vaccination but after vaccination only BALB/c mice have increased levels of IL-13. Although IFN-γ (C) rises in both strains after vaccination the level of IFN-γ post vaccination is higher in BALB/c mice. Each point represents a single mouse and the bar represents the mean.

6.2.5. Loss of IFN-γ signalling leads to lower cell recruitment after vaccination.

Because protection in the RA cercariae vaccination model is reduced in IFN-γR deficient mice (Smythies *et al.*, 1992) we chose to determine the expression of Arginase1, AMCase, Fizz1 and Ym1 in this setting. IFN-γ receptor (IFN-γR) deficient mice and their wild type (WT) controls were inoculated with a single dose of RA cercariae as detailed in Chapter 2. 129 mice were used because the IFN-γR deficient mice were available on this background. We also compared the response of C57BL/6 mice in the same experiment as much of the data regarding this model system has been gathered in this strain. In terms of cell recruitment there were no differences in the number of cells recovered from the lungs of any of the groups prior to vaccination (Figure 29). Vaccination led to a rise in the number of cells recovered from the BAL fluid of all three groups with the largest rises in the two WT groups.

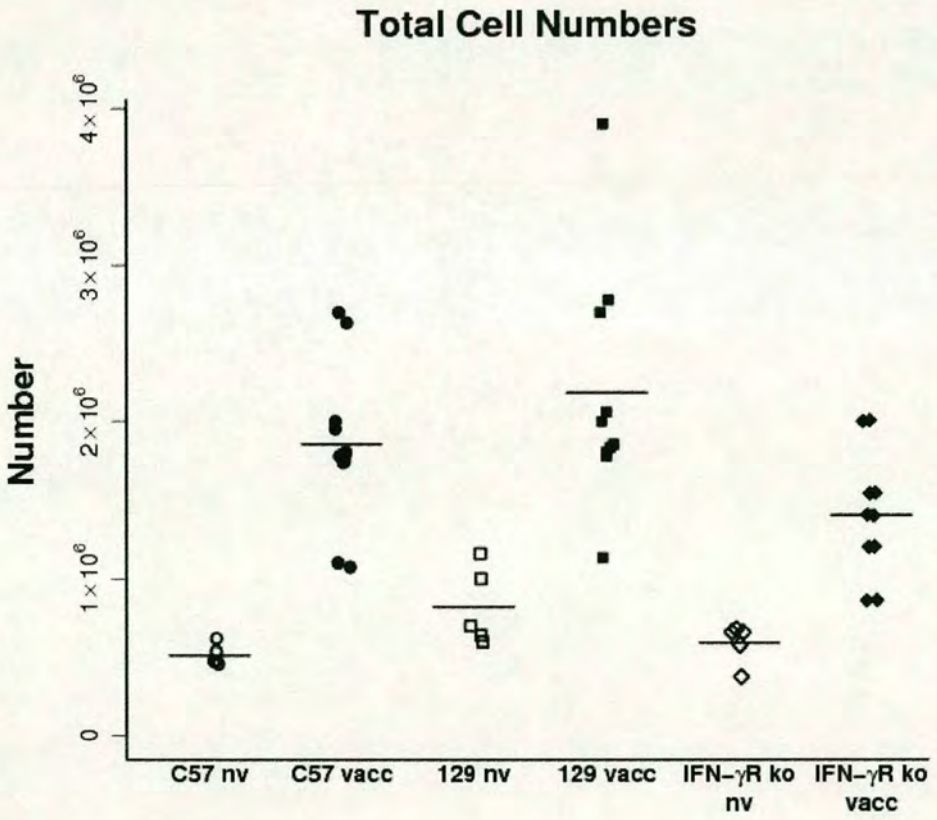

Figure 29: Total cell recovery from the lungs of C57BL/6,129 and IFN-γR deficient mice before and 28 days after vaccination with RA *S. mansoni* cercariae. Lungs were lavaged as described in Chapter 2. After addition of trypan blue viable cells were counted in 10µl of BAL fluid using a haemocytometer. Each point represents an individual mouse with the bar showing the mean. Data for the infected mice is from two separate experiments.

In all three groups macrophages represented the largest number of cells recovered from the lungs before and after vaccination (Table 5). Prior to vaccination the only significant statistical difference was a greater number of lymphocytes in the lungs of the 129 WT mice compared to either of the other groups. The profile of the cellular response after vaccination was broadly similar across the three groups with significant rises in the numbers of macrophages, eosinophils and lymphocytes recovered from the lungs ($p < 0.05$). The WT groups (C57BL/6 and 129) recruited more macrophages and eosinophils than the IFN-γR deficient group ($p < 0.05$).

Strain	Macrophages	Eosinophils	Lymphocytes
C57BL/6 nv	$5.1+/-0.29x10^5$	$0.02 +/- 0.002x10^5$	*0*
C57BL/6 vacc	$17.7+/-0.56x10^5$	$2.2 +/-0.09x10^5$	$0.5+/-0.2x10^5$
129 nv	$8 +/-1x10^5$	$0.1 +/- 0.08x10^5$	$0.06 +/- 0.004x10^5$
129 vacc	$19.9 +/-3.4x10^5$	$2.8 +/- 0.7x10^5$	$1.6 +/- 0.04x10^5$
IFN-γ R ko nv	$5.7 +/- 0.6x10^5$	*0*	*0*
IFN-γ R ko vacc	$12.2 +/-1.5x10^5$	$1.2 +/- 0.3x10^5$	$0.5+/-0.01x10^5$

Table 5: Differential cell counts from the BAL fluid of mice before and after vaccination with RA *S. mansoni* cercariae. Cells types were counted after cytospin using standard staining techniques (see Chapter 2). Two fields of at least 150 cells were counted per mouse. The percentage of different cell types was calculated from the total number of cells (Figure 29) and this percentage used to calculate the total numbers of the different cell types. Cell numbers are shown +/- s.e.m.

6.2.6. Loss of IFN-γ responsiveness leads to lower Fizz1 and Ym1 expression in airway cells of vaccinated mice

In addition to Fizz1 and Ym1 expression we also examined the expression of the other Th2-dependent proteins associated with helminth induced pulmonary inflammation; acidic mammalian chitinase (AMCase) and Arginase1 (Chapter 5). As detailed above the immune response in WT C57BL/6 and BALB/c mice after vaccination only is a mixed Th1/Th2 response. However the BAL cells from IFN-γR deficient mice exposed to challenge after vaccination were reportedly skewed towards a Th2 cytokine expression profile (Wilson *et al.*, 1996). We therefore predicted that the expression of Th2 induced proteins would be higher in the BAL cells from these animals than in WT mice. To assess this we examined the gene expression profile of BAL cells and homogenised lung tissue by real time RT-PCR.

Prior to vaccination there was detectable level of Ym1 mRNA in all three groups of mice although this was higher in the C57BL/6 mice than in either of the other two groups (Figure 30A). Fizz1 mRNA

expression was seen in the 129 mice prior to vaccination but not in the C57BL/6 or the IFN-γR deficient mice (Figure 30B). There were also low levels of both Arginase1 (Figure 30C) and iNOS mRNA (Figure 30D) detectable in the BAL cells of all three groups prior to vaccination. There was a robust rise in Ym1, Fizz1 and Arginase1 mRNA in the BAL cells of the WT mice after vaccination indicating that there was a Th2 response in this compartment (Figure 30A, B, C). An increase in iNOS expression (Figure 30D) in the WT mice indicated that there was also a Th1 component to the response in the BAL cells. The lack of iNOS upregulation in the IFNγR deficient mice confirmed that these mice cannot mount a Th1 response after vaccination. The iNOS data for the WT mice is in agreement with previously published data showing a rise in both iNOS expression and activity in vaccinated mice (Coulson & Wilson, 1988). The lack of a Th1 response might be expected to promote a more robust Th2 response. Thus it was a surprise to observe that the lack of a functional IFN-γ receptor had an inhibitory effect on the expression of Ym1 and Fizz1 (Figure 30A & B). Although mRNA for these genes was upregulated in response to vaccination in the BAL cells of the IFNγR deficient mice the increase was significantly less than that seen in the WT mice (Figure Figure 30A &B). In contrast Arginase1 upregulation was equivalent in both the WT groups and the IFN-γR deficient group (Figure 30C). Thus whilst the Th2 response seemed to be intact there was a blunting of the Ym1 and Fizz1 response. AMCase expression was undetectable in the BAL cells (data not shown).

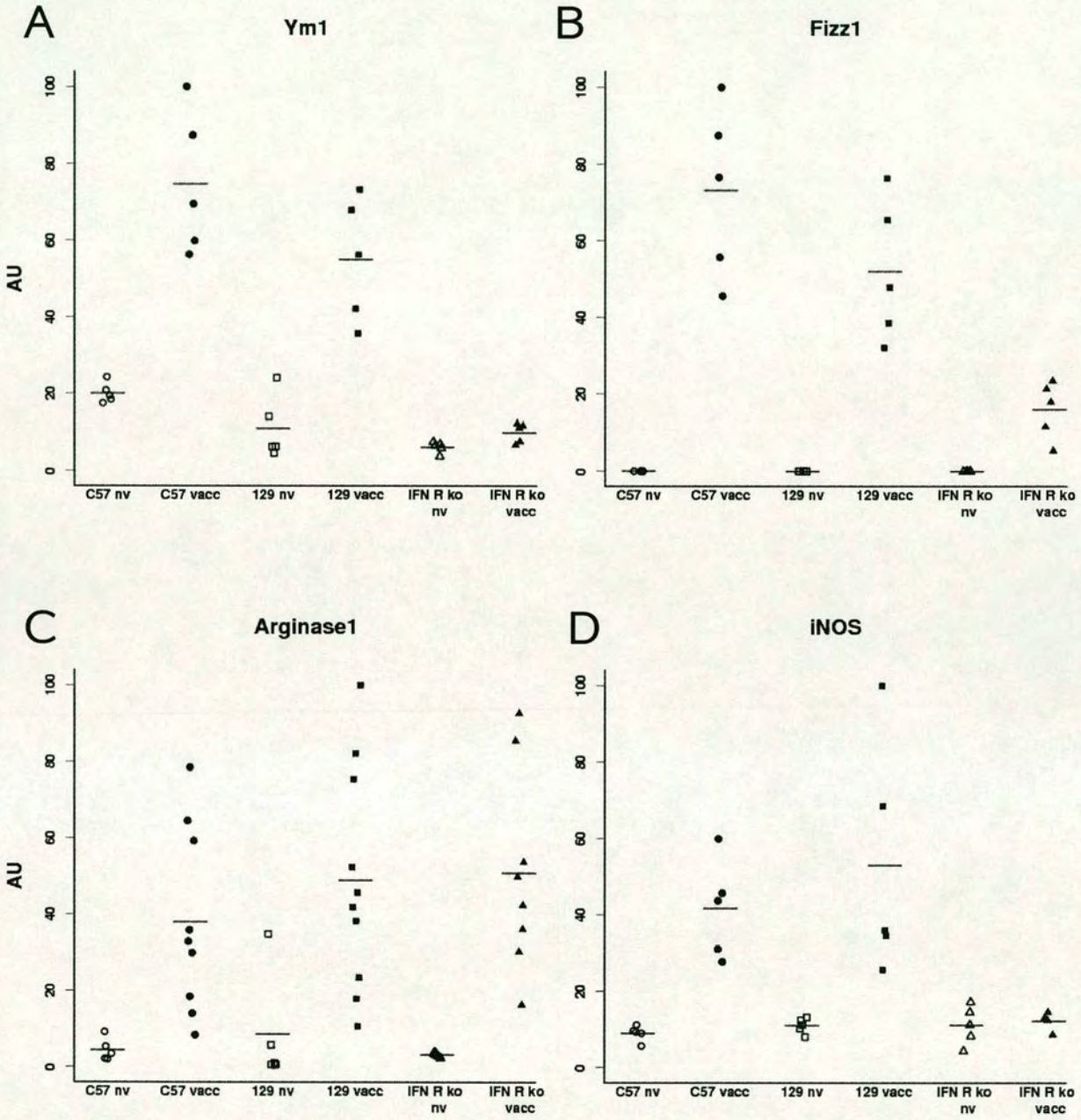

Figure 30: Real time RT-PCR reveals that Ym1 (A), Fizz1 (B) Arginase1 (C) and iNOS (D) are all expressed in airway cells after vaccination with RA *S. mansoni* cercariae. Ym1, Fizz1 and iNOS all demonstrate a dependence on IFN-γ. Each point represents an individual mouse with the bar showing the mean. Ym1, Fizz1 and Arginase1 data from infected mice was collected from two separate experiments.

In order to examine whether protein quantity reflected mRNA levels we carried out western blotting on the BAL fluid from naïve and vaccinated mice (Figure 31). Ym1 was detectable in the BAL fluid of mice from all three groups prior to vaccination (31A). After vaccination the signal for Ym1 protein rose in both the C57BL/6 mice and the 129 mice. There was no apparent increase in protein levels in the IFN-γR deficient mice. Fizz1 protein was not detectable in the BAL fluid of mice prior to vaccination (31B). After vaccination protein was seen in the BAL fluid of all three groups and levels were highest in the C57BL/6 and 129 groups.

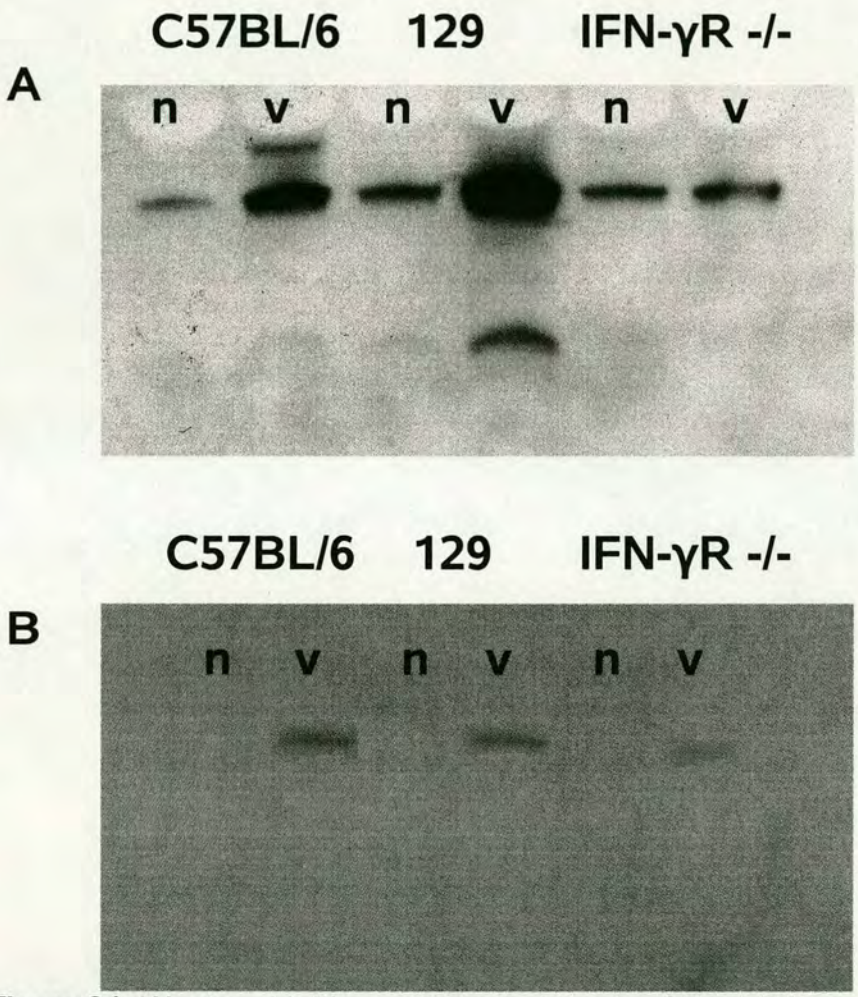

Figure 31: Western blotting reveals that levels of Ym1 (A) and Fizz1 (B) protein in the BAL fluid reflect gene expression. C57BL/6 and 129 WT mice have elevated levels of both proteins in BAL fluid after vaccination. In contrast the level of Ym1 protein in IFN-γR deficient mice is relatively unaffected by vaccination and the signal for Fizz1 protein is lower than that seen in the WT mice. Fizz1 protein was not detected in any of the groups prior to vaccination.

6.2.7. Macrophages are responsible for Ym1 expression after vaccination

The reduced Ym1 expression could be in part explained by reduced macrophage numbers in the IFN-γR deficient mice. We thus chose to identify the cells responsible for Ym1 expression in this system. Immunostaining of cytospin slides reveals clear expression of Ym1 by large cells present in the BAL of WT 129 vaccinated animals (Figure 32A). The cells expressing Ym1 were identified as macrophages by differential staining of separate cytospin preparations taken at the same time (data not shown). The immunostaining data indicate that there is lower protein expression of Ym1 in the IFNγR deficient mice (Figure 32B) and that the increase in expression and secretion in the WT mice is not simply a result of the greater cell recruitment in the these mice.

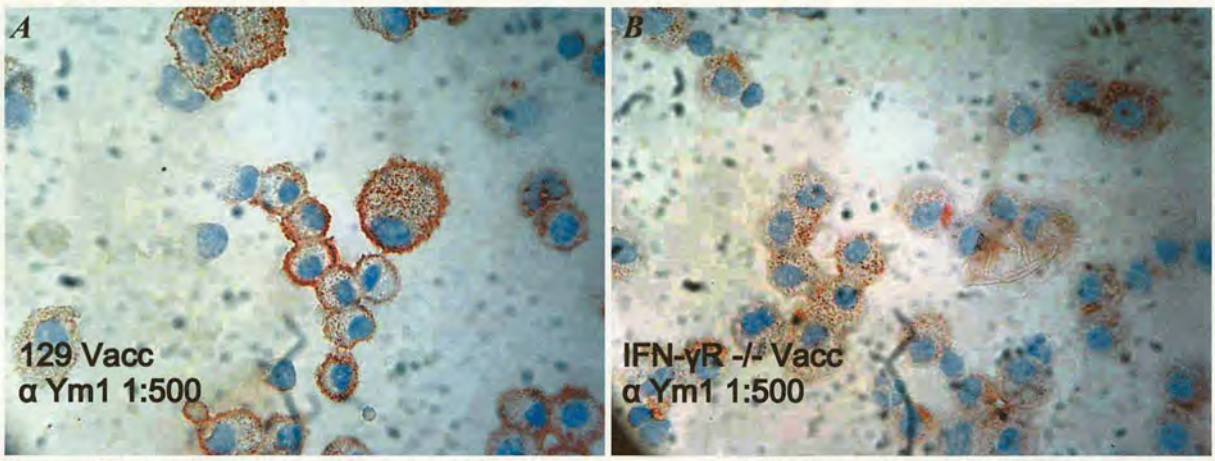

Figure 32: Immunohistochemical staining reveals that Ym1 protein in 129 WT and IFN-γR deficient mice is present in large cells in the BAL fluid. There is a stronger signal from the 129 WT mice consistent with the expression and western blotting data presented above. The granularity seen in these figures is an artefact of cytospin.

6.2.8. Loss of IFN-γ responsiveness leads to lower Ym1, Fizz1 and AMCase expression in the lungs of vaccinated mice

To confirm that the IFN-γ dependence for Ym1 and Fizz1 expression in BAL cells was also true of the lung tissue we used real time RT-PCR to examine homogenised lung tissue. In the case of Ym1 we found that mRNA was detectable in the lung tissue of both WT groups prior to vaccination (Figure 33A). Unlike the BAL cell data (Figure 30A) there was no noticeable difference in the pre vaccination level between the C57BL/6 and 129 mice. We could not detect Ym1 mRNA in the IFN-γR deficient mice before vaccination suggesting that even naïve levels of Ym1 expression are regulated by IFN-γ

receptor signalling. Whilst there was a large rise in the level of Ym1 mRNA detectable in the lung tissue of the WT mice after vaccination the level in the IFN-γR deficient groups was barely elevated (Figure 33A). We could not detect Fizz1 mRNA in the lung tissue of any of the mice prior to vaccination (Figure 33B) but afterwards there was a rise in mRNA expression for Fizz1 in both WT strains; however IFN-γR deficient mice again demonstrated little response (Figure 33B). AMCase mRNA was also undetectable in the lung tissue of unvaccinated mice (Figure 33C) but after vaccination mRNA levels increased in all three groups although the lowest level of expression was again found in the IFN-γR deficient mice (Figure 33C). In a previous experiment we had not observed a rise in AMCase after vaccination (Figure 27C) but that experiment had a much smaller sample size. We were not able to detect Arginase1 mRNA expression in the lung tissue. Together the mRNA and protein expression data from these experiments confound our expectation that a lack of IFN-γ would increase the magnitude of a Th2 response and lead to higher expression of alternative activation markers.

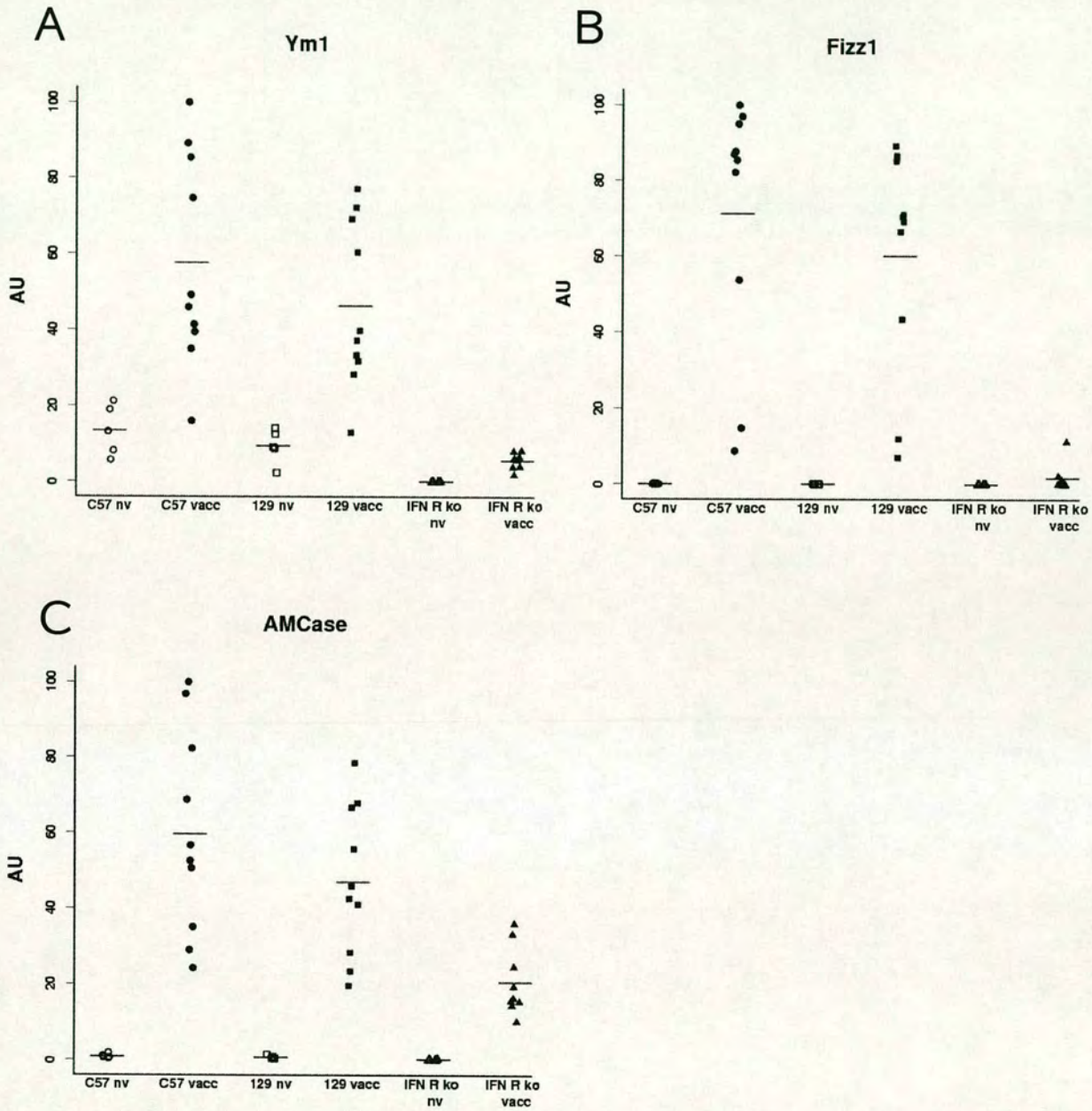

Figure 33: Real time RT-PCR reveals that the expression of Ym1 (A), Fizz1 (B) and AMCase (C) mRNA is increased in lung tissue after vaccination with RA *S mansoni* cercariae. As in the airway cells (Figure 32) there is a dependence on INF-γ signalling. We were unable to detect Arginase1 expression in the lung tissue of mice before or after vaccination (data not shown). Each point represents an individual mouse with the bar showing the mean. Data from infected mice was collected from two separate experiments.

6.2.9. Loss of IFN-γ responsiveness does not result in a runaway Th2 response after vaccination

In order to assess whether the loss of IFN-γ responsiveness leads to a higher Th2 response after vaccination only, we examined the cytokine profile in the lungs of vaccinated mice. We carried out ELISA for the Th1 cytokine IFN-γ and the Th2 cytokines IL-4, IL-5 and IL-13 on BAL fluid recovered from the lungs of vaccinated mice. In both WT and IFN-γR deficient mice there is a rise in both IL-4 and IFN-γ (Figure 34A & B). Thus, as previously reported (Wynn *et al.*, 1996) there is a mixed Th1 / Th2 response in the lungs following vaccination with RA larvae; IL-5 (Figure 32C) also rises upon vaccination in the both WT and IFN-γR deficient mice but the rise is much less pronounced in the IFNγR deficient mice. This suggests that there is an enhancement rather than an inhibition of the Th2 response by IFN-γ in this context. IL-5 is required for the mobilisation of eosinophils from the bone marrow (Hitoshi *et al.*, 1991) and less eosinophils were recruited into the lungs of the vaccinated IFN-γR deficient group compared to the vaccinated WT mice (Table 5). Interestingly the level of IL-13 in the WT and IFN-γR deficient groups (Figure 32D) was equivalent and did not change upon vaccination. This is consistent with data from Wynn et al where the IL-13 response was assessed by real time RT-PCR in whole lung tissue (Wynn *et al.*, 1996) and with the data presented above (Figure 28) for C57BL/6 mice. We did not assess the kinetics of the IL-13 response and the unchanging level we see may reflect a return to basal levels to prevent fibrotic damage.

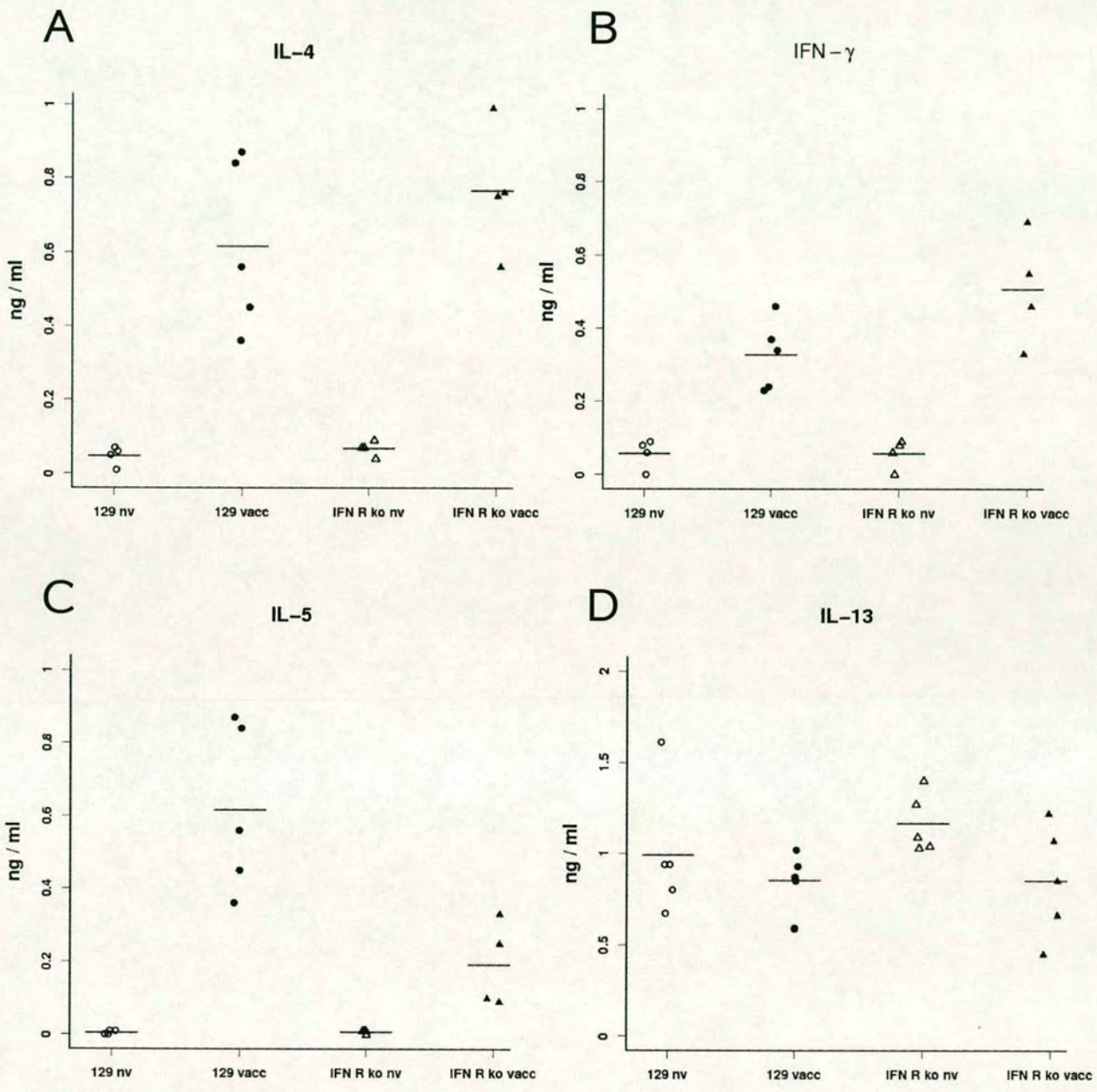

Figure 34: ELISA of BAL fluid reveals that there is a mixed Th1 / Th2 response in the lungs of vaccinated mice. IL-4 (A) levels are elevated after vaccination in both the 129 WT and IFN-γR deficient mice. The same is true of the Th1 cytokine IFN-γ (B). IL-5 (C) is also elevated in both groups after vaccination but this rise is less pronounced in the IFN-γR deficient group. IL-13 (D) is present in the BAL fluid of both groups and vaccination does not affect the level of this cytokine. Each point represents an individual mouse with the bar showing the mean.

6.2.10. IFN-γR deficient mice have a higher level of the IL-13 decoy receptor, IL-13Rα2, in the BAL fluid

Ym1 and Fizz1 are regulated by IL-4 and to a lesser extent by IL-13 through the IL-4 receptor (IL4Rα) and STAT-6 (Welch *et al.*, 2002), (Liu *et al.*, 2004a), (Stutz *et al.*, 2003). Given the robust expression of IL-4 in both the WT and IFN-γR deficient mice, the unexpected suppression of these genes in IFN-γR deficient mice suggested that IL-13 activity might play a role. Although we saw equivalent levels of IL-13, the amount of available IL-13 can be altered by the level of the IL-13 decoy receptor, IL-13Rα2 (Zhang *et al.*, 1997a). This receptor has been implicated in the local regulation of IL-13 levels by promoting the internalisation of IL-13 without activating the STAT-6 signalling pathway (Kawakami *et al.*, 2001) and increased levels of the receptor have been shown to affect the liver granuloma size in *S. mansoni* infected mice (Wynn *et al.*, 2004a). Thus in order to more accurately assess the levels of active IL-13 we also quantified the levels of IL-13Rα2 by ELISA (Mentink-Kane *et al.*, 2004).

The IL-13Rα2 levels were higher in the IFN-γR deficient animals irrespective of vaccination status. Elevated levels of this receptor would lead to a decrease in the active IL-13 available in the lung. Thus in contrast to the previously published observations on the cytokine response after both vaccination and challenge (Wilson *et al.*, 1996) the Th2 response in the IFN-γR deficient mice seems to be equivalent or even reduced relative to the WT mice after vaccination only. These differences may be accounted for by the fact that the original observations were carried out on re-stimulated cells after vaccination and challenge whereas in this study we measured cytokines only after a single vaccination and in BAL fluid rather than from re-stimulated cells.

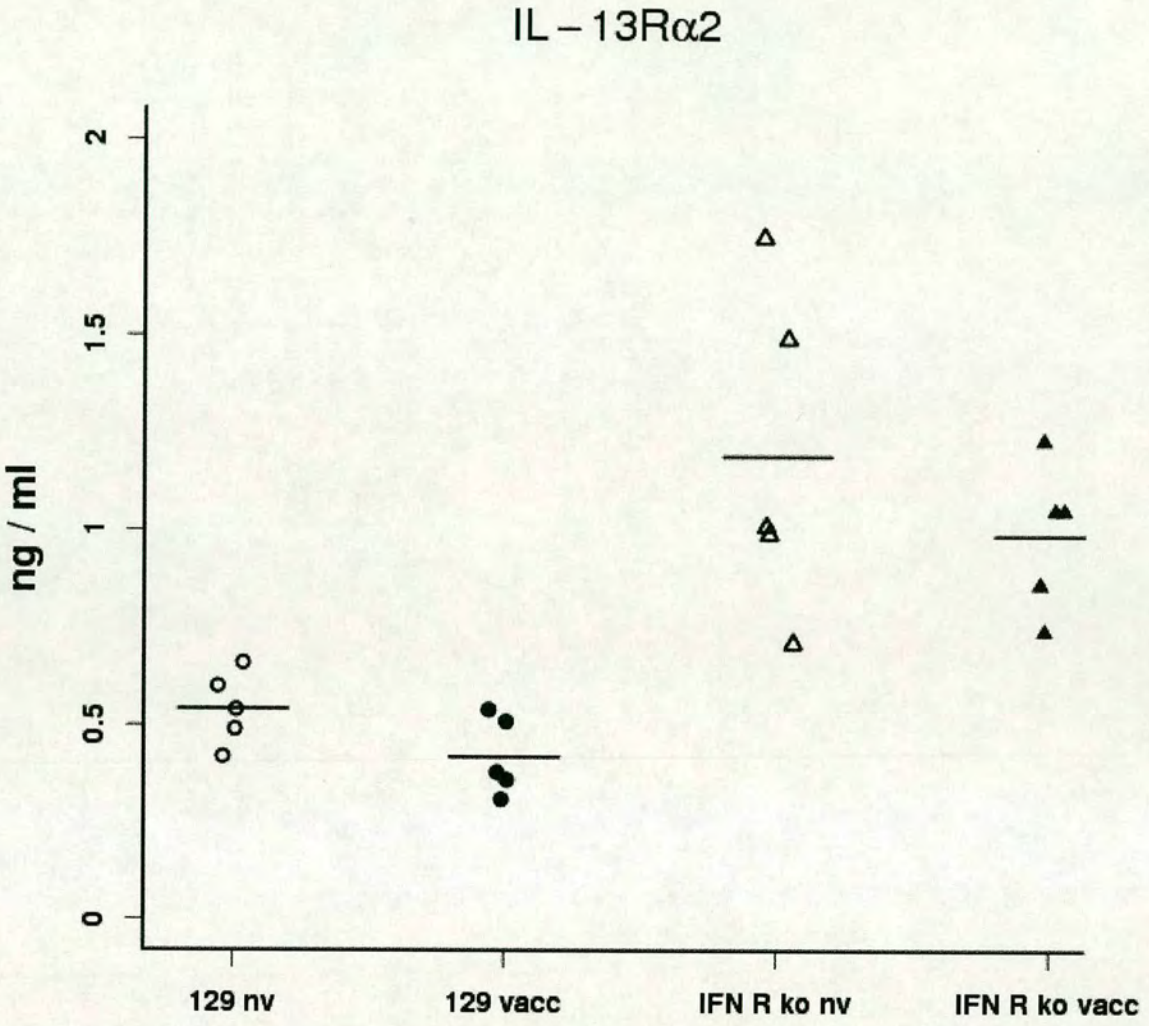

Figure 35: The level of the IL-13 decoy receptor (IL-13Rα2) is higher in the BAL fluid of IFN-γR deficient mice. Using ELISA (Mentink-Kane *et al.*, 2004) we measured the level of soluble IL-13Rα2 and found that vaccination did not affect levels but absence of the IFN-γ receptor did. The IFN-γR deficient mice had approximately double the amount of IL-13Rα2 protein in BAL fluid than that seen in WT mice. Each point represents an individual mouse with the bar showing the mean.

6.2.11. The expression of IL-13Rα2 mRNA does not reflect the protein level detectable in BAL fluid

The pattern of IL-13Rα2 expression measured using real time RT-PCR did not reflect the ELISA data we obtained from the BAL fluid. In the WT animals (Figure 36A) there was expression of mRNA for this receptor prior to vaccination in both the BAL cells (Figure 36A) and the lung tissue (Figure 36B) and levels were significantly increased after vaccination in both compartments examined. In contrast there was no expression of mRNA for IL-13Rα2 in either compartment in the IFN-γR deficient mice (Figure 36A and 36B). Although there was a small trend towards an increase after vaccination in the IFN-γR deficient animals this was much lower than that seen in the WT animals. The smaller rise in IL-13Rα2 message in the IFN-γR deficient animals may reflect a reduced requirement for expression because of the higher level of receptor protein in these animals.

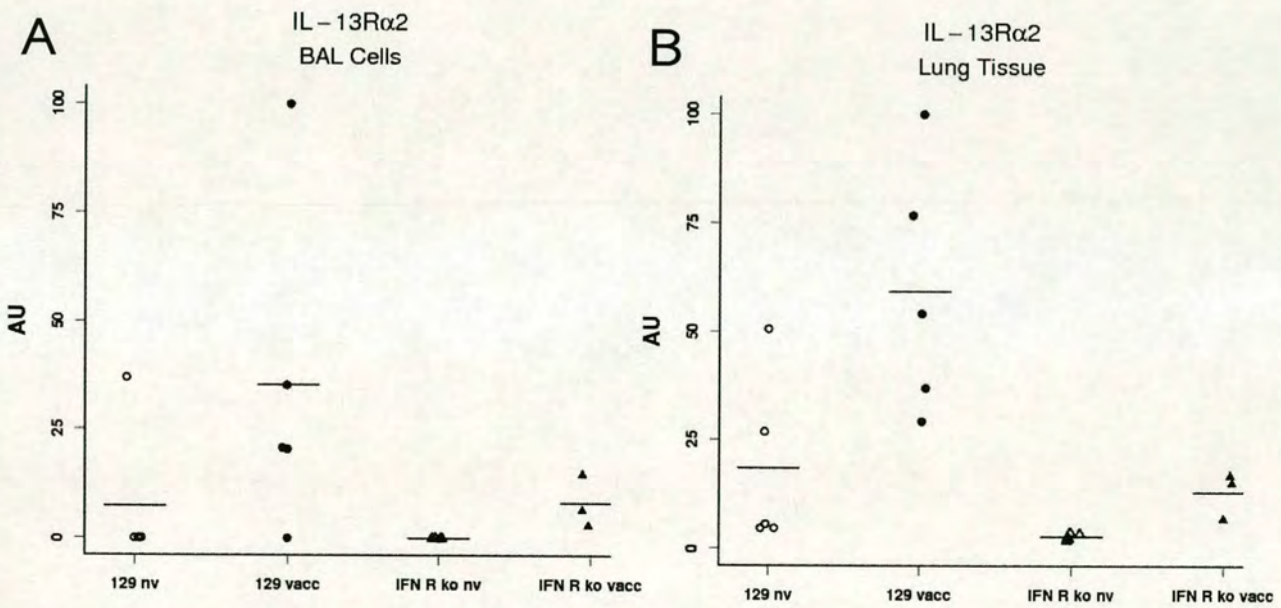

Figure 36: Real time RT-PCR reveals that the expression of IL-13Rα2 in the BAL cells (A) and the lung tissue (B) do not reflect the protein levels found by ELISA. Each point represents an individual mouse and the horizontal bar indicates the mean.

6.3. Discussion

Vaccination with irradiated *S. mansoni* cercariae leads to an effector response on infection that physically blocks the onward migration of the parasite through the lung vasculature and is heavily dependent on a Th1 response (Smythies *et al.*, 1992), (Wilson *et al.*, 1996) although there is a requirement for a Th2 response also (Anderson *et al.*, 1998), (Mountford *et al.*, 2001). This blockage is the result of an infiltrate of immune cells that gather round the lung stage schistosomulae upon challenge infection (Coulson & Wilson, 1988). Alveolar macrophages in this system upregulate Ym1 after vaccination and challenge, and this finding led us to examine the impact of the Th1 cytokine IFN-γ on this hitherto Th2 associated protein. We found that there were differences in the Fizz1 and Ym1 responses after vaccination in C57BL/6 and BALB/c mice and that, contrary to our expectations, the expression of both Fizz1 and Ym1 but not Arginase1 were lower in IFN-γR deficient mice than in their WT counterparts suggesting that IFN-γ signalling was needed for the optimal expression of these Th2 proteins.

6.3.1. Alveolar macrophages may function in tissue repair rather than as anti-parasite effector cells

Our previous work and the work of others has shown that macrophages and other APCs are the main sources of Fizz1 and Ym1 in response to parasite infection (Loke *et al.*, 2002), (Raes *et al.*, 2002), (Nair *et al.*, 2005). Macrophages are the main cell type in the airways of mice both prior to and after vaccination and alveolar macrophages vigorously express Ym1 after vaccination (Figure 32). In the post vaccination context the main insult to the lung tissue is the presence of schistosomulae in the airways (Crabtree & Wilson, 1986). These schistsomulae are surprisingly unaccompanied by any cellular reaction until sometime after the parasites entry into the lungs (Crabtree & Wilson, 1986) even in the face of an inflammatory reaction which may be due to tissue damage (Mastin *et al.*, 1985). In contrast intravascular challenge larvae are met with a rapid and robust cellular response with lymphocytes and monocytes / macrophages invading the interstitial space between the alveolar epithelium and the vascular endothelium. The macrophages in these focal aggregates contained para-crystalline inclusions which were subsequently identified as Ym1 (Crabtree & Wilson, 1986), (R.A Wilson – pers comm) indicating an alternative activation phenotype. Thus the role of the macrophages in the both the post vaccination and post challenge effector response may not be to directly attack the trapped larvae but to provide Ym1, and perhaps other molecules which would help in tissue repair or on the binding of inflammatory foci tightly around the parasite.

Alveolar macrophages have been shown to have schistosomulicidal activity in vitro (Xu & Xu, 1991) and macrophages activated by IFN-γ upregulate iNOS and produce NO. This is one mechanism by which alveolar macrophages could cause the death of schistosomulae as they pass through the lung. There is no evidence for direct NO effector activity in the response after vaccination (Coulson *et al.*, 1998) although this molecule may function in the modulation of the immune response (James *et al.*, 1998). Alveolar macrophages are not the only potential source of NO as endothelial cells are able to synthesise this molecule via the enzyme endothelial nitric oxide synthase (eNOS) (Thippeswamy *et al.*, 2006), (Dudzinski *et al.*, 2006). Thus there is a potential second source of NO from endothelial cells which could mediate damage to schistsomulae as they pass through the blood vessels. However it has been reported that IFN-γ, TNF-α and IL-1β (all Th1 associated cytokines) lead to a reduction in eNOS expression in pulmonary artery endothelial cells (Zhang *et al.*, 1997b). This suggests little role for NO derived from alveolar macrophages or other sources in the effector mechanism against lung stage schistosomulae after vaccination. This is consistent with the finding that schistosomulae trapped within the effector foci which develop in the lungs show little signs of physical damage (Crabtree & Wilson, 1986).

6.3.2. *Eosinophil recruitment after vaccination may contribute to healing*

The other immune effector cell recruited into the lungs after vaccination is the eosinophil (Tables 4 & 5). In contrast to data on the cellular population after vaccination and challenge (Wilson *et al.*, 1996) IFN-γR deficient mice recruited less eosinophils that their WT counterparts (Table 5) most likely as a result of reduced IL-5 (Figure 34). The findings that ablation of the eosinophil response with anti-IL-5 does not affect resistance to challenge infection (Sher *et al.*, 1990) and that large numbers of eosinophils are recruited in vaccinated and challenged IFN-γR deficient mice but that these mice have greater worm burdens (Wilson *et al.*, 1996) argues against a role for eosinophils in a direct effector role against the parasite or that eosinophils are required to set up the immune reaction effected by this vaccination system.

Eosinophils could however represent part of the inflammatory response caused by the entry of RA schistosomulae into the alveoli. The role of eosinophils in tissue repair was discussed briefly in Chapter 4, in particular their role as producers of TGF proteins. In the context of the lung, eosinophils have long been linked to the pathophysiological processes at work in bronchial asthma and in particular the contribution of granule associated basic proteins, reactive oxygen species and lipid mediators to lung damage (Walsh, 2001). However the putative role for eosinophils in tissue repair indicates that they may also contribute to the repair of damaged lung tissue and indeed there is a

reduction of airway eosinophil numbers and markers of tissue remodelling (including collagen precursors and extracellular matrix proteins) in IL-5 deficient and IL-5 receptor deficient mice subjected to an allergic asthma protocol (Cho *et al.*, 2004), (Tanaka *et al.*, 2004) but these markers of remodelling are upregulated in IL-5 overexpressing mice (Tanaka *et al.*, 2004). Thus the eosinophilia seen in response to RA schistosomulae in the lungs could be partly responsible for the repair of the damage induced in the alveolae.

Recent evidence suggests the eosinophils are a source of both IL-4 and IL-13 (Gessner *et al.*, 2005). Whilst we found that the level of IL-13 in the WT and IFN-γR deficient mice was equivalent both before and after vaccination this does not rule out a role for IL-13 from eosinophils contributing to a local interaction to drive Ym1 and Fizz1 expression. Thus the lower eosinophil numbers in the IFN-γR deficient mice may result in lower eosinophil derived IL-13 leading to reduced Fizz1 and Ym1.

6.3.3. *Reduced Ym1 and Fizz1 may indicate reduced lung damage in IFN-γR deficient mice*

Ym1 and Fizz1 have been identified in many tissue injury contexts including lung damage (Hung *et al.*, 2002), (Li *et al.*, 2005), (Wagner *et al.*, 2004), (Nair *et al.*, 2005). One possible explanation for the reduced expression of these proteins in IFN-γR deficient mice is that lack of IFN-γ responsiveness would lead to a lower level of Th1 mediated immune damage and thus the requirement for proteins involved in tissue repair would be reduced. In this study we have shown that IFN-γR deficient mice do not upregulate iNOS in response to vaccination. There is also a lower level of TNF production in IFN-γR deficient mice than in WT mice in response to challenge after vaccination (Wilson *et al.*, 1996). These two findings point to a depressed Th1 immune response and suggest that IFN-γR deficient mice may suffer less inflammatory immune mediated damage than WT mice after lung trauma. The movement of irradiated schistosomula into the alveoli results in an inflammatory response (Mastin *et al.*, 1985). As a result of impaired Th1 macrophage activation less tissue damage may occur and tissue repair proteins like Fizz1 and Ym1 may not be induced to the levels seen in WT mice.

Despite the lower levels of Ym1 and Fizz1 the expression of Arginase1, a marker of Th2 activation was identical in the BAL cells of both wild type and IFN-γR deficient mice. Arginase1, whilst an established marker of alternative macrophage activation can also be upregulated by IL-10 (Corraliza *et al.*, 1995). Whilst in the wound healing model we saw a strong dependence on IL-4 for Arginase1 production in the RA cercariae vaccination model we are looking later after trauma and thus the cytokines induced at this point may drive Arginase1 production. Arginase1 has an established role in

wound healing (Witte *et al.*, 2002) and although IFN-γ is known to suppress Arginase1 expression (Hesse *et al.*, 2001) the presence of lung damage would require the upregulation of proteins involved in healing including Arginase1. Indeed in a model of pulmonary fibrosis arginase1 and 2 were found to play an important roles (Endo *et al.*, 2003). The finding that Arginase1 expression, and indeed the Th2 response in general, is not higher in the IFN-γR deficient mice might indicate that this response is limited to facilitate repair but not predispose the tissue to fibrosis. This suggestion is supported by our observation of reduced IL-5 and a reduced eosinophil response in the IFN-γR deficient mice. If eosinophils can potentiate debilitating tissue remodelling then their activity would be expected to be tightly regulated no matter what the source of pulmonary damage.

6.3.4. *IFN-γ may control Th2 associated proteins through the IL-13Rα2 receptor*

The data presented above, illustrating a raised level of IL-13Rα2 in the absence of IFN-γ signalling suggests that the the activity of IL-13 may be regulated by IFN-γ through modulation of the IL-13 decoy receptor levels. IL-13 is a very profibrogenic cytokine, much more potent than IL-4 in this regard and has been shown to promote lung fibroblast growth, a property enhanced by IL-1β (Ingram *et al.*, 2004) and an important component of the healing response in tissue damaged by schistsomulae. The potentially damaging pro-fibrotic effects of IL-13 are tightly regulated through the IL-13Rα2 receptor (Wynn, 2004). Binding of IL-13 to this receptor leads to internalisation but not signalling (Kawakami *et al.*, 2001) and thus IL-13Rα2 can decrease the activity of IL-13. Indeed lack of the receptor leads to higher IL-13 activity (Wood *et al.*, 2003). There is good evidence that IFN-γ is involved in the control of IL-13Rα2 levels. Daines and Hershey found that in human cells IFN-γ can positively control IL-13Rα2 levels by allowing the release of intracellular stores (Daines & Hershey, 2002). However Wynn et al found that in a murine system the presence of P-selectin decreased the level of IFN-γ and this increased the level of IL-13Rα2 and inhibited IL-13 mediated fibrotic damage (Wynn *et al.*, 2004a). The observations presented here suggest that an absence of IFN-γ signalling leads to increased IL-13Rα2 and would support the data of Wynn et al i.e. decreased IFN-γ signalling results in higher levels of the decoy receptor. The level of IL-13Rα2 was unaffected by vaccination in both groups of mice. Therefore it is the presence or absence of IFN-γ responsiveness in the system rather than a response to the schistosomula which drives the increased expression of the decoy receptor. The higher IL-13Rα2 level in the IFN-γR deficient mice may then be a means of keeping the potentially damaging effects of IL-13 under control in the absence of a Th1 response. Two observations support a role for the IL-13Rα2 decoy receptor. Firstly naïve IFN-γR deficient mice expressed lower Ym1 than WT 129 mice

consistent with their higher IL-13Rα2 levels (Figure 35). Secondly Arginase1 was unaffected by a lack of IFN-γ signalling and was found to be more strictly IL-4 dependent in BAL cells after *N. brasiliensis* infection (Figure 19C) than Fizz1 or Ym1.

The real time RT-PCR data we present for IL-13Rα2 levels confound the ELISA results in that the message for IL-13Rα2 rises in response vaccination in both 129 WT mice and IFN-γR deficient mice. This suggests that there is a degree of post translational control and despite the response at the level of message the level of available receptor protein does not change.

IFN-γ may also directly control the levels of Fizz1 and Ym1, proteins previously thought to be Th2 controlled. Lack of IFN-γ has been shown to downregulate the wound healing process in the Calu-3 human lung epithelial cell line and lack of IFN-γ led to less migration along an artificial wound front. Reduced migration was also seen when IL-4 and / or IL-13 were added to the system. This effect was reversed by addition of IFN-γ (Ahdieh *et al.*, 2001). Ym1 may play a role in the migration of cells into damaged areas through its ability to bind ECM components (Hung *et al.*, 2002). Thus the lack of IFN-γ in the Calu-3 model may be a result of a loss of cues, Ym1 being among them to guide cells along the wound front.

6.3.5. *Differences in C57BL/6 and BALB/c immune responses*

BALB/c mice have been characterised as biased towards a Th2 response. Thus it was somewhat surprising to find that despite elevated levels of Th2 cytokines compared to C57BL/6 mice the expression of both Fizz1 and Ym1 was comparatively lower in BALB/c mice after vaccination. Interestingly the reduced Fizz1 and Ym1 expression was seen in the context of a robust IL-13 response in the BALB/c mice which was missing from C57BL/6 mice. Thus in contrast to our data from IFN-γR deficient 129 mice BALB/c mice display reduced Fizz1 and Ym1 responses in the face of increased IFN-γ activity. It has been reported that BALB/c mice are less susceptible to pulmonary fibrosis and that this phenotype correlates with reduced expression of Arginase1 from lung tissue (Misson *et al.*, 2004). Thus despite their Th2 bias BALB/c mice perhaps have a greater control over the potentially fibrotic aspects of the Th2 response than C57BL/6 mice perhaps through modulation of this response with Th1 cytokines. Unfortunately the level of IL-13Rα2 was not investigated in this experiment. If levels of this receptor were raised then it would support our argument that modulation of IL-13 activity leads to changes in Fizz1 and Ym1 responses in tissue damage. If however the IL-13Rα2 receptor expression was equivalent to or lower than that seen in C57BL/6 mice then it would suggest the IFN-γ can directly control Fizz1 and Ym1 in the lung.

As discussed previously IL-10 is capable of modulating both the Th1 and Th2 responses and it has been reported that resistant BALB/c mice make a higher IL-10 response after *Litomosoides sigmodontis* infection than susceptible C57BL/6 mice (Le Goff *et al.*, 2002). Since the Th2 cytokine response of BALB/c mice is greater than that of the C57BL/6 mice and IL-10 suppresses cytokine production (Grutz, 2005), an increased IL-10 response seems unlikely to be the reason for the lower Th2 cytokine and IFN-γ driven gene expression we have found in BALB/c mice. Other differences that could arise between these strains include an decreased sensitivity to Th2 cytokines in BALB/c mice. Thus more IL-4 or IL-13 may be required for a lesser effect.

6.3.6. Conclusions

- Fizz1 and Ym1 are expressed in a trematode model.
- Alveolar macrophages express Ym1 after vaccination with irradiated *S. mansoni* cercariae.
- The upregulation of Fizz1 and Ym1 may be related to tissue repair rather than a direct anti-parasite response.
- IFN-γ may modulate Fizz1 and Ym1 expression through IL-13Rα2 levels and thus IL-13 activity.
- The regulation of the Fizz1 and Ym1 response in the lung after vaccination differs in C57BL/6 and BALB/c mice.

Chapter 7 - Discussion

In this thesis we have shown that Fizz1 and Ym1 are upregulated in response to tissue damage and both nematode and trematode infection. In Chapter 3 we used our well characterised *B. malayi* implant system to show that maximal and sustained expression of both Fizz1 and Ym1 rely on the adaptive immune response. Early expression in this system may be driven by an innate requirement to repair tissue and in Chapter 4 we showed that this injury response is highly dependent on the Th2 cytokine IL-13. We broadened the scope of Fizz1 and Ym1 expression in helminth infection in Chapter 5 showing that these genes are expressed in an acute infection and that mouse strain differences may underlie contradictory expression patterns we have seen in the past. Finally in Chapter 6 we examined the expression patterns of these genes in response to a trematode model system and demonstrated a surprising dependence on IFN-γ for full expression in this system.

7.1. In *B. malayi* infection macrophage derived Ym1 and Fizz1 may be important in the effector response

In the *B. malayi* implant system, *N. brasiliensis* infection and the *S. mansoni* vaccination model we have shown that macrophages produce large quantities of Ym1 by immunostaining. In terms of an anti-parasite effector response the implications of these findings are not yet known. Certainly, as discussed in chapters 5 and 6, the pulmonary responses seen in *N. brasiliensis* infection and *S. mansoni* vaccination may relate more to tissue repair than to parasite control. Data from the *B. malayi* implant model suggests that an adaptive Th2 response is required to maintain high levels of Fizz1 and Ym1 expression and there is a large increase in the protein levels for both, co-incident with the timing of the adaptive response becoming active.

However a role for these proteins in parasite killing is not readily indicated as even after this adaptive response becomes active *B. malayi* in the peritoneal cavity seem to be unaffected; microfilariae are produced and the worms recovered from the peritoneal cavity at day 21 seem to be viable. It is possible that the Ym1 response we find in NeMΦ is related to the repair of damage caused by the parasite in the peritoneal cavity. There is a precedent for this hypothesis in that Ym protein (the distinction between Ym1 and Ym2 could not be made) is continually expressed in a chronic but futile regenerative response in damaged olfactory epithelium (Giannetti *et al.*, 2004). Interestingly the dynamics of Ym protein production in the injured olfactory epithelium are similar to those seen in the *B. malayi* model with a large increase in Ym1 protein seen a week after the initial injury (Giannetti *et al.*, 2004).

However the requirements for Th2 cytokines in the sustained Ym1 expression in the olfactory injury model are unknown and the source of the Ym1 was not macrophages but epithelial cells.

Work done on the kinetics of murine *Brugia pahangi* infection may shed some light on an effector response involving Ym1. Intraperitoneal injection of *B. pahangi* L3 gives rise to adult worms and microfilaria production in the peritoneal cavity and to a cellular response dominated by macrophages. By 5-6 weeks post infection multinucleated macrophages, eosinophils and fibroblasts were closely associated with the worms and microfilariae and there was deposition of collagen around the parasite (Mackenzie *et al.*, 1985b). In the *B. malayi* implant system microfilaria at 21 days display patchy staining for Ym1 (Figure 5) and some worms recovered from the peritoneal cavity at this timepoint which have granulomatous material deposited around them stain strongly for Ym1 (Figure 6). However we have not been able to demonstrate a close association between macrophages and microfilaria or adult worms. Nonetheless a mechanism such as that described above, involving the isolation of the parasite is highly reminiscent of tissue repair and could well involve Ym1.

The role for Fizz1 in the adaptive response to *B. malayi* in the peritoneal cavity may be similar to that proposed for Fizz2 in infection with gastrointestinal nematodes (Artis *et al.*, 2004). In our implant model we do not see *B. malayi* in the bloodstream of mice and this is also the case in murine infection with other *Brugia* species (Mackenzie *et al.*, 1985a). The lifecycle of *B. malayi* involves a haematophagus arthropod vector and so microfilaria must be able to migrate from the lymphatics to the bloodstream to complete the lifecycle. In the murine implant model the peritoneal wall provides a barrier to microfilarial entry into the lymphatic system but not a complete barrier as there are lymphatic stomata scattered throughout the peritoneal wall (Shinohara, 1997), (Azzali, 1999). These stomata are small at around 5μm (Azzali, 1999) but it may be possible for *B. malayi* microfilariae to migrate into the lymphatic vessels through these openings. Following the hypothesis put forward by Artis et al (Artis *et al.*, 2004), Fizz1 may act to block sensory organs on microfilaria an prevent migration from the peritoneal cavity to the lymphatics. The ability of Fizz1 to modulate the phenotype of various cells (Holcomb *et al.*, 2000), (Blagoev *et al.*, 2002), (Liu *et al.*, 2004b) also indicates that this protein may have a role in the differentiation of either macrophages or another cell type in response to *B. malayi* infection.

7.2. Tissue repair and the immune response

The finding that Fizz1 and Ym1 are upregulated early in the response to surgical trauma (Chapter 4) indicates that these proteins are important in this response. The immune system is involved

immediately and intimately in the response to a wound (Park & Barbul, 2004) when signals from platelets and damaged endothelium allow circulating neutrophils to attach to and move through the vessel walls in a process called diapedesis (see (Granger & Kubes, 1994)). As well as engaging in phagocytosis these neutrophils upregulate genes for signalling molecules including those that bring macrophages into the wound (e.g. macrophage inflammatory protein 1α – MIP-1α, monocyte chemotactic peptide-1 – MCP-1), growth factors (e.g. vascular endothelial growth factor – VEGF-A) and other cyokines (e.g IL-8) (Gillitzer & Goebeler, 2001). Thus the initial immune response sets the tone for not only subsequent immune effector recruitment but also for repair of tissue and the growth of cells.

The arrival of macrophages signals the transition from the inflammatory phase of wound repair to the proliferative phase. Initially macrophages finish off the job of wound debridement and phagocytose apoptotic neutrophils and other dead or dying cells. Macrophage infiltration into the wound is a key step in proper healing and if prevented there is a failure in debridement and a fibrotic response (Leibovich & Ross, 1975). Macrophages also modulate both the immune and cellular responses secreting molecules which are anti-inflammatory and which promote the migration of fibroblasts and endothelial cells (e.g. transforming growth factor-α and β – TGF-α / β, VEGF-A, platelet derived growth factor – PDGF) into the wound area (Mutsaers *et al.*, 1997). This mixture of macrophages, fibroblasts and endothelial cells is termed granulation tissue (Werner & Grose, 2003). Whilst the endothelial cells go about the process of vascularisation, fibroblasts lay down the collagenous network required to provide a bed for the repaired tissue (Midwood *et al.*, 2004). Some of these fibroblasts differentiate into myofibroblasts and begin the process of matrix contraction to bring the wound edges together. These resident cells then apoptose leaving a collagen rich scar which is slowly remodelled over time.

The finding that Ym1 protein is expressed in the absence of Ym1 mRNA in IL-4Rα deficient mice but that these mice seem to heal effectively indicates that either sustained Ym1 expression is not required for healing or that Ym1 is dispensable for effective healing. This latter hypothesis is likely to be the case since healing is a basic requirement for all organisms and it is likely to be multiply redundant and not rely on any one pathway.

7.3. Sources of pre-formed Ym1

The finding that Ym1 mRNA expression is ablated in IL-4Rα deficient mice but not in IL-4 deficient mice indicates that the expression of Ym1 message requires signalling through this receptor and that

IL-13 is the cytokine driving expression (Chapter 4). The identification of Ym1 on or in platelets in Chapter 5 indicates that these cell fragments may be a source of pre-formed Ym1. Platelets are a rich source of platelet derived growth factor (PDGF) and TGF-β which play an important roles in early tissue repair (Hosgood, 1993). It would be expected that they would also contain other molecules involved in the early repair events such as Ym1. The finding of pre-formed Ym1 in platelets merits further investigation. The other cell type which is a candidate for containing pre-formed Ym1 is the neutrophil. Ym1 has been identified in immature neutrophils during haematopoesis (Hung *et al.*, 2002) and in the granules of neutrophils in aged mice and a mouse model of chronic granulomatous disease (Harbord *et al.*, 2002). The formation of Ym1 during haematopoesis may not require type 2 cytokines and so expression of Ym1 protein may proceed normally during the early phases of wound healing.

7.4. The immune system in fibrosis

The process of wound healing bears many similarities to the processes involved in fibrotic diseases which can be the result of over active immune responses. After large wounds or if there is a failure in the healing process a build up of matrix in either the scar tissue or healthy tissue can result in a loss of normal architecture and function. This is the process which leads to liver pathology in schistosomiasis. Liver pathology of this type is thought to involve macrophages and they are present in *S. mansoni* induced granuloma, mycobacterial granulomatous lesions (Sandor *et al.*, 2003) and in liver lesions after carbon tetracholride (CCl_4) dosing (Duffield *et al.*, 2005). The role of macrophages in the latter CCl_4 model illustrates the double edged nature of immune cell involvement in healing. In this model if macrophages were depleted once the damage phase was underway there was less scarring and destruction of tissue architecture; if, however, macrophages were depleted during the recovery phase then there was a failure to clear the matrix material and increased tissue damage (Duffield *et al.*, 2005). Thus although there is an important role for the immune response after trauma this response can lead to fibrosis and tissue damage. These findings essentially echo those of Leibovich on the role of macrophages in cutaneous wounds (Leibovich & Ross, 1975) and underscore the double edged nature of immune involvement in tissue repair.

The importance of the AAMΦ phenotype in *S. mansoni* granuloma formation was underscored by studies showing that IL-4/IL-10 doubly deficient mice had smaller pulmonary granulomas after i.v. injection of *S. mansoni* eggs than WT mice. This correlated with reduced Arginase1 activity in the lung tissue (Hesse *et al.*, 2001). Furthermore the same authors showed that inhibition of ODC, the enzyme responsible for the conversion of ornithine to polyamines, increased the fibrosis associated with granuloma (Hesse *et al.*, 2001). This effect was thought to arise because the inhibition of ODC would

preferentially shift ornithine metabolism to the OAT pathway thus generating more proline and providing more precursor for collagens and a fibrotic response. The generation of proline in macrophages was shown to be dependent on the Th2 cytokine driven level of Arginase1 (Hesse *et al.*, 2001).

7.5. The importance of IL-13 in fibrosis

We have shown that the expression of the Th2 associated genes Fizz1 and Ym1 can be driven by both IL-4 and IL-13. In the *B. malayi* implant model the upregulation of these genes in IL-4 deficient BALB/c mice is driven by a compensatory IL-13 production by the spleen (Nair, 2003) and in vitro both IL-4 and IL-13 can drive Fizz1 and Ym1 expression in macrophages (Nair *et al.*, 2003). In the early response to surgical trauma whilst IL-4Rα signalling is required for the expression of Fizz1 and Ym1 mRNA, IL-4 is not (Figures 11 & 13), implying that IL-13 controls this expression. The involvement of these genes in the early response to a surgical wound and the strong positive staining of granulomatous material in the lungs of *N. brasiliensis* infected mice (Figure 21) indicate that Ym1 has a role in the processes of tissue repair and perhaps granuloma formation. The involvement of IL-13 in granuloma formation and fibrosis is well established through both *S. mansoni* infection (Chiaramonte *et al.*, 1999), (Chiaramonte *et al.*, 2003), (Mentink-Kane *et al.*, 2004) and pulmonary models (Kolodsick *et al.*, 2004), (Belperio *et al.*, 2002). IL-13 is a key regulator of fibroblast activity, promoting collagen manufacture by these cells (Wynn, 2004). Fizz1 is also involved in fibroblast regulation promoting the differentiation of fibroblasts to myofibroblasts (Liu *et al.*, 2004b) and the persistence of these cell types can promote fibrosis instead of healing (Wynn, 2004).

The data presented in chapter 6 indicate that the level of Fizz1 and Ym1 expression can be controlled through IL-13Rα2 modulation of IL-13 activity. The IL-13Rα2 receptor has been considered a decoy receptor for IL-13 since its discovery. The initial characterisation of this receptor as a soluble receptor found in body fluids (Zhang *et al.*, 1997a) together with its short cytoplasmic domain (Donaldson *et al.*, 1998) suggested that it functioned to mop up excess IL-13 and prevent pathology such as fibrosis arising. This role for IL-13Rα2 was supported by studies showing increased IL-13 activity when IL-13Rα2 was absent (Wood *et al.*, 2003) or a decreased fibrotic response when the receptor was present (Chiaramonte *et al.*, 1999), (Wynn *et al.*, 2004a), (Mentink-Kane *et al.*, 2004). However recent work indicates that IL-13 signalling though IL-13Rα2 can promote the expression of TGF-β a key mediator of fibroblast function. IL-13 upregulated IL-13Rα2 on the surface of monocytes by a STAT-6 dependent mechanism. The transcription factor activation protein-1 (AP-1) then activated the transcription of TGF-β and promoted collagen production (Fichtner-Feigl *et al.*, 2006). It should be

noted that the increase in the fibrotic response in the study by Fichtner-Feigl et al was brought about by cell surface expression of IL-13Rα2 whereas the reduction in fibrosis in the studies by Chiaramonte et al and Mentink-Kane et al were the result of soluble IL-13Rα2 mopping up IL-13. Thus IL-13Rα2 may function in both an anti and pro-fibrotic capacity.

The data presented in this thesis has unravelled some of the early events that shape the NeMΦ phenotype and also illuminated the involvement of the Th2 associated proteins Fizz1 and Ym1 in early wound repair. Furthermore we have shown that IL-13 is important for this early response to trauma. Surprisingly, given the previously Th2 dominated regulation of these two proteins we have also demonstrated a dependence on IFN-γ signalling. This regulation by the prototypical Th1 cytokine may however involve the modulation of IL-13 activity through changes in the level of the IL-13Rα2 acting as a decoy receptor for IL-13.

Appendix I - Intracellular Cytokine Staining for Fizz1 and Ym1

In order to provide a rapid method for separating the NeMΦ population from other cells in the peritoneal cavity and to more accurately determine the co-expression of other markers of alternative activation we attempted to use the antibodies raised against the Fizz1 and Ym1 peptides to carry out intracellular cytokine staining on the PECs from *B. malayi* implanted mice or PEC recruited into the peritoneal cavity using thioglycollate. After retrieval from the peritoneal cavity the cells were subjected to a standard intracellular cytokine staining protocol (Coligan *et al.*, 2002). In several experiments the profile of the total cellular population from the peritoneal cavity after *B. malayi* implant was the same (Figure A1.1 A shows a typical population). Having previously shown that the macrophage population expressed the highest levels of both Fizz1 and Ym1 we identified this popualtion using either forward scatter / side scatter characteristics or by positive staining for the the F4/80 antibody.

We then further analysed this population for positive staining for Fizz1 and Ym1. Unfortunately the high affinity of NeMΦ for IgG frustrated our efforts. Whilst we were routinely able to show a positive staining in NeMΦ for both proteins (Figure A1.1 B & C) we also found that these cells bound non specific antibody better than thioglycollate recruited cells (Figure A1.1 D) and were thus unable to demonstrate specificity. This was the case with both anti-Fizz1 and anti-Ym1 sera, purified IgG and despite the use of a range of blocking agents.

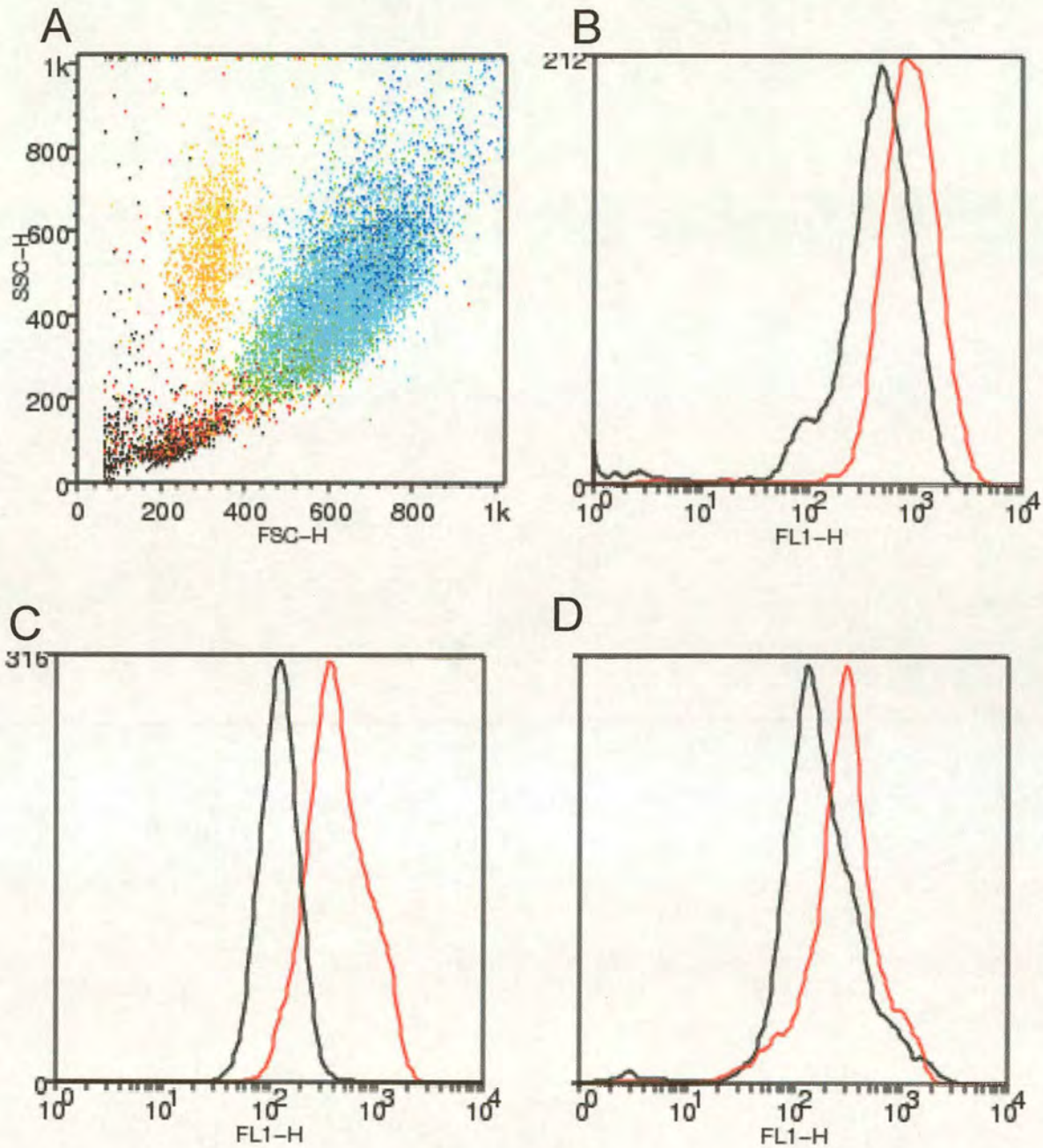

Figure A1.1: Intracellular cytokine staining of the PEC population recruited through *B. malayi* implant or i.p. thioglycollate injection. The peritoneal cell population contains a population of large cells which stain positively with the F4/80 antibody, indicated here by blues and greens (A). We gated on this F4/80+ve population and analysed these cells for positive staining with the anti-Fizz1 and anti-Ym1 antibodies (B & C respectively). Whilst *B. malayi* recruited cells (red curves) stained more intensely for Fizz1 (B) and Ym1 (C) than thioglycollate recruited cells (black curves), they also stained more intensely for non specific antibody (D).

Appendix II - Expression of Recombinant Ym1

Recombinant Ym1 protein was expressed using the pET system (Novagen) which utilises the viral T7 promoter to produce strong expression of the gene of interest. Using the primers detailed below we generated full length Ym1 DNA with Bam H1 restriction sites (bold text) inserted at each end to facilitate insertion into the expression vector.

Forward – 5' – TGGG***GGATCC***GTACCAGCTGATGTGCTACT – 3' positions 76 to 94 of Ym1

Back – 5' – GTAAA***GGATCC***TCAATAAGGGCCCTTGCA – 3' positions 1192 to 1209 of Ym1

The Ym1 gene contains a sequence that codes for a signal peptide (positions 12 to 75 of the Ym1 coding sequence - accession number M94584 NCBI Nucleotide database) and allows secretion of the protein in the mouse. In order to ensure the correct expression and folding of Ym1 in the bacterial system the primers did not include this signal sequence. Agarose gel electropheresis of the PCR product treated with Bam H1 yielded products of the expected length. Figure (A2.1) shows the structure of the construct.

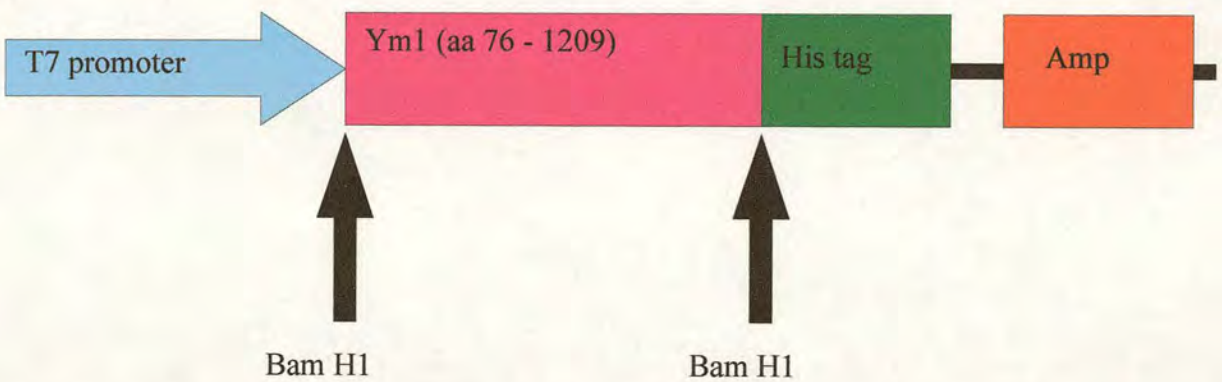

Figure A2.2: Using Bam H1 restricition sites Ym1 was inserted into the pET 21b expression vector. This vector allows expression to be driven through the T7 promoter and facilitates the fusion of a histidine tag to the carboxy terminus of the protein.

After checking that the Ym1 insert had the correct sequence and was inserted in the correct orientation we used this construct to transform BL21 DE3 *Escherichia coli* cells and induced expression of Ym1 by addition of IPTG to the culture. The culture was sampled prior to induction and at regular intervals

afterwards to determine whether Ym1 was expressed. Figure A2.2 shows that Ym1 protein was indeed expressed rapidly after the addition of IPTG to the culture media.

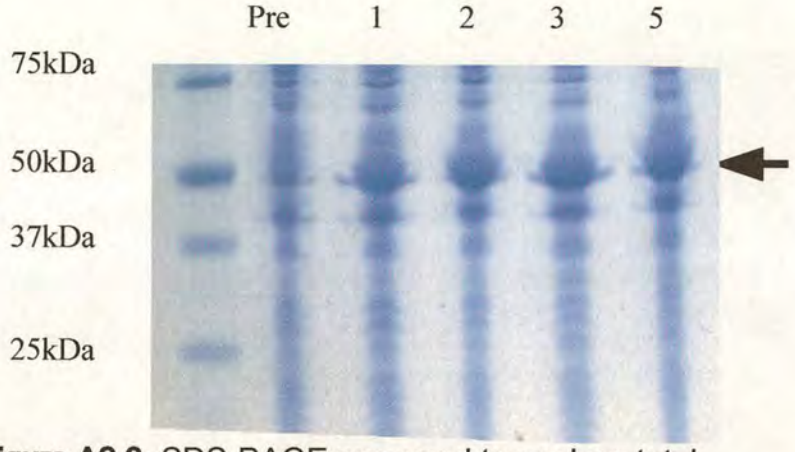

Figure A2.3: SDS-PAGE was used to analyse total proteins in BL21 DE3 cells before and at regular intervals after IPTG induction of protein expression. 1 hour after induction a large band appears at approximately 48 kDa (arrowed). This represents Ym1. Numbers across the top of the gel show hours after induction.

We examined the detergent soluble and insoluble fractions of the cells and found that most of the Ym1 protein was in the insoluble fraction (Figure A2.3). In order to try and improve the solubility of Ym1 protein we decided to try modulating the activity of the T7 promoter. In Bl 21 DE3 cells the activity of this promoter is controlled by the IPTG induction of the T7 RNA polymerase which has been incorporated into the genome of this bacterial strain and is under the control of the lac UV5 promoter. The addition of lactose or the lactose analog isopropyl-beta-D-thiogalactopyranoside (IPTG) results in production of the T7 RNA polymerase and this polymerase binds the T7 promoter site and the inserted gene is transcribed (Brown, 1996). The T7 promoter results in rapid and strong transcription of the inserted gene. One strategy we tried to reduce the rate of protein formation and therefore minimise the amount of Ym1 in the insoluble fraction of the cells was to lower the temperature of the culture at the point of induction from 37°C to 30°C. However neither this nor dropping the temperature further to 23°C had any effect.

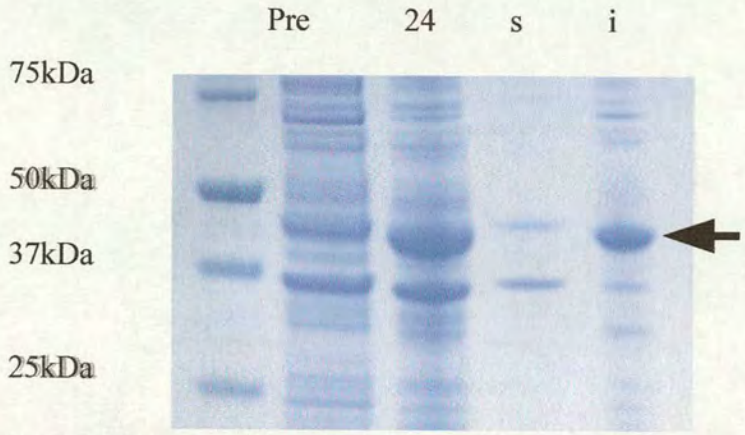

Figure A2.4: The expression of Ym1 in BL21 DE3 cells is mainly in the insoluble fraction (arrowed). Pre = before induction; 24 = 24 hours post induction; s = soluble fraction; i = insoluble fraction.

We also attempted to modulate the activity of the T7 promoter through chemical means. We transformed the BL21 AI (Invitrogen) *E. coli* strain. This bacterial strain carries the T7 RNA polymerase gene under the control of the arabinose-inducible *araBAD* promoter. The addition of different concentrations of arabinose to the culture media means that the level of T7 RNA polymerase and hence the activity of the T7 promoter can be tightly controlled. However despite different concentrations of arabinose Ym1 protein remained in the insoluble fraction of the cells. We purified the inclusion bodies from the cells using the B-PER detergent reagent (Pierce) and were able to solubilise this in a high concentration of urea. Although this was of no use for functional studies western blotting did show a strong reactivity for Ym1 in this solution and so it provided a useful positive control for the presence of Ym1.

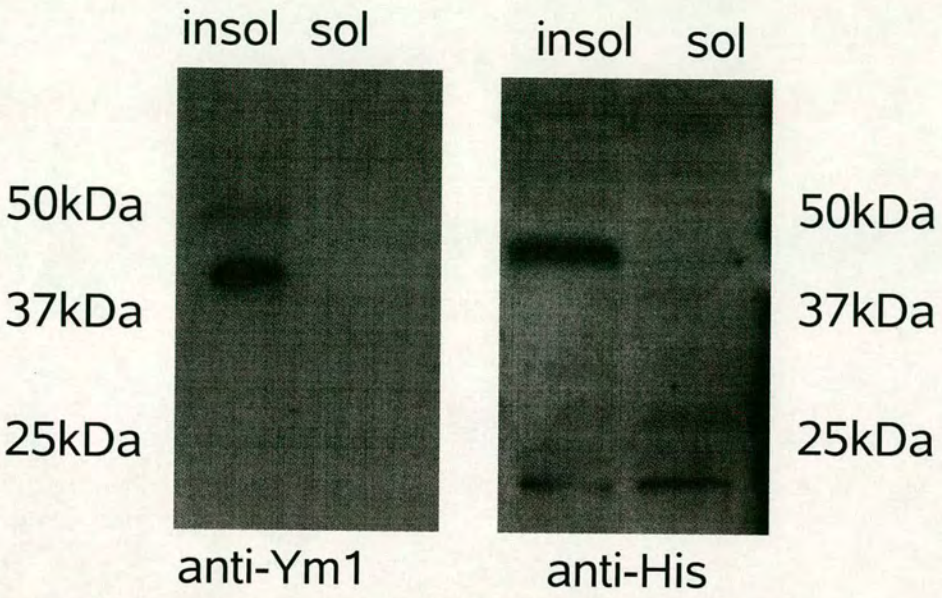

Figure A2.5: Western blotting using the anti-Ym1 (left) and anti-His (right) antibodies confirms that Ym1 expression is limited to the insoluble fraction of BL 21 AI cells after induction of protein expression.

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