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RT-PCR Analysis of Cytokine Expression in Murine Lymph Node and Lung Tissue Following Exposure to the Irradiated *Schistosoma mansoni* Vaccine.

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DEDICATION

This thesis is dedicated to my Mother and Father for their constant support, and to Lucy and Sonia for their friendship.

ABSTRACT

Vaccination of C57BL/6 mice with irradiated cercariae of *Schistosoma mansoni* results in the generation of up to 70% resistance to challenge infection. Protection is dependent on the development of $CD4^+$ Th1 cells in the skin draining LN, and recruitment of these cells to the lungs post-vaccination (Mountford *et al.,* 1992). The effector mechanism operates in the lungs and consists of the formation of foci, rich in $CD4^+$ T cells and macrophage, around migrating challenge parasites. Inflammatory cytokines play an important role in this model. Furthermore, resistance is IFNγ dependent, as demonstrated by a 90% abrogation of immunity upon ablation of this cytokine (Smythies *et al.,* 1992b).

The cytokine expression levels in lung and lymph node tissue following exposure to the irradiated vaccine have been investigated at a semi-quantitative and quantitative level by reverse transcription-PCR. Analysis of total RNA isolated from the skin draining LN after vaccination has demonstrated that an early Th1 response occurs, closely followed by increases in the expression of Th2-type cytokines. Following challenge of previously vaccinated mice, the level of IFNγ and IL-2 expression in LN tissue is much reduced. In contrast, IL-4 expression increases sharply above the level detected post-vaccination, displaying a strong anamnestic response.

Lung tissue shows a mixed Th1/Th2 response after vaccination with all cytokines under test peaking at day 21. Post-challenge, it is the inflammatory cytokines, such as IFNγ, TNFα, IL-12 and IL-1β, which increase to levels approaching those detected post-vaccination. Although Th2 cytokines are detectable, expression is much lower than before challenge resulting in a higher Th1:Th2 cytokine ratio than that observed following vaccination. The kinetics of the effector response were further analysed by the sampling of a synchronous challenge time course. The data obtained in this experiment confirmed the profiles of cytokine expression detected after percutaneous challenge. In addition, these samples showed significant expression of inducible nitric oxide synthase (iNOS), the enzyme responsible for nitric oxide production, at the time of peak inflammatory cytokine expression.

The induction responses were further investigated by the vaccination of BALB/c and C57BL/6 mice and the cytokine mRNA detected in lung and LN tissue of both strains compared. Results indicate that BALB/c mice show a tendency to develop a more Th2-biased cytokine response upon vaccination, compared to the mixed response seen in C57BL/6 animals. The higher Th2 cytokine profiles parallel the lower resistance measurements obtained for the former strain. In addition, vaccination of IFN$\gamma R^{-/-}$ mice has demonstrated that these animals show an increased Th2 cytokine profile compared to wild type animals of the same and C57BL/6 strains, corresponding to a reduction in resistance. The cytokine profiles obtained for all the above systems are described and related to immunity in the irradiated *Schistosoma mansoni* vaccine model.

CONTENTS

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

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ABBREVIATIONS

APC	Antigen presenting cell
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
BCG	*Mycobacterium bovis* strain Bacillus Calmette-Guerin
BSA	Bovine serum albumin
c.p.m	Counts per minute
CC	Challenge controls
cDNA	Complimentary DNA
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribose nucleic acid
dNTP	Deoxy nucleoside triphosphates
DTH	Delayed type hypersensitivity
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FCS	Foetal calf serum
fg	Femtograms
gDNA	Genomic DNA
GMEM	Glasgow minimal essential medium
HPRT	Hypoxanthine-guanine phosphoribosyl transferase
i.v.	Intravenous
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
KO	Knockout
krad	Kilorad
M	Molar
MHC	Major histocompatability
min(s)	Minute(s)
ml	Millilitres

mRNA	Messenger RNA
ng	Nanograms
NK	Natural killer
NO	Nitric oxide
NOS	Nitric oxide synthase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pg	Picograms
^{32}P	32-phosphorus
RNA	Ribose nucleic acid
RT	Reverse transcription
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SE	Standard error
SSC	Sodium chloride/sodium citrate solution
Tc	T cytotoxic cell
TE	Tris-EDTA
TES	Tris-EDTA-SDS
TGF	Transforming growth factor
Th	T helper cell
TNF	Tumour necrosis factor
µg	Micrograms
µl	Microlitres
UV	Ultra violet
VC	Vaccinated and challenged
WT	Wild type

CHAPTER ONE

Introduction

Part 1. Immunity to Schistosomiasis

1.1 Background to the Disease

Schistosomiasis is a widespread parasitic disease caused by trematode blood flukes of the genus *Schistosoma*. There are now 76 countries in which schistosomiasis is endemic, resulting in an estimated 200 million infected people (Cheever, 1993, Bergquist *et al.,* 1994) and 1 million deaths annually (Warren, 1989). Three main species of *Schistosoma* are responsible for infection in man: *S. mansoni* is prevalent in Africa, South and Central America; *S. japonicum,* which is limited to Asia; and *S. haematobium*, responsible for schistosomiasis in Africa and the Middle East. The first two species reside in the mesenteric veins of their host and release eggs into the gut tissues. From here the eggs pass through to the gut lumen and are excreted. The third parasite inhabits the vesical plexus of the bladder and eggs are released in the urine.

The schistosome life cycle involves primary mammalian and intermediate fresh water molluscan hosts. Free swimming miracidia hatch from eggs excreted into water, releasing the stage infective to snails. In the snail, asexual reproduction occurs and results in the release of a second free swimming stage, the cercaria, in response to light. Cercariae infect their primary host by skin penetration, upon which their swimming tails are shed and transformation into the migratory stage, the schistosomulum, takes place. These larvae migrate via an intravascular route, possibly using secretions from the acetabular and head glands to cross the dermis and access blood vessels, to the hepatic portal system or the bladder (Wilson, 1987). Once in the liver the worms mature, pair up and migrate to their final destination in the mesenteric veins of the gut (*S. mansoni, S. japonicum*), or vesical plexus of the bladder (*S. haematobium*). Here oviposition of 300 to 3000 eggs per day, depending on species, commences.

The presence of worm pairs in the host causes little pathology; it is the host's immune response against the eggs, of which over half get trapped in local tissues or

washed away with blood flow, which results in the characteristic pathology of the disease (Von Lichtenberg, 1987). In *S. mansoni* infections eggs are trapped in the pre-sinusoidal portal venules (Cheever, 1993), where the release of antigen from mature eggs results in an intense local inflammatory response and the formation of fibrous lesions, or granulomas (Boros and Warren, 1970). As egg burden in the liver increases with repeated high level infection, portal hypertension, hepatomegaly and oesophageal varices form, eventually leading to liver failure (Warren, 1972).

Various control measures have been implemented in attempts to clear infection in endemic populations (Mott, 1987). Stategies such as improving sanitation and health education have had limited success, mainly due to the economic costs involved. The use of molluscicides, besides damaging the local environment, is expensive and can lead to the development of resistant snails and reinvasion of a cleared area. There are chemotherapeutic drugs available for widespread treatment, and these have been effective in reducing prevalence in some areas (Mahmoud *et al.*, 1983). Drugs such as Praziquantal (broad range) and Oxamniquine (*S. mansoni* only) eliminate worm burdens but do not reverse existing pathology or prevent reinfection, and therefore successive doses are required to control infection (WHO Report, 1989). The cost and practical problems of repeated treatment make it impractical to control infection significantly in third world countries with chemotherapy. Hence the preferable long term solution is the development of an economically viable vaccine able to prevent infection with a single dose.

Research into potential vaccines is centred on the study of the host immune response to parasite antigen candidates and attenuated models. Although the use of a live vaccine in humans may have dangerous implications, an understanding of the immune mechanisms required for resistance can only aid in the design of an antigen-based vaccine. Development of age-related protective immunity does occur in human populations (Butterworth *et al.*, 1988), and the differences in reinfection levels of children and adults cannot simply be accounted for by exposure patterns alone (Butterworth, 1994). However, the mechanisms of human resistance are not known, and since manipulation of this system is impossible the majority of research is carried out on animal models.

1.2 Experimental Models of Schistosomiasis

Various animal models exist for the laboratory study of immunity to schistosomiasis. Different species are used depending on the nature of the research, each host having a distinct relationship with the parasite. Non-human primates provide a model close to that of human infection. However, for practical reasons, the majority of research is based on rodents. Both rats and mice can support human-infective species of *Schistosoma*, but mice are permissive hosts and display similar pathology to human infection (McLaren and Smithers, 1987). In contrast, rats are semi-permissive hosts and eliminate *S. mansoni* worms around 28 days post-infection. *S. mansoni* is the most prevalent of the human-infective species and its life cycle can be easily maintained in laboratory conditions. This makes schistosomiasis mansoni the most researched form of the disease. The following studies will focus exclusively on the immunity induced in the murine host following exposure to *S. mansoni*.

1.3 Infection of Mice with Normal Parasites

1.3.1 Migration and Development

Between 30% and 50% of the numbers of cercariae penetrating the skin will survive to reach maturity in the hepatic portal vasculature (Miller and Wilson, 1980). During penetration of the mammalian host skin the cercariae lose their tails and begin transformation into schistosomula. This process takes less than 1 hour to complete *in vivo* (Cousin and Stirewalt, 1981), and involves changes in respiration and tegument (outer membrane) structure (Hockley and McLaren, 1973). Once in the skin, the epidermal base is reached within 30 minutes (Wheater and Wilson, 1979) but the basement membrane provides a barrier which takes longer to cross (Wilson and Lawson, 1980). The majority of parasites locate and penetrate post-capillary venules and migrate to the lungs via the vasculature (Miller and Wilson, 1978). However, 10% to 20% leave the skin by entering the lymphatics and join the blood circulation via the thoracic duct (Wheater and Wilson, 1979, Mountford *et al.,* 1988). The

duration of stay in the skin is estimated, on average, to be between 2 and 5 days (Miller and Wilson, 1978).

By day 4 post-infection the first parasites can be found in the lungs (Miller and Wilson, 1980). During the next few days the schistosomula undergo a period of elongation accompanied by rhythmic extension and contraction (Wilson *et al.,* 1978), and loss of mid-body spines (Crabtree and Wilson, 1980), thought to aid passage through the lung capillary beds. The larvae exit the lungs through the pulmonary vein, are carried to the left ventricle of the heart and are distributed to the systemic organs in proportion to cardiac output (Wilson *et al.,* 1986). Those parasites reaching the hepatic portal system lodge there and mature into blood feeding adult schistosomes. Parasites distributed to other organs negotiate the capillary beds, return to the lungs and repeat the process of systemic migration. The trapping efficiency of the liver for schistosomula is 72% to 86% (Wilson and Coulson, 1986) and it is estimated, by portal perfusion, that the entire recoverable adult population can accumulate in two to three circuits of the blood system (Wilson, 1987). Accumulation in the liver is complete by day 21 post-infection (Wilson *et al.,* 1986). The signals responsible for termination of migration are probably environmental, the hepatic vessels having a low blood pressure and providing nutrient-rich surroundings (Wilson *et al.,* 1978). Between 28 and 35 days post-infection the parasites in the hepatic portal system pair and migrate to the mesenteric veins (Wilson, 1987); here egg laying begins with production of approximately 300 eggs per worm pair per day. The immune response resulting in the formation of granulomas around eggs trapped in the liver will be discussed in section 2.8.

1.3.2 Maturation

The fate of the 50% to 70% of parasites which penetrate the skin but fail to mature was the subject of fierce debate until the advent of autoradiographic tracking techniques. Extraction of skin phase parasites by mincing and incubation suggested that up to 65% of penetrating cercariae died in the skin (Clegg and Smithers, 1968, Smithers and Gammage, 1980). However, parasites could not be detected in the skin in significant numbers by histological methods after migration through this organ is complete (Wheater and Wilson, 1979). Furthermore, 63% of skin penetrants were

present in the lungs, as detected by quantitative histology 6 to 7 days post-infection (Mastin *et al.,* 1983). Compressed organ autoradiography proved to be the most accurate method for following the migration of previously labelled parasites, since whole organs can be assayed for radioactive counts without the need for a parasite extraction step. This technique demonstrated that up to 78% of skin parasites can be detected in the lungs by day 7 post-infection (Mangold and Dean, 1983) and, by day 14, 90% of penetrating parasites can be detected in organs other than the skin (Wilson *et al.,* 1986). Thus it would seem that the majority of parasite loss during primary infection of naive mice occurs after migration from the skin.

The lungs are now generally accepted as the major site of schistosomulum attrition in naive mice (Mangold and Dean, 1986). The number of parasites eventually maturing in the hepatic portal system is much lower than the number detected in the lungs during migration (Georgi *et al.,* 1983), and hence elimination could occur in the lungs, during migration to the liver or in the liver itself. The latter possibility was ruled out by injection of lung stage schistosomula directly into the hepatic portal system (Mangold *et al.,* 1986, Coulson and Wilson, 1988). High levels of maturation were obtained indicating that this organ provides a stable environment for worm development. Final evidence for the lungs being the major site of parasite loss came with whole body autoradiography (Wilson *et al.,* 1986). When all tissues were sampled at day 21, after recruitment is complete, parasites detected in organs other than the portal system, were distributed in the ratios 3.9 : 1.9 : 1 in the lungs, systemic organs and skin respectively.

The method of parasite trapping in the lungs is thought to be a non-specific mechanism. Histological and ultrastructural examinations of the lungs over the migratory period have indicated that the first schistosomula to arrive are intravascular and attract little inflammatory response (Mastin *et al.,* 1983, Crabtree and Wilson, 1986a). However, with time more parasites can be found in the alveoli, presumably by rupturing the capillaries as they try to migrate alongside the delicate blood/air barrier. Each parasite entering the alveoli attracts an inflammatory focus (Von Lichtenberg *et al.,* 1985, Crabtree and Wilson, 1986b). The trapped parasites are not damaged by the immune response, and if extracted from the lung tissue and introduced into the hepatic portal system of naive mice are capable of full maturation

(Coulson and Wilson, 1988). Conversely, parasites recovered from donor mice and introduced via the trachea into the lungs of naive mice have difficulties in crossing the alveolar barrier to complete their migration through the vasculature. Hence parasite attrition in the lungs of mice exposed to a normal infection would appear to be due to the inability of schistosomula to re-enter the vasculature once diverted into the alveoli, leading to probable death by starvation.

1.3.3 Concomitant Immunity

Concomitant immunity is the term used to describe the phenomena by which secondary infections in mice with existing adult worm burdens fail to reach maturity in the liver (Smithers and Terry, 1969). This acquired resistance of up to 100% (Dean *et al.,* 1981c) would seem to indicate an immune mechanism which can eliminate migrating parasites but leave the resident adult worm population unharmed. Further work indicated that resistance correlated closely with egg burden (Dean *et al.,* 1978a), and that single sex infections failed to give protection (Dean *et al.,* 1978b, Bickle, 1982 Wilson *et al.,* 1983). These findings led to the 'leaky liver' hypothesis (Wilson *et al.,* 1983, Wilson, 1990) in which the impaired integrity of the liver, due to pathology in chronically infected mice, results in the accumulation of parasites in locations unfavourable to schistosomula maturation, for example the lungs. It has been shown that mice with chronic infections develop altered hepatic vasculature (Cheever and Warren, 1964) which results in a reduced trapping ability of the liver. Thus there is no increase in hepatic worm burden, giving the impression of immunity. It should be noted that even the lowest infection possible in a mouse of one worm pair is equivalent to a heavy worm burden in man (McLaren and Smithers, 1987). This condition also accounts for the seemingly innate resistance of 129 strain mice to schistosome infections (Mitchell *et al.,* 1984). A high proportion of these mice have natural portal shunts which, following infection with *S. mansoni*, result in the direction of liver stage worms to the lungs (Coulson and Wilson, 1989). In conclusion, concomitant immunity in the mouse is most likely not an immune effector mechanism, but rather a consequence of liver pathology caused by the immune response against eggs produced from an already resident adult population.

1.4 The Irradiated Cercariae Vaccine

1.4.1 Parasite Attenuation

A major advance towards a vaccine for schistosomiasis occured when it was discovered that cercariae, attenuated by gamma irradiation, were incapable of maturing (Villella *et al.,* 1961). Vaccination of mice with these parasites induced resistance without the danger of liver pathology. Several different methods of attenuation have been used including chemicals (Bickle and Andrews, 1983), ultraviolet light (Dean *et al.,* 1983), and X-rays (Hsu *et al.,* 1981). However it is gamma radiation from a ^{60}Cobalt source which has proved most popular. The dose of radiation is vital in inducing resistance (Minard *et al.,* 1978a); a low dose will allow migration to the liver and possible pathology, a high dose may prevent entry to the vasculature completely. Although the optimal dose of irradiation in the UK is 20 krads (Bickle *et al.,* 1979a), in the USA a higher dose of 50 krads is preferred. This discrepancy may be due to differences in parasite maintenance or irradiation conditions. However, a recent report in the USA has found that parasites attenuated with 15 krads of irradiation give higher protection than those exposed to the original 50 krad dose (Reynolds and Harn, 1992).

The resistance induced using attenuated parasites varies from 30 to 80% depending on the strain of mouse (James *et al.,* 1981). Strains such as C57BL/6 routinely develop around 70% resistance to reinfection after a single vaccination. Multiple vaccination of mice results in little increase above this limit (Minard *et al.,* 1978, Bickle *et al.,* 1979b, Dean, 1983). The ceiling limit on protection may be due to many immunological factors and will be discussed later. The optimal radiation dose appears to be one which leads to termination of migration in the lungs (Mastin *et al.,* 1983). Following a single percutaneous vaccination of C57BL/6 mice with 500, 20 krad irradiated parasites, resistance peaks between days 17 and 24, and persists at a high level for at least 15 weeks (Ratcliffe and Wilson, 1991, Minard *et al.,* 1978a).

1.4.2 The Effects of Optimal Irradiation on Parasite Development and Maturation

The ability of attenuated parasites to impart resistance has been investigated without finding any major differences between irradiated and normal larvae. Early

ultrastructural studies concluded that there were no morphological variations between normal and irradiated lung stage worms (Mastin *et al.,* 1985a). Furthermore, no differences in the antigens secreted by 3 hour schistosomula derived from normal and irradiated cercariae have been detected (Simpson *et al.,* 1985). Recent studies on UV-attenuated parasites have shown modification of some carbohydrate antigens on newly transformed cercariae (Wales *et al.,* 1992) and inhibition of early protein synthesis. Although these effects have not been described for gamma irradiation techniques as yet, the comparison of *in vivo* and *in vitro* cultured parasites attenuated in this way has highlighted some minor changes in neuromuscular function (Harrop and Wilson, 1993). Scanning electron microscopy of lung stage parasites revealed muscular constrictions along the length of the worms and reduced motility which may account for the truncated migration of attenuated larvae.

The main difference between normal and attenuated larvae is the slower migration of vaccinating parasites (Mangold and Dean, 1984). Only 13% of healthy parasites are left in the skin by day 5 post-infection, whereas 43.4% of the irradiated larvae remain after vaccination (Mountford *et al.,* 1988). Normal parasites also negotiate the skin-draining lymph nodes (sdLN) quicker than their irradiated counterparts, 3% of the former remaining at day 7 compared to 10.4% of the latter. Although the vaccinating parasites can remain in the LN for up to 2 weeks, death in this site is negligible and most of the attenuated larvae eventually reach the lungs. Quantitative histological studies have shown that 57% of irradiated larvae reach the lungs by day 7 and persist there until day 21 post-vaccination, by which point 81% are dead (Mastin *et al.,* 1983). Thus it would seem that protection is induced by persistence in the skin-draining LN and lungs, these migration characteristics being the major difference between normal and attenuated parasites. The increased exposure of LN cells to parasite antigen in an environment geared towards antigen processing and presentation may generate an effective immune response. The detection of a proportionally greater amount of parasite antigen in the LN of vaccinated mice compared to mice with normal infections (Mountford *et al.,* 1988) supports this theory.

1.5 Induction of Immunity

1.5.1 The Nature of Immunity

The immune response induced by vaccination and challenge has been an area of intense research over the last 10 years. Early experiments demonstrated that immunity was transferable to a naive partner during parabiosis (Dean *et al.,* 1981a), and that resistance is usually only effective against challenge with the same species of schistosome (Cheever *et al.,* 1983, Bickle *et al.,* 1985). Thus immunity is based on transferable factors, such as cells and antibodies, specific for parasite antigens. By investigating mouse strains deficient in certain types of immune response the role for various cell types in protection has been demonstrated. Vaccinated athymic (nu/nu) mice show no resistance to challenge with *S. mansoni* (Sher *et al.,* 1982) but can be reconstituted with thymus grafts. Thus T lymphocytes would appear to play a pivotal role in protection. Confirmation of this finding came with the ablation of $CD4^+$ T cell population by *in vivo* administration of an antibody directed against this subset of T lymphocytes (Kelly and Colley, 1988, Vignali *et al.,* 1989), a treatment which completely abrogated immunity. It is interesting to note that anti-CD4 treatment only reduces resistance by 70% in animals vaccinated twice before challenge (Vignali *et al.,* 1989), and immunity is not diminished at all in mice vaccinated five times (Kelly and Colley, 1988). This would seem to indicate a changeover from cell-mediated to antibody-mediated immunity with multiple vaccinations.

Evidence for T cell and macrophage involvement in immunity came from vaccination of the PN strain mouse which shows poor resistance upon reinfection (James and Sher, 1983). These mice have normal antibody titres but are unable to activate macrophages due to a defect in T cell function and lack of IFNγ production (James *et al.,* 1986). The importance of this one cytokine, IFNγ, was further demonstrated by ablation experiments in which anti-IFNγ antibody administered during challenge abrogated immunity by up to 90% (Sher *et al.,* 1990a, Smythies *et al.,* 1992b). An increase in eosinophilia in the lungs with this treatment suggested that these cells play no role in resistance. Furthermore, ablation of the Th2 cytokines IL-4 and IL-5 result in the reduction of serum IgE and eosinophilia, but has no effect

on immunity implying that these factors are not vital components of protection (Sher *et al.,* 1990a).

A role for B lymphocytes in protection has also been implied after depletion of this cell type, via administration of an anti-μ chain antibody, resulted in reduced resistance (Sher *et al.,* 1982). However, it was later found that this antibody could also deplete specific and non-specific T cell responses (Kim *et al.,* 1984), and thus these results are inconclusive. The advent of B cell-deficient mice (Kitamura *et al.,* 1991) may provide the answer to B cell involvement. Humoral responses have been shown to play a less important role than $CD4^+$ T cells in the single vaccination regime. Schistosome-specific antibody titres show no correlation to host immunity, and serum from mice exposed to a single vaccination cannot mediate protection when transferred to naive recipients (James *et al.,* 1981, Roberts *et al.,* 1988). In contrast, administration of immune serum from animals receiving multiple vaccinations to naive animals results in some resistance to infection (Mangold and Dean, 1986, McLaren and Smithers, 1988). Furthermore, the IgG1 isotype, which is frequently associated with humoral immune responses, seems especially important in this model (Delgado and McLaren, 1990). An increase in the production of interleukin-4 (IL-4) and IL-5 in the skin-draining LN of multiply-vaccinated mice is consistent with the presence of IgG1 antibody in the serum, all the above being components of a Th2 type response (see section 2, Caulada-Benedetti *et al.,* 1991, Finkleman *et al.,* 1990). However, splenocytes from multiply-vaccinated mice can still produce IFNγ and activate macrophages, indicative of the presence of a Th1 cell population and implying compartmentalisation of T-helper responses (Caulada-Benedetti *et al.,* 1991). Thus the type of immune mechanism responsible for protection appears to shift from a cell-mediated to humoral response upon multiple exposure to irradiated parasites.

1.5.2 Site and Exposure Time Requirements in the Induction of Immunity

In order to determine the *in vivo* exposure time necessary for live parasites to induce immunity in the host, several approaches have been used. Terminating the vaccinating infection by drug treatment at various times post-exposure has demonstrated that resistance is decreased if the parasites do not survive for at least 7

to 11 days (Bickle *et al.,* 1982). In addition, removing the exposure site, either the ear (Bickle *et al.,* 1982), or tail (Mangold and Dean, 1984), has also shown that the presence of the vaccination site is needed for at least 4 days, and for maximum resistance should be left intact for 8 to 11 days. Conclusions drawn from this work suggest that the presence of live parasites is required during the first 2 weeks of a vaccinating infection for induction of the protective response.

The parasite stage required for induction has also been investigated. Dermal innoculation of 8 day lung schistosomula, extracted from donor mice, induces equal resistance to percutaneous vaccination with irradiated cercariae (Coulson and Mountford, 1989). This indicates that cercarial antigen is not necessary for successful vaccination. However, vaccinating intradermally with 3 to 4 week old schistosomula results in lower resistance than skin or lung stage parasites (Sher and Benno, 1982, Dean *et al.,* 1981b), possibly due to the loss of migratory potential.

1.6 Immune Responses Following Vaccination

1.6.1 The Skin-draining Lymph Nodes

As described above, the two key sites considered to be necessary for successful vaccination are: the lymph nodes through which a minority of parasites migrate and persist, and the lungs where migration terminates. Vaccinating parasites persist longer in the skin and LN than their normal counterparts (Mangold and Dean, 1984, Mountford *et al.,* 1988). The dependence of the protective response on parasite migration through the LN was demonstated by administering parasites into mice via routes which bypass the nodes (Coulson and Mountford, 1989). Vaccination by the intravenous injection of day 8 schistosomula imparted a much reduced level of protection compared to parasites injected intradermally. In addition, excising the draining LN five days prior to vaccination decreased immunity by 60%, the residual resistance presumably being due to other mechanisms operating independently of the LN (Mountford and Wilson, 1990). Furthermore, lymphadenectomy at various timepoints post-vaccination resulted in higher resistance the later this operation was performed, indicating that the LN play an early role in induction. Vaccination with

hyper-irradiated (80 krad) cercariae induces no resistance, even though small numbers of parasites reach the draining LN and elicit a transient response similar to that produced by normal larvae (Constant *et al.*, 1990). In contrast to 20 krad irradiated larvae, these weaker parasites do not migrate as far as the lungs. Combining a vaccination with 80 krad irradiated cercariae and intravenous injection of day 8 schistosomula eight days later results in the induction of protection, even though independently the two regimes have little effect (Coulson and Mountford, 1989, Mountford *et al.*, 1992). Thus it would seem that both antigen presentation in the LN and parasite migration to the lungs are vital for the generation of a protective response.

Analysis of the axillary LN cell populations has revealed that cell numbers peak at day 14 post-vaccination, with a 13.5 fold increase over the cell number detected in naive mice (Constant *et al.*, 1990). Proliferation also peaks at this time and is higher in vaccinated mice compared to normal infection controls. These observations could be explained by the persistence of parasites in the nodes, and hence the increased amounts of antigen present (Mountford *et al.*, 1988). Further, there is an increase in B cell proliferation and recruitment to the axillary LN which leads to a dramatic change in the T:B cell ratio from 4:1 to 1:1 (Constant and Wilson, 1992). By comparing the total numbers of T and B cells to their individual proliferation values, it can be concluded that there is a higher proportion of T cells proliferating even though there is an increase in B cell number. Upon further investigation into the LN cellular composition it was discovered that this increase in B cells is caused by recruitment and retention, as suggested by low B cell numbers in the efferent LN vessels (Constant and Wilson, 1992). The role of this cell type during induction is unclear, but B cells may act as antigen presenting cells (APCs) for development of the immune response in the LN (Janeway *et al.*, 1987).

Research into the immune response induced by vaccination took a new direction after the discovery that $CD4^+$ T cells exist in two distinct subsets, termed T-helper (Th) 1 and Th2, each of which secretes different cytokine patterns and results in the development of contrasting immune mechanisms (see section 2 for a full description, Mosmann and Coffman, 1987). Fundamental to the distinction of these subsets is the production of IFNγ by Th1 cells and IL-4 by Th2 cells. Ablation experiments

directed against $CD4^+$ and $CD8^+$ cells during vaccination have demonstrated that the majority of IFNγ is secreted by the $CD4^+$ cell population in the axillary nodes (Pemberton *et al.,* 1991). IFNγ protein secretion peaks in cultures of sdLN cells at day 5, when the maximum number of parasites and parasite-released material are present. The decrease in IFNγ release after day 5 may be explained by a fall in parasite material and an influx of B cells into the nodes. However, a second peak of IFNγ secretion occurs around day 18, possibly stimulated by the release of antigens from dead or dying parasites (Pemberton *et al.,* 1991). In comparison with normal infection, cultured LN cells from vaccinated mice secrete higher levels of IFNγ. In addition, the increased numbers of the $CD4^+$ cell type present in the LN of vaccinated mice compared to control animals (Pemberton *et al.,* 1991, Constant *et al.,* 1990) results in an overall 24 fold increase in the capacity for IFNγ production at day 22 post-vaccination. Vaccinated nodes also produce IL-2, IL-3, IL-4 and IL-10 in response to secondary *in vitro* stimulation with specific antigen (Pemberton *et al.,* 1991, Pemberton and Wilson, 1995). In contrast, cells from control animals given a normal infection secrete little IL-4 and IL-3, but do produce significant levels of IL-2. Thus the cytokine profiles are not clearly of Th1 or Th2 phenotype, but consist of a mixed, or Th0 type response (see section 2) in which the high levels of IFNγ may promote the generation of Th1-like memory cells (Pemberton *et al.,* 1991, Coulson and Wilson, 1993, Mountford *et al.,* 1992).

The response elicited in the sdLN varies depending on the exposure regime. Repeated infection, either multiple vaccinations or vaccination and challenge, on the same area leads to the generation of a Th2 type response, characterised by IL-4 and IL-5 production (Pemberton and Wilson, 1995, Caulada-Benedetti *et al.,* 1991). Alternatively, a single exposure appears to favour a Th0/Th1 profile. Vaccination and challenge on the same site leads to downregulation of the IFNγ production in nodes previously secreting high levels of this cytokine, and an increase in IL-4, an effect reversed by the neutralisation of IL-10 (Pemberton and Wilson, 1995). Thus the absence of any anamnestic Th1 response in the LNs may be due to the effects of IL-10, acting via antigen presenting cells to anergize the Th1 population whilst leaving the Th2 response free to develop (Fiorentino *et al.,* 1991a, Flores Villanueva *et al.,* 1993, Gajewski *et al.,* 1994).

1.6.2 The Lung and Mediastinal Lymph Node.

Footpad inflammation assays have demonstrated that a schistosome-specific lymphocyte pool present in the circulation expands between day 10 and day 17 post-vaccination (Menson and Wilson, 1989, Ratcliffe and Wilson, 1991). The peak response to schistosome antigen after vaccination occurs at day 17, and decreases again by day 35. The peak response to lung-stage parasites is weaker and occurs earlier at day 10 (Ratcliffe and Wilson, 1991), and both responses can be abrogated by prior *in vivo* administration of anti-CD4 monoclonal antibody. These results correspond with data showing that mononuclear cell recruitment to the lungs occurs between days 10 and 16 post-vaccination, thus coinciding with the decrease in peripheral responses detected by DTH footpad assays (Ratcliffe and Wilson, 1991). Irradiated parasites start arriving in the lungs from day 4 post-vaccination onwards, and terminate their migration here (Mangold and Dean, 1984, Mastin *et al.,* 1983, 1985a). Cell accumulation around the worms can be detected between days 13 and 21 post-vaccination (Mastin *et al.,* 1983, 1985b) as cells are recruited from the circulation to parasites trapped in the alveolar spaces.

Broncho-alveolar lavage (BAL), which samples cells present in the airways of the lung, has shown that mononuclear infiltration into the alveoli begins at day 14 and peaks at day 21 post-vaccination (Aitken *et al.,*1988, Menson *et al.,* 1988). The influx of cells results in a 6 fold increase in macrophage recovery and a 58 fold increase in lymphocyte numbers (Menson *et al.,* 1988), as compared to a baseline comprising of 90% macrophages in naive mice (Aitken *et al.,* 1987). The recruited cell populations persist for at least 10 weeks post-vaccination (Aitken *et al.,* 1988). A transient pulmonary eosinophilia also occurs, peaking at day 21, when these cells account for 30% of the leucocyte influx (Menson *et al.,* 1988). Although there is no difference in the cell number recovered at 2 weeks post-infection by BAL from mice exposed to vaccinating and normal parasites (Aitken *et al.,* 1988), by week 5 the absolute cell numbers recovered from vaccinated mice are much higher, reflecting the transient nature of the pulmonary response in a normal infection.

Cytokine protein production by BAL cells is very limited and appears to be derived from the Th1 subset of $CD4^+$ T cells (Smythies *et al.,* 1992a and b, Mountford *et al.,* 1992). Neutralisation experiments have defined $CD4^+$ cells as

producers of the increased levels of IFNγ detected in culture supernatents post-vaccination. These cells also produce IL-3, but little IL-2 or IL-4, and appear incapable of proliferation (Smythies *et al.,* 1992a, Wilson *et al.,* 1996). In contrast, splenocytes from vaccinated mice taken at day 14 or 21 can respond to antigen by proliferating (Mountford *et al.,* 1992, Smythies *et al.,* 1992a), but display different kinetics of cytokine production. IFNγ secretion is only detectable in splenocyte cultures after 72 hours, compared to 24 hours for BAL cells. Thus it seem splenocytes cannot mount a strong recall response upon secondary stimulation *in vitro,* unlike cells recovered from the lungs. This may be explained by the absence of parasites or antigen in the spleen after vaccination (Mountford *et al.,* 1988) and demonstrates the local, as opposed to systemic, nature of the induced immune response.

Further evidence demonstrating the recruitment of schistosome specific T cells to the lungs was obtained by analysis of cells recovered by lavage for the expression of memory characteristics (Coulson and Wilson, 1993). BAL cells from the lungs of vaccinated mice are capable of mounting a stronger response to mitogen or schistosome antigen than splenocytes from the same individual. This observation is explained by the upregulation of the p55 subunit of the IL-2 receptor on the surface of these cells, indicative of previous activation. In addition, higher numbers of cells recovered from the airways express CD44, a marker associated with the memory phenotype, compared to LN or peripheral blood lymphocytes, which show only a low percentage of memory cells. In contrast, the reverse is true for CD45RB expression, a marker which is lost upon activation, demonstrating the naive status of the majority of LN and circulating lymphocytes. Therefore the lymphocyte population recruited to the lungs post-vaccination appears to be of the memory/effector phenotype.

Flow cytometric analysis of the alveolar macrophages recovered by BAL has demonstrated an increase in Ia (MHC II) expression and a decline in F4/80 between days 14 and 21 (Menson and Wilson, 1990). These expression profiles are indicative of macrophage activation, and indeed alveolar macrophages are capable of mounting an oxidative burst between days 14 and 21 post-vaccination (Menson and Wilson, 1989). This upregulation of macrophage activity coincides with IFNγ production from Th cells recovered from the mediastinal LN, and from day 21 BAL cells,

restimulated *in vitro* with schistosomal antigen (Menson and Wilson, 1990, Smythies *et al.,* 1992a). Indeed, the above cytokine has been implicated as a major activation signal for macrophage cytotoxic functions and inflammatory cytokine release, both *in vitro* and *in vivo* (Murray *et al.,* 1985).

The results obtained from BAL cells are confirmed by analysis of lymphocyte populations extracted from the LN draining the lungs. Peak proliferation in the mediastinal LN occurs at day 21 and persists until after day 35 post-vaccination (Constant *et al.,* 1990, Constant and Wilson, 1992). This coincides with detectable levels of parasite protein (Mountford *et al.,* 1988), and increased secretion of IFNγ from cultured cells at day 22 (Pemberton *et al.,* 1991). In summary, lymphocytes which are recruited to, and persist, in the lungs appear to be of the short term memory/effector Th1 phenotype capable of elevated IFNγ secretion, and may act to arm this organ against further infection.

1.7 Immune Responses Following Challenge

1.7.1 Immune Responses in the Skin-draining LN after Challenge

The involvement of the LNs draining the exposure site appear less important after challenge than during vaccination (Lewis and Wilson, 1982). There is no anamnestic Th1 type response, and the increase in IL-4 and IL-10 cytokine release from cultured LN cells indicates a tendency towards Th2 development (Pemberton and Wilson, 1995). Indeed, the circulating T cell population can be depleted by whole body irradiation (Vignali *et al.,* 1988, Aitken *et al.,* 1987) with no deleterious effects on resistance. This implies that any cell populations which may be elicited in the LN, and recruited to the lungs after challenge, are not required for effective challenge elimination (Aitken *et al.,* 1987).

1.7.2 The Site of Parasite Elimination

The organ in which challenge parasites are eliminated has been in dispute for many years. There are two main contested theories, involving attrition in the skin and lungs, with evidence available supporting both (reviewed by McLaren, and

Wilson and Coulson, respectively, 1989). However, it is agreed that the elimination target is the lung stage worm (Ward and McLaren, 1988). With the development of more accurate techniques the data supporting lung phase immunity has overwhelmed that of skin phase and a brief resume of the experiments leading to the current opinion will be described.

Mincing and incubation studies have detected equal numbers of parasites in the lungs of previously vaccinated and control mice (Minard *et al.,* 1978). In addition, histological studies indicate that there is no difference between the numbers of parasites leaving the skin of vaccinated or control mice (Mastin *et al.,* 1983), and that parasites detected in the skin of previously vaccinated animals show no damage (Mastin *et al.,* 1983, Von Lichtenberg *et al.,* 1985). Intravenous injection of challenge parasites into the lungs gives little or no reduction in resistance (Dean *et al.,* 1981b), and 30-50% of injected worms are eliminated in the lungs (Mangold *et al.,* 1986, Dean *et al.,* 1981b). These data imply that it is the lungs which represent the major migratory hazard for larvae. In support of this, injection of schistosomula downstream of the lungs, into the superior mesenteric vein, results in 80% maturation of challenge parasites (Mangold *et al.,* 1986).

Histological sections of lung tissue from vaccinated and challenged mice have demonstrated the presence of focus formation around migrating parasites (Von Lichtenberg *et al.,* 1985), with no visible damage to the actual worm trapped in the centre. Autoradiographic tracking studies have provided information on the timing of parasite arrival in the lungs following challenge. Equal numbers of challenge parasites reach the lungs of vaccinated mice as in naive controls (Dean *et al.,* 1984, Wilson *et al.,* 1986). However, in the former group migration is delayed and fewer parasites leave the lungs to continue systemic migration and portal accumulation (Wilson *et al.,* 1986). By day 21, when normal migration would be complete, a large proportion of challenge parasites can still be detected in the lungs. Challenge elimination is thought to start around days 7-12 and continue until at least day 35 (Wilson *et al.,* 1986).

Although some parasite attrition may occur in the skin, the data described above indicate that the majority of challenge elimination takes place in the lungs. In

accordance with this current consensus of opinion, the immune response upon challenge will be described for lung phase immunity only.

1.7.3 The Pulmonary Effector Mechanism

On the arrival of challenge parasites in the lungs of previously vaccinated mice an anamnestic inflammatory response occurs, rich in $CD4^+$ T cells and macrophages, resulting in focus formation around each parasite (Kambara and Wilson, 1990, Crabtree and Wilson, 1986b, Aitken *et al.,* 1988). Increases in cell numbers recoverable by BAL have been reported (Aitken *et al.,* 1988), although other studies find no such influx (Menson *et al.,* 1988, Ratcliffe and Wilson, 1992) and the effects of whole body irradiation described above suggest no further recruitment is necessary for protection.

Cytokine assays on recoverable BAL populations have implicated IFNγ as the major cytokine involved in the effector mechanism. Peak IFNγ secretion occurs at day 14 post-challenge and returns to baseline levels by day 22 (Smythies *et al.,* 1992a). At peak response, this protein can be detected by ELISA without the need for secondary stimulation, but at no time can IL-2 or IL-4 be detected in culture supernatants by bioassay. However, more recent ELISA data demonstrates a low level of IL-4 production (Smythies *et al.,* 1993, Smythies *et al.,* 1996a). Proliferation levels were also reduced in BAL cell cultures post-challenge, comparable to levels detected after vaccination. Coincidental with peak IFNγ release, alveolar macrophages show increased MHC II expression (Menson and Wilson, 1990) and are capable of mounting an oxidative burst, consistent with cell activation (Menson and Wilson, 1989). These immunological effects occur over a period of time corresponding to challenge parasite retention in the lungs (Wilson *et al.,* 1986). In comparison, inflammation in challenge control mice is delayed by around 1 week and thus materialises too late to prevent worm migration and maturation.

As described earlier (section 1.5.1), anti-IFNγ antibody administered from days 4-16 post-challenge can reduce resistance by 90% (Smythies *et al.,* 1992b). BAL cell populations from these treated mice show increased numbers of eosinophils at day 14, although this cell type has previously been shown to play no role in resistance (Sher *et al.,* 1990a). The foci around migrating parasites in IFNγ-ablated mice were

larger, but looser in composition, and contained many giant cells. It is possible that the increase in maturation of challenge parasites following anti-IFNγ treatment is caused by the ablity of parasites to navigate through the loose foci to continue migration. Thus IFNγ may play a role in adhesion molecule (such as LFA-1 and ICAM-1) upregulation, reducing the ability of cells in IFNγ-neutralised animals to adhere and form a compact focus (Smythies *et al.,* 1992b, Dustin *et al.,* 1986, Dustin and Springer, 1988).

As mentioned above, parasites arriving in the lungs attract a focus of inflammation (Von Lichtenberg *et al.,* 1985). By day 11 post-challenge these foci have increased in size and surround schistosomula trapped both in the capillaries and alveoli, in contrast to normal infection where only parasites in the alveolar spaces attract foci, (Crabtree and Wilson, 1986b). With time, more parasites can be found in alveoli, possibly due to the large foci blocking migration routes. Although macrophages have the ability to kill schistosomula *in vitro* (reviewed by James, 1986), these studies often involve peritoneal macrophages and newly, mechanically transformed parasites, and do not reflect the true *in vivo* situation in the lungs. However, C57BL/6 macrophages are capable of larvacidal activity against skin phase and post-lung stage worms (James, 1986, Pearce and James, 1986, James and Glavern, 1989, James and Hibbs, 1990). More recently, nitric oxide (NO) synthesis by macrophages has been shown to kill newly transformed parasites *in vitro* (James and Nacey, 1993). No reports as yet demonstrate the killing of lung stage worms *in vitro*, and in support of this, an experiment blocking NO synthesis *in vivo* after challenge reported no effect on resistance (Smythies *et al.,* 1996b). However, a possible requirement for NO release during challenge elimination has been reported recently when prolonged suppression of NO synthase activity *in vivo* resulted in an increase in worm burden of 12-22% (Wynn *et al.,* 1994a). Furthermore, inducible nitric oxide synthase (iNOS) expression can be detected *in situ* in the foci surrounding the parasites (Wynn *et al.,* 1994a).

Although these observations may indicate a role for macrophage larvicidal activity *in vivo*, evidence in support of the theory that parasites trapped in the lungs are resistant to the cytotoxic environment surrounding them is strong. Ultrastructural studies show no visual damage to the parasites, and no evidence of worm death up to

day 35 post-challenge (Crabtree and Wilson, 1986b). Furthermore, trapped parasites extracted from the lungs at day 17 are able to migrate and fully mature when introduced into a naive host (Coulson and Wilson, 1988, Wilson and Coulson, 1989). This would suggest that the effector mechanism is unable to harm trapped parasites, and elimination occurs through an indirect mechanism which prevents completion of migration and maturation (Smythies *et al.*, 1993).

1.8 Summary of the Induction and Effector Mechanisms Leading to Protection

Protective immunity appears to be mediated by two major migration characteristics of optimally irradiated cercariae. The slower, truncated migration of these parasites and their persistence in the skin-draining LN results in increased antigen exposure in the LN during vaccination, compared to a normal infection. The cytokine profiles induced during this extended stay in the LN are Th1 in phenotype, and more intense than those observed following exposure to normal parasites. The cell population elicited in the LN is recruited to the lungs, presumably attracted by parasites unable to migrate further, and arms that organ against the subsequent arrival of challenge parasites. Thus, upon secondary infection the schistosome-specific Th1 cell population can react quickly to antigenic stimulation. This interaction stimulates the release of IFNγ from the $CD4^+$ cell population, resulting in macrophage activation. Further secretion of inflammatory cytokines by macrophages and T cells might be responsible for the observed rapid focus formation, and hence blocking of the exit route from the lungs.

Trapped parasites probably eventually die by starvation, although cytotoxic mechanisms may play a role at later times. In this model the actual cause of death would appear to be irrelevant, it being the focus itself that prevents worm migration and maturation into an adult, egg laying population. It should be noted that the vaccination regime does not provide 100% immunity; some parasites manage to negotiate the lungs and proceed with migration to the portal system. The factors influencing the differentiation pathways of T-helper subsets, and the effect of the

subsequent release of cytokines on the immune response to parasitic infection will be discussed in detail in the next section.

Part 2. Th1 and Th2 Cell Subsets: Cytokine Secretion and Related Function.

2.1 Background

In 1968 it was found that lymphocyte cell populations fall into two separate categories: B cells and T cells, maturing from the bone marrow and thymus respectively (Mitchell and Miller, 1968). Subsequently, in 1975, the T cell compartment was further divided into two types recognisable by different cell surface markers (Cantor and Boyse, 1975). Cells bearing the CD8 suface marker (originally designated lyt-2 in the mouse) displayed a cytolytic function and thus were termed cytotoxic T (Tc) cells. Cells positive for CD4 (formally lyt-1 and then L3T4 in the mouse) were designated T-helper (Th) cells due to their ability to aid antibody production by B cells.

In 1986 cellular immunology took a further major step forward when two distinct cellular subsets were described for murine $CD4^+$ T cell clones (Mosmann *et al.,* 1986, Mosmann and Coffman, 1987, Mosmann and Coffman, 1989). These subsets, named Th1 and Th2, cannot be distinguished by any surface marker but only by their differing patterns of cytokine secretion. Th1 clones produce predominantly IFNγ, IL-2 and TNFβ upon secondary stimulation *in vitro* (Mosmann and Coffman, 1987, Mosmann and Coffman, 1989, Cherwinski *et al.,* 1987, Mosmann *et al.,* 1991, O'Garra and Murphy, 1994) leading to delayed-type hypersensitivity reactions usually indicative of cell-mediated immunity (Cher and Mosmann, 1987). Th2 clones secrete IL-4, IL-5, IL-6, IL-10 and the recently discovered IL-13 (Cherwinski *et al.,* 1987, Fiorentino *et al.,* 1989, Doherty *et al.,* 1993). This combination of cytokines induces the humoral side of immunity involving B cell help for antibody production and immediate hypersensitivity reactions such as those responsible for allergic inflammation (Tony *et al.,* 1985, Kim *et al.,* 1985).

Although initially Th subsets were only described for murine T cell clones, cells displaying Th1- and Th2-type characteristics have now been reported *in vivo* in both

man and mouse (Romagnani, 1991, 1994, Scott, 1991, Scott and Kaufman, 1991). When Th1 and Th2 secretion patterns were first reported, human clones did not show the clear distinctions observed with cells isolated from mice. Primary analysis of human clones revealed a mixed cytokine profile involving cytokines from each subset expressed by the same population. However, later screening of a panel of human T cell clones did detect segregation of cytokine production into patterns similar to those seen in mouse T cell populations (Romagnani, 1991, 1994). The initial results implying that T-helper subsets are a murine phenomenon may have arisen due to differences in the methods used to derive T cell clones in mice and man. Murine clones are obtained after vigorous immunization and manipulation; these procedures are impossible to carry out in humans and thus clones are established from normal, healthy individuals. Thus early clones were not derived from cells undergoing an intense immune response. However, a panel of human clones displaying T-helper subset characteristics was isolated from patients previously exposed to *Mycobacterium tuberculosis* or *Toxocara canis*, resulting in Th1 clones specific for purified protein derivative (PPD), or Th2 clones responding *T. Canis* excretory/secretory antigen respectively (Del Prete *et al.,* 1991, Romagnani, 1991, 1994).

The discovery of the existence of either Th1 or Th2 extremes *in vivo* promoted the theory that each subset has the ability to cross-regulate the other, resulting in the generation and dominance of very different immune responses. Many murine models of human disease are now studied to gain insight into the development and role of Th1/Th2 polarisation *in vivo*. Thus the cytokine profiles exhibited during the induction and execution of a mechanism to deal with various invading organisms have been segregated into Th1 and Th2 responses. However, evidence is now emerging to suggest that this dogma is too rigid and should be relaxed to take into account cytokine production patterns observed in $CD8^+$ T cells, and the mixed cytokine profiles produced by individual Th cells (Kelso, 1995, Assenmacher *et al.,* 1994, Mosmann and Sad, 1996). It is becoming clear that the involvement of T-helper subsets in infection is not as simple as initially thought, although there are some systems which demonstrate clear cut Th1 and Th2 dichotomy. The following sections aim to provide a current review of T-helper cell immunology and its relation

to infection, the investigation of which provides the basis of the research carried out in this thesis.

2.2 Cytokine Secretion by Other Cell Types

Although cytokines are thought to originate predominantly from $CD4^+$ T cells, it is becoming increasingly clear that many other cell types can produce the cytokines necessary for Th cell development. The production of cytokines early in an immune response may be vital in influencing the direction of T-helper cell development, and hence the outcome of infection. With this in mind, intense investigation into the cell types responsible for cytokine production during the initial stimulation and differentiation of T cell subsets has shown that many cells other than $CD4^+$ can secrete a wide range of cytokine profiles.

2.2.1 $CD8^+$ T Cells.

The cytotoxic arm of the T cell compartment can secrete a Th1-like profile of cytokines such as IFNγ, GM-CSF, IL-3, TNFβ and TNFα (Kelso and Glasebrook, 1984, Taguchi *et al.,* 1990). Although some clones derived from $CD8^+$ cells also produce IL-2, this is not found universally. The expression of this range of cytokines can induce a similar type of DTH response to that observed upon $CD4^+$ T cell polarisation into a Th1 population (Fong and Mosmann, 1990). Furthermore, $CD8^+$ cells have recently been shown to secrete IL-4 and IL-5 in comparable amounts to Th2 cells under certain *in vitro* conditions (Taguchi *et al.,* 1990, Salgame *et al.,* 1991, Seder *et al.,* 1992). These data indicate that $CD8^+$ T cells might also segregate into Type 1 and Type 2 subsets, as defined by their cytokine secretion profiles.

2.2.2 B Cells

Normal and Ly-1^+ (CD5) B cells have been found to express IL-10 mRNA (O'Garra *et al.,* 1990, Murphy *et al.,* 1993,Velupillai *et al.,* 1994). Message levels were most abundant in the Ly-1 subset, and cytokine expression from this cell type may be responsible for B cell-mediated immunoregulation or suppression (O'Garra

et al., 1990). In addition to IL-10, murine peritoneal B cells have been shown to express IL-6, TNFα and TNFβ (O'Garra *et al.,* 1990). Furthermore, both human and murine B cells can express the p35 and p40 components of IL-12 to produce biologically active protein (D'Andrea *et al.,* 1992, Mengel *et al.,* 1992) although B cells are not considered to be the major producers of this cytokine *in vivo* (Trinchieri, 1995).

2.2.3 Monocytes and Macrophages

Both human and murine monocytes have been shown to produce IFNγ, IL-1, TNFα, IL-10 and IL-12 (Essner *et al.,* 1989, de Waal Malefyt *et al.,* 1991). These cytokines are released coincident with macrophage activation; hence this cell type plays a central role in the development of DTH and inflammatory responses. IL-12 production is both spontaneous and inducible in adherent human peripheral blood mononuclear cell populations (D'Andrea *et al.,* 1992), the effect of which can stimulate IFNγ production and proliferation in natural killer (NK) and T cells (Chan *et al.,* 1991, Perussia *et al.,* 1992). Thus monocyte-derived IL-12 might play an initial role as the stimulus for Th1 differentiation (see section 2.4). Macrophage secretion of IL-10 appears to be later than the typical DTH cytokines such as IFNγ and TNFα (de Waal Malefyt *et al.,* 1991). This IL-10 release is partially inhibited (70%) by IL-4, as are other macrophage-derived cytokines (Essner *et al.,* 1989, te Velde *et al.,* 1990). In addition, endogenous IL-10 can depress expression of IL-1, IL-6, IL-8 and TNFα as well as its own message (de Waal Malefyt *et al.,* 1991). Thus macrophages appear to posess an inbuilt, self-regulatory inhibitor which is produced once the inflammatory response has established, presumably as a means of regulating the potentially host-damaging molecules produced during a DTH reaction.

More recently, macrophages have been shown to secrete IL-15, an IL-2-like cytokine which can stimulate T and NK cell proliferation (Doherty *et al.,* 1996). IL-15 expression is more resistant to inhibitors of macrophage cytokine release such as IL-4 and IL-13, and IL-10 appears to have no effect on production of this cytokine.

2.2.4 Mast Cells and Basophils

Murine and human mast cells and basophils are capable of secreting IL-4 and have

long been thought of as the driving source of this cytokine behind Th2 development (Bradding *et al.,* 1992, Plaut *et al.,* 1989, Ben-Sasson *et al.,* 1990a). However, cytokine expression in mast cells appears to require cross-linking of surface Fc receptors and thus IL-4 production may not occur early enough to provide naive cell priming. In addition to IL-4, murine mast cells can express IL-3, IL-5 and TNFα when triggered by IgE or IgG cross-linking (Latour *et al.,* 1992).

2.2.5 Natural Killer Cells

Natural killer cells can release significant quantities of IFNγ and TNFα, which may contribute to Th1-like responses (Aste-Amezaga *et al.,* 1994). The secretion of IFNγ is stimulated by IL-12 from macrophages (see above), and is thought to provide the early source of IFNγ required for Th1 differentiation.

2.2.6 Eosinophils

Eosinophils have been associated with IL-5 mRNA expression in a human tissue from patients with Coeliac disease (Desreumaux *et al.,* 1992). This cytokine may be acting as an autocrine differentiation and activation factor, since IL-5 has been shown to induce eosinophil differentiation and proliferation (Yamaguchi *et al.,* 1988). Furthermore, IL-5 can activate cytotoxic mechanisms in this cell type resulting in the release of mediators (Lopez *et al.,* 1988). In addition to IL-5, human eosinophils appear to secrete an increasing list of cytokines. At present these include: IL-3 (Kita *et al.,* 1991), IL-6 (Hamid *et al.,* 1992), TNFα (Costa *et al.,* 1993) and IL-4 (Moqbel *et al.,* 1995). Many of these cytokines have been associated with lung inflammatory conditions, and research into expression by murine eosinophils is still somewhat incomplete in comparison.

2.3 Functions of Th1 and Th2 Subsets as a Result of Their Respective Cytokine Secretion

2.3.1 B cell help

Cytokine secretion from Th2 cells positively regulates B cell function and the

production of antibodies, predominantly of the IgG1, IgE and IgA isotypes (Tony *et al.,* 1985, Kim *et al.,* 1985, Coffman *et al.,* 1986, Finkelman *et al.,* 1988, reviewed by Mosmann and Coffman, 1989, Finkelman *et al.,* 1990). Th1 cells and IFNγ can also provide some help for antibody production; however, the help is restricted to switching to IgG2a isotype production (Snapper and Paul, 1987, Finkelman *et al.,* 1990). Furthermore, this switch is not solely dependent on IFNγ since antibody ablation of this cytokine *in vitro* only partially inhibits the generation of an IgG2a response (Snapper and Paul, 1987, Coffman *et al.,* 1988, Finkelman *et al.,* 1990), and IFNγ added to Th2 cell cultures is not sufficient to change antibody isotypes from IgG1 to IgG2a.

One of the major cytokines responsible for antibody production, especially the induction of IgE, is IL-4. This cytokine can have an effect on the function of B cells, T cells, mast cells and macrophages (Mosmann *et al.,* 1986, Mosmann and Coffman, 1987, Finkelman *et al.,* 1990). The dependence of IgE isotype switching upon IL-4 has been established both *in vivo* and *in vitro* (Coffman and Carty, 1986, Coffman *et al.,* 1986, 1988, Finkelman *et al.,* 1990) and correlates with an increase in IgG1 production (Isakson *et al.,* 1982). The presence of these isotypes during an immune response is now generally regarded as a marker of Th2 subset activation. However, the production of IgG1 is less dependent on IL-4 than IgE switching, and ablation of IL-4 has little effect on the isotype (Mosmann and Coffman, 1989). In addition, IgG1 can be produced during a Th1 response, whereas IgE is rarely present in these situations.

Thus Th1 and Th2 subsets show some degree of characteristic isotype regulation; Th1 responses leading to IgG2a production and Th2 cytokines promoting switching to IgE with IgG1. In general, immune responses involving high antibody titres are regarded as polarised towards the Th2 phenotype, with IgE production indicating the presence of high IL-4 levels and little IFNγ and Th1-related cytokine expression.

2.3.2 Th2 Cell Function

The cytokines released by Th2 cells, namely IL-4, IL-5, IL-10 and IL-13, lead to an increase in IgE, mastocytosis and eosinophilia, and an inhibition of macrophage activation (Mosmann *et al.,* 1986, Mosmann and Coffman, 1989, Fiorentino *et al.,*

1991b, Doherty *et al.,* 1993). This combination of immune mechanisms results in an immediate type hypersensitivity response similar to that seen in allergy and asthma (Romagnani, 1994), with high antibody titres (see above section) and inflammation caused by eosinophil and mast cell degranulation.

2.3.3 Th1 Cell Function

The major response associated with the cytokines released during Th1 subset activation is classed as DTH (Cher and Mosmann, 1987, Mosmann *et al.,* 1991). Only Th1 cell clones can elicit the footpad swelling characteristic of DTH when injected with antigen, and many inflammatory responses *in vivo* correlate with Th1 cytokine production (reviewed by Romagnani, 1991). Cytokines involved in inflammation include IFNγ, IL-1β, TNFα and TNFβ, which can be produced by Th1 -type T cells and macrophages, with the exception of IL-1β which is only produced by the latter cell type (Mosmann, 1988). IFNγ is a principal activator of macrophage cytotoxicity, affecting the production of nitric oxide which in turn inactivates the enzymes critical for DNA replication, the citric acid cycle and mitochondrial respiration (James and Hibbs, 1990, Gazzinelli *et al.,* 1992a). The release of inflammatory cytokines from activated macrophages and Th1 cells can be severely downregulated by the Th2 cytokines IL-4, IL-10, IL-13 and TGFβ (see cross regulation section).

A important factor in the development of a DTH response is the accumulation of cells at the site of infection. Recent studies into the possible mechanisms reponsible for the recruitment of Th1 cells to the area of inflammation have implicated RANTES (Regulated upon Activation of Normal T cells Expressed and presumably Secreted, Rathanaswami *et al.,* 1993) as an important chemokine in human DTH reactions. This chemokine is a selective recruiter for human monocytes and memory T cells (Schall *et al.,* 1990, Rathanaswami *et al.,* 1993, Devergne *et al.,* 1994), yet its role in murine responses has not been fully evaluated. RANTES is released by macrophages, possibly acting as a self-perpetuating factor for granuloma formation by those macrophages already at the site (Dervergne *et al.,* 1994) . In addition, endothelial cells, activated T cells, epithelial cells (Schall *et al.,* 1990) and fibroblasts (Rathanaswami *et al.,* 1993) can all secrete this chemokine. RANTES expression is

upregulated by the Th1 cytokines IFNγ and TNFα, which are produced by macrophages and T cells, and IL-1β from macrophages (Rathanaswami *et al.,* 1993, Arend *et al.,* 1989, Donnelly *et al.,* 1991). In contrast, RANTES is not expressed in tissues undergoing a humoral immune response, and is downregulated by IL-4 directly, and by IL-4 and IL-10 indirectly via inhibition of IL-1β, TNFα and IFNγ release by macrophages and Th1 cells (Essner *et al.,* 1989, te Velde *et al.,* 1990, Rathanaswami *et al.,* 1993). RANTES has also been shown to attract eosinophils *in vitro*, but since these cells are not usually present in natural DTH reactions this effect may be inhibited by other factors *in vivo* (Alam *et al.,* 1995). Moreover, it has recently been demonstrated that human eosinophils can express and secrete RANTES, and thus an autocrine role for this chemokine in allergic inflammation might yet be defined (Ying *et al.,* 1996).

2.3.3 $CD8^+$ T Cell Help

Both Th1 and Th2 cells can release cytokines that provide help for cytotoxic T cells and cause an upregulation in the expression of MHC class II on some APCs (Mosmann and Coffman, 1987, 1989). Most notably, IL-2 provides help for $CD8^+$ cell expansion and upregulation, a cytokine which is absent or expressed at very low levels by $CD8^+$ T cells themselves.

2.4 Differentiation of Th1 and Th2 Cell Populations.

Early studies involving T cell clones in both murine and human systems found that some clones could not be segregated into either Th1 or Th2 types, but secreted a mixed cytokine profile (Romagnani, 1991, 1994, Mosmann and Coffman, 1987, 1989). It was later proposed that these clones might be displaying an earlier phenotype of Th1/Th2 differentiation than established clones, and at this stage IFNγ, IL-2, IL-4 and IL-5 can all apparently be secreted by the same cell (Firestein *et al.,* 1989, Gajewski and Fitch, 1988, Openshaw *et al.,* 1995). These mixed T-helper type cell populations were designated Th0, and since their discovery the search for the factors and conditions necessary to promote their development into a primary

response of the Th1 or Th2 type has been intense. Basic questions to be addressed included whether both Th1 and Th2 cells differentiate via a Th0 phenotype but segregate due to differing cytokine and APC exposure, or if naive cells are precommitted as precursor Th1 and Th2 cells (Th1p and Th2p) to become one subset or the other upon maturation (Mosmann and Coffman, 1989). The factors leading to the dominance of one T-helper subset over the other and the proposed differentiation pathways are intricate, hence an overview of the current hypothesis is described below.

2.4.1 Growth Requirements of Th Cell Clones

Investigations into the growth of T cell clones *in vitro* have indicated differences in the timing and responsiveness of the two cell subsets to IL-2 and IL-4, the cytokines produced by Th1 and Th2 cells respectively. Both types of clone can proliferate vigourously in response to IL-2, and to a lesser extent IL-4. However, the Th1 response to IL-4 is low, and weakens with time. In contrast, Th2 cells respond to the effects of IL-4 longer than Th1 cell clones but still require IL-2 for growth (Fernandez-Botran *et al.,* 1986, 1988). The dependence of $CD4^+$ T cells on IL-2 for proliferation has been further demonstrated by the generation of transgenic mice containing the Herpes Simplex Virus thymidine kinase (HSV-tk) gene linked to the IL-2 promotor (Minasi *et al.,* 1993). Culture of cells from these animals in the presence gancyclovir to prevent the production of endogenous IL-2 results in abrogation of proliferation in response to superantigen.

The response of T-helper subsets to their own cytokines may indicate that proliferation is under autocrine control at the population level (Mosmann and Coffman, 1989, Fernandez-Botran *et al.,* 1988). In addition to cytokine stimulation, costimulatory molecules play an important role in primary growth responses. Signalling through the B7/CD28 pathway is required for naive T cell development both *in vitro* and *in vivo*, although the addition of IL-2 appears to bypass the need for accessory cell presence during primary stimulation (Seder *et al.,* 1994). The importance of costimulation in the differentiation of Th cell subsets is described in section 2.4.5

2.4.2 The Role of Cytokines in Th Cell Differentiation

Two main approaches have been used to investigate the development of Th cells from naive precursors. The first involved *in vitro* culture of naive T cells or Th0 clones in the presence of mitogen and various cytokines or anti-cytokine antibodies. However, recent transgenic mouse technology has permitted the development of animals with a modified αβ T cell receptor (TCR) specific for an antigen that the animal would not normally encounter *in vivo*, for example ovalbumin (Hseih *et al.,* 1993, O'Garra and Murphy, 1994 , Seder *et al.,* 1992, Scott, 1993a). This results in the isolation of true naive cells which can then be stimulated either *in vitro* or *in vivo*, reproducing a more natural pathway of activation in the presence or absence of various cytokines.

The major cytokines implicated in T-helper cell differentiation are IL-4 with regard to Th2 cell development (Swain *et al.,* 1990, Croft and Swain, 1995, LeGros *et al.,* 1990, Seder *et al.,* 1992a, O'Garra and Murphy, 1994), and IFNγ and IL-12 for Th1 development (Gajewski and Fitch, 1988, Hsieh *et al.,* 1993, Seder *et al.,* 1993, Manetti *et al.,* 1993, 1994). The cell types responsible for the initial production of these cytokines required for subset differentiation have also been extensively studied. The *Leishmania major* infection model in mice has proved an ideal system in which to analyse the development of T cell subsets due to the two extremes of response seen in mice infected with the parasite. Self-healing strains produce a strong DTH response with Th1 cytokine secretion, whereas non-healing mice die due to disease exacerbation as a result of Th2 cytokine profiles (Heinzel *et al.,* 1989, 1993b, Scott, 1993a, Reiner and Locksley, 1995). This model will only be discussed with strict relevance to the cytokine factors necessary for subset development in this section, the model as a whole will be discussed more thoroughly in section 2.6.

2.4.3 IL-4 and Th2 Development

As mentioned above, IL-4 is crucial to the development of a Th2 cell population in both *in vitro* and *in vivo* systems (Swain *et al.,* 1990, LeGros *et al.,* 1990, Scott, 1993a and Vella *et al.,* 1992, Vella and Pearce, 1992). The cell type acting as the primary source of this cytokine is still not clear, although possiblities include T cells themselves, mast cells and basophils. There is some evidence to suggest that naive T

cells ($CD44^{LO}$) can spontaneously produce IL-4, especially if repeatedly stimulated with antigen (Vella *et al.,* 1992, Croft and Swain, 1995). This contradicts earlier findings that only $CD44^{HI}$ cells can produce cytokines other than IL-2 (Budd *et al.,* 1987), although these differences may be due to differing levels of sensitivity in the cytokine assays. However, mast cells and basophils can both secrete IL-4 and may provide the initial burst of the cytokine to initiate Th2 development (Ben-Sasson *et al.,* 1990a, Mosmann *et al.,* 1991, Bradding *et al.,* 1992, Scott, 1993a, Mosmann and Sad, 1996). Furthermore, a population of cells bearing the markers NK1.1, CD4, and CD44 has been isolated which can produce IL-4 without the requirement for initial IL-4 presence (Yoshimoto and Paul, 1994).

Although the development of both Th2 and Th1 cell populations requires IL-2, exogenous IL-4 directs development towards a Th2 phenotype and decreases the number of IFNγ and IL-2 producing effectors (Swain *et al.,* 1990, LeGros *et al.,* 1990, Macatonia *et al.,* 1993). The presence of high levels of IL-4 in primary culture conditions can reduce the number of IFNγ producing cells, resulting a population displaying predominantly Th2 characteristics (Swain *et al.,* 1990, LeGros *et al.,* 1990). In addition, studies involving the *in vitro* stimulation of naive $CD4^+$ T cells from αβ TCR transgenic mice have shown that the presence of IL-4 determines the development of these cells into IL-4-secreters (Seder *et al.,* 1992a, Macatonia *et al.,* 1993). Thus it is generally accepted that IL-4 is critical for the polarisation of naive $CD4^+$ T cells into an IL-4-producing Th population.

2.4.4 IFNγ, IL-12 and Th1 Development

Although the direct involvement of IFNγ in the generation of Th1 cell populations has been more difficult to demonstrate than the role for IL-4 in Th2 development, there is some evidence to suggest that this cytokine can promote Th1 cell polarisation by inhibition of Th2 proliferation (Gajewski and Fitch, 1988, Seder *et al.,* 1993). IFNγ has been reported to drive naive T cells towards a Th1 pattern of cytokine secretion in some instances, but is ineffective at activating resting memory cells (O'Garra and Murphy, 1994). Furthermore, addition of IFNγ to primary cultures of αβ TCR transgenic T cells is not sufficient for the development of a Th1 cytokine-secreting cell population (Hseih *et al.,* 1993). More recently, IL-12 has been

implicated as the Th1 equivelent to IL-4 (Hsieh *et al.,* 1993, D'Andrea *et al.,* 1992, Sher *et al.,* 1993). IL-12 is produced by macrophages, dendritic cells and B cells (D'Andrea *et al.,* 1992, Trinchieri, 1995), and can act to stimulate IFNγ production from T cells and NK cells so driving development towards a Th1 population (Perussia *et al.,* 1992, Seder *et al.,* 1993, Manetti *et al.,* 1993, 1994, Hsieh *et al.,* 1993). The association between IL-12 and Th1 cell induction may have developed through innate response mechanisms, since it has been shown that pathogens which induce Th1-like profiles upon infection also induce IL-12 production upon infection, for example in *Staphylococcus aureus* (D'Andrea *et al.,* 1992) and *Toxoplasma gondii* (Sher *et al.,* 1993).

The macrophage is thought to be the major cellular source of IL-12 during the initiation of a primary response (reviewed by Scott, 1993b, Trinchieri, 1995). IL-12 produced from macrophages can act synergistically with TNFα and IL-1β, which are also produced by these cells, to give optimal proliferation of Th1 cells (Trinchieri and Scott, 1994). In addition, IL-12 might act both directly through receptors on the T cell itself, and indirectly via IFNγ production by NK cells (Scharton and Scott, 1993, Hsieh *et al.,* 1993). Acting independently of IFNγ, IL-12 can reduce IgE levels by Th2 stimulated B cells and can suppress Th2 development *in vivo* (Manetti *et al.,* 1993). IFNγ from NK and T cells can stimulate a positive feedback mechanism for more IL-12 production from macrophages (reviewed by Trinchieri, 1995). Furthermore, there is some evidence to suggest that IFNγ may be required for the initial stimulation of IL-12 secretion from macrophages (Trinchieri, 1993, 1995) and can upregulate transcription of both the p35 and p40 subunits required for biologically active IL-12 (Ma *et al.,* 1996).

Although IFNγ appears to have a positive effect on the expression of IL-12, the ability of IFNγ gene-disrupted mice to produce IL-12 would seem to indicate that this cytokine is not an absolute requirement (Wynn *et al.,* 1995b). Furthermore, addition of IL-12 to primary cultures of naive T cells results in the development of Th1 clones independently of accessory cell presence (Seder *et al.,* 1993, Macatonia *et al.,* 1993). In contrast, IFNγ appears to have little effect in either APC-dependent or independent priming. Further evidence supporting the proposal that macrophages provide the initial burst of IL-12 comes from studies involving primary stimulation

of T cells in the presence of various APCs. If B or dendritic cells are used as the APCs, exogenous IL-12 is required to promote Th1 development whereas macrophages require no additional cytokine presence (Hsieh *et al.,* 1993). IL-12 produced by macrophages has also been shown to stimulate cytokine production by NK cells independently of T cell presence, either alone, or in combination with TNFα and IL-2 (Gazzinelli *et al.,* 1993).

IL-12 secretion can be inhibited by IL-4, IL-10 and TGFβ, presumably through the downregulatory effects of these cytokines on macrophage cytokine expression (Fiorentino *et al.,* 1991b, Tripp *et al.,* 1993, D'Andrea *et al.,* 1993). IL-4 appears to have a dominant role over IL-12, since Th2 development can occur in the presence of IL-12 (Trinchieri, 1993, Trinchieri and Scott, 1994, Reiner *et al.,* 1994, Reiner and Locksley, 1995). Moreover, combining IL-12 and IL-4 in primary cultures leads to development of a Th2-like population (Seder *et al.,* 1993, Hsieh *et al.,* 1993).

With human T cell lines, clones which usually develop into Th2 cytokine producers upon *in vitro* culture can be induced to produce more IFNγ and reduced levels of IL-4 if derived in the presence of IL-12 (Manetti *et al.,* 1993). Th1-type clones derived in the presence of anti-IL-12 display a Th0 phenotype, whereas transient IFNγ production can be observed in established Th2 clones cultured with IL-12, although the Th2 phenotype is not permanently reversible (Manetti *et al.,* 1994).

Finally, an alternative early source of IFNγ for Th1 development might be γδ T cells, which have been detected within the first three days of *Listeria* innoculation into the peritoneal cavity (Hiromatsu *et al.,* 1992). Furthermore the presence of these cells in lesions induced by infection with *Leishmania major* implies that they may contribute to an early burst of IFNγ to increase IL-12 expression and hence Th1 polarisation (Scott, 1993b).

2.4.5 The Influence of Costimulatory Molecules and Antigen Presenting Cells on Th1/Th2 Development

The interaction of APC with naive T cells is thought to play an important role in the determination of the outcome of primary stimulation. The factors responsible for stimulation include: the primary engagement of the TCR with MHC class II

displaying antigen, and a secondary signal from costimulatory molecules present on the surface of the APC. The importance of accessory signals during the *in vivo* induction of an immune response is becoming evident from numerous studies on costimulatory molecules such as the B7 family, ICAM-1 and some cytokines (reviewed by Linsley and Ledbetter, 1993, Jenkins, 1994, Linsley, 1995, Allison and Krummel, 1995).

In vitro studies have indicated that naive T cells require interaction with professional APCs displaying high levels of costimulatory molecules for activation (Larson *et al.,* 1994, Murphy *et al.,* 1994, Croft *et al.,* 1994, Croft and Swain, 1995, Kawamura and Furue, 1995). Thus dendritic cells, Langerhans cells and activated macrophages and B cells can act as superior APCs for naive T cells, whereas resting macrophages and B cells are inefficient at promoting expansion (Croft and Swain, 1995).

Although the involvement of B7 on APCs and CD28 on T cells was thought initially to be a simple model of primary stimulation, the discovery of families of receptors and ligands with differing affinities and expression profiles has revealed a more complicated mechanism. There are now two known members of the B7 family, B7-1 and B7-2, with a possible third under investigation (Kawamura and Furue, 1995). B7-1 and B7-2 are expressed by macrophages, dendritic and Langerhans cells, and B and T lymphocytes (Hathcock *et al.,* 1994); both these molecules can provide costimulatory signals for naive T cells independently of each other. Further, B7-2 is constitutively expressed on most APCs and can be upregulated to high levels by IL-4 and IFNγ (Kuchroo *et al.,* 1995, Kawamura and Furue, 1995, Morokata *et al.,* 1995, Larsen *et al.,* 1994). In contrast, B7-1 is expressed upon cell activation and is upregulated *in vitro* by the action of IL-4, and inhibited by IFNγ (Kawamura and Furue, 1995). Both B7-1 and B7-2 are downregulated by IL-10, possibly explaining the inhibitory effects this cytokine has on the capacity of macrophages to act as APCs (Ding *et al.,* 1993, Kawamura and Furue, 1995).

Similarly there are two main receptors for these ligands on T cells, CD28 and CTLA-4. CD28 is expressed constitutively at high abundance, but has a low affinity for both B7-1 and B7-2 (Linsley, 1995, Allison and Krummel, 1995, Kuchroo *et al.,* 1995). In contrast, CTLA-4 is highly inducible but has low abundance before

activation, and binds B7-1 and B7-2 with a greater affinity than CD28. Thus it initially appeared that CD28 and CTLA-4 may cooperate in the activation of T cells. However, recent research into the functions of the components of this costimulatory pathway has highlighted an inhibitory role of CTLA-4 on T cell expansion. The main evidence for this has come from the generation of CTLA-4 gene-disrupted mice (Waterhouse *et al.,* 1995). These animals exhibit extensive lymphoproliferation, with infiltration of activated lymphocytes into the major organs of the body. It would therefore seem that CTLA-4 acts as a natural negative regulator of the T cell response (Allison and Krummel, 1995). Furthermore, overlap between the function of CD28 and CTLA-4 also appears unlikely due to the observation that CTLA-4 cannot compensate for the reduced levels of stimulation in CD28 gene-disrupted mice (Shahinian *et al.,* 1993). These animals have some limited T cell activation and DTH responses, however, reduced IL-2 levels appear to correlate with diminished T cell help for antibody class switching.

The roles of B7-1/-2 and CD28/CTLA-4 in the induction of the primary immune response has been investigated *in vitro* and *in vivo*. Some published data describe the development of Th2-like cells following *in vitro* stimulation of naive or Th0 cells in the absence of costimulatory signals (Gajewski *et al.,* 1994, Croft and Swain, 1995). Populations displaying Th0 cytokine secretion profiles lose the ability to secrete Th1 cytokines such as IL-2, and progress to develop Th2 characteristics (IL-4 and IL-5 release) when stimulated by TCR cross-linking alone (Gajewski *et al.,* 1994). Furthermore, this population subsequently becomes Th2-like in the calcium levels required for intracellular signalling pathways. Th1 cells typically show an increase in Ca^{2+} when activated via the TCR. However, Th2 clones exhibit higher basal levels of Ca^{2+} and cytokine synthesis appears relatively insensitive to changes in this ion (Gajewski *et al.,* 1989, 1994, Quill and Schwartz, 1987).

Repeated stimulation, or the presence of high levels of antigen can overcome the need for costimulation or exogenous cytokine presence (Croft and Swain, 1995). Naive T cells stimulated *in vitro* under these conditions appear able to produce enough endogenous IL-4 to facilitate the development into a Th2 population. In contradiction to this *in vitro* finding, *in vivo* manipulation of the B7 costimulation pathway has demonstrated that Th2 cell development is more dependent on accessory

molecules than Th1 (Corry *et al.,* 1994). Administration of CTLA-4Ig fusion protein to block B7 interaction with T cells can abrogate susceptibility to *Leishmania major* infection in BALB/c mice if given in the first week. However, this treatment has no effect on the development of a protective response in C57BL/6 animals. Other *in vivo* evidence supporting the requirement of costimulation for Th2 cell polarisation includes: the observation that CTLA-4Ig transgenic mice, which express extremely high levels of soluble CTLA-4, develop diminished Th2 populations, and the reduced ability of CD28 knockout animals to mount antibody-mediated responses (Ronchese *et al.,* 1994, Corry *et al.,* 1994, Shahinian *et al.,* 1993).

Alternative pathways in the *in vitro* stimulation of naive T cells have been reported which do not encompass costimulation with CD28/B7, but depend on the addition of exogenous cytokines. Cell populations displaying Th2 characteristics can be derived *in vitro* by culturing with exogenous IL-2 (Seder *et al.,* 1994). Furthermore, APCs deficient in CD28 can provide priming for Th2 differentiation if additional IL-2 and IL-4 are incorporated into the culture medium, or the addition of IL-2 alone can result in the generation of Th1 cells (Seder *et al.,* 1994).

Much of the investigation into T cell costimulation has been carried out *in vitro* on naive Th0 cell clones and established Th1/Th2 cell lines. Thus the conditions and outcome of manipulation may not reflect the true *in vivo* situation. Some reports have described the induction of anergy in Th1 clones stimulated in the absence of costimulatory molecules (Gajewski *et al.,* 1990, Jenkins, 1994). In contrast, established Th2 clones are reported to require no costimulation if cultured at high density, and can rapidly secrete Th0-like cytokine profiles upon the engagement of the TCR alone through the autocrine production of IL-1 (Zubiaga *et al.,* 1991, Croft and Swain, 1995). Thus cytokine secretion from memory-type cells may not require the same high costimulatory signals as naive T cell activation, and APCs displaying lower levels of B7, such as resting macrophages and B cells, may be able to provide all the signals necessary for reactivation (Croft *et al.,* 1994). However, this is disputed by other data detailing the induction of anergy in both murine and human Th1 and Th2 cells stimulated in the absence of B7/CD28 (Yi-qun *et al.,* 1996, Gimme *et al.,* 1993), although recently activated murine T cells seem able to forego costimulation (Yi-qun *et al.,* 1996).

The differential regulation of Th1 and Th2 activation by costimulatory molecules has been reported with reference to the timing of the secondary signal. In the activation of Th1 clones, CD28 engagement induces autocrine IL-2 production and cell expansion. In contrast, Th2 cells require IL-4 for activation. However, in order to respond to increased IL-4 levels the cells require IL-1, presumably derived from the APC (McArthur and Raulet, 1993). Thus in Th2 clones costimulation results in the increased responsiveness to IL-4, and B7/CD28 is not required if exogenous IL-1 is added to the cultures. Optimal stimulation of human and murine Th1 clones can be achieved with the addition of IL-12 into the culture medium (Murphy *et al.,* 1994, Kubin *et al.,* 1994). Thus IL-12 appears to synergise with costimulatory signals through the B7/CD28 pathway in the stimulation of Th1 development.

Finally, investigations into the possible differential roles of the costimulatory molecules have shown that B7-1 and B7-2 can induce Th1 and Th2 differentiation respectively (Kuchroo *et al.,* 1995). The addition of anti-B7-1 antibody during *in vitro* stimulation results in the generation of a Th2-like population with IL-4 secretion, whereas anti-B7-2 results in IFNγ secretion. This has been confirmed *in vivo* by the abrogation of encephalomyelitis with the administration of anti-B7-1 to reduce Th1 development and promote healing Th2 cytokine secretion (Kuchroo *et al.,* 1995).

2.4.6 Th1 and Th2 Development: The Hypothesis

The current dogma for differentiation of T-helper phenotypes involves progression of T cells through various stages of activation and cytokine secretion. Upon primary stimulation of naive T cells, IL-2 is the only cytokine produced, closely followed by low levels of IL-4 secretion, both these cytokines are required for T cell proliferation and growth (reviewed by Mosmann and Coffman, 1989, Croft and Swain, 1995 Mosmann and Sad, 1996). This stage in development has been summarised as a T-helper precursor cell (Thp). The next stage of differentiation is characterised by secretion of a mixed Th1/Th2 profile of cytokines including IL-2, IL-3, IL-4, IL-5, IL-10 and IFNγ with cells designated as possessing a Th0 phenotype (Firestein *et al.,* 1989). This pattern of secretion appears to be short-lived, especially *in vivo*, and the cell population rapidly progresses into Th1- or Th2-type secretors,

secretors, depending on the cytokine environment, type of APC and costimulation signal. Th1 and Th2 cells can return to the restricted IL-2 secreting phenotype when they become memory cells with the cell markers CD45RB^{lo}, CD44^{hi}, L-Selectin^{lo} (Mosmann and Moore, 1991). Memory cells can be directed to develop into Th1 or Th2 populations rapidly upon secondary stimulation with APC and antigen, possibly with less restricted costimulatory requirements.

Based on the similarity in intracellular Ca^{2+} profiles upon activation, some reports have suggested that precursor Thp cells can develop directly into Th1-type populations (Gajewski *et al.*, 1994). Th0 cells show an intermediate calcium profile between that for Thp and Th2 cells, indicating that Th0 may represent a step in Th2 development rather than a stage between Th1 and Th2 differentiation. The precommitment of Thp cells to either Th1 or Th2 subtypes before primary stimulation was a convincing hypothesis for immune response development, but has recently been disputed by analysis of transgenic animals with the HSVtk gene situated behind the promoter for the IL-4 gene (Kamagowa *et al.*, 1993). When cells from these mice are activated *in vitro* in the presence of gancyclovir any cell producing IL-4 is eliminated, and thus the role of IL-4-producing cells in T cell differentiation can be defined. Results from these investigations have indicated that IL-4- and IFNγ-secreting cells originate from the same precursors which can express IL-4, implying that Th1 and Th2 cells may differentiate from the same cell population.

2.5 Cross Regulation of the Th1 and Th2 Responses

Th1 and Th2 subsets represent the extremes of $CD4^+$ T cell polarisation, resulting in DTH or antibody-based immune responses respectively. These responses are mutually exclusive, which implies that a counter regulatory mechanism must exist between them. IFNγ has been found to inhibit Th2 effector function by suppressing proliferation of this subset (Gajewski and Fitch, 1988). However, it appears that IFNγ has no effect on cytokine secretion from Th2 cells, and hence the decreases observed in cytokine secretion are due to the reduction in cell number rather than

inhibition of gene transcription. IL-12 may also have the capability to reduce IL-4 levels and thus inhibit Th2 population development (Manetti *et al.,* 1993, Finkelman *et al.,* 1994, Trinchieri, 1995), although it has recently been observed that murine Th2 cells lose the ability to respond to IL-12 receptor signalling and thus any effect must occur early during differentiation (Szabo *et al.,* 1995).

In response to the regulation of Th2 populations by IFNγ and IL-12, the Th1 subset can be downregulated by IL-4 produced by Th2 cells, and IL-10 produced by Th2 cells and macrophages (reviewed by Mosmann and Moore, 1991, Mosmann and Coffman, 1989, Moamann and Sad, 1996). IL-10 is thought to be the major regulatory cytokine involved in modulating the Th1 and inflammatory immune responses, and can execute this affect via a variety of mechanisms. This cytokine can suppress Th1 activity by inhibiting the production of IFNγ and IL-2, seemingly via inhibition of macrophages and their inflammatory cytokine release (Fiorentino *et al.,* 1989, 1991a, 1991b, Moore *et al.,* 1990). The *in vitro* downregulation of Th1 populations only occurs when macrophages are the acting APC; there is no effect when the macrophages are replaced with B cells. The ability of macrophages to induce a Th2 response is not impaired, and therefore IL-10 may be acting on the expression of costimulatory molecules and independently of TCR-MHC interactions (Fiorentino *et al.,* 1991a, Ding *et al.,* 1993, Kawamura and Furue, 1995). This observation might explain the lack of effect on Th2 cell induction, a process thought to require less costimulation than Th1 populations (Croft and Swain, 1995). Not all inhibition by IL-10 can be attributed to macrophages; inhibition may also affect other APC populations such as dendritic cells. However, B cells are not inhibited by IL-10, and indeed the Ly-1/CD5 subset of this population has been shown to produce this cytokine (O'Garra *et al.,* 1990, Murphy *et al.,* 1993). IL-10 inhibition of T cell proliferation has also been reported in human systems (Taga and Tosato, 1992), although the production of this cytokine by both human Th1 and Th2 cells implies that a more general suppression of the immune response occurs rather than regulation solely of Th1 popularions (Romagnani, 1994).

It is well documented that IFNγ can act as a potent upregulator of macrophage activation and the resulting inflammatory cytokine release (Fiorentino *et al.,* 1991a, 1991b, Gautham *et al.,* 1992, Gazzinelli *et al.,* 1992a). IL-10, and to a lesser extent

IL-4, can inhibit IL-1, IL-6 and TNFα production in macrophage cell lines and peritoneal macrophages (te Velde *et al.,* 1990, Essner *et al.,* 1989, Fiorentino *et al.,* 1991a and 1991b). The downregulation of TNFα may also act to inhibit the Th1 response, since TNFα is a cofactor for the production of IFNγ from murine T and NK cells, which in turn is thought to be important in the polarisation of a Th1 population. IL-10 inhibition of IFNγ production can be overridden by the addition of exogenous TNFα (Fiorentino *et al.,* 1991a,1991b). However, TNFα stimulation of human T cells is insufficient to stimulate the secretion of IFNγ, and thus it is unlikely that the major inhibitory effect of IL-10 acts via the downregulation of TNFα (D'Andrea *et al.,* 1993). An effect of IL-10-mediated downregulation of IFNγ is the inhibition of NO production by macrophages, so reducing microbicidal activity (Gazzinelli *et al.,* 1992b).

IL-10 can also inhibit IL-12 production by macrophages and dendritic cells (D'Andrea *et al.,* 1993, Trinchieri and Scott, 1994). This in turn will downregulate the production of IFNγ. Thus, in conjunction with the downregulatory effects upon the costimulatory family of B7 molecules and cofactors TNFα and IL-1 (Kubin *et al.,* 1994), IL-10 can inhibit IFNγ release and hence Th1 development. IL-10 suppression can be overridden in human systems by addition of IL-12 and IL-1 (D'Andrea *et al.,* 1993).

The ability of IL-10 to depress the inflammatory response has been put to good use by the Epstein Barr Virus (EBV), which contains an open reading frame in the viral genetic code homologous to IL-10 (Moore *et al.,* 1990). When translated, this protein can inhibit IFNγ production *in vitro* by both isolated murine T cells and human PBMC, mainly from NK cells in the latter population (Hsu *et al.,* 1990). Thus production of this IL-10 homologue by B cells infected with the virus may protect these cells from host attack during viral replication (Mosmann and Moore, 1991). In addition to depressing cytokine production by macrophages and $CD4^+$ T cells, IL-10 can also act to downregulate Th1-like cytokine production from murine $CD8^+$ (Mosmann and Moore, 1991) and NK cells (Sher *et al.,* 1992) resulting in suppression of all sources of Th1-like cytokines.

IL-4 is the other major Th2 cytokine thought to exert control over Th1 responses and thus enhance the expansion of Th2 cells. This cytokine can downregulate IFNγ

synthesis and T cell proliferation (Peleman *et al.,* 1989, Vercelli *et al.,* 1990, Martinez *et al.,* 1990) and inhibit macrophage cytotoxic functions and cytokine expression (Essner *et al.,* 1989, te Velde *et al.,* 1990, Sher *et al.,* 1992, Oswald *et al.,* 1992, Gautham *et al.,* 1992). In addition, IL-4 can regulate IL-12 production, and thus the development of a Th1 response, via the inhibition of cytokine release from monocytes and macrophages (Doherty *et al.,* 1993, Trinchieri, 1993, 1995). Hence IL-4 has become the major marker for Th2 cell polarisation and ongoing antibody-mediated effector responses.

A recently discovered cytokine with IL-4 properties, IL-13, has also been found to have suppressive effects on cell-mediated immunity (Minty *et al.,* 1993, Doherty *et al.,* 1993). This cytokine is produced by Th2 cells and inhibits both murine and human macrophage cytotoxicity and inflammatory cytokine release. In addition, human IL-13 can upregulate IgE production from B cells, although this function has not been described in the mouse (McKenzie *et al.,* 1993). Murine IL-13 suppresses monokine release in response to IFNγ or LPS (lipopolysaccharide) stimulation, thus decreasing IL-12 and iNOS production at the transcription level (Doherty *et al.,* 1993). This reduction in cell activity has been illustrated by enhanced survival of *Leishmania major* parasites in macrophages cultured *in vitro* in the presence of IL-13 (Zurawski and DeVries, 1994).

Finally, TGFβ produced by macrophages, T and B cells can have a general downregulatory effect on macrophage cytokine production, lymphocyte proliferation and NK cell effector function (Silva *et al.,* 1991). This cytokine can act on many components of the immune response, but in contrast to early *in vitro* studies suggesting that TGFβ can promote Th1 cell expansion (Swain *et al.,* 1991), recent data has implied that the converse is true (Schmitt *et al.,* 1994). In fact, TGFβ can strongly inhibit IFNγ production in response to IL-12 stimulation, and prevent IL-12-induced Th1 development. Furthermore, TGFβ can inhibit NK cell activity; thus depletion of this cytokine in mice infected with *Leishmania major* can lead to enhanced Th1 responses and resistance in susceptible strains (Su *et al.,* 1991, Barral-Netto *et al.,* 1992).

2.6 Models of Parasitic Disease: The Relevance of Th1 and Th2 Subsets

2.6.1 Introduction

By the very nature of parasitic infections, the immune response against the invading organism is strong and sustained. This provides the stimulation necessary to make parasitic models ideal *in vivo* systems in which to study the induction, polarisation and cross-regulation of T-helper subsets. The ease of manipulation provided by animal models has permitted the detailed examination of induction and effector mechanisms generated upon infection, and allowed the development of various vaccination strategies. During these investigations, an abundance of information on the immunology of T-helper cells has been described. Many parasitic infections have been found to provoke the development of one Th subset over the other. This has lead to the general observation that intracellular invaders such as protozoa, bacteria and viruses induce cell-mediated Th1-type responses, whereas multicellular organisms such as helminths lead to the development of humoral Th2 responses (reviewed by Scott and Kaufman, 1991, Scott *et al.,* 1989, Sher *et al.,* 1992, Sher and Coffman, 1992, Romagnani, 1994). The immune mechanism generated upon infection is not always in the best interest for survival of the host, the inappropriate production of cytokines sometimes leading to pathology rather than cure. This section will briefly discuss the induction of T-helper subsets following infection with various pathogens, with special emphasis on the induction and effector mechanisms employed in the murine models of leishmaniasis and schistosomiasis. Both these systems have provided valuable information on the mechanisms underlying Th1 and Th2 polarisation. In fact, exposure of mice to *Leishmania major* remains the most defined model of T-helper subset differentiation during parasite-induced immune responses. Hence this model has become the paradigm against which Th1/Th2 segregation during other infections is compared.

2.6.2 Infections Resulting in the Generation of Th2 profiles

Typical immediate hypersensivity responses induced by the promotion of Th2 cytokines occur with helminths such as *Nippostrongylus brasiliensis*, *Trichinella spiralis* and *Trichuris muris* (Svetic *et al.,* 1993, Scott and Kaufmann, 1991, Else *et*

al., 1991, 1994, Finkelman *et al.,* 1991). These parasites promote high serum IgE, and blood and tissue eosinophilia via increased IL-4 and IL-5 levels respectively (Coffman *et al.,*1989, Finkelman *et al.,* 1991, Cox and Liew, 1992), and the generation of a Th2-based mechanism is responsible for protection. In some cases, blocking Th2 cytokine production with anti-cytokine antibodies, or administering Th1-promoting cytokines such as IL-12, leads to the abrogation of resistance. For example, in *Heligosomoides polygyrus, T. spiralis and T. muris* infections, ablating IL-4 increases worm survival and renders resistant strains susceptible (Else *et al.,* 1994, Sher and Coffman, 1992). Similarly, administration of IFNγ or IL-12 early after infection with *N. brasiliensis* can exacerbate infection (Finkelman *et al.,* 1994, Sher and Coffman, 1992). The induction of a Th2 response following patency of *Schistosoma mansoni* infection in mice will be described in detail in section 2.8.

2.6.3 Infections Resulting in the Generation of Th1 Responses

The majority of research into the differentiation of Th1 populations during parasitic infection has been carried out in association with the development of protection following exposure of mice with *Leishmania major* (Scott, 1989, 1991, Locksley and Scott, 1991, Reiner and Locksley, 1995). The effects of this parasite on the host are directly related to the type of Th response induced, and this will be discussed as a model for the investigation of T-helper subsets in detail below. Other infections known to generate strong cell-mediated immunity include *Mycobacterium tuberculosis* and *leprae* (Scott and Kaufmann, 1991, Walker *et al.,* 1992, Mosmann and Sad, 1996) and *Toxoplasma gondii* (Sher and Coffman, 1992, Mosmann and Sad, 1996). In these infections parasite control and killing is mediated by IFNγ activation of macrophages and the release of toxic compounds (Liew *et al.,* 1990, 1991, Gazzinelli *et al.,* 1992a, 1992b, 1993, Oswald *et al.,* 1994c), and blocking either IFNγ or IL-12 can have a deleterious effect on the outcome of disease (Scott *et al.,*1989, Gazzinelli *et al.,* 1993, Walker *et al.,* 1992, Yamamura *et al.,* 1991).

There are cases when the nature of the immune response generated to cope with the invading parasite causes severe consequences for host survival. Human infection with falciparum malaria can result in TNFα levels elevated to such an extent that they become lethal, an effect also observed in murine malaria (Grau *et al.,* 1987, Sher

and Coffman, 1992). Similarly, following *S. mansoni* infection, the Th2 immune response mounted against eggs deposited in the liver results in the formation of granulomas, which in turn leads to liver damage (reviewed by Sher and Coffman, 1992). It is also important to note that some infections rely on the production of Th1-like cytokines from $CD8^+$, rather than $CD4^+$, T cells, since depletion of this cell population during infection results in reduced protection, for example *T. gondii* and *Trypanosoma cruzi* (Gazzinelli *et al.,* 1991, Tarleton, 1990, Sher and Coffman, 1992).

2.7 Leishmaniasis

2.7.1 Background

Leishmaniasis is a protozoal disease transmitted by the bite of the sandfly when the promastigote stage present in the saliva invades host macrophages. The infectious stage transforms into an amastigote within the macrophage, and undergoes a period of replication before rupturing the cell and infecting other macrophages in the vicinity. The resulting disease mainfests itself as various types of cutaneous tissue necrosis, and the most invasive strain, *L. donovani* progresses internally to cause visceral leishmaniasis. This latter infection in humans is closely mimicked by the infection of mice with *L. major*. The study of this disease in mice has led to the discovery of strains resistant and susceptible to infection, with phenotypes which correlate closely to the type of T-helper cell response mounted (reviewed by Scott, 1989, Reiner and Locksley, 1995). Self-healing strains include C57BL/6, B10 and C3H/HeN, in which infection progresses no further than a cutaneous lesion and heals with no treatment. Susceptible strains such as BALB/c cannot control infection, and the cutaneous lesion progresses into the visceral tissues to become lethal.

The very different responses to infection have been associated with the induction of different cell populations. Ablation of the $CD4^+$ T cell type with specific antibody in susceptible mice results in the ability of these animals to self-heal (Heinzel *et al.,* 1989, Scott, 1989). In contrast, neither the $CD8^+$ or NK cell compartments are crucial for disease resolution in C57BL/6 mice. The following two sections describe

the cytokines involved in resistance and susceptibility to murine infection with *Leishmania major*.

2.7.2 The Th1 Response and Resistance to L. major

In resistant animals, healing of lesions is concomitant with high IFNγ production at both the mRNA and protein level, and low or undetectable levels of IL-4, from LN cells (Heinzel *et al.,* 1989). Administration of recombinant IL-4 or anti-IFNγ can lead to exacerbation of the disease, temporarily or permanently respectively (Chatelain *et al.,* 1992, Belosevic *et al.,* 1989, Sadick *et al.,* 1990). To ablate the IFNγ response and affect the outcome of the disease, the antibody treatment has to be given within the first week of infection, indicating that it is the differentiation of the Th1 population that must be altered (Scott, 1991). Likewise, suppression of macrophage activation by IL-4 treatment later in infection is not sufficient for reversal of protection in resistant animals (Scott, 1989, Scott *et al.,* 1989, Sadick *et al.,* 1991, Reiner and Locksley, 1995).

Although it has been shown that early IFNγ is required for resistance, C57BL/6 mice do not produce high levels of this cytokine during the first week of infection but are nonetheless resistant (Reiner *et al.,* 1994). Thus research has concentrated on possible differences between healer and non-healer strains in an attempt to define the crucial step in induction of immunity which is absent in BALB/c mice. In some strains, such as C3H/HeN, NK cells have been shown to provide an early source of IFNγ (Scharton and Scott, 1993). However, BALB/c mice have a low NK cell response, possibly due to downregulation by early IL-4 and TGFβ. The presence of IL-13 in both resistant and susceptible mouse strains during the first 4 days of infection may also enhance IFNγ release from NK cells. This cytokine has properties similar to IL-4, with the surprising exception that it can promote some IFNγ production from murine NK cells (Doherty *et al.,* 1993).

The protection afforded by the Th1 response has been associated with the activation of macrophages to a state capable of killing the amastigote parasites (Nathan *et al.,* 1983, Green *et al.,* 1990, Liew *et al.,* 1990, 1991). This is further demonstrated by the ability of IL-4 and TGFβ to inhibit macrophage activation, consequently leading to increased levels of parasitaemia (Barral-Netto *et al.,* 1992).

Studies into the cytotoxic nitrogen products released by activated macrophages have highlighted the importance of NO in amastigote killing (Green *et al.,* 1990, Liew *et al.,* 1990, Reiner and Locksley, 1995). Although the crucial difference in the survival pattern of BALB/c and B10 mice is the release of NO in the latter (Hsieh *et al.,* 1995), these differences do not appear to correlate with an early production of either Th1 or Th2 cytokines, which are similar in resistant and susceptible strains (Reiner *et al.,* 1994). Furthermore, BALB/c macrophages are capable of releasing NO when driven by a Th1 population generated by the addition of exogenous cytokines (Reiner *et al.,* 1994, Hsieh *et al.,* 1995). Thus, the differences in the outcome of infection observed between these strains are apparently due to the eventual default pathway of Th subset differentiation; self-healing mice producing a Th1 response resulting in macrophage activation and NO production, and non-healers producing a Th2 reponse rich in IL-4 and IL-10 resulting in inhibition of macrophage activation. The dependence of resistant mice upon the production of NO was proved beyond doubt with the generation of iNOS-deficient mice (Wei *et al.,* 1995). These mice, on the MF1 background, are normally resistant to infection with *L. major*. However, upon exposure of animals with a deleted portion of the inducible NOS gene, and hence a non-functional enzyme, the lesions become visceral and lethal. Thus, it can be concluded that this macrophage product is essential for control of parasitaemia and self-cure in murine leishmaniasis.

2.7.3 The Th2 Response and Progression of Disease

The cytokine responses of susceptible mice to infection correspond to a characteristic Th2 phenotype: high IL-4 and IgE antibody titres, low IFNγ and no DTH reaction. LN tissue sampled from infected BALB/c mice displays high levels of IL-4, IL-1β and IL-10 expression with little IFNγ message (Heinzel *et al.,* 1989, 1991). Furthermore, a single dose of anti-IL-4 administered to susceptible mice at the time of infection results in the healing of cutaneous lesions, self-cure and protection against further infection (Chatelain *et al.,* 1992). Presumably the long lasting effects of an antibody which only has a half-life of 4.5 days is due to the suppression of IL-4 during a crucial stage of Th2 development, allowing the expansion of the Th1 subset which subsequently remains as a memory population.

The administration of recombinant IFNγ leads to temporary improvement in the progression of leishmaniasis in susceptible hosts, but the effects are not permanent (Scott, 1991). In addition, the administration of T cell clones of either Th1 or Th2 in nature, and specific for leishmanial antigen can either cure or exacerbate disease in BALB/c mice respectively (Scott *et al.,* 1988), again demonstrating the complete dependence of pathology on the expression of Th2 cytokines.

The high level of IL-2 secretion in susceptible mice has initiated research into this non-Th2 cytokine and its role in disease exacerbation. IL-2 is required for the development of both Th1 and Th2 subsets (LeGros *et al.,* 1990, Ben-Sasson *et al.,* 1990b), and ablation of this cytokine results in self-healing of 80% of infected mice (Heinzel *et al.,* 1993a). Moreover, during this treatment an increase in the level of IFNγ and a reduction in IL-4 production by LN cells is observed. Furthermore, the IFNγ secretion can be traced to both $CD4^+$ and $CD8^+$ cell populations, an effect not observed in anti-IL-4 treated BALB/c mice. The IL-2 production is limited to B cells in BALB/c mice, in contrast to C57BL/6 animals where both T and B cells produce this cytokine (Heinzel *et al.,* 1991). This production of IL-2 may explain the reversal of progressive disease when B cells are ablated. Ablation of IL-2 in resistant mice has no effect on the development of Th1 cells, possibly due to the lower levels of IL-2 required by Th1 cells for proliferation, and the synergistic effect of small amounts of IL-2 with IL-12 in promoting Th1 expansion (Heinzel *et al.,* 1993a, 1993b).

Contrary to earlier studies which reported the complete segregation of IFNγ and IL-4 mRNA expression in resistant and susceptible strains using the Northern blot technique (Heinzel *et al.,* 1989), more recent research has indicated that both types of mice produce a Th0 response during the first week of infection (Morris *et al.,* 1992, Scott,1991, Reiner *et al.,* 1994). A high percentage of IL-4-producing T cell clones can be derived from BALB/c mice at 1 week post-infection, and this value continues to rise up to week 8. A similar panel of clones is obtained at week 1 from self-healing mice. However, by week 8 the proportion of clones expressing a Th2 phenotype has fallen and those cells secreting IFNγ are dominant (Morris *et al.,* 1992). Furthermore, this cytokine production has been shown to originate from the $CD4^+$ T cell compartment in both strains of mice (Scott *et al.,* 1989, Scott, 1991). Analysis of the mRNA production in LN tissue from susceptible and resistant strains

demonstrated that both types of mice produce IFNγ, IL-2, IL-4, IL-10 and IL-12 within the first 4 days of infection (Reiner *et al.,* 1994). Resistant mice then start to downregulate the Th2 cytokine synthesis whereas non-healing mice do not, despite the production of IL-12, resulting in the development of a strong Th2 response. A possible explaination for this observation is proposed by a recent report indicating that BALB/c mice lose the ability to generate IL-12-induced Th1 responses (Güler *et al.,* 1996). As infection progresses the capacity of susceptible mice to respond to IL-12 is reduced, presumably allowing upregulation of Th2 cytokine expression. This data is supported by the finding that Th2 cells are unable to respond to IL-12 at the intracellular signalling level (Szabo *et al.,* 1995). The onset of IL-12 unresponsiveness may also account for the inability of this cytokine to alter an established Th2 response in BALB/c mice when administered after the first week of infection (see section below, Heinzel *et al.,* 1993a).

2.7.4 The Importance of IL-12 in Leishmania *Infection*

After the discovery of IL-12 and its role in the differentiation of Th1 populations, the effect of administering or blocking this cytokine in both susceptible and resistant strains of mice infected with *L. major* was investigated. Recombinant IL-12 given early to BALB/c mice can lead to complete cure (Heinzel *et al.,* 1993a, Sypek *et al.,* 1993), whereas anti-IL-12 can exacerbate the diseased state of the resistant mice if given at 1 week post-infection (reviewed by Trinchieri and Scott, 1994, Scott, 1993b, Locksley, 1993).

There is some debate as to whether IL-12 is required only during the early response, or is also important at later time points. As mentioned above, administration of IFNγ cannot reverse the outcome of infection in susceptible mice on a permanent basis (Sadick *et al.,* 1990). However, IL-12 appears able to do so, suggesting that it is manipulation of T-helper subset differentiation which is important. An early role for IL-12 in the development of protection is contradicted by the ability of promastigotes to evade induction of pro-inflammatory cytokine mRNA upon initial infection (Reiner *et al.,* 1994). Furthermore, *in vitro* culture of macrophages with *L. major* parasites fails to result in IL-12 production. Although stimulation of murine peritoneal macrophages *in vivo* by injection of parasites into

the abdominal cavity followed by cell harvesting and *in vitro* stimulation can induce the release of IL-12, this may reflect the effect of secondary activation rather than primary interaction with promastigotes (Vieira *et al.,* 1994). The discrepancies between *in vivo* and *in vitro* systems may reflect the lack of costimulatory molecules during artificial stimulation. Alternatively, delayed expression of IL-12 may only be detected when the second round of macrophage infection with amastigotes occurs (Reiner *et al.,* 1994, Vieira *et al.,* 1994). It has been shown that a lipopolysaccharide produced by promastigotes can inhibit IL-12 production by macrophages *in vitro*. In fact the timing of IL-12 production, around day 7 post-infection, coincides with amastigote release from ruptured cells and the reinfection of further macrophages (Reiner *et al.,* 1994). Thus this parasite stage may be more resistant to cellular attack and not require immune evasion strategies. Finally, due to the effectiveness of IL-12 in the induction of Th1 responses and protection, its role as an adjuvant in vaccination has been investigated. Administration of IL-12 and leishmanial antigen has been shown to increase NK cell activity and IFNγ release, and as such this cytokine has potential in the development of a vaccine (Afonso *et al.,* 1994).

2.8 Schistosomiasis

2.8.1 Introduction

Infection of mice with *Schistosoma mansoni* has further highlighted the crucial effect of T-helper subset polarisation on the outcome of parasitic exposure. Several models are currently under investigation, each resulting in the induction of very different cytokine profiles. The three major models include liver granuloma formation following normal infection, the development of synchronous egg granulomas in the lungs, and the irradiated vaccine model. Each of these systems will be discussed below in relation to the cytokine profiles produced during the immune response. The polarisation of Th1 and Th2 subsets in these models is not as extreme as that observed during *Leishmania major* infection. Thus the results obtained are often contradictory and difficult to interpret, and further research is

required to unravel the complicated interplay of the Th1 and Th2 cytokines in schistosomiasis.

2.8.2 The Formation of Liver Granulomas Following Normal Infection

Following infection of mice with normal cercariae, a strong Th2 cytokine response develops which appears to be induced by the deposition of eggs in the liver (Pearce *et al.,* 1991, Grzych *et al.,* 1991). Typical characteristics of this response include high serum IgE, eosiniphilia, and a seemingly Th2-associated pathology around eggs trapped in the liver (reviewed by Scott *et al.,* 1989, Sher and Coffman, 1992). It has been reported that prior to worm maturation and the onset of egg laying, a response rich in Th1 cytokines develops (Pearce *et al.,* 1991). However, after oviposition this response is downregulated, presumably by the enhanced levels of Th2 cytokines which can be detected (Pearce *et al.,* 1991, Gryzch *et al.,* 1991, Sher *et al.,* 1992).

The majority of research into the immune response induced by normal infection has concentrated on characterising the cytokines involved in the granulomatous response against eggs trapped in the liver. This consequence of schistosome infection results in the pathology associated with the disease, and hence an understanding of the immune response may aid the development of a vaccine to prevent the damage caused by granuloma formation. Approximately half the eggs produced by an adult worm pair get washed downstream to the liver by blood flow. There they attract a small focus of $CD4^+$ T cells (Mathew *et al.,* 1986), which in turn promotes cellular recruitment and formation of a granuloma comprising of $CD4^+$ T cells, $CD8^+$ T cells, macrophages, eosinophils, neutrophils, giant cells, mast cells, fibroblasts and some B cells (Moore *et al.,* 1976). The developmental progression of granuloma formation can be divided into a distinct sequence of events with respect to growth and cytokine profiles (Boros, 1994): a primary response, peak granuloma formation, and immunomodulation of the responses.

a) Primary Granuloma Development

Primary responses in the liver begin around week six of infection with a switch from Th1 to Th2 cytokine production (Pearce *et al.,* 1991, Grzych *et al.,* 1991). The stimulus for this change in response appears to be the onset of oviposition, although

there are conflicting reports concerning the involvement of Th1-type cytokines in the formation of the liver granulomatous response (reviewed by Boros, 1994). Some studies have demonstrated that single sex infections result in the induction of a mixed Th1/Th0 response with production of IFNγ, IL-2 and IL-4 and only marginal secretion of the other Th2 cytokines (Grzych *et al.,* 1991, Vella and Pearce, 1992, Chensue *et al.,* 1993). This would suggest that it is the presence of egg antigen which promotes the expression of Th2 cytokines; an observation supported by the rapid expression of IL-4 in a $CD4^{-ve}$ cell population upon egg injection into the peritoneal cavity (Sabin and Pearce, 1995). Furthermore, two oligosaccharides derived from eggs have been shown to induce IL-10 and prostaglandin E2 production by B cells *in vitro*, both of which can downregulate the Th1 response (Velupillai and Harn, 1994).

By six to eight weeks post-infection, IL-4 and IL-5 mRNA can readily be detected in liver tissue and isolated granulomas (Grzych *et al.,* 1991). In addition, egg antigen stimulates higher Th2 cytokine responses than adult worm antigen from splenocytes in culture. Cytokines predominant in the development of granulomas at this early stage include IL-4, IL-5, IL-10, TNFα, IL-1β, TGFβ and low levels of IFNγ (Wynn *et al.,* 1993, Boros, 1994), with message for all these cytokines readily detectable and IL-4, IL-5 and IL-10 displaying the highest levels of expression (Wynn *et al.,* 1993). IFNγ production declines around week eight post-infection. However, this cytokine appears to maintain a regulatory role in the granulomatous response, since the administration of an anti-IFNγ antibody results in the exacerbation of granuloma development. In contrast, treatment with recombinant IFNγ can reduce the size of established granulomas (Chensue *et al.,* 1992, Boros, 1994).

b) Peak Granuloma Development

Eight to twelve weeks after infection, granuloma development peaks, coinciding with high levels of IL-4 and IL-5 which promote increased levels of serum IgE and eosinophilia respectively (Grzych *et al.,* 1991). During this vigorous stage there is little IFNγ release; IL-4 is the dominant cytokine detectable at this time and may act to downregulate the IFNγ and Th1 cell responses (Henderson *et al.,* 1991, Chensue *et al.,* 1992). Administration of anti-IL-4 antibody can reduce granuloma growth, and

somewhat surprisingly causes a reduction in IL-2 levels (Chensue *et al.,* 1992, Boros, 1994). The involvement of this Th1 cytokine has been under much debate due to conflicting reports. The advent of the reverse transcription-polymerase chain reaction (RT-PCR) has permitted the detection of this cytokine during the acute stage of granuloma formation (Wynn *et al.,* 1993). Although Northern blot analysis has repeatedly failed to detect any trace of IL-2 in the liver (Henderson *et al.,* 1991), this is probably due solely to the lower sensitivity of this technique compared to RT-PCR. Furthermore, a role for this cytokine in granuloma formation is supported by the finding that anti-IL-2 antibody treatment results in a reduction in granuloma size and fibrosis (Cheever *et al.,* 1992), and that IL-2 can exacerbate lesion development in $CD4^+$-ablated mice (Mathew *et al.,* 1990). Repressed granuloma development after anti-IL-2 treatment corresponds to a reduction in IL-5 levels and eosinophilia, an observation supported by data describing IL-2-dependent eosinophilia (Yamaguchi *et al.,* 1990). However, the reduction in this cell type is not responsible for the reduced size of the granulomas, since anti-IL-5 treatment has no effect in this model (Sher *et al.,* 1990b). Recent studies on the infection of IL-4 gene-disrupted mice have implied that this cytokine is not responsible for hepatic pathology (Pearce *et al.,* 1996). Infection of these knockout animals did not result in the expected reduction in granuloma development, contradicting earlier studies involving ablation of this cytokine with anti-IL-4 antibody (Chensue *et al.,* 1992). Possible explainations for the discrepancies observed between the two experimental systems might include compensation for gene loss by the operation of redundant mechanisms in knockout animals.

In summary, expression of the Th2 cytokines is dominant during peak granuloma development. However, in addition there is a requirement for IL-2 in the vigourous growth of the lesions. The association of IL-2 and IL-4 release is perhaps not so surprising in that IL-2 is a requirement for the development of Th2 responses (LeGros *et al.,* 1990, Ben-Sasson *et al.,* 1990b, Cheever *et al.,* 1992). In addition, IFNγ appears to play a control function in the granulomatous response at this stage, since ablation of this cytokine with specific antibodies results in exacerbation of granuloma development and an increase in Th2 cytokine production (Chensue *et al.,* 1992, 1994).

c) Immunomodulation of the Egg Granuloma

During the period of sixteen to twenty weeks post-infection, the granulomatous response becomes immunomodulated and existing granulomas reduce in size (Stadecker, 1992, Boros *et al.,* 1975). IL-4 and IL-2 production from the granuloma is greatly reduced at this stage, along with increased expression of IFNγ (Chensue *et al.,* 1993, Henderson *et al.,* 1992). The source of IFNγ remains unclear, although $CD8^+$ cells may be responsible for the production of cytokines able to inhibit the Th2 response in the absence of a Th1 $CD4^+$ cell population. During this period of suppressed activity, the administration of IL-4 results in an upregulation of granuloma formation around new eggs arriving in the liver, a response similar to that seen upon injection of IL-2 (Mathew *et al.,* 1990, Yamashita and Boros, 1992, Boros, 1994). In addition, recombinant TNFα treatment in immunomodulated animals leads to vigorous granuloma formation, and both IFNγ and IL-2 can enhance production of TNFα from granuloma macrophages (Joseph and Boros, 1993). High levels of IL-10 can be detected in splenocytes isolated from immunomodulated mice and may possibly be acting in its capacity as a downregulatory molecule, especially in the inhibition of macrophage cytokine and toxic mediator production (Gazzinelli *et al.,* 1992b, Sher *et al.,* 1992, Mosmann and Moore, 1991).

In contradiction to the above data suggesting that Th1 cytokine release may be responsible for immunomodulation, macrophages isolated from mice displaying downregulated responses are unable to stimulate schistosome antigen-specific Th1 cell clones, resulting in anergy (Flores Villanueva *et al.,* 1994, Stadecker, 1992). In contrast, egg antigen presentation to Th2 clones is unaffected. This observation implies that granuloma APCs become suppressive to Th1 clones, promoting the development of a Th2 response during this late stage of the granuloma development. This effect may be caused by inhibitory cytokines such as IL-10, or via costimulation in the absence of B7/CD28, and can be reversed by the introduction of IL-2 into the cultures (Flores Villaneuva *et al.,* 1994). Thus these data disagree with Th1-mediated immunomodulation in two ways. Firstly, these results imply that it is Th1 cytokine secretion which induces granuloma formation, and that immunomodulation is the result of downregulation of these cytokines. Secondly, that Th2 cytokines are responsible for the immunomodulation of the Th1 response, and hence the reduction

in granuloma development and size. However, IL-4 gene-disrupted mice are capable of immunomodulation, hence arguing against a role for this Th2 cytokine in the suppression of the granulomatous response (Pearce *et al.*, 1996).

2.8.3 The Synchronous Lung Egg Granuloma Model

The analysis of granuloma development in the lungs has increased in popularity over the last few years because it permits the study of the kinetics of cytokine release. Thus the sequential expression of cytokines upon injection of a standard number of eggs and their subsequent arrival in the lungs has been determined. RT-PCR studies at day one after intravenous injection can detect IFNγ, IL-1β and IL-6 mRNA in lung tissue, coincident with small influxes of mononuclear cells (Wynn *et al.*, 1993). By day three, IL-2, IL-4 and IL-10 mRNAs are detected and mononuclear cell accumulation begins. The last cytokines found to be upregulated in this study are IL-5 and TNFα, coincident with peak expression of the earlier cytokines and preceding maximum granuloma formation by eight days (Wynn *et al.*, 1993). As with liver granuloma formation, administration of anti-IL-4 or anti-IL-2 antibodies leads to diminished granuloma size, a reduction in IL-4 and IL-5 expression, and increased IL-10 production, possibly due to the removal of IL-4 inhibition of macrophage cytokine production. In contrast, ablation of IL-12 or IFNγ with specific neutralising antibodies leads to an enhanced Th2 response and a subsequent increase in granuloma size (Wynn *et al.*, 1994b). The study of this model has shown that the induction of IL-12 can be blocked by ablating IFNγ or NK cells, implying that IL-12 expression is dependent upon the presence of IFNγ (Wynn *et al.*, 1994b, Gazzinelli *et al.*, 1993). In addition, ablation of this cell type results in reduced IFNγ mRNA and an increase in granuloma size. Thus the source of early IFNγ required for IL-12 expression may be NK cells, and possibly macrophages themselves (de Waal Malefyt *et al.*, 1991, Scharton and Scott, 1993).

Administration of recombinant IL-12 with either a primary or secondary egg infection inhibits granuloma formation, and correlates to increased levels of IFNγ, IL-2, IL-12 and, surprisingly, IL-10 at the mRNA level (Wynn *et al.*, 1994b). The other Th2 cytokines, IL-4, IL-5, IL-6 and IL-13, all decrease presumably due to the suppressive effects of the upregulated Th1 response upon Th2 cytokine secretion

(Gajewski and Fitch, 1988, Seder *et al.,* 1993). Increases in IL-10 mRNA associated with upregulation of Th1 cytokine expression have been reported numerous times (Wynn *et al.,* 1993, 1994a, 1995a, 1995b, Finkelman *et al.,* 1994). This may be explained by the downregulation of inhibitive Th2 cytokines, and the corresponding activation of cytokine production from macrophages. Thus IL-10 may be upregulated as a consequence of general upregulation of cytokine secretion by activated macrophages. Alternatively, the detection of mRNA for IFNγ and IL-10 in the same $CD4^+$ T cell may account for the observed link in expression of these cytokines (Assenmacher *et al.,* 1994). In addition to the above egg model, IL-12 has also been shown to reduce Th2 responses induced upon egg injection into the footpads of mice, if given at early time points (Oswald *et al.,* 1994b).

Injection of eggs into athymic mice has highlighted the T cell-independent production pathways of IFNγ, IL-6, IL-10 and IL-12 mRNA (Wynn *et al.,* 1993). Granuloma development in these animals is virtually absent, even in the presence of high levels of TNFα, indicating that this cytokine alone is not sufficient for the induction of the granulomatous response. The above cytokines may be expressed by macrophages, NK cells and γδ T cells, all of which are present in athymic mice (Yoshikai *et al.,* 1992, Rocha *et al.,* 1992). However, the production of IL-4 and IL-5 is completely absent, demonstrating that these cytokines have a solely T cell source in this model.

A recent study of egg-induced lung granuloma formation in IFNγ gene-disrupted mice has investigated the ability of IL-12 to stimulate T cells directly in the absence of endogenous IFNγ (Wynn *et al.,* 1995b). Surprisingly, IL-12 administration to these animals results in exacerbation of the Th2 response and granuloma formation. Following infection, TNFα, TNFβ and IL-2 message are all upregulated in $IFN\gamma^{-/-}$ mice, along with the IL-12 p40 chain. This implies that IL-12 does not require IFNγ for self regulation or Th1 cytokine induction and can act directly on T cells, an observation supported by other reports (Seder *et al.,* 1993, Macatonia *et al.,* 1993). The increase in expression of the Th2 cytokines may be IL-2 driven in the absence of IFNγ inhibition, resulting in an increased granulomatous response. This correlates with observed increases in eosinophilia and IgE levels, indicative of the requirement of IFNγ for the IL-12-driven downregulation of these factors (Wynn *et al.,* 1995b).

In support of this work, $CD4^+$ T cells have been shown to default to the Th2 developmental pathway in IFNγ gene-disrupted mice (Wang *et al.,* 1994).

2.8.4 The Attenuated Vaccine Model

The kinetics of basic cytokine secretion in the attenuated vaccine model have already been established at the protein level and described in sections 1.6 and 1.7. However, the manipulation of this system in immunological research, and relevance to Th1 and Th2 subset development will be discussed below. Murine exposure to *S. mansoni* has provided a system for the investigation of Th cell subsets due to the segregating cytokine profiles observed following normal or a vaccinating infection. Patency of a normal infection induces a Th2 environment not favourable to resistance and resulting in pathology, as described above in section 2.8.2. In contrast, exposure to irradiated cercariae results in the development of a Th1 response capable of arming against exposure to normal parasites. Although this system appears to generate dichotomy superficially similar to that observed following infection with *Leishmania major*, the polarisation of cytokine profiles into Th1 and Th2 subsets is not as extreme, and the exact mechanism of resistance not as well understood.

a) The Cytokine Phenotype Required for the Induction of a Protective Response

The protective response induced with the attenuated vaccine model is dependent upon the generation of an IFNγ-producing population of $CD4^+$ T cells in the skin-draining LN (Pemberton *et al.,* 1991, Mountford *et al.,* 1992). As a proportion of the applied parasites migrate through the LN draining the exposure site, high levels of IFNγ can be detected in culture supernatents from cells stimulated *in vitro*. However, no IL-2, and only minimal levels of IL-4 and IL-10 protein, are detectable post-vaccination (Pemberton *et al.,* 1991, Pemberton and Wilson, 1995).

Following vaccination, the cytokines most readily detectable after culture of cells washed from the lung airways are IFNγ and IL-3; there are very low detectable levels of IL-2, IL-4 or IL-5 protein (Smythies *et al.,* 1992a, Wilson *et al.,* 1996). In addition, the lack of proliferation observed in this isolated cell population upon secondary stimulation would indicate that these cells are of the Th1 memory/effector phenotype (reviewed by Smythies *et al.,* 1993). In fact, this was later shown to be

the case, with cells washed from the airways of vaccinated mice bearing high levels of the surface maker CD44 and low levels of CD45RB, a phenotype associated with short term memory cells (Coulson and Wilson, 1993).

The administration of IL-12 during vaccination has been shown to increase the expression of Th1 cytokines and induce an elevated level of immunity compared to vaccination alone (Wynn *et al.,* 1995a). The increase in IFNγ and IL-12 expression corresponds to a decrease in the levels of IL-4 and IL-5 mRNA, as detected by RT-PCR. Although IL-12 administered during challenge infection has no effect on the already raised resistance and Th1 message levels obtained by the early treatment, the residual amounts of Th2 cytokine mRNA are lower than those seen in the group given recombinant cytokine with vaccination only. Thus IL-12 may exert its effect by directing the induction response and by reducing any IL-4 inhibition of the effector mechanism. There is no effect on disease outcome if this cytokine is administered during a normal infection (Wynn *et al.,* 1995a).

b) The Cytokines Involved in the Effector Mechanism

Post-challenge, the efficiency of the effector mechanism can be almost completely abrogated by the neutralisation of IFNγ (Sher *et al.,* 1990a, Smythies *et al.,* 1992b). This results in increased Th2 cytokine expression and reduced Th1 and IL-10 mRNA levels in treated mice (Wynn *et al.,* 1993, 1994a). In contrast, immunity is not affected by the ablation of IL-4 or IL-5 (Sher *et al.,* 1990a). These ablation experiments support data describing the high level of IFNγ protein secretion during the effector response (Smythies *et al.,* 1992a). The inflammatory cytokine TNFα, produced by Th1 cells, Th2 cells, and macrophages appears to have no influence in the protection mechanism since specific ablation of this cytokine has no effect on resistance (Smythies *et al.,* 1993).

Analysis of the lung effector response at the RT-PCR level has uncovered a more complicated picture than previously described by protein studies. Not only do vaccinated and challenged mice express higher levels of IFNγ, IL-2 and TNFα mRNA than challenge control animals, but IL-4, IL-5, IL-10 and IL-13 expression is also elevated above the naive baseline(Wynn *et al.,* 1994a). In addition, IL-10 and IL-13 mRNA can be detected at higher levels in previously primed mice than in

naive animals upon challenge infection. The major difference observed between infection of vaccinated and naive mice is the timing of the Th1 and Th2 responses. Vaccinated and challenged mice mount earlier IFNγ and IL-2 responses compared to the control group, with IL-10 following the IFNγ expression profile and not behaving like a Th2 cytokine, as reported previously (Wynn *et al.,* 1993, 1994a, Finkelman *et al.,* 1994). This increased expression of IL-10, an inhibitor of inflammatory cytokine release from macrophages and Th1 cells, may possibly be due to the increased activation of macrophages in the presence of IFNγ. Thus IL-10 may be expressed as a consequence of general macrophage activation, also indicated by the increase in TNFα and IL-1β expression in the effector response (Wynn *et al.,* 1994a).

The upregulation in the expression of cytokines required for macrophage activation and cytokine release in lung tissue from vaccinated and challenged mice might indicate that the toxic intermediate nitric oxide, also produced by macrophages, plays a role in the effector mechanism. It has been shown that IFNγ, IL-1β, IL-2 and TNFα can all act to upregulate the expression of the iNOS gene, the enzyme responsible for NO production from L-arginine (Oswald *et al.,* 1994a, 1994c). All these cytokines are present in the lungs post-challenge and thus NO might be expected to play a role in parasite elimination. Indeed, iNOS mRNA is upregulated above baseline values at day 17 post-challenge (Wynn *et al.,* 1994a), at a time when parasites are trapped in the lungs (Coulson and Wilson, 1988). In addition, blocking NO production by the administration of an arginine analogue results in an overall increase in worm burdens of 33% (Wynn *et al.,* 1994a). The importance of NO in this model is discussed further in chapter four.

In conclusion, vaccination of mice with optimally irradiated cercariae of *Schistosoma mansoni* leads to the generation of a predominantly Th1-type response. This can be manipulated to develop further towards Th1 or Th2 polarisation by the administration of IL-12 or ablation of IFNγ, respectively. Normal infection progresses towards a Th2 polarisation quicker than a vaccinating exposure in which IFNγ predominates above a mix of cytokines to promote the development of inflammatory foci around migrating parasites. Although the data described above addresses the same questions on the expression of cytokine mRNA as those considered in this thesis, these recently published investigations were performed

concurrently with the analysis reported in this DPhil. Differences between the data described above and that detailed in the subsequent chapters will be discussed as relevant.

2.9 Aims of This Study

From the above two sections it can be concluded that the generation of a Th1 population in the skin-draining LN, and the recruitment of these cells to the lungs is necessary for the production of protective IFNγ in this model. The aims of this study were to investigate in detail the response generated following exposure of mice to the irradiated *Schistosoma mansoni* vaccine. The method chosen for this investigation was RT-PCR, a very sensitive technique for the detection of mRNA without the need for *in vitro* secondary stimulation as required with cytokine protein detection systems. In chapter two a full description of the precise methods chosen and adapted for the analysis of cytokine mRNA, both at a semi-quantitative and quantitative level, is described. In subsequent chapters these methods are used to investigate the cytokine profiles induced in the lungs and LN of mice exposed to various exposure regimes.

Chapter three concentrates on the cytokine patterns detected during the induction mechanism in the skin-draining LN following vaccination and challenge. The expression levels of the major Th1 and Th2 cytokines are described with relevance to the type of T-helper subset induced during vaccination. In chapter four the cytokines expressed during cell recruitment to the lungs post-vaccination, and the subsequent effector mechanism are examined. In addition, at a time of peak response post-vaccination, sorted cell populations isolated from the lung airways are analysed to define the cell types reponsible for the major cytokine expression at this time. The expression of inducible nitric oxide synthase post-challenge is also investigated to determine whether the pattern of mRNA transcription has any correlation with peak cytokine production. Finally, the kinetics of cytokine expression in the lungs is analysed following the arrival of a synchronous pulse of parasites administered by intravenous challenge.

The consequence of host strain on cytokine production during the induction mechanism is studied in chapter five. The cytokine responses occuring in the LN and lungs of C57BL/6 and BALB/c mice over a short timecourse are described. The final experimental chapter deals with the development of a screening technique for the segregation of homozygous gene-disrupted mice from heterozygous and wild type littermates. This procedure is applied to the screening of IFNγ receptor-deficient animals, with subsequent analysis of a homozygous and wild type population for the profiles of cytokine expression induced following vaccination and challenge.

In conclusion, this thesis provides a detailed analysis of the cytokine profiles induced in the LN and lungs of mice exposed to cercariae of *Schistosoma mansoni*. The use of RT-PCR has facilitated the analysis of many cytokines not previously detectable by protein assay due to sensitivity problems or the lack of available antibodies.

CHAPTER TWO

The Development of Semi-Quantitative and Competitive PCR Based Techniques for the Analysis of Cytokine mRNA

Introduction

Until recently, the study of cytokine release in immunological research has been based on the detection of protein secretion from cultured cells. The main disadvantage of this technique is the requirement for *in vitro* stimulation before culture supernatants can be assayed, quite possibly resulting in a distortion of the true *in vivo* response. An alternative to cytokine protein analysis involves the detection of cytokine mRNA from fresh tissue without *in vitro* stimulation. This approach provides a snapshot of the immune response occurring *in vivo* at the time of sampling. In addition, analysis of mRNA can detect the presence of a cytokine which may be absent in culture supernatants due to uptake and utilisation by cells bearing receptors. Thus, mRNA profiles can provide an accurate account of cytokine production in any organ from which RNA can be isolated. However, care must be taken with the interpretation of results. The analysis of gene expression at the message level is problematic if the protein of interest has a constitutively expressed, stable mRNA which is always present in the tissue. In these cases, the presence of mRNA does not necessarily mean that the active protein is secreted from the cell. This problem does not arise when protein expression is regulated at the transcription level, with no storage of mRNA or post-transcriptional regulation. The majority of cytokine genes fall into the inducible category, and are not constitutively expressed (Dallman *et al.,* 1991a, 1991b). On the whole, cytokine mRNAs are very unstable, with short half lives and low copy numbers (Shaw and Kamen, 1986). This means, that for successful analysis, sampled tissue must be fixed rapidly to prevent RNA degradation once the immunological stimulus for production is removed.

Previous research has investigated the presence of cytokine mRNA in parasitic models of infection by Northern blotting. However, this technique requires large amounts of mRNA and lacks the sensitivity essential for the detection of these relatively rare messages. Systems in which Northern blotting has proved effective at detecting cytokine mRNAs are based on acute immune responses induced by high levels of antigen, such as invasion of LN tissue by *Leishmania major* (Heinzel *et al.,* 1989), or the granulomatous response to egg antigen during *Schistosoma mansoni*

infection (Henderson *et al.,* 1991, 1992). In both these experimental systems, the advent of more sensitive techniques has uncovered a more complicated picture than originally described based on results from Northern blotting. In addition, Northern blot analysis has failed to detect any cytokine expression (IFNγ, IL-2, IL-4 and IL-5) in LN and spleen RNA isolated from mice infected or vaccinated with cercariae of *S. mansoni* (Constant, 1991, Betts, unpublished observations), even though *in vitro* culture of isolated cells has indicated that at least some of these cytokines can be detected at the protein level in this model (Constant *et al.,* 1990, Mountford *et al.,* 1992, Pemberton *et al.,* 1991, Pemberton and Wilson, 1995). The differences in detection seen between the *S. mansoni* vaccination model and the *Leishmania* and egg granuloma models may be due to the presence of a constant source of stimulation in the latter two systems. In contrast, following vaccination of mice with irradiated cercariae the parasites only reside transiently in the LN, and there is no evidence for parasite presence in the spleen (Mountford *et al.,* 1988).

Other methods of mRNA analysis include the RNAse protection assay and *in situ* hybridisation. However, these methods also possess drawbacks. RNAse protection is more sensitive than Northern blotting, but requires relatively large quantities of mRNA and thus is difficult to apply to small organ or biopsy samples. *In situ* hybridisation is incredibly sensitive, detecting mRNA species at the single cell level, but is technically very demanding to quantify and requires stringent control measures to prevent false positive results. Faced with these less than satisfactory mRNA detection systems, the majority of cytokine research was carried out at the protein level following *in vitro* culture.

The advent of the Polymerase Chain Reaction (PCR) (Saiki *et al.,* 1988, Rapolee *et al.,* 1988), once linked to a reverse transcription (RT) reaction, has permitted the analysis of cytokine gene expression in systems where traditional methods of detection have failed. The PCR procedure amplifies a DNA template sequence up to detectable levels using a DNA polymerase enzyme stable at high temperatures, and a temperature cycling program to alternate strand denaturing and DNA synthesis. This reaction can produce a doubling of template DNA with every round of the temperature cycling profile, resulting in reaction kinetics resembling an exponential

growth curve. However, by the very nature of the reaction, true quantification is difficult to achieve. PCR is a very sensitive technique and makes the detection of any rare message or DNA possible. However, this sensitivity can lead to a variety of associated problems in that it allows amplification of any contaminating DNA, such as previously amplified PCR product, present in the RNA sample or reaction components. To prevent these problems arising, all pre-PCR manipulations can be carried out in a clean area separate from the product handling area, such as a flow hood, and reagents and equipment can be kept as DNA-free as possible. Aliquoting of primer and enzyme stocks can also aid the prevention of expensive bulk contamination.

In spite of all the above drawbacks, PCR has become one of the most widely used tools for the analysis of mRNA due to the sensitivity and ease of the assay. The research described in this thesis is based on RT-PCR detection of murine cytokine expression by the application of methods adapted from the many published protocols to provide semi-quantitative and quantitative data. For semi-quantitative analysis, the majority of protocols described in the literature rely on the comparison of products derived from RT-PCR of test and control RNA samples using the same primer pair (Murphy *et al.,* 1993, O'Garra and Vieira, 1992, Dallman *et al.,* 1991a). However, to investigate the relationship between the levels of different cytokine production within the same sample, a more complicated PCR procedure is required, involving the addition of competitive templates as described later.

To achieve a semi-quantitative result in cytokine mRNA analysis, several methods have been described. One of the earliest assesses the PCR product generated after increasing numbers of cycles (reviewed by Dallman *et al.,* 1991a, Montgomery and Dallman, 1991, Dallman *et al.,* 1991b). Product from each PCR tube is sampled at intervals of five cycles, dot blotted and hybridised with end-labelled probes specific for each primer pair. Autoradiographs of the probed products are then inspected visually to detect any differences in cytokine expression. This method is especially useful in distinguishing variations in cytokine expression in different tissues. Analysis at a single cycle number may miss the exponential range of the PCR, with some samples having reached saturation, whilst others contain barely detectable

product. For semi-quantitative assessment of the mRNA level in a sample, PCR product must be examined during the exponential phase of the reaction. Analysis at multiple cycle numbers allows the position of this phase to be determined, and provides a good estimate of the comparative levels of RNA expression between samples.

A second published method for the analysis of PCR product involves the use of a diluted standard RNA sample (Murphy *et al.,* 1993, O'Garra and Vieira, 1992). RNA isolated from cell lines expressing the cytokine of interest is serially diluted to give a standard curve against which test samples can be compared. Duplicate test samples are expressed as arbitrary units derived from the standard curve. The housekeeping gene HPRT (hypoxanthine-guanine phosphoribosyltransferase) is amplified to indicate any RNA degradation or loss, and can be used as a correction factor for differing levels of starting RNA, since the effect of activation on expression of this gene is minimal (Murphy *et al.,* 1993).

Finally, a quicker, although less accurate, way to analyse mRNA by RT-PCR is by single cycle number PCR with detection of probed products by autoradiographic densitometry (Svetic *et al.,* 1991, Wynn *et al.,* 1993, 1994a, 1995a). The values obtained in this way are often presented as fold increases over baseline, or percentage of the maximum value obtained. This approach is now emerging as the most popular method for rapid analysis of gene expression at the RNA level. However, data displayed in this way provides no information on the baseline level of expression of different cytokines.

The semi-quantitative analysis of mRNA in this thesis is based on the multiple cycle number PCR described above, modified to quantify the amount of radioactive probe bound to the PCR product obtained for each cycle number. Furthermore, the application of regression analysis to the data obtained has been used to determine an accurate value for the level of cytokine mRNA across an infection time course. This extra manipulation allows the PCR amplifications to be monitored for uniform efficiency using a given primer pair.

The expression of different cytokines in a single RNA sample can be analysed by quantitative, competitive PCR. Several methods have been published describing

PCR in the presence of control templates, each with some advantages and drawbacks. One of the disadvantages common to most techniques of quantification is the large number of tubes required for each sample under test, limiting analysis to a few specific samples of special interest. In order to quantify products derived from PCR, a competitive control template can be incorporation into the same tube as the test sample. In this way all reaction variables, except for the amount of starting material, are kept identical. Differences between methods lie in the type of internal control added to the mixture, and whether these constructs control for the RT reaction and PCR, or only for the PCR itself.

One of the most complex original quantitative PCR methodologies was based on the incorporation of an RNA competitor at the start of the RT-PCR analysis (Wang *et al.,* 1989). A synthetic gene was constructed containing a tandem array of cytokine primer pairs designed to generate a product differing in size to that derived from sample RNA. The artificial gene was transcribed with RNA polymerase, and the synthesised RNA collected, purified and quantified by spectrophotometry. The artificial and sample RNA were then added together, serially diluted, and PCR carried out. This procedure generated a standard curve of the construct RNA PCR product against which the test sample could be correlated. A correction factor for the differences in product length was also applied to account for the increased incorporation of ethidium bromide or radioactivity into longer products during the detection step. The end result gave the level of test RNA in molecules at the start of the amplification procedure. This procedure accounts for all components of the RT-PCR reaction, and because the same primers are used to amplify the test and competitive templates, primer efficiency is constant. However, the reaction is complex and requires the construction of a completely novel template for analysis of the RNA of interest. In addition, the test and control samples possess different sequences internal to the two primers, and thus it might be argued that amplification may proceed at different rates for each template, although this could be tested by examining the efficiency of the PCR for the sample and competitive templates independently from each other.

Subsequent methods have tried to strike a balance between accuracy and ease of

setting up the system. For example, the use of genomic DNA as the competitor molecule provides a product naturally different in length which also partly controls for differences in DNA synthesis efficiency, but does not incorporate the RNA reverse transcription step (Gilliland *et al.,* 1990, Carding *et al.,* 1992). Genes with no small introns can be altered by the insertion of a unique restriction site into the genomic competitor so that product can be distinguished by digestion (Gilliland *et al.,* 1990). The genomic DNA is then added in a dilution series to constant amounts of test sample cDNA, and the dilution at which both products are equal in band intensity after probing and densitometry correlates to the point at which both starting templates are equal in concentration. This method can be further quantified by applying a correction factor for the addition of double stranded DNA competitor compared to single stranded sample cDNA (Carding *et al.,* 1992). However, the synthesis of unique competitors for each gene of interest can make the procedure time-consuming to establish.

A more recent competitive PCR protocol combines the convenience of a single artificial competitor for all genes under test, with the accuracy of both templates containing the same sequence for amplification (Reiner *et al.,* 1993, 1994). PCR primers are designed to span a small intron; thus competitive control templates derived from genomic DNA can be separated by size from the test cDNA following PCR. For genes with no appropriate introns, the primers are chosen so that sequence internal to the primers contains a unique restriction site to allow distinction between gDNA- and cDNA-derived PCR products after digestion. All competitive templates are cloned into a single vector and purified to a known concentration. This generates a single competitor which can be used for all the cytokines of interest. The competitor DNA is coamplified in a dilution series with constant amounts of test cDNA and the different size products analysed by gel electrophoresis. Alternatively, to reduce sample number, the competitor can be added at a single, previously determined concentration and the relative intensity of the sample band compared with that of HPRT, which is used as a control for equal amounts of RNA in each sample under test. This method does not control for the reverse transcription step,

but reduces the cost of quantitative PCR considerably. Thus a variety of methods are now available for the accurate analysis of cytokine mRNA by RT-PCR.

In conclusion, PCR has increased the possibilities, and sensitivity of cytokine mRNA analysis over that previously available through Northern blotting (Gilliland *et al.,* 1990, Wang *et al.,* 1989). The full potential of this method is still evolving with the adaptation of non-radioactive ELISA-type assays to increase sample throughput without decreasing accuracy (Tanaguchi *et al.,* 1994). The methods described in the following chapter are adapted from the multiple cycle number PCR approach (Dallman *et al.,* 1991b), and the DNA competitive construct PCR for a more accurate quantification (Wang *et al.,* 1989). Many of the more recent RT-PCR methods described above (Wynn *et al.,* 1993, Carding *et al.,* 1992, Reiner *et al.,* 1993, 1994) were not published when this work was initiated, and thus the procedures described in the following chapters have been adapted independently throughout the course of research. This chapter is designed to provide a detailed account of the methods used in subsequent chapters, along with the problems encountered and an overview of the use of RT-PCR as a tool for cytokine analysis in the present research climate.

Materials and Methods

Tissue Sampling

The tissue from which RNA was to be extracted was either snap frozen in liquid nitrogen, or submersed directly into homogenisation buffer containing guanidinium thiocyanate to inhibit RNAse action (4M guanidinium thiocyanate, 0.1M Tris HCl pH 7.5, 1% β-mercaptoethanol added just prior to homogenisation). Tissues were removed from the body using dissection equipment washed and soaked in diethyl pyrocarbonate-treated water (DEPC-H_2O), a treatment which inhibits RNAse activity.

Total RNA Extraction

RNA extraction was performed using a previously published method adapted for the isolation of DNA-free total RNA from small amounts of tissue (Sambrook *et al.*, 1989). Whole organs were homogenised in 5mls of buffer (as above) with a tissue shearer (Ystral, Dottingen, Germany) at full speed for 2 minutes. Sodium lauryl sarcosinate (tradename Sarcosyl, BDH, Poole, UK) was then added to a final concentration of 0.5% and the cell lysate centrifuged at 3500 rpm for 25 mins at room temperature in a Beckman bench top refrigerated centrifuge. The supernatant was layered onto 3.5 mls of 5.7M CsCl/ 0.01M EDTA (pH 7.5) in a Beckman open top tube, and sample tubes were balanced accurately by weight using homogenisation buffer. The total RNA was pelleted over the CsCl gradient by centrifugation at 20 ^{o}C for 24 hours at 32,000 rpm in a Beckman ultracentrifuge with an SW41 swing-out rotor.

The homogenate and CsCl were removed from above the RNA pellet, and the base of the tube containing the RNA removed with a hot razor blade. The pellet was washed with 70% ethanol and allowed to dry at room temperature. The RNA was resuspended overnight at 4^{o} C in 150µl TES (Tris EDTA/ 0.1% SDS) by gentle shaking, and then transferred to a sterile microfuge tube. Any remaining RNA in the base of the centrifuge tube was washed out with 50 µl TE (Tris EDTA) and added to the resuspended RNA. An equal volume, 200 µl, of phenol/chloroform/

isoamylalcohol mix (25:24:1) was added to the RNA, vortexed well and the aqueous phase removed to a fresh tube. The interface was back-extracted with 200 µl of TE and the two aqueous phases added together. A chloroform extraction was performed to remove any traces of phenol left in the aqueous phase and the RNA was then precipitated at -20°C overnight with 0.1 volumes of 3M sodium acetate and 3 volumes of 100% ethanol.

Finally, the precipitated RNA was collected by centrifugation at 12,000g at 4 °C for 30 mins, the pellet washed with cold 70% ethanol, respun, air dried and resuspended in 100 µl DEPC-H_2O. The yield of total RNA was estimated by spectrophotometry at 260 nm and confirmed, along with the intact nature of the RNA, by 1% agarose gel electrophoresis of between 1-3 µg of isolated RNA. An absorbence reading at 280 nm was performed to monitor the protein contamination of the RNA, and for samples with a protein:RNA ratio higher than 1:1.5, the phenol extraction step was repeated.

Extraction of Total RNA from Small Numbers of Cells

A mini-prep based on the above method scaled down to a 100 µl CsCl volume, as published (Brenner *et al.,* 1991), was used to prepare RNA from small numbers of cells (10^2 to 10^6). However, the carrier ribosomal RNA used in this extraction obscured visualisation of the PCR product unless the cDNA generated by reverse transcription was first treated with DNAse-free RNAse H. In addition, this method could not be relied upon to yield RNA in 100% of extractions performed, and thus for further experiments involving small numbers of cells an RNA isolation kit was purchased (Micro RNA isolation kit, Stratagene, Cambridge, UK), and used in accordance with the manufacturer's instructions.

Reverse Transcription-PCR

To avoid problems with contamination all pre-PCR procedures were carried out in a laminar flow hood distant from the PCR product analysis area, using filter pipette tips and sterile technique. For all RT-PCR methods applied throughout this thesis the same basic reaction mix was used and is described below. The annealing temperatures and magnesium chloride concentrations were optimised empirically to

produce a single product for all sets of primers, and the specific conditions and primer sequences are described in subsequent chapters. A standard amount of total RNA was reverse transcribed in 1x RT buffer (50 mM Tris HCl pH 8.3, 75 mM KCl, 3mM $MgCl_2$ and 10mM DTT) containing 200U Molony-Murine Leukaemia virus (M-MLV) RT (Gibco BRL, Paisley, Scotland), 1 mM dNTP mix, 100 ng oligo dT primer and 20U RNAsin (Promega, Madison, WI). The reaction was incubated at 37°C for 45 mins to generate the cDNA, and then the RT enzyme heat-inactivated at 95°C for 5 mins. The product was stored on ice or at -20°C until further use.

The amplification reaction was carried out in 1x PCR buffer (10 mM Tris HCl, 50 mM KCl, 1.5 mM $MgCl_2$), 0.2 mM dNTP mix, 0.5 μM of sense and antisense primer and 2U Taq DNA Polymerase (Promega). The temperature program used for each primer pair varied in annealing temperature but in general the profiles used were very similar: 92 °C for 1 min; 55, 60 or 65 °C for 1 min; 72 °C for 2 mins. Primer pairs were picked by hand from the published cDNA or gene sequences, or obtained from previously published work and are described in the relevant chapters. Where possible, primers were positioned to span an intron so that any contaminating genomic DNA would not be amplified, or could be distinguished by size from the product derived from mRNA. Positive and negative controls, including RT and PCR template blanks, were run on each amplification to monitor reagent contamination. In addition, controls for contamination during RNA isolation, and the presence of genomic DNA in the test RNA, were run at the start of each new experiment. This was achieved by RT-PCR of an RNA preparation blank, and amplification of RNA without a reverse transcription step respectively. These controls ensured that each PCR was specific for the message in the sample under test.

The analysis of longitudinal time courses was carried out using a multiple cycle number approach (Dallman *et al.,* 1991b), as discussed in the introduction, to obtain accurate estimates of the amount of message present in a sample. In the following chapters total RNA for each organ at a time point was pooled, with equal quantities donated by each individual sample, and duplicates run for each pool to give a total of six PCR tubes per pool per cytokine. The cycle numbers chosen for each primer set were dependent upon the abundance of that particular message in the organ under test, and were determined empirically. However, sampling was always carried out at

intervals of 3 cycles to ensure that the whole analysis took place on the exponential phase of the PCR reaction.

Slot and Southern Blotting

In order to detect the amount of specific PCR product generated, samples were either slot or Southern blotted, and probed with oligonucleotide probes recognising sequences internal to the two primer positions. Since slot blotting does not allow the distinction of size, product for all new primer pairs was first Southern blotted to check for a single product and for probe specificity. After initial PCR optimisation, samples from multiple cycle analysis were slot blotted for ease of handling the large numbers of products generated, and the grid format permitted accurate quantification by phosphorimager.

Southern blotting was carried out using standard procedures (Sambrook *et al.*, 1989, Mason and Williams, 1990). Briefly, after separation on a 2% agarose gel the PCR product was denatured in 1M NaCl / 0.5M NaOH solution for 45 mins and then washed and neutralised in 1M Tris HCl / 1.5M NaCl for 45 mins. The blotting stack was assembled with Zeta probe GT nylon membrane (BioRad, Hemel Hempstead, Herts, UK) and the DNA transferred by capillary action in 10 x SSC (standard sodium chloride/sodium citrate solution). The membrane was fixed with 0.4M NaOH and washed in 2 x SSC before air drying and baking at 80°C for 30 mins under vacuum.

Slot blotting was carried out using a vacuum assisted blotting apparatus (BioRad) with methods modified from the manufacturers instructions. Samples of 5 µl from each product were denatured by the addition of a 95 µl volume of denaturation mix to give a final concentration of 0.4 M NaOH / 10mM EDTA, followed by boiling for 10 mins. After cooling on ice , the DNA was neutralised with an equal volume of ice-cold 2M ammonium acetate and loaded on to pre-wetted nylon membrane. The samples were applied to the membrane using a partial vacuum and the wells washed through with 0.4M NaOH to denature the DNA on the membrane. Finally, the membrane was washed in 2 x SSC and air dried before baking at 80°C for 30 mins in a vacuum oven.

Probe Labelling and Hybridisation

Blotted PCR products were hybridised with short, specific, radioactively labelled oligonucleotide probes. The probes were designed from the published gene/cDNA sequences or picked from previously published work (see Chapters Three and Four for specific information on sequences and relevant references). The labelling reaction was carried out in a 25 µl volume containing 10 pmoles of probe, 10 U T4 Polynucleotide kinase (T4PNK, Promega) and 10 pmoles [γ-^{32}P]ATP (5 µl of 3000 Ci/mmol, NEN Dupont). The reaction mix was incubated at 37 ^{o}C for 45 mins, and the enzyme then heat- denatured at 70 ^{o}C for 5 mins. The labelled probe was diluted up to a final volume of 100 µl and the unincorporated bases removed by spin column chromatography over G-50 sephadex packed into a 1ml syringe. A 1µl aliquot of each pre- and post-spun probe solution was added to 1 ml of scintillant (Ultima Gold, Packard, Meriden, CT) and the counts obtained by β-scintillation counting to estimate the incorporation efficiency and specific activity for each probe. Approximately 10^7 counts per minute (cpm) were required for each probing in 20 mls buffer.

Membranes were prehybridised with an SDS blocking buffer (1 mM EDTA, 7% SDS, 0.5M Na_2HPO_4) at 65 ^{o}C for 5 mins and then hybridised at 42 ^{o}C overnight in 20mls of a fresh aliquot of the same solution containing labelled probe at approximately $2x10^5$ cpm/ml. The blots were washed twice at room temperature in 1 mM EDTA/ 40 mM Na_2HPO_4 pH 7.2/ 5% SDS for between 3 and 5 mins, and then a further wash for a similar period of time at a temperature 1 ^{o}C below the temperature of dissociation (T_d) was carried out in a solution containing 1% SDS. The washing temperature was calculated using the following formula: $T_d = 2(A+T) + 4(G+C)$.

Washed blots were wrapped in clear film and exposed to a storage phosphor screen for between 0.5 and 24 hours dependent upon the specific activity of the probe, and the level of bound radioactivity as assessed by a hand held monitor. The screen was scanned on a phosphorimager (SF, Molecular Dynamics, Sevenoaks, Kent, UK) and the resulting image analysed to obtain counts for individual blotted samples. Rectangles or grids of the correct size were drawn around the band or slots in the displayed image. The phosphorimager counts within each cell were obtained, corresponding to a known sample and cycle number.

Data Analysis and Statistics

For multiple cycle PCR analysis, the six counts obtained for each sample were plotted as a linear regression and estimates for product at a given cycle number calculated from this line. The regression equation derived for each time point was tested for significance against the slopes of samples amplified in the same PCR and primer group by obtaining the standard error of the difference between lines (standard statistical test). A lack of significance indicated that all the PCR reactions for a given primer pair are amplifying at the same efficiency. Error bars derived from the 95% confidence limits for each equation (1.96 x SD, as calculated by computer or from standard statistical texts) were added to the estimated value of a specific cytokine at a given cycle number within the analysis range. Error bars that did not overlap indicated a significant difference with $p \leq 0.0025$ (0.05^2).

For simple comparisons at a few time points, PCR was carried out for a single cycle number and the mean of the individual samples plotted. All time courses were adjusted for differences in the housekeeping gene, HPRT, expression to account for variations in the amount of starting material added to each RT-PCR.

Competitive PCR

The above methods can only be used to compare the amount of a single cytokine present in different samples across a time course. For analysis of different cytokines within a sample, an internal control template of known concentration must be co-amplified in the same tube as the test sample using the same primer pair. Quantitative PCR analysis in subsequent chapters involves the incorporation of a competitive template into the PCR reaction. This artificial construct (kindly synthesised and donated by Mr Kevin Page, Glaxo Group Research, Stevenage, after a method first described by Wang *et al.*, 1989) contained a tandem array of primer sequences and was designed to result in generation of a product different in size from that derived from cytokine mRNA. Primer sequences incorporated into the construct included IFNγ, IL-2, IL-4, IL-5, IL-10 and HPRT. The competitor was serially diluted between 1μg and 10 fg, and 5μl of each dilution added to a constant amount of test template, derived from 1μg or 0.25μg of total RNA for lung and LN tissue respectively. PCR was carried out as described previously. However, the construct

was designed with primer sequences different to those already in use for the semi-quantitative PCR and thus the amplification conditions were slightly different, requiring an annealing temperature of 55°C and a cycle number of 30 or 35 depending on the sample and cytokine under test.

All test RNA was pooled to reduce tube number, and initially titrated using a 1:10 dilution series of plasmid DNA, followed by a 1:4 range if a finer degree of accuracy was required. The products were separated by 2% agarose gel electrophoresis in the presence of ethidium bromide, and the point at which product derived from test cDNA equaled that from plasmid determined by visual inspection. At the point of equivalence, the amount of plasmid incorporated into the PCR mix pre-amplification provides an estimate of the initial amount of cDNA (derived from specific cytokine mRNA) present before amplification.

Naive Baseline Values

Naive SPF animals maintained in isolator conditions were sampled at days 0, 14, 21, 35 and 50 post-delivery. RNA was extracted from the lungs and LN and analysed by semi-quantitative PCR for the basal level of cytokine expression. The results from this experiment demonstrated that no discernible drift in baseline values could be detected across the standard duration of the vaccination and challenge period. Thus in subsequent experiments naive mice were only sampled at day 0 and 35 to coincide with the vaccination and challenge procedures. The average naive value obtained for these mice is depicted as a dotted line in chapters three and four.

Analysis of Results

Extraction and Analysis of Whole Tissue Total RNA

Total RNA was isolated from whole tissue with careful consideration for the prevention of contamination with DNA. Furthermore, procedures were maximised for recovery of RNA from small quantities of tissue. Fig. 1 shows a typical agarose gel stained with ethidium bromide and the corresponding autoradiograph obtained following hybridisation. For each new primer pair, the PCR conditions were optimised and the amplification of a single product confirmed using gel electrophoresis and Southern blotting. The latter detection system was used to assess primer and probe specificity; in this example it can be seen that only one clean band is obtained after RT-PCR of a dilution series of LN total RNA using HPRT primers. In addition, the greater sensitivity of Southern blotting over ethidium bromide staining can be seen. Once the specificity of the primer and probe sequences had been verified, analysis could be carried out by slot blotting to increase sample throughput (Fig. 2). This blotting method allowed easy visualisation of the multiple PCR products derived in each experiment, whilst providing a time-saving, cost-effective alternative to Southern blotting. In addition, many samples could be blotted onto a small piece of membrane, so increasing the number of blots probed with one labelling reaction. In this example of the HPRT analysis of LN RNA, differences in the product amounts detectable at each increasing cycle number can be seen. For each cycle number, duplicates of each sample are run to give a total of six PCR products per sample per cytokine.

The counts obtained for each slot by phosphorimager analysis were analysed by linear regression. As demonstrated in Fig. 3a, the phosphorimager count for each slot- blotted product was transformed ($\log_{10}$), and a regression line fitted to the six values per RNA sample. The equation was plotted and the 95% confidence limits determined (Fig. 3). These limits increase toward the ends of the value range; thus the best estimate from the regression equation is obtained near the centre point. All regression lines derived for samples to be compared to each other were tested for significant differences between slopes by calculating the standard error of the difference between the slopes. This ensured that the PCR efficiency was comparable

between samples. The error bars added to each point on the final graph (Fig. 4) were derived from the 95% error value associated with the corresponding regression line used to estimate the values. Table 2 shows the two errors added on to each regression parameter in order to obtain the 95% error bar. Where possible, all cytokines within an experiment were shown at the same estimated cycle number, for example by substituting x =25 or 27 the estimated values should be in the linear range for all cytokines under test. An example of a final graph of data subjected to full statistical analysis is shown in Fig. 4. The vertical axis is read as phosphorimager counts (ie. units of energy released from the phosphor screen as electrons excited by β–emissions from the radioactive probe return to ground state upon laser scanning), and is shortened to counts in subsequent experimental chapters and should not be confused with radioactivity counts. This data for HPRT is estimated at 25 cycles from the day 0 regression (Fig. 3). In addition, values for days 2 and 5 post-vaccination (derived in the same way) are plotted in this example. The 95% confidence limits on these bars overlap and thus the vertical position of the regression equations, and hence estimated values, are not significantly different from each other. This is not unexpected with the analysis of HPRT which is constitutively expressed. Although correction factors were small (Fig. 4), the corresponding cytokine data from the same RNA samples was adjusted for completeness. For RNA samples displaying variable amounts of HPRT, an adjustment must be made to the relevant cytokine data to ensure that any differences reported are due to cytokine expression and not total RNA content of the original samples. HPRT adjustment was carried out for all samples in each experimental time course.

Competitive PCR

The inclusion of a competitive template into the reaction tubes allows the actual level of cDNA derived from the cytokine mRNA under test to be determined. An example of a competitive PCR result is shown in Fig. 5. At the higher concentrations of plasmid added to the test cDNA the only product generated is derived from construct DNA (lanes 1 and 2). As the serial dilution continues along the test samples, PCR product derived from cytokine cDNA becomes evident and eventually becomes the only visible product (lanes 6,7, 8 and 9). The dilution at which both

product bands are of equal intensity (lane 4) provides the point of equivalence at which both templates have consumed approximately equal amounts of PCR reagents and thus are in equal abundance in the reaction. However, this value in pg cannot be related back to the exact level of mRNA in the sample due to the lack of control for the RT reaction, and differences in the sequence length between the 3' and 5' primers in the test and control templates. In addition, the estimate obtained by visual inspection is somewhat crude without access to a gel scanner. Differences in band intensity, as judged by ethidium bromide staining, could be seen easily with a ten and four fold dilution series. However, the ability to convert band fluorescence into a numerical figure would allow a more accurate cross-over point to be determined graphically. Samples cannot be hybridised with labelled probes and analysed by densitometry or phosphorimaging due to the differing internal sequences situated between the primer pair in the test and competitive templates.

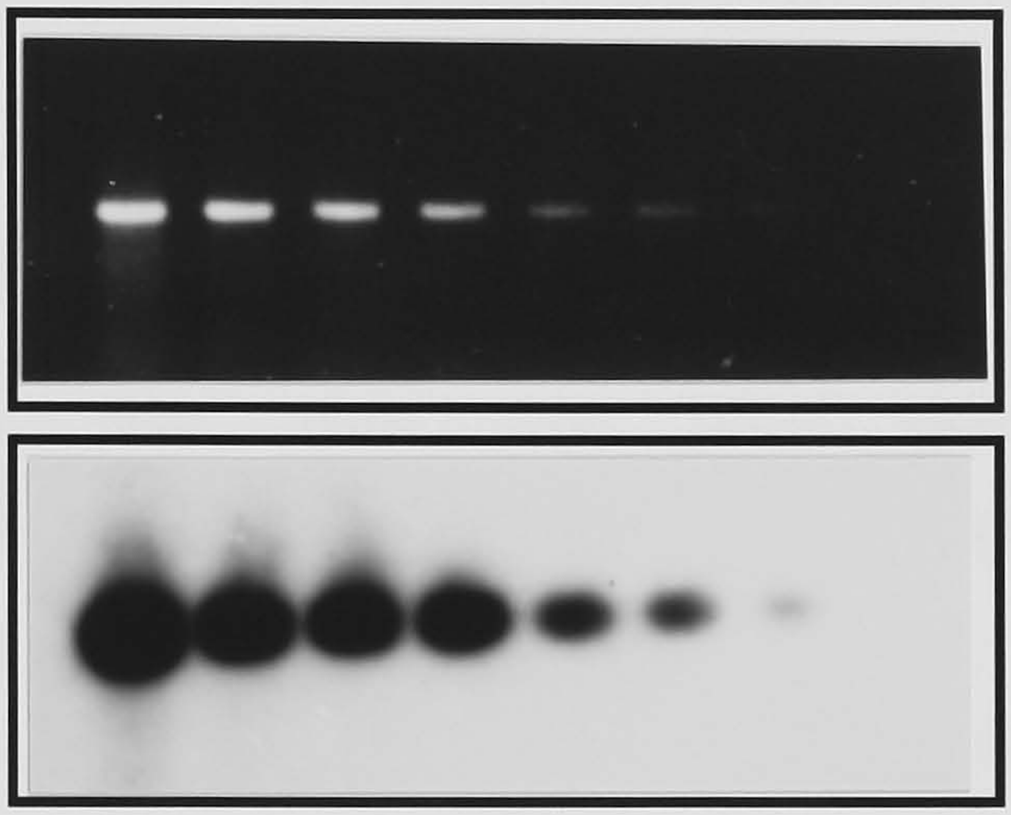


Fig. 1. An example of an agarose gel stained with ethidium bromide, showing HPRT PCR product derived from a doubling dilution series of LN total RNA. Directly below is the corresponding autoradiograph. PCR was carried out for 30 cycles and the products run on a 2% agarose gel followed by Southern blotting and hybridisation with a ^{32}P-end-labelled oligonucleotide probe.

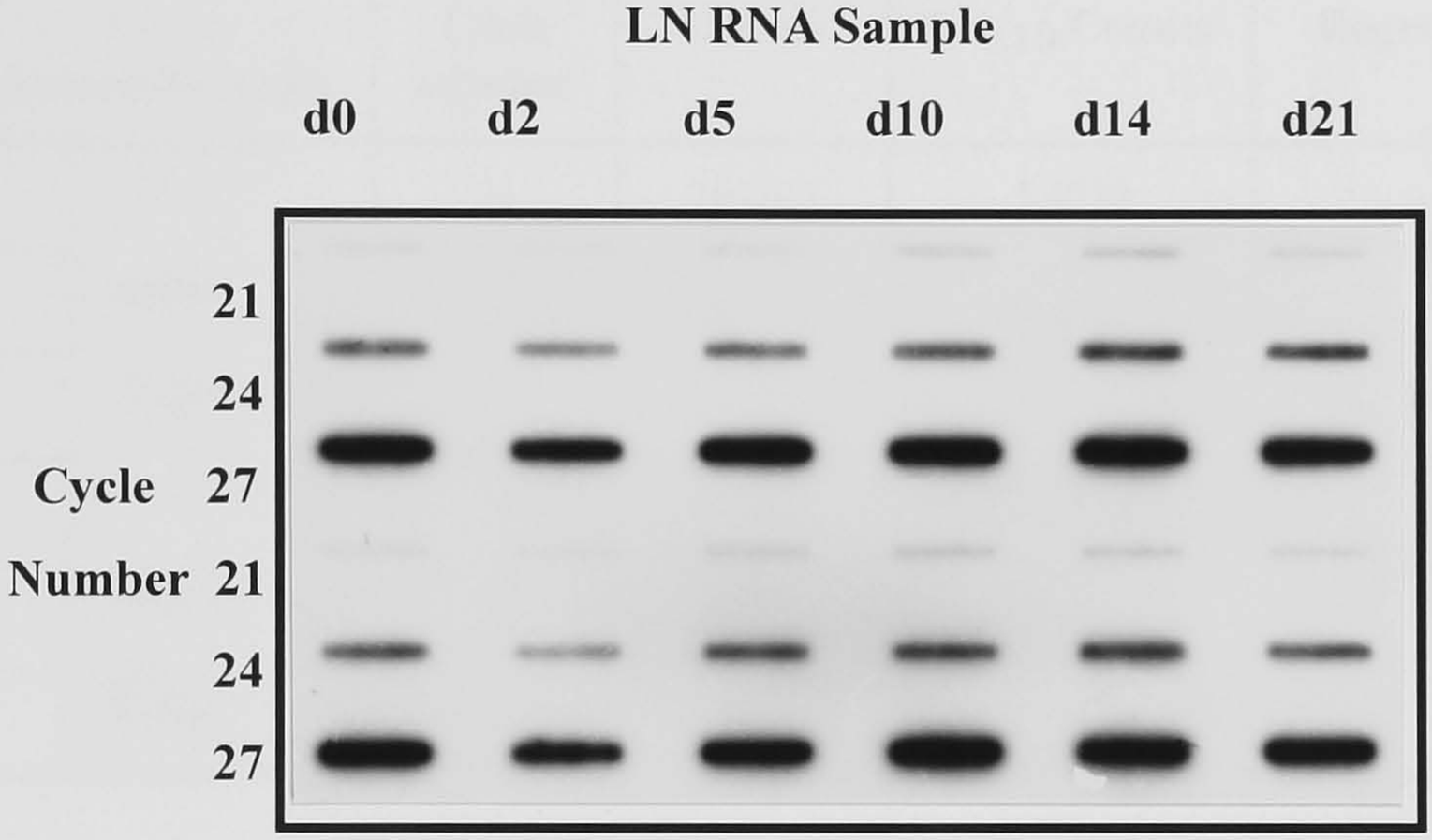
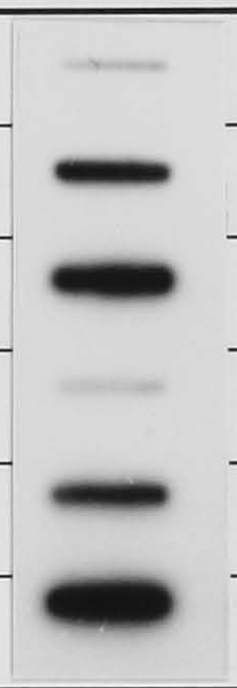


Fig. 2. A typical slot blot layout, after hybridisation and autoradiography, showing the differences in product obtained with increasing cycle number. Each RNA sample was subjected to amplification in duplicate for 3 cycle numbers.

Fig. 3. A worked example of the derivation of a regression line from counts obtained by phosphorimager analysis of a probed slot blot.

a) **Table 1**. Regression analysis of the phosphorimager counts obtained from slot blotted HPRT PCR product derived from day 0 LN RNA.

Day 0 Autoradiograph	Cycle number	Counts	Log_{10} Counts	Regression Equation
	21	264200	5.4219	
	24	996861	5.9986	
	27	1779475	6.2503	log y = 0.1567x + 2.146
	21	230146	5.3620	(±SE)
	24	963652	5.9839	
	27	2576006	6.4109	

b) The regression line derived from the above equation, demonstrating the position of the 95% confidence limits for the day 0 sample.

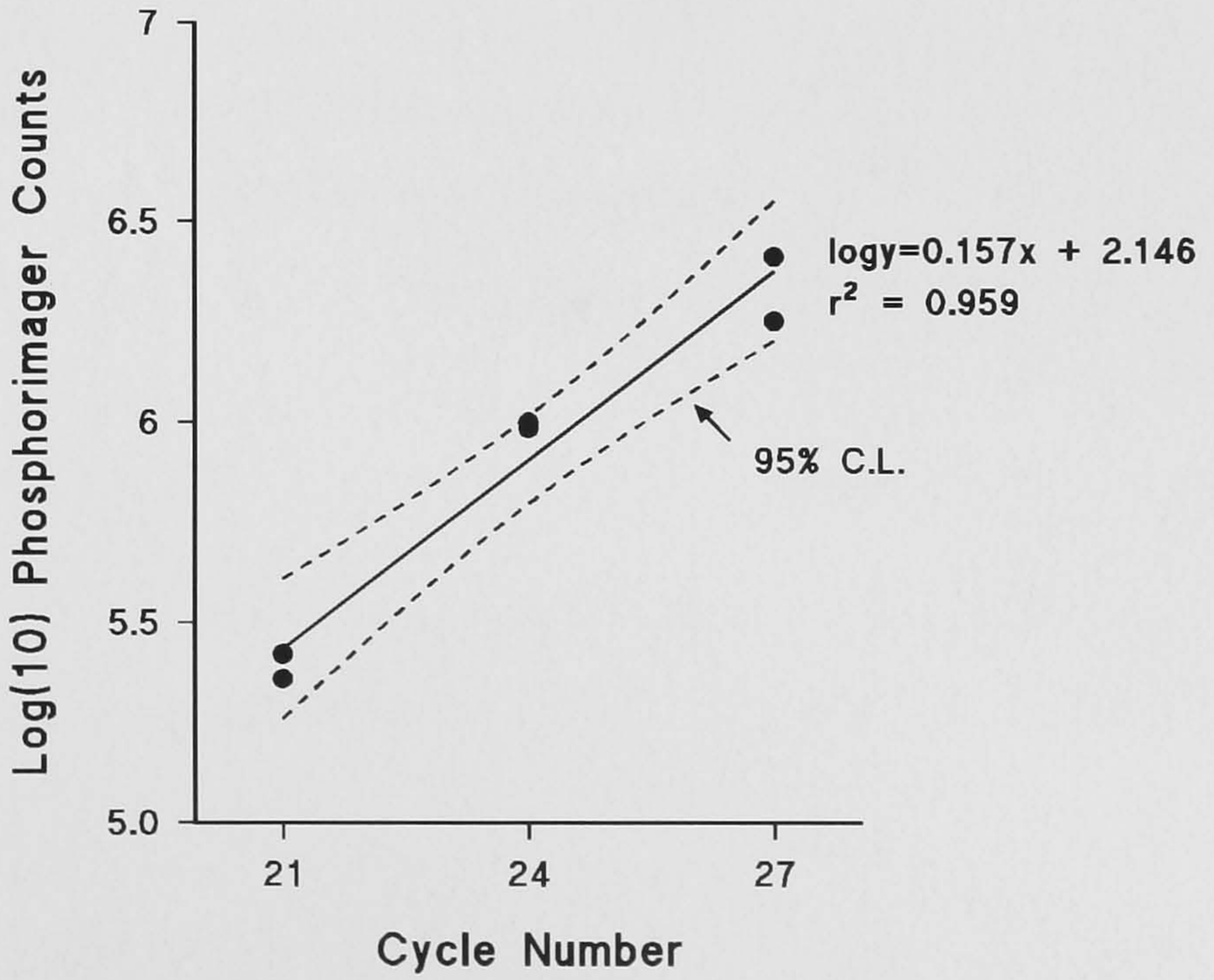


Table 2. Breakdown of the equation errors for the regression line shown above, demonstrating the standard error and 95% confidence limit values added to each parameter. The addition of confidence limits to each estimated point permits the significance of that point compared to other samples to be determined.

Using the regression equation, and errors shown below, the estimated HPRT count value for the day 0 sample at 25 cycles is **1,149,145 ± 141,427**.

Equation Component	Basic Value	± SE	± 95% Confidence Limits
m	0.1567	0.0161	0.0448
c	2.146	0.389	1.081

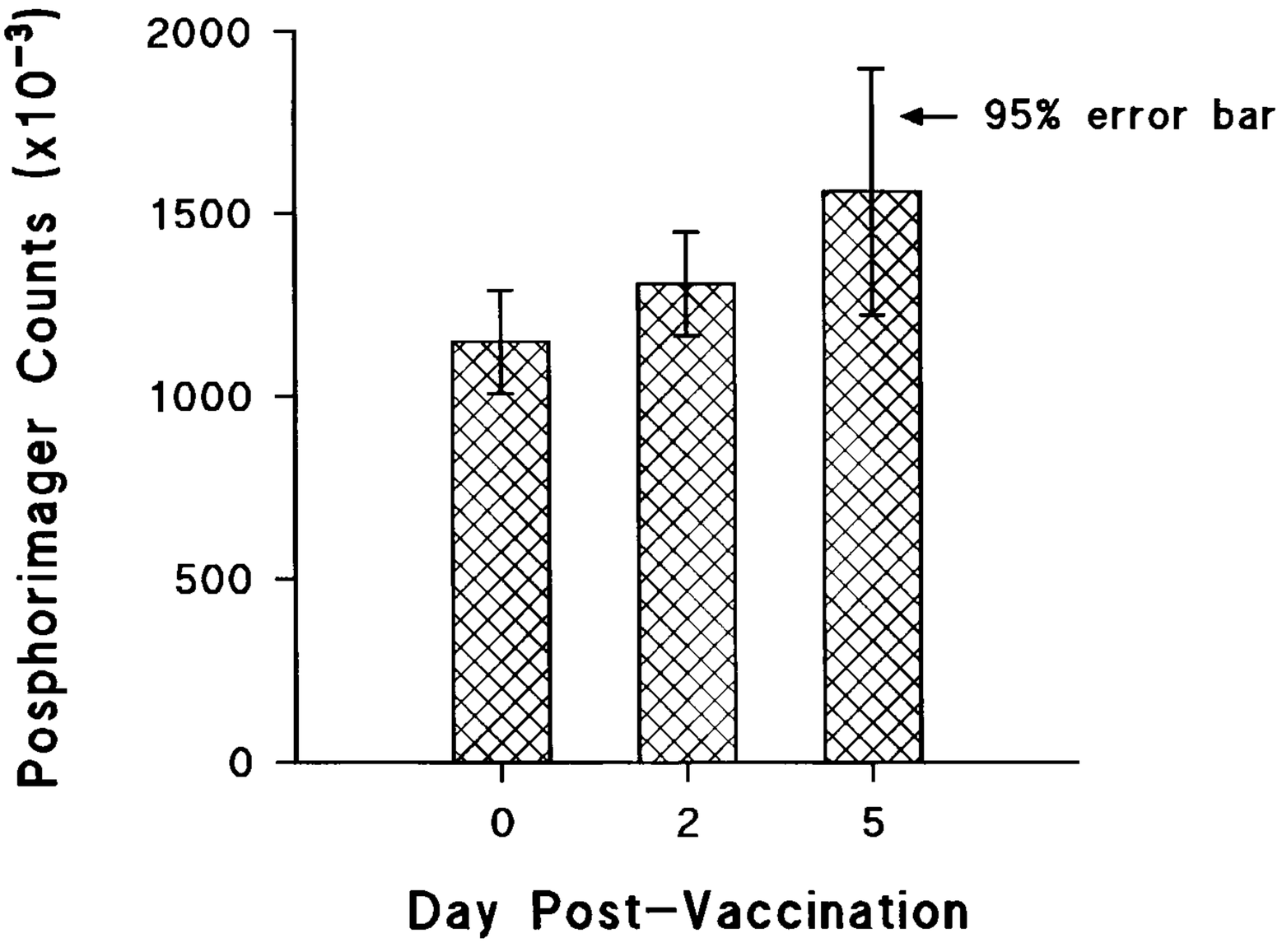


Fig. 4. Final data presentation of the estimated values of HPRT derived as above for days 0, 2 and 5. The 95% error bars overlap for these values, and thus the estimated values are not significantly different from each other. However, the small correction factors (see table below) are applied to all cytokine data from LN samples at days 0, 2 and 5. These factors are worked out for all samples under test from the same pool of RNA, and used to adjust all cytokine assay data derived from that pool. Thus data in subsequent chapters is shown corrected for differences in HPRT mRNA levels.

	Day 0	**Day 2**	**Day 5**
HPRT Estimated Value	1,149,145	1,307,149	1,558,056
Correction Factor	1	0.88	0.74

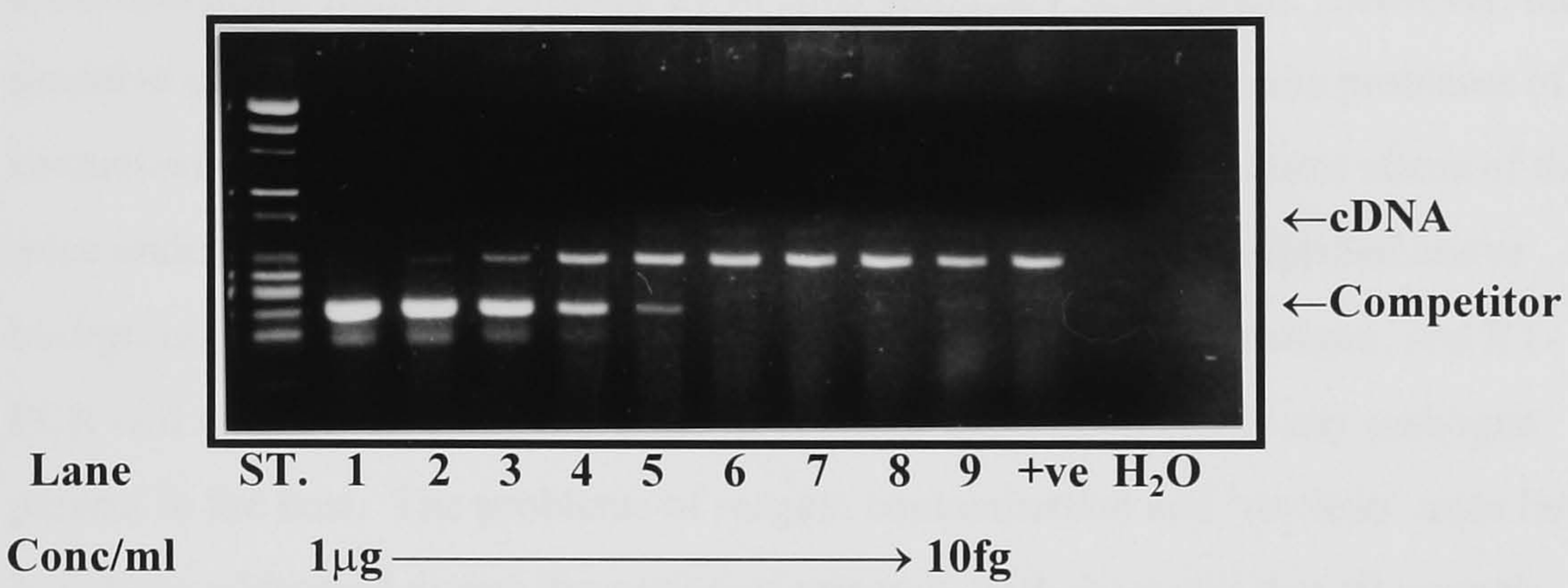


Fig. 5. An example of competitive PCR for IFNγ on naive C57BL/6 lung RNA. The smaller plasmid product decreases in intensity as the larger sample cDNA-derived product increases. The PCR was carried out as described in materials and methods, and the products separated by 2% agarose gel electrophoresis alongside a DNA ladder (ST). The point of equal band intensity in this sample is at the 1ng/ml dilution (lane 4), corresponding to the addition of 5pg of competitive construct, added in 5μl, to the reaction mix.

Discussion

This chapter describes the basic RT-PCR protocols applied to the analysis of cytokine mRNA profiles following vaccination and challenge of mice with *S. mansoni*, as detailed in the following experimental chapters. The study of the cytokine profiles induced *in vivo* after exposure to parasites required the use of a sensitive technique like RT-PCR, which is able to detect rare events without distortion of the immune response by *in vitro* secondary stimulation. However, the sensitive nature of PCR makes this detection system vulnerable to the problems of contamination. This is especially relevant with regards to the immune status of the mice under test, since specific schistosome responses cannot be amplified above background cytokine production by *in vitro* culture with parasite antigen, and RT-PCR will detect even small increases in cytokine expression due to any pathogen present in the host. The problems of reagent contamination and 'unclean' mice have both been addressed during the period of research, with the result that all animals used in this thesis were specified pathogen free (SPF), and housed in isolator conditions to prevent non-specific infection. Furthermore, all pre-amplification manipulations were carried out in a sterile environment spatially separated from the product handling area to maintain a clean cytokine mRNA detection system.

The semi-quantitative PCR method used in the following chapters allows direct comparison of the cycle number at which product for various samples can first be detected, and thus distinguishes those samples which contain higher levels of starting template (Dallman *et al.*, 1991a, 1991b, Montgomery and Dallman, 1991). This method was chosen over others published (see introduction) because it did not require the generation of standard curves using cell lines constitutively expressing the cytokine of interest, a potential source of contamination. In addition, this technique permitted easy comparison between samples for the same cytokine. As research progressed, the method was adapted to provide a more accurate estimate of the amount of product generated from the cytokine mRNA content of a sample by the inclusion of phosphorimager counting and regression analysis. In early experiments four tubes per sample were used to generate the regression line. However, analysis

carried out in this way often required repeating due to this minimum of points resulting in regression lines of varying efficiency. Later experiments were carried out by amplifying duplicates of each sample at 3 different cycle numbers to give six regression points. This resulted in more accurate data presentation, with additional statistical tests performed to incorporate the 95% confidence limits of the regression analysis. Due to the expense of the PCR technique, sample repeat size could not be as large as initially hoped.

Exposure to a storage phosphor screen and scanning by phosphorimager was preferred to autoradiography and densitometry due to the high sensitivity and increased linear detection range obtained with the former. Autoradiographic film darkens in a linear relationship over two orders of magnitude, thus samples may appear saturated at the top cycle number, and barely detectable at the lowest. The phosphorimager can detect radioactivity with a linear relationship over five orders of magnitude, allowing accurate detection of the product over the nine rounds of amplification covered by the three cycle numbers sampled during analysis. With PCR working at 100% efficiency on the exponential phase of the reaction, product can potentially increase eight fold per three cycles, giving an overall increase of 512 fold over nine cycles. This range cannot be accurately quantified from densitometry of autoradiographs. Moreover, due to the increased sensitivity of phosphorimager analysis compared to autoradiography, membranes need only be exposed for one tenth the time of autoradiographic film increasing sample throughput.

RNA analysis by RT-PCR in this way required the application of careful practical handling and control measures. This method cannot provide reliable data on the relative levels of cytokine expression across a time course if the PCR has reached the plateau phase, where amplification is limited by lack of reaction components and the accumulation of inhibitors. Thus careful assessment of the linear range of the reaction must be carried out before semi-quantitative analysis is undertaken. Furthermore, the regression analysis is only valid if the slopes derived from the equation for each sample do not differ significantly from each other.

To ensure that as many as possible of the parameters involved in this analysis are kept constant, RNA samples to be compared to each other were run on the same RT-PCR amplification, using the same master mix and heating block. In addition,

samples were slot blotted and probed together to control for DNA denaturation, probe labelling and hybridisation efficiency. Where the number of samples under investigation exceeded available equipment, RT-PCR was carried out with the same batch of enzyme on consecutive days and the membranes probed together in a single hybridisation. Controls for PCR and blotting efficiency were included on each run where two or more blots contributed to a single graph. However, these controls only allow comparisons to be made between samples probed in a single hybridisation. Due to markedly different end-labelling efficiencies resulting in probes with different specific activities, no comparisons can be made between the phosphorimager count values obtained for the same cytokine (e.g. IFNγ) in different experiments.

Competitive PCR involves the inclusion of a control template of some description into the same reaction tube as the sample under test so that both DNAs are amplified in exactly the same conditions. Early attempts at quantifying PCR involved the addition of a primer pair for a housekeeping gene into the test reaction (Chelly *et al.*, 1988). However, this method utilises distinct primer and target template sequences, with all test samples related back to the expression of the housekeeping gene. Thus a strict comparison between the expression of different cytokine genes cannot be made. In general, more recent methods have developed templates with primer sequences identical to those of the test sample, and consisting of either an artificial RNA/DNA construct or modified genomic DNA. The competitive template used throughout this thesis consists of a tandem array of cytokine primer sequences cloned into a pGEM plasmid. The primers were inserted in an order designed to generate products of different sizes than those derived from cytokine mRNA. The theory behind the generation and use of the competitive template are similar to those previously described (Wang *et al.*, 1989, Gilliland *et al.*, 1990, Reiner *et al.*, 1993), with a drawback in that product cannot be easily detected other than by visual inspection under UV illumination. This problem could be addressed by the inclusion of radioactivity into the PCR reaction, either as labelled bases or via the use of radiolabelled primers. Alternatively, a gel scanner could be used to quantify the bands seen directly from the gel. However, these options were not available at the time of analysis and thus all competitive PCR data is derived from human judgement, and hence is also subject to human error. Therefore, due to the

limitations of this technique at present, the increases in cytokine expression derived by competitive PCR do not necessarily correspond to the fold increases observed for the same samples following semi-quantitative PCR analysis.

Although many published competitive PCR methods claim to allow absolute quantification of product by relating the competitive template quantity to the number of cytokine mRNA molecules in the test sample, very few actually take into the consideration the reverse transcription reaction and the differences in product length. The quantification of cytokine expression down to the exact number of mRNA molecules present was deemed unnecessary in this study, thus results from the competitive PCR in the experimental chapters are displayed in picograms of control template required to attain equivalence with the test sample. In addition, the relationship between the message for a certain cytokine and the relative potency of the active protein remains unclear (Trinchieri, 1993), hence accurate quantification at the mRNA molecule level has limited relevance to understanding the *in vivo* situation. In spite of the limitations, if handled with care, competitive PCR provides a valuable tool for comparing the expression level of different cytokines in samples from a variety of situations.

The following four chapters describe the application of the RT-PCR technique in the analysis of the *in vivo* cytokine responses induced in mice after various vaccination and challenge exposure regimes. The results obtained provide a detailed description of the cytokines expressed during the induction and effector mechanisms, in the skin draining LN and lungs respectively, which constitute a protective response (Chapters Three and Four). In addition, the differences in these responses caused by variations in genetic background, such as those occurring naturally in different strains of mice or artificially induced by gene deletion, are investigated (Chapters Five and Six).

CHAPTER THREE

Responses in the Skin-Draining Lymph Nodes Following Vaccination and Challenge of C57BL/6 Mice with Cercariae of *S. mansoni*

Introduction

Vaccination of C57BL/6 mice with 500 radiation-attenuated cercariae of *Schistosoma mansoni* leads to the generation of high levels of resistance against challenge infection (Dean, 1983). This immunity is $CD4^+$ T cell mediated, with little input from $CD8^+$ T cells, as determined by *in vivo* ablation studies (Vignali *et al.,* 1989). The importance of the skin draining lymph nodes in the induction of protection has been established by lymphadenectomy (Mountford and Wilson, 1990), and vaccination with irradiated parasites by intravenous injection (Coulson and Mountford, 1989), both of which treatments bypass this site and result in reduced resistance. The slower, truncated migration and persistence in the LN of a proportion of the irradiated parasites (Wheater and Wilson, 1979, Mountford *et al.,* 1988), results in prolonged exposure of antigen presenting cells to intact parasites and related antigens during a vaccinating, compared to normal, infection. It is thought that this increased exposure leads to the generation of a Th1 population and establishment of a protective immune response (Mountford *et al.,* 1988, 1992). Following both normal infection and vaccination, the number of schistosomula in the LN peaks at day 5, with 11-16 % of infecting parasites detectable in that location (Mountford *et al.,* 1988). However, although irradiated larvae persist in the nodes of vaccinated mice for up to two weeks, 10.4% still being present after day 7, under 3% of healthy parasites remain after normal infection.

The effectiveness of vaccinating parasites at inducing an immune response is demonstrated by cell population studies. *In vitro* proliferation of cells isolated from axillary LN peaks at day 5 post-vaccination (Pemberton *et al.,* 1991, A. Finlay, unpublished observations) and is of similar magnitude in both normal and vaccinating infections. However, the proliferative response is more persistent after vaccination than that observed after exposure to normal parasites (Pemberton *et al.,* 1991). There is also a significant elevation in total cell number in the LN of vaccinated mice, with a 13.5 fold increase over naive controls detectable at day 14 (Constant *et al.,* 1990). In comparison to the eight fold increase in T cell number, the number of B cells present in the LN dramatically increases by twenty eight fold, thus effectively reducing the ratio of T:B cells from a naive value of 4:1, to almost 1:1

(Constant and Wilson, 1992). In spite of this large increase in the B cell population of the LN, T cells still slightly exceed B cells in absolute numbers over the extent of the time course. The elevation in B cells appears to be due to selective retention of this cell type in the nodes, since no preferential recruitment or proliferation can be detected (Constant and Wilson, 1992). However, there is an absence of B cells in the efferent lymph flow, possibly accounting for the accumulation of these cells from day 7 post-vaccination onwards. These increases in B cell number and T cell proliferation coincide with parasite residence, and increased levels of released antigenic material, in the LN (Mountford *et al.,* 1988). In vaccinated mice the cell number remains elevated above background until day 35, whereas after normal infection the LN cell population starts to decline at day 14 and reaches naive levels again by day 21 (Constant *et al.,* 1990, Mountford *et al.,* 1992, Pemberton and Wilson, 1995).

The rise in cellular activity is matched by upregulation of cytokine expression by LN cells stimulated *in vitro*. The Th1 cytokine interferon-γ (IFNγ) dominates cytokine protein secretion profiles at all times post-vaccination (Pemberton *et al.,* 1991), with higher and more prolonged expression than cultured LN cells from mice exposed to normal parasites (Pemberton *et al.,* 1991, Mountford *et al.,* 1992). Two main peaks of IFNγ protein production occur, one at day 5 and the other at day 18 post-vaccination. The first corresponds to the residence of maximum numbers of parasites in the nodes, the second to the release of large amounts of parasite antigen, presumably from dying parasites which have failed to exit the LN and continue migration (Pemberton *et al.,* 1991). Anti-CD4 ablation *in vitro* has an almost total inhibitory effect on IFNγ release upon secondary stimulation, indicating that this cytokine is produced predominantly by the $CD4^+$ cell population with very little secretion attributable to $CD8^+$ T cells (Pemberton *et al.,* 1991). Production of the other typical Th1 cytokine, IL-2, after vaccination is low at all time points, and similar to levels detected after a normal infection. Th2-type cytokines have also been detected at elevated levels after vaccination, indicating the mixed Th1/Th2, or Th0, characteristics of the response in the sdLN. IL-4 protein is released at low levels at day 4 and increases at day 10 post-vaccination, whereas IL-10 secretion displays the opposite kinetics, peaking at day 4 and decreasing by day 10. In general, cytokine

levels detected after exposure to normal infection are similar to those following vaccination, but of lower magnitude and a more transient nature (Pemberton and Wilson, 1995).

Analysis of events after challenge of vaccinated mice has demonstrated the importance of the secondary exposure site in determining the type of immune response generated upon infection with normal parasites. However, this secondary response seems unable to alter the effector mechanism operating in the lungs, since whole body irradiation performed prior to challenge infection has no influence on resistance (Aitken *et al.,* 1987, Vignali *et al.,* 1988). If mice are vaccinated and challenged on the same site, and thus through the same LN, reduced cell proliferation is observed with no indication of an anamnestic response displaying Th1 characteristics (Pemberton and Wilson, 1995). In fact the opposite is true, with culture supernatants exhibiting variable reductions (73-96%) in the post-vaccination level of IFNγ secretion, and an increase in IL-4 secretion (Pemberton and Wilson, 1995). This reduction in IFNγ levels is less evident if challenge is performed on a site remote from the vaccination area (Pemberton and Wilson, 1995, Caulada-Benedetti *et al.,* 1991). IL-2 production is slightly raised above background levels in mice challenged on a remote site, or in control groups. However, LN cells from mice receiving challenge infection on the same site as vaccination exhibited almost undetectable IL-2 levels, again emphasising the lack of an anamnestic Th1 response in the LN which have already seen parasites. In contrast to the Th1 cytokines, IL-4 protein secretion is higher post-challenge than that observed post-vaccination in groups of mice challenged on a remote or the same site. Furthermore, IL-4 reaches maximal levels more rapidly than in challenge control supernatants, indicative of an anamnestic Th2-type response. The dominance of IL-4 in LN cultures from mice vaccinated and challenged on the same site implies that the priming vaccinating infection induces anergy, or an inhibition in the development of Th1 IFNγ-secreting cells after vaccination. Alternatively, any Th2 cell precursors remaining in the LN from after vaccination may expand upon secondary stimulation in the absence of inhibitory levels of IFNγ protein and, once established, the Th2 response will then prevent expression of any developing Th1 cytokines (Mosmann and Moore, 1991, Tanaka *et al.,* 1993).

In both same and remote-site exposure regimes, IFNγ production from cultured cells can be restored, and IL-4 secretion reduced, by the *in vitro* neutralisation of IL-10 (Pemberton and Wilson, 1995). The level of IL-10 protein expression post-challenge is similar to that induced after exposure to irradiated parasites (Pemberton and Wilson, 1995). This protein can be released by macrophages and some types of B cells (Mosmann *et al.,* 1991, Moore *et al.,* 1991, O'Garra *et al.,* 1990, Murphy *et al.,* 1993), and may act along with IL-4 to enhance the development of a stronger Th2 response post-challenge via the inhibition of macrophage and Th1 cytokine release (Fiorentino *et al.,* 1989, 1991a, 1991b, Flores Villaneuva *et al.,* 1993). At day 6 post-challenge, the LN from mice receiving a vaccination on the same site displayed a two-fold increase in B cell numbers compared to challenge control animals (Pemberton and Wilson, 1995). This factor may also influence the bias of challenge infection towards a Th2 type response if B cells can act as the primary APC for Th2 cell populations (Gajewski *et al.,* 1991, Janeway *et al.,* 1987).

Multiple vaccination regimes have been shown to downregulate Th1 and increase Th2 cytokine production in the sdLN with increasing exposure to the same site (Caulada-Benedetti *et al.,* 1991). Multiple vaccination results in little or no increase in the resistance generated by a single exposure. Moreover, protection is transferable to naive recipient mice with immune serum indicating that soluble factors such as antibodies are now involved, an effect not seen with single vaccination serum (Kelly and Colley, 1988, Mangold and Dean, 1986). However, the resistance in recipient animals is not comparable to the levels seen in donor mice, indicating that the mechanism responsible for resistance is not entirely humoral . In contradiction to this, hyperimmunized mice treated with anti-CD4 antibody do not display reduced resistance, implying that $CD4^+$ T cells are not essential for resistance (Caulada-Benedetti *et al.,* 1991). In combining the above two observations, a more plausible explanation for these discrepancies could be that ablation of the circulating $CD4^+$ population does not necessarily deplete those T cells already resident in the lungs awaiting the arrival of challenge parasites (Caulada-Benedetti *et al.,* 1991). In summary, the above experiments indicate that upon multiple exposure to *S. mansoni* parasites a Th2-like response, involving antibodies, develops and the Th1 response seen with a single vaccination is reduced, but not completely switched off.

Based on previously published reports, a more complete investigation of the cytokine profiles following vaccination and challenge was designed. To minimise any artifacts induced by *in vitro* stimulation, analysis of cytokine gene expression was performed using Reverse Transcriptase-PCR (RT-PCR). This method allows detection of cytokine mRNA *ex vivo,* providing an accurate characterisation of the immune status of the LN at the time of excision. The resulting cytokine mRNA profiles detected across the time course supported earlier findings of a dominant Th1 response post-vaccination with a sharp increase in Th2 cytokines message following challenge. Apparent reductions in some cytokine messages below the naive baseline values were investigated to determine if B cell accumulation in the LN may act to dilute messages detectable from other, less abundant, cell populations.

Materials and Methods

Experimental Animals and Parasites

A Puerto Rican isolate of *Schistosoma mansoni* was routinely passaged through MF1 outbred mice and albino *Biomphalaria glabrata* snails. Female C57BL/6 specific pathogen free (SPF) mice, purchased from B&K Universal (Grimston, Hull, UK), were housed in isolator conditions to maintain SPF status throughout the experiment.

Exposure Regimes

Mice weighing 17 to 20g were vaccinated percutaneously on the left flank with 500 20 krad ^{60}Co gamma-irradiated cercariae (radiation source provided by the Department of Radiobiology, Cookridge Hospital, Leeds) and then 35 days later challenged with 200 normal cercariae on the opposite flank. The axillary and inguinal LN draining the exposure site were excised at days 0, 2, 5, 10, 14, 21 and 35 post-vaccination, and at days 5, 10 and 15 post-challenge. LN were removed directly into an RNAase inhibitor (4M guanidinium thiocyanate solution) or snap frozen in liquid nitrogen and stored at -80 ^{o}C until use. A second time course sampling at days 0, 1, 2, 5, 10, 14, 21 and 35 post-vaccination was carried out for repeat analysis.

Resistance data was calculated using the worm burdens obtained by hepatic portal perfusion of groups of 5 vaccinated (VC) and 5 naive (CC) mice from the first experiment at 35 days post-challenge. Resistance was calculated from the respective worm burdens as follows : %R = [(CC-VC)/CC]x100.

Total RNA Isolation

Axillary and inguinal LN from groups of 3 mice sampled at each time point were pooled and homogenised in guanidinium thiocyanate solution (4M guanidinium thiocyanate, 1% 2-mercaptoethanol) using a tissue shearer, and the total RNA extracted as previously described in chapter two (Sambrook *et al.*, 1989). The RNA yield was measured spectrophotometrically and verified by 1% agarose gel

electrophoresis in the presence of ethidium bromide (0.5 mg/ml), which also confirmed the intact nature of the preparation.

Semi-Quantitative RT-PCR Detection of Cytokine mRNA

Analysis of the levels of cytokine mRNA in samples from the longitudinal time course was adapted from a previously published method (see Chapter Two and Dallman *et al.,* 1991, Montgomery and Dallman, 1991). A titration series of RNA dilutions was carried out to determine the optimal amount of starting material for the RT-PCR. Based on these results, 0.25μg of total LN RNA was subjected to reverse transcription and PCR as previously described. Briefly, total RNA was reverse transcribed and the resulting cDNA amplified in duplicate for three different cycle numbers previously determined to lie in the exponential phase of the reaction. The cycle numbers chosen for each cytokine were dependent on the individual kinetics of the primer pairs, and were spaced at intervals of three cycles. Thus amplification for the cytokines assayed in LN RNA was carried out for the following number of cycles: HPRT, 21, 24, 27 ; IFNγ, 24, 27, 30 ; IL-2, 27, 30, 33 ; IL-4, 27, 30, 33 ; IL-5, 27, 30, 33 ; IL-10, 24, 27, 30 ; IL-12, 24, 27, 30 ; IL-13, 27, 30, 33. An annealing temperature of 60°C proved optimal for all the cytokine primers except IL-10, where annealing was performed at 55°C. Positive and negative controls, including PCR and RT-PCR reagent blanks, and a control for the presence of genomic DNA in the sample, were run on each amplification to control for the potential contamination of reaction components and RNA samples with PCR products from previous experiments. Furthermore, where possible, primer pairs were designed to span an intron to distinguish product derived from any contaminating genomic DNA. Primer sequences for HPRT, IL-5 and IL-13 were based on previously published work (Murphy *et al.,* 1993, Montgomery and Dallman, 1991, and Wynn *et al.,* 1993 respectively). Primers for additional cytokines were designed from published gene/cDNA sequences and were as follows: IL-2 sense GTCAACA GCGCACCCACTTCAAGC, antisense GACAGAAGGCTATCCAATCTCCTCAG (Kashima *et al.,* 1985); IL-4 sense GCTAGTTGTCATCCTGCTCTTC, antisense GCTCTTTAGCTTTCCAAGGAAGCT (Noma *et al.,* 1986); IL-10 sense GAAGCTGAAGACCCTCAGGA, antisense TCCCTGGATCAGATTTAG (Moore

et al., 1990) ; IL-12 p40 chain sense CCATCTCCTGGTTTGCCATCGTT, anti-sense GGAGCAGCAGATGTGGAGTGGC (Schoenhaut *et al.,* 1992). The housekeeping gene HPRT was reverse transcribed and amplified for each set of samples to ensure equal quantities of starting total RNA were used in each reaction.

In order to determine the contribution of B cell RNA to the total LN RNA content RT-PCR was carried out with primers specific for the IgM μ chain constant region, as designed from the published cDNA sequence (Auffray and Rougeon, 1980) as follows: sense, CGACATCTTCCTTAGCAAGTCCGC, anti-sense, CAGCAGGT ACACAGCAGGTGGATG. Due to the high level of expression of this message, the cycle numbers used for semi-quantitative PCR were 18, 21 and 24, with an annealing temperature of 60°C. In addition the T cell marker CD4 was amplified as an indication of the number of T helper cells present in the LN, with the following primers designed from the published cDNA sequence: sense, CTGGTTCGGC ATGACACTCTCAGTG, antisense, ACACTGCCACAGCCCTGTCTCAGG (Tourvieille *et al.,* 1986).

Detection and Analysis of PCR Product

The amounts of specific PCR product obtained for each sample were determined by slot blotting 5μl aliquots onto nylon membrane (Zeta Probe, Bio Rad). Baked membranes were hybridised with ^{32}P-end-labelled oligonucleotide probes specific for regions of the PCR product internal to the two primer sequences. Probe sequences were either adapted from previously published work (IFNγ from Gray *et al.,* 1983, IL-4 and IL-5 modified from Montgomery and Dallman, 1991, IL-10 and HPRT modified from Murphy *et al.,* 1993, IL-13 modified from Wynn *et al.,* 1993) or designed from the published cDNA sequences as follows: IL-2, TCCAAGTTC ATCTTCTAGGCACTG (Kashima *et al.,* 1985); IL-12, TCAGGCGTGTCAC AGGTGAGGTT (Schoenhaut *et al.,* 1992); IgM, GGAATCTGCCTTCACCACAG (Auffray and Rougeon, 1980); CD4, GTTGCCAGAACCAGCAAACTGAAG (Tourvieille *et al.,* 1986). Hybridised membranes were washed at 1°C below the dissociation temperature of the probe, and exposed to a storage phosphor screen for subsequent phosphorimager analysis of bound radioactivity.

At each time point a linear regression was performed with the six repeats for each sample (two for each cycle number), and the amount of product at a given cycle number estimated as previously described (Chapter Two). Values obtained for the cytokines were normalised for differing RNA content using the estimated HPRT values. The adjusted values were then plotted to give a profile of cytokine expression with time post-exposure to irradiated and normal parasites. The 95% error bars of the estimated values were plotted by analysing the confidence and standard error limits of the regression line. The regression line slope value was also analysed for significant differences to confirm that comparable samples had undergone PCR amplification of the same efficiencies.

Competitive PCR

The above method permits only the comparison of given cytokine differences between samples and not the relative levels of different cytokines with respect to each other. To achieve this objective, a competitive construct, comprising of primer sequences for a set of cytokines, of known dilution must be amplified simultaneously, in the same tube as the sample under test. Such a template (kindly constructed and donated by Mr. K Page, Glaxo Research, Greenford, Middx) consisting of primer sequences in tandem array was used to provide a more accurate estimate of the amount of mRNA present in naive mice, and at two peak response time points following either vaccination or challenge (as described in Chapter Two). Briefly, test LN RNA was reverse transcribed and then amplified in the presence of a dilution series of competitive template. Cytokines analysed included IFNγ, IL-4 and IL-10. The mixture of different sized products was separated by 2% agarose gel electrophoreisis in the presence of ethidium bromide, and the point of equivalence at which both products gave equal band intensities determined by visual inspection under UV light.

LN Total RNA Content Determination

The total weight and RNA content of the LN draining the exposed flank were investigated in a third vaccination time course sampled at similar time points to the

second experiment. The axillary and inguinal LNs draining the left flank were harvested and processed as a pair for each of the 3 animals per time point. Analysis was carried out on pooled pairs of LN from each mouse so that the total weight and RNA increase could be assessed without small discrepancies in vaccination site favouring parasite migration through one node over the other. The nodes were weighed, teased apart and the RNA content estimated by a reaction with orcinol reagent, eliminating the need for any isolation and purification stages during which RNA might be lost (Cerotti *et al.,* 1954). The CsCl isolation procedure for total RNA extraction, as used in the above section, produces high yields of intact, DNA-free RNA. However, the extraction is not quantitative between sampling days. The orcinol reaction provides an estimate of the RNA present in an organ, but the RNA preparation is crude and of no further use. The system detects the RNA pentose present in a sample by an orcinol/HCl/$CuCl_2$ colour reaction measurable by spectrophotometry. The teased LN were extracted twice with absolute alcohol and twice with diethyl ether. The dried residue was then incubated in cold 2% perchloric acid at 4°C for 20 minutes. The residue was centrifuged down, the supernatant discarded, and the incubation repeated. The LN residue was then incubated in 250µl 10% perchloric acid at 70°C for 20 minutes, the supernatant collected and the procedure repeated. The supernatants were pooled and boiled for 40 minutes with an equal volume of 0.004M $CuCl_2.H_2O$ in conc. HCl/20 mg/ml orcinol. The colour was extracted twice with 500µl isoamyalcohol and an optical density reading taken at 675nm with a reaction blank and a set of RNA standard dilutions at known concentrations. A regression line was plotted using the standard RNA OD readings and from this the RNA content of the LN samples estimated.

Results

Cytokine Profiles Observed in the Axillary and Inguinal LN Following Vaccination and Challenge.

Previously published work indicated the predominance of Th1 type cytokines in supernatants obtained by *in vitro* culture of leucocytes from the skin draining LN after vaccination (Pemberton *et al.,* 1991, Pemberton and Wilson, 1995, Mountford *et al.,* 1992). Although the analysis of cytokine protein after secondary stimulation of isolated cells provides information on the potential cytokine secretion upon exposure to antigen, it does not reveal the true immune response occurring in the LN at the time of sacrifice. To address this problem, the more sensitive technique of RT-PCR was applied to investigate the *in vivo* responses occurring in the skin draining LN following exposure to *S. mansoni*. However, although RT-PCR analyses the cytokine mRNA present in the tissue *ex vivo*, this molecule is not the final active form of the cytokine and thus care should be taken when extrapolating data to the *in vivo* secretion of protein.

The data shown are representative of two experimental time courses. For all data, the values shown are estimates at 25 cycles, derived from regression analysis, with error bars showing the 95% confidence limits attributable to the regression equation. The slopes of all the regression lines for each cytokine were not significantly different from each other. All data points for which the error bars do not overlap are significantly different ($p \leq 0.0025$) from each other.

The profiles of the classic Th1 cytokines, IFNγ and IL-2, can be seen in Fig. 1. Both these cytokines rise sharply after vaccination, peaking significantly ($p \leq 0.0025$) above baseline at day 2, and falling almost to baseline levels by day 5. For IFNγ there is a second peak around day 14 post-vaccination; however, the level of message detected at this time point is lower than that seen at day 2. Both cytokines show no increase above naive values after challenge at day 35 in vaccinated and challenged animals (VC). Thus there is no anamnestic Th1 activity in the axillary and inguinal LN upon challenge of previously vaccinated mice. It should be stressed that the challenge parasites exit the skin through nodes on the opposite flank to those exposed

to vaccinating larvae, and that both sets of nodes (left and right) exhibited similar cytokine levels at day 35 for IFNγ, IL-2 and IL-4, reflecting the general immune status of the mouse rather than a localised response due to previous exposure. But insufficient RNA was recovered from the challenge flank LN at day 35 post-vaccination to carry out RT-PCR analysis for all the cytokines under investigation. Thus, data is plotted with the naive baseline indicated with a dotted line and no direct connection can be made between day 35 post-vaccination and day 5 post-challenge samples due to the switch to nodes on the other side of the body.

In challenge control animals (CC) there is a very slight, but not significant, increase in IFNγ and IL-2 mRNA above baseline values on day 5 after infection (day 40 on Fig. 1). In fact, after challenge, these cytokines are higher in CC mice than the VC group, but neither group reaches the elevated level of Th1 expression observed following vaccination.

The Th2 cytokines, IL-4 and IL-5, exhibit a completely different pattern of expression compared to the Th1 cytokines (Fig. 2). Post-vaccination, IL-4 mRNA shows no early increase, only peaking at day 10 and declining to a relatively uniform, but still elevated, level until challenge at day 35. Upon exposure to normal parasites, levels of IL-4 message rise sharply in both vaccinated and naive mice, peaking at day 10 post-challenge and then falling by day 15 (days 45 and 50 respectively of Fig. 2). The response in the VC group is of higher magnitude than that for CC animals, indicating a strong anamnestic response for this cytokine, and in both groups IL-4 levels peak 2-3 fold higher than following vaccination. After an initial lag, IL-5 message falls sharply below the naive baseline after vaccination and remains low up to day 35, an effect also seen for several other cytokines and discussed in detail below. Following challenge infection, IL-5 mRNA increases above background at day 5 in VC animals, before falling again at days 10 and 15. CC mice also show a rapid fall in IL-5 expression similar to that observed following vaccination. However, by day 15 values have recovered to above baseline and reach levels comparable to those seen in VC mice at day 5 post-challenge.

The other cytokines assayed, IL-10, IL-12 and IL-13, all display a sharp decrease in expression post-vaccination, with message levels falling below those obtained for naive LN (Figs. 3 and 4). IL-10 mRNA peaks briefly, although not significantly, at

day 5 after vaccination and falls gradually below the naive background level for the remainder of the post-vaccination time course. Furthermore, mRNA levels remain below the naive value after challenge, with both VC and CC animals displaying a slight decline in IL-10 expression with time. A similar response is seen for IL-12 expression, although the peak is earlier at day 2, corresponding with peak IFNγ expression, and the fall sharper than that observed for IL-10. Finally, the sharpest decline after vaccination is seen for IL-13 mRNA, with no peak before the message drops below the baseline. The same decrease is observed in both treatment groups following challenge.

Competitive PCR

A more accurate estimate of the amount of cytokine message for IFNγ, IL-4 and IL-10 was obtained for day 2 post-vaccination and day 10 post-challenge samples by quantitative PCR (Table 1). The point of equivalence for IFNγ product derived from the competitive plasmid and the sample cDNA was over 100 fold higher (50-500pg) at day 2 than the values obtained for IL-4 and IL-10 (0.5 and 5 pg respectively). However, after challenge of vaccinated mice the pattern is reversed with IL-4 message approximately 10 fold higher, at 5 pg, than IFNγ and IL-10 mRNA, both of which had points of equivalence corresponding to 0.5 pg of competitive template. Perhaps surprisingly, IL-10 displayed slightly higher levels of expression post-vaccination, with a 10 fold decrease following challenge. This is in contradiction to the increase in the other Th2 cytokine, IL-4, observed at this time. Lack of time and reagents limited this analysis to a 1:10 dilution series, and thus some cross-over points are expressed as a range of values if the exact point of equivalence was not covered by a single dilution.

Total RNA Content of the LN Following Vaccination

The production of cytokines by a minority of cells in an organ could lead to any message produced being swamped by the proliferation or influx of a more common cell type. To test this hypothesis two investigations were carried out involving the quantification of total RNA present in the nodes, and the analysis of the proportion of this RNA relating to B and T cells. Using a vaccination regime similar to that used

for cytokine mRNA analysis, the axillary and inguinal LN draining the vaccinated flank were excised and pooled for weighing and RNA content measurement. Following vaccination, the weights of the combined LN increased sharply at days 1 and 2, reaching a peak around days 10-14 before falling to near naive values at day 35 (Fig. 5). Surprisingly the total RNA content followed a similar pattern initially, but then reached a plateau and remained elevated with no decrease observed even at day 35 post-vaccination.

B and T Helper Cell Contribution to mRNA in the LN

RT-PCR data for the constant region of the μ chain of the IgM antibody indicates that there is an increase in the amount of IgM message detected in the LN following vaccination (Fig. 6a). The significant increase in expression observed at day 2 post-vaccination falls rapidly at day 5 and then remains slightly elevated until day 35, at which point a second, smaller peak occurs. After exposure to challenge parasites, no increase in IgM expression is observed in either VC or CC groups. In fact, the levels detected are below the naive baseline. Analysis of CD4 mRNA demonstrated an initial rise up to day 5 post-vaccination, followed by a steady decline until day 21 (Fig. 6). Similar to IgM expression, CD4 mRNA then increases at day 35. There is no detectable increase in CD4 expression above background post-challenge in previously vaccinated or control animals.

Resistance Data

Mice previously vaccinated with 500 irradiated cercariae were highly protected against a challenge infection compared to naive controls, demonstrating a resistance of 73%. The resistance was calculated from the worm burdens (CC = 46.75 ± 5.36, VC = 12.6 ± 1.86) using the equation given earlier. This result confirms data obtained previously by left flank vaccination followed by right flank challenge in which resistances of 66.8% and 79.3% were obtained (Pemberton and Wilson, 1995).

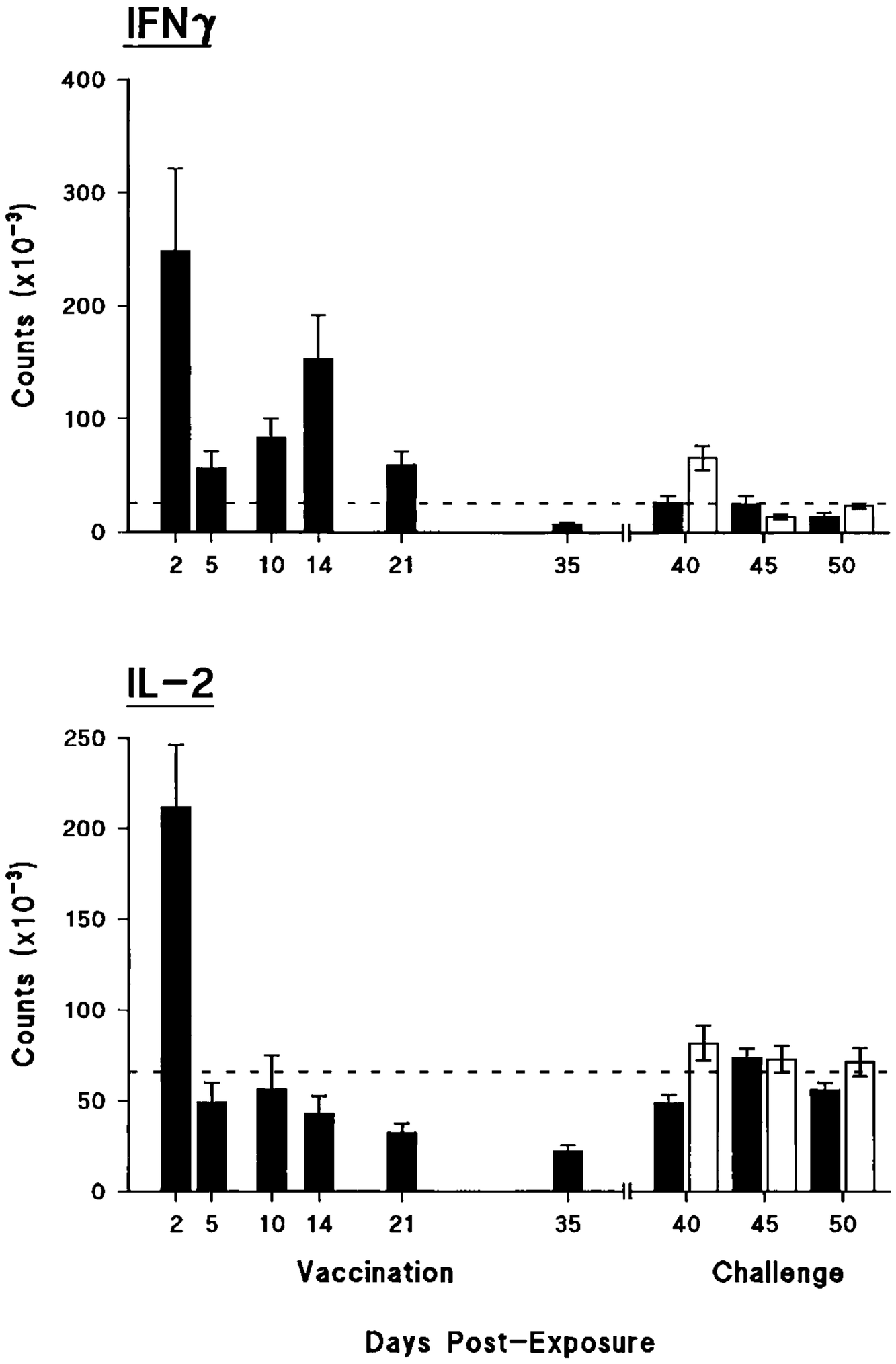


Fig. 1. The levels of IFNγ and IL-2 cytokine mRNA expression in combined axillary and inguinal LN tissue following percutaneous vaccination and challenge (■) or challenge alone (□). Values shown are estimates obtained at 25 cycles from regression equations derived using multiple cycle number PCR, and are adjusted for differences in HPRT levels between samples. The naive baseline level is represented by a dotted line determined as the average cytokine expression in uninfected mice sampled at days 0 and 35 of the vaccination time course.

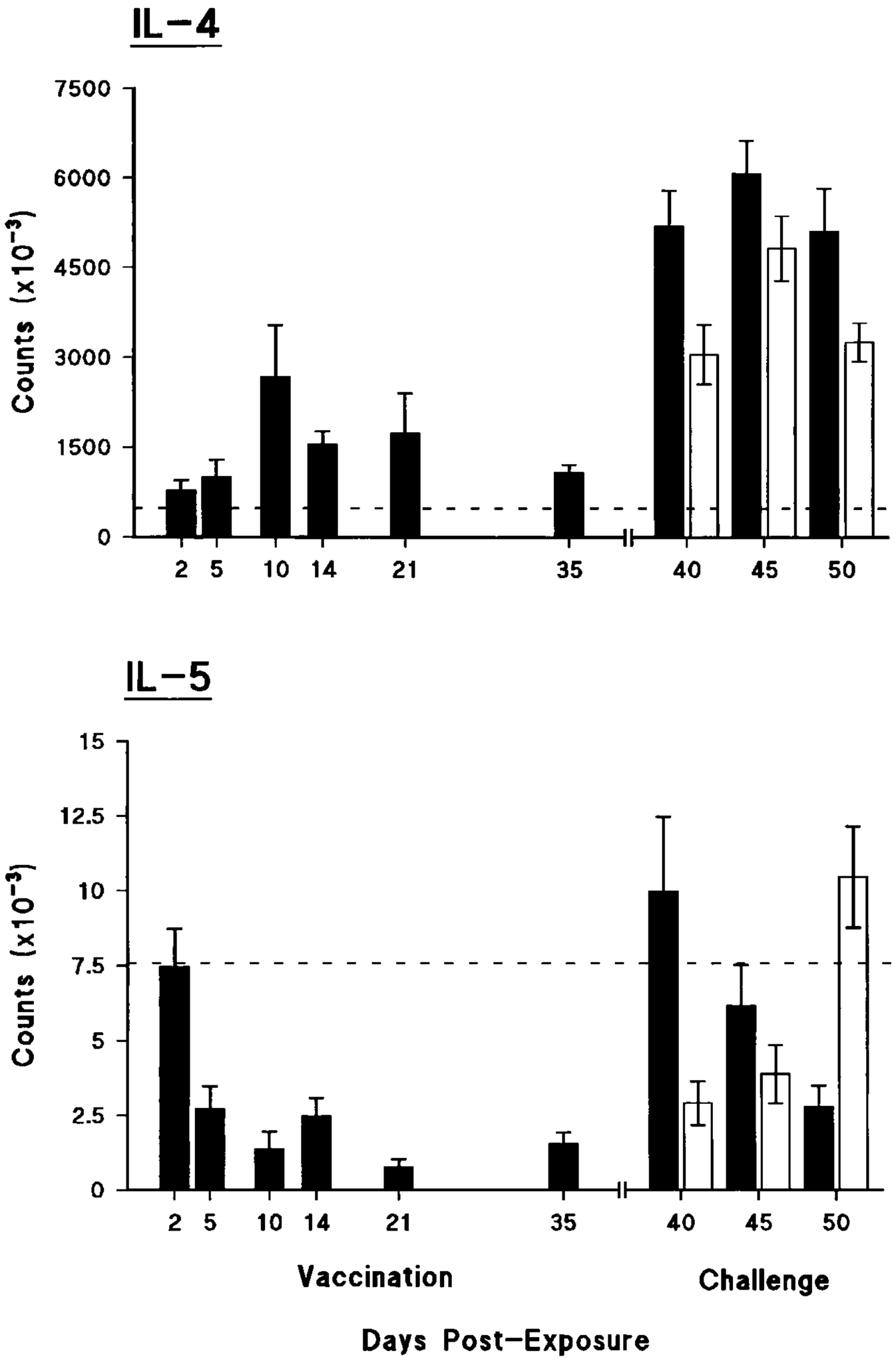


Fig. 2. Levels of IL-4 and IL-5 mRNA expressed in combined axillary and inguinal LN tissue following vaccination and challenge (■) or challenge alone (□). Values shown are as described for Fig. 1.

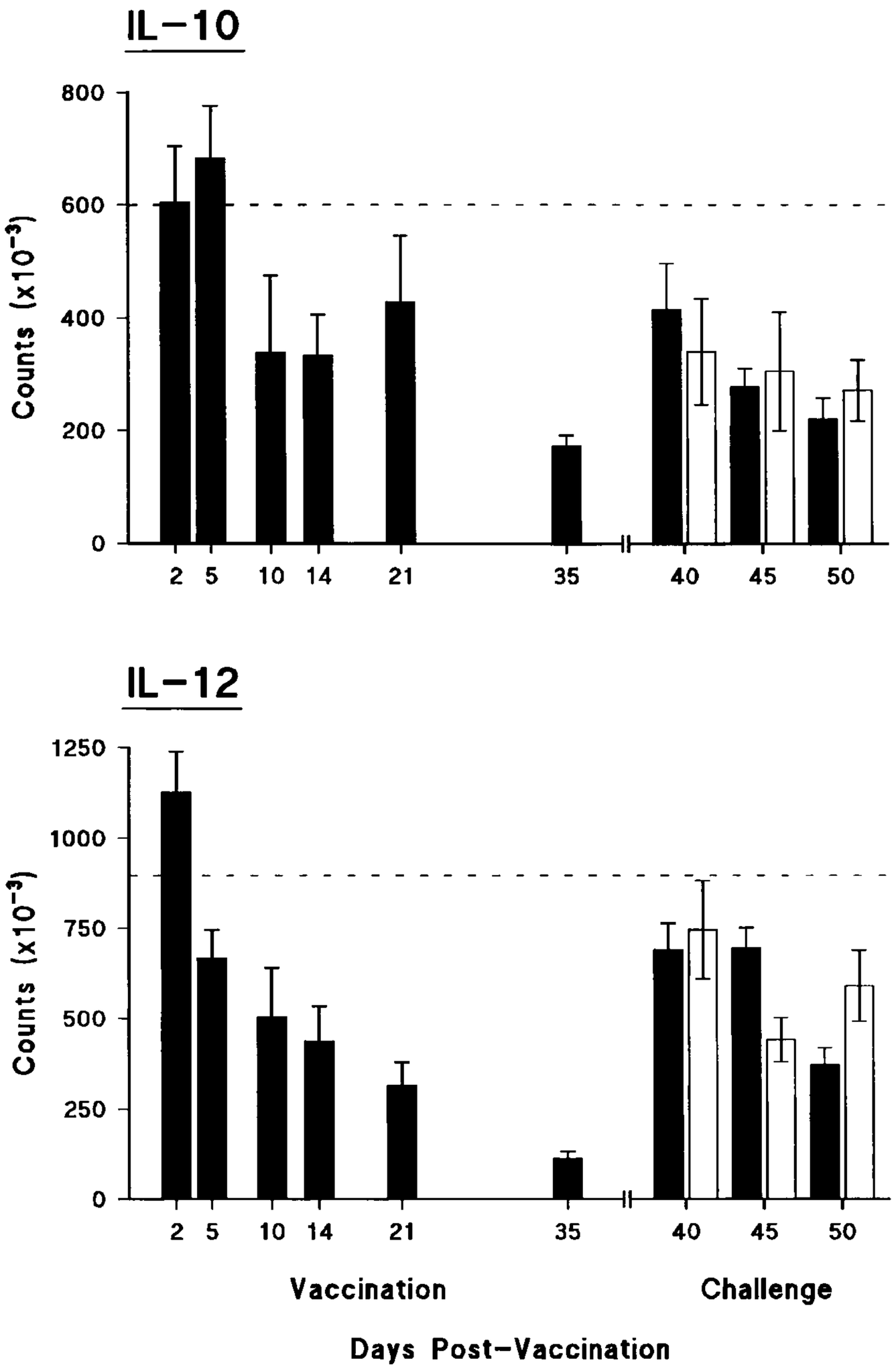


Fig. 3. Expression of the regulatory cytokines, IL-10 and IL-12, in combined axillary and inguinal LN tissue following vaccination and challenge (■) or challenge alone (□). Values shown are as described for Fig. 1.

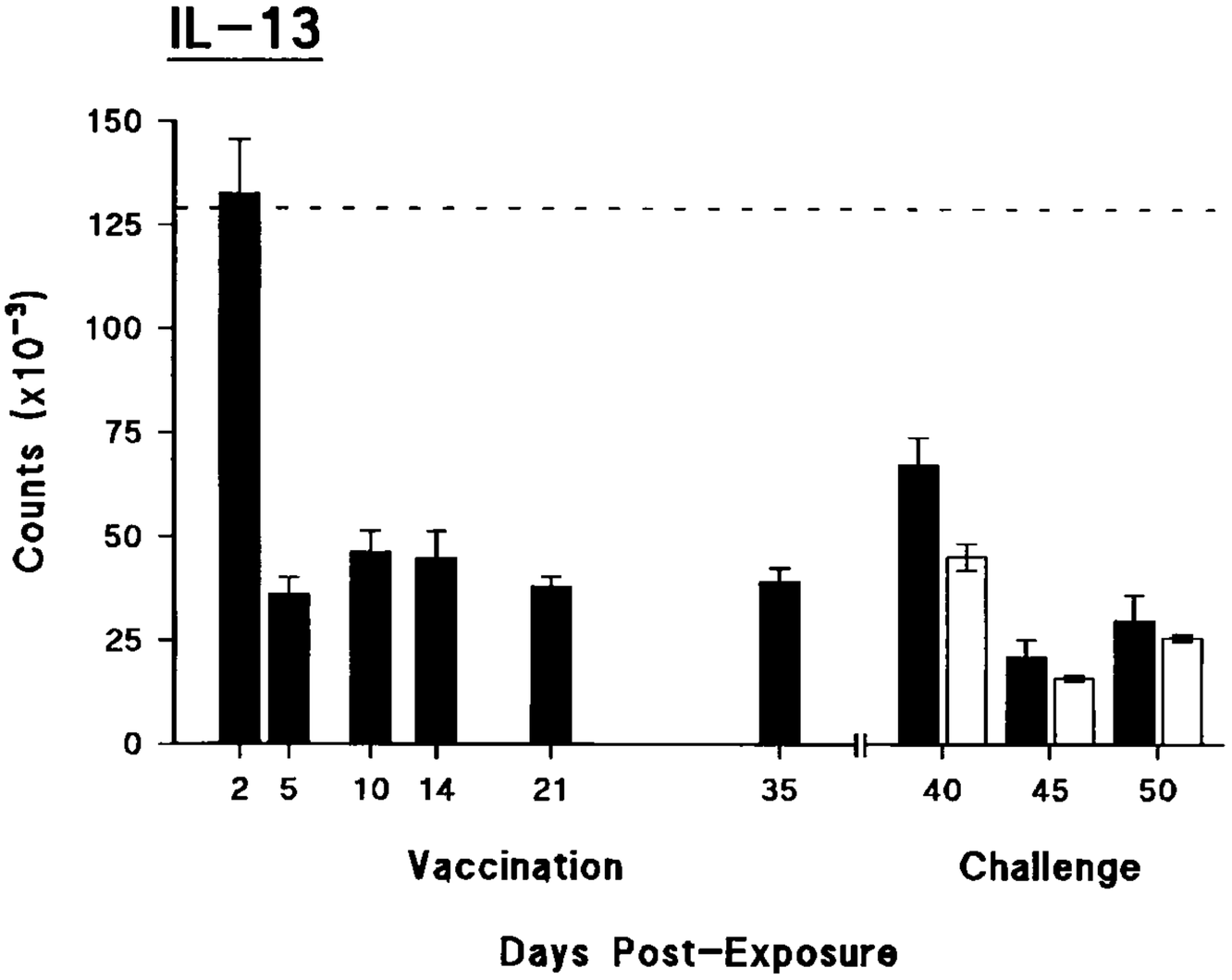


Fig. 4. Expression of the Th2 cytokine IL-13 in LN tissue following vaccination and challenge (■) or challenge alone (□). Values shown are as described for Fig. 1.

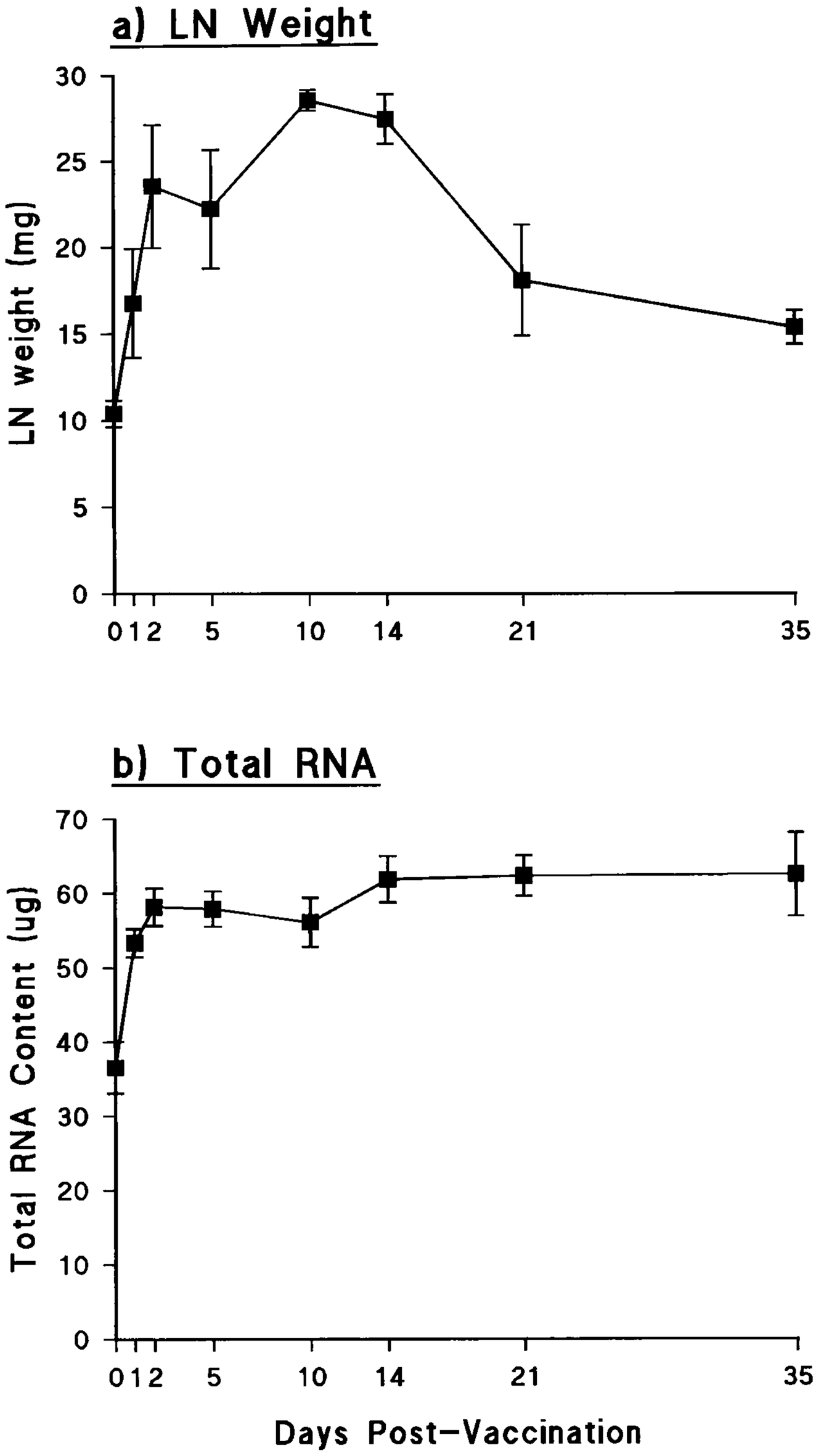


Fig. 5. Profiles of combined axillary and inguinal LN weight (a), and total RNA content (b), following vaccination on the left flank with 500 radiation attenuated cercariae. After weighing, the LN were teased apart, extracted in organic solvents and boiled with orcinol to give a colourmetric reading of the ribose content of the tissue. Values shown are the mean ± SE for three animals per sampling time.

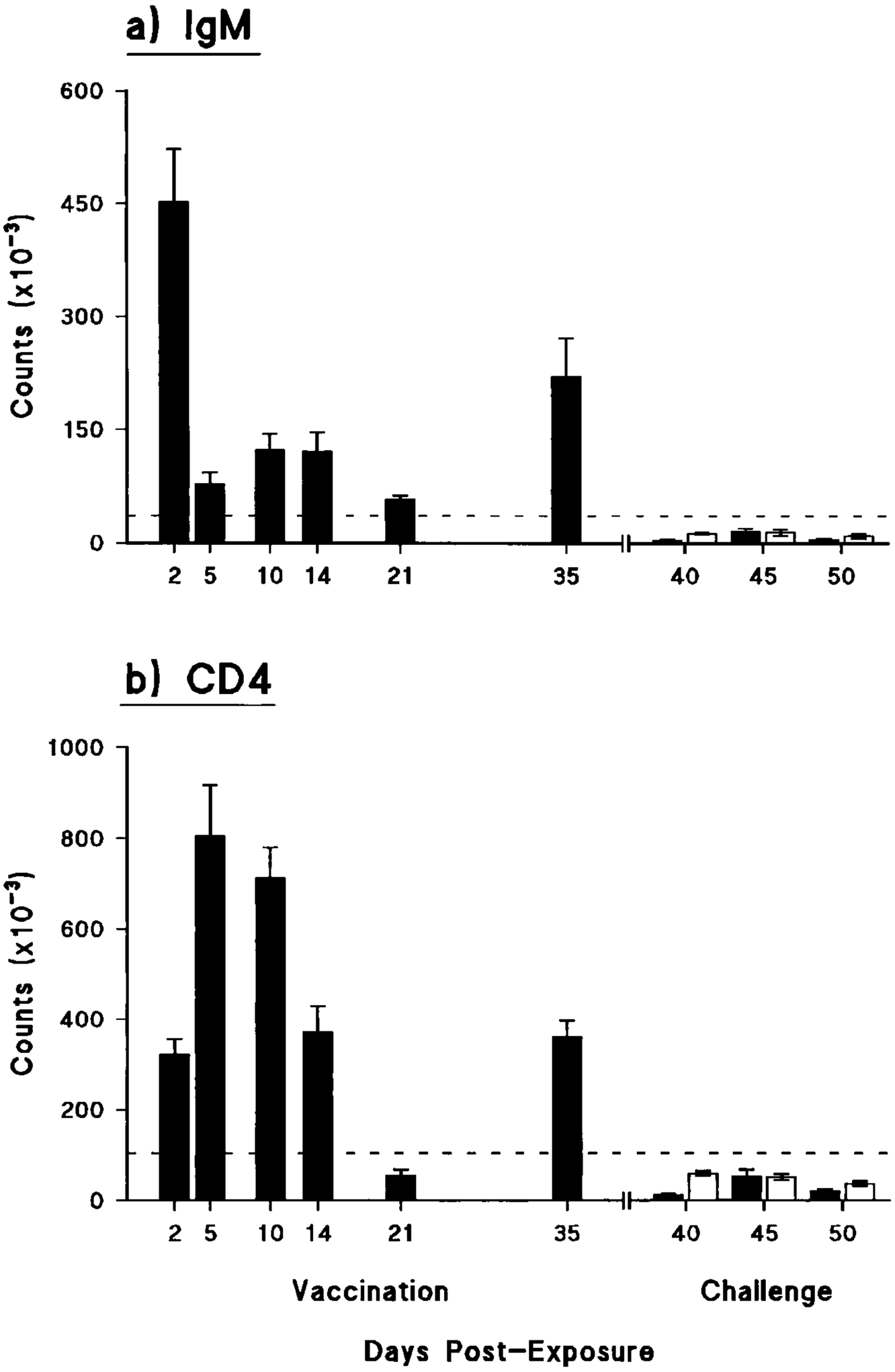


Fig. 6. The expression mRNA for the μ heavy chain constant region (a) and CD4 (b) mRNA in combined axillary and inguinal LN tissue following vaccination and challenge (■) or challenge alone (□). Values shown are as described for Fig. 1.

Table. 1. The quantity of competitive plasmid DNA added to naive, day 2 PV and day 10 PC samples in order to obtain PCR products of equivalent band intensities, as determined by visual inspection, when co-amplified with the cDNA under test. Values shown are pg of plasmid DNA added in a 5µl volume to a PCR tube containing cDNA derived from 0.25 µg total LN RNA.

	IFNγ	**IL-4**	**IL-10**	**HPRT**
Day 0 (Naive)	0.5 pg	0.05 pg	ND	50 pg
Day 2 post-vaccination	50-500 pg	0.5 pg	5 pg	50 pg
Day 10 post-challenge	0.5-5 pg	5 pg	0.5 pg	50 pg

ND = not detectable

Discussion

The cytokine messenger RNA profiles presented above provide an interesting overview of the events occurring in the skin draining LN following vaccination and challenge with *S. mansoni*. These results support data previously published on the *in vitro* secretion of cytokine protein following secondary stimulation (Pemberton *et al.*, 1991, Pemberton and Wilson, 1995). However, this chapter also highlights differences between the profiles observed at the protein and mRNA level, especially with cytokines such as IL-10, which can be produced by non-T cells (Mosmann and Moore, 1991). These discrepancies may be due to a variety of factors. Secondary stimulation *in vitro* does not account for cells utilising released cytokine protein, thus low levels of a protein detected by ELISA may not reflect actual production. In addition, this study involves the analysis of a wider variety of cytokines than already assayed by cell stimulation in culture. Thus RT-PCR provides a more complete picture of the Th1 and Th2 responses occurring in the sdLN, with the analysis of IL-12 and IL-13 which cannot be readily measured at the protein level at present.

Following vaccination with 500 irradiated cercariae of *S. mansoni,* mRNA levels for the Th1 cytokines IFNγ and IL-2 in the sdLN are elevated at day 1 and peak at day 2 post-exposure. This is earlier than the previously reported increase in IFNγ protein at day 4 (Pemberton *et al.,* 1991, Pemberton and Wilson, 1995); although no earlier time points were sampled in the above report, and thus an early peak may have been overlooked. In addition, the differences in the kinetics of mRNA transcription and protein secretion may account for any small differences in timing of peak expression. The rapid increase in IFNγ at day 2 coincides with a small increase in IL-12 message at this time point. IL-12 has been shown to induce IFNγ secretion from NK and T cells (Seder *et al.,* 1993), and this early elevation in expression may be responsible for inducing Th1 cytokine production in the LN soon after parasite stimulation of resident antigen presenting cells. IFNγ message remains elevated above background until day 21 with a another, smaller, peak at day 14, closely matching data obtained on protein production (Pemberton *et al.,* 1991). The magnitude and persistence of IFNγ expression may reflect the prolonged residence of

parasites in the LN for at least 2 weeks after vaccination (Mountford *et al.,* 1988, 1992). An early peak of IL-2 expression coincides with elevated levels of IFNγ mRNA, and has not been reported before, again possibly due to the absence of early sample points. The negligible levels of IL-2 seen across the remainder of the post-vaccination time course correspond to protein data as reported above (Pemberton and Wilson, 1995). This high expression of Th1 cytokines is in conflict with published data indicating that prolonged antigen exposure can lead to Th2 dominance in the skin draining LN after vaccination with irradiated cercariae of *S. mansoni* (Caulada-Benedetti *et al.,* 1991). However, this discrepancy may be explained by differences in sampling times and exposure regimes, the above published study having assayed cytokine levels at 4 weeks post-vaccination, long after migrating parasites have left the nodes. In addition, an *in vitro* antigen presentation system has demonstrated the development of Th2-like clones with increasing antigenic load (Gajewski *et al.,* 1989).

Th1 cytokine response detected in the LN coincides with the arrival of the first migrating parasites in this organ. Irradiated larvae can be detected in this tissue from day 1 onwards, peaking in numbers at day 5 (Mountford *et al.,* 1988). Furthermore, parasite-released material can be detected from day 2 after vaccination. The first peaks in mRNA for IL-4 and IL-10 correspond to the peak in parasite numbers resident in the nodes, occurring later than the very early expression of Th1 cytokines. A delay in IL-4 protein release, compared to IFNγ, has been demonstrated in cultured LN cells (Pemberton and Wilson, 1995, Mountford *et al.,* 1992) and is supported by the mRNA data presented here; IL-4 peaks at day 10 post-vaccination, 8 days later than IFNγ. The increase in Th2 cytokines after levels of Th1 cytokines have started to subside indicates that IFNγ, and also elevated IL-12 expression, may have an inhibitory effect on the expression of Th2 cytokine genes during the initial response. Alternatively, it may be the stimulation of IL-4 production which acts to suppress further Th1 secretion, although later increases in IFNγ tend to indicate that Th2 inhibition does not occur. The downregulation of IL-4 and IL-10 by IFNγ and IL-12 has been demonstrated in both *in vitro* and *in vivo* systems (Gajewski and Fitch, 1988, Gajewski *et al.,* 1989, Heinzel *et al.,* 1993a; 1994, Manetti *et al.,* 1993), and

thus the complex counter-regulatory mechanisms existing between the T helper subsets may be exerting an effect in this parasitic model.

The sharp decline in the level cytokine message detected after day 5 post-vaccination may be due to a dilution effect caused by a retention of B cells in the LN after day 7 (Constant *et al.,* 1990, Constant and Wilson, 1992). This cell accumulation might result in a relative increase of non-T cell RNA in the nodes and consequently, the proportion of cytokine transcripts from T cells or macrophages sampled in the 0.25 μg added to the RT-PCR reaction would be reduced. In addition, if the B cells present in the LN were activated and producing high levels of antibody then the dilution factor caused by this increase would have a significant effect on the detection of non-B cell messages. To test this hypothesis the weight and total RNA content of the combined axillary and inguinal LN were analysed, and a marker for B cell presence assayed by RT-PCR. As expected, LN weight increases with time post-vaccination, peaking at days 10 to 14. This is indicative of an increase in cell number. The RNA content of the LN also increased after vaccination, but in contrast to the LN weights, remained elevated until the end of the experiment at day 35. This may indicate an increase in cell activation and transcription, possibly attributable to Ig production, since the reduction in LN weight indicates that the number of cells in the nodes is actually falling around this time. The sustained increase in total RNA detected in the nodes coincides with the decline in cytokine message observed until day 35.

The sharp increase in IgM expression observed at day 2 post-vaccination, followed by a rapid decline, does not explain the steady decrease seen in IL-5, IL-10, IL-12 and IL-13 expression. Thus this marker may represent the activation of young and resting B cells already present in the LN rather than the accumulation of this cell type into the nodes. IgM expression switches to the IgG isotype upon cell activation, possibly explaining the apparent fall in expression. The profile obtained in this PCR would seem to indicate that B cell retention in the LN is not the cause of the downward trend observed in the mRNA levels for IL-5, IL-10, IL-12 and IL-13. However, upon further investigation, a fall in CD4 message was detected from day 10 post-vaccination onwards, even though the $CD4^+$ T cell population has been reported to stay elevated in the nodes for up to 28 days following exposure (Constant

et al., 1990). This indicates that a dilution effect of the proportion of T helper cell mRNA in the LN may occur. Alternatively, cytokine expression may be turned off after stimulation during vaccination, and inhibition of cytokine release can be caused by factors such as TGFβ. However, the downregulation of expression for certain cytokines to below naive baseline values would tend to indicate that these genes are constitutively expressed, and this is thought not to be the case (Dallman *et al.,* 1991a, 1991b). The true nature of the apparent reduction in some cytokine gene expression remains undefined. Moreover, decreases in IL-10 expression of up to 5 fold below baseline have also been observed in the LNs of mice infected with *Leishmania major* (Reiner *et al.,* 1994). The analysis of the LN cell populations by RT-PCR using primers for a more constitutive B cell marker, such as the kappa light chain expressed for all antibody class production, may highlight an increase in RNA from this cell type. However, in order to investigate the precise cytokine expression levels generated by the LN cell compartments, cell populations could be sorted and assayed individually by RT-PCR. In this way, compensation can be applied for any dilution effects attributable to large increases in cell types not responsible for the majority of cytokine expression.

In conclusion, the presence of both Th1 and Th2 cytokine mRNA in the LN following vaccination defines the presence of a mixed, or Th0, cell population. However, the Th1 cytokine expression occurs earlier, and is of higher magnitude than Th2 cytokine expression, as determined by competitive PCR. Although this early expression of IFNγ confirms data obtained by protein analysis, the presence of some of the other cytokines described by this study (IL-2, IL-5, IL-12) has not been reported previously.

Challenge with 200 normal parasites on the opposite flank to vaccination, through nodes which have not previously seen migrating parasites, induces strong IL-4 and IL-5 cytokine responses with very little increase in IFNγ or IL-2 mRNA. A possible explanation for the low Th1 cytokine values obtained in primed and control animals following challenge is that the day 5 sample point is on the downward slope of an earlier peak, similar to that seen after vaccination. The early increase in Th1 subset cytokine mRNA observed post-vaccination is replaced by IL-4 expression after challenge. Thus it would seem that there is no anamnestic Th1 response, but a strong

IL-4 anamnestic effect leading to the establishment of a predominantly Th2 profile upon multiple exposure. This type of Th1 inhibition has been reported previously when mice are multiply-vaccinated, or when challenge and vaccination have been applied to the same site (Caulada-Benedetti *et al.,* 1991, Pemberton and Wilson, 1995, respectively). The above data, and that presented here, indicates that repeated exposure to intact parasites can lead to the downregulation of the initial Th1 response and an increase in Th2 cytokine gene expression.

The absence of Th1 cell activity following challenge may be attributable to the anergy of this cell subset. The ability of Th1 type cells to become anergized has been reported to be related to the stimulation of this subset in the absence of any costimulatory signals, such as B7/CD28 (Gajewski *et al.,* 1990, 1994, Jenkins, 1994). The increased presence of B cells in the LN after vaccination may lead to this cell type acting as the predominant APC for cell stimulation following challenge. Resting B cells lack high levels of B7 expression (Croft and Swain, 1995). Hence activation in the absence of this molecule may lead to the induction of anergy in the Th1 population, resulting in preferential stimulation of the Th2 population and a subsequent increase in IL-4 release (Gajewski *et al.,* 1994, Jenkins *et al.,* 1990). Th2 cells can resist anergy to a higher extent than Th1 cells; in fact stimulation of Th0 cell populations in the absence of costimulatory molecules can lead to an increase in Th2 characteristics (Zubiaga *et al.,* 1991, Gajewski *et al.,* 1994) and a corresponding decrease in Th1-like cells. However, the role of costimulatory factors is not well established, and in contradiction to the above reports memory T cells of both subset-type have been reported as requiring lower levels of costimulation than naive cells (Croft *et al.,* 1994, Croft and Swain, 1995, Yi-qun *et al.,* 1996). Thus the mechanism by which Th1 cytokine expression is reduced post-challenge remains to be defined. An alternative explanation may be simply that the absence of IFNγ and IL-12, both of which can have an inhibitory effect on the proliferation and cytokine production of Th2 cells, allows the expansion of any residual Th2 population remaining in the LN after vaccination.

The lower expression of IFNγ post-challenge has been confirmed by the analysis of day 2 post-vaccination and day 10 post-challenge RNA by competitive PCR. IFNγ message levels are approximately 100 fold higher following exposure to

irradiated parasites than on secondary infection. Interestingly, IL-10 also has reduced expression post-challenge, corroborating data obtained by multiple cycle number PCR analysis. The pattern of IL-10 gene expression in lung tissue has been reported previously to follow that of IFNγ (Wynn *et al.,* 1993, 1994a), an observation possibly caused by the increase in Th2 cytokines exerting an inhibitory effect on macrophage activation and cytokine release (Fiorentino *et al.,* 1991a). This mechanism may result in a decrease in macrophage-derived IL-10 by a form of self-regulation. In contrast, IL-4 profiles are opposite in nature, with a 10 fold increase in mRNA levels observed following challenge similar to that seen in data obtained from the longitudinal RNA analysis. Thus the competitive PCR method allows detailed analysis of the level of expression of mRNA for a given cytokine, and comparison between different cytokine messages within the tissue. However, this method cannot account for possible differences in potency of the cytokines under analysis. Hence caution must be applied when interpreting data where comparisons are made between cytokines, since although IFNγ dominates the post-vaccination responses, naive C57BL/6 mice also show a much higher basal level of expression of this cytokine compared to IL-4 and IL-10.

The increase in the typical Th2 cytokines post-challenge does not appear to have any effect on the resistance mechanism. This is further demonstrated by the administration of sub-lethal whole body irradiation prior to challenge (Aitken *et al.,* 1987, Vignali *et al.,* 1988), a treatment which prevents the development of a new T cell response but has no effect on the existing memory T cell population resident in the lungs of previously vaccinated mice; following challenge of irradiated mice the effector mechanism is left intact. Thus it can be inferred that the predominant Th1 response generated after vaccination is sufficient for protection, and any other immune responses generated upon challenge are superfluous.

In conclusion, vaccination with irradiated cercariae of *S. mansoni* promotes the development of a dominant, but not exclusively, Th1 population with some expression of Th2 cytokines at later time points post-vaccination. These data demonstrate that the vaccinating dose of 500 irradiated parasites results in higher Th1 cytokine responses than an infection with 200 normal cercariae. Furthermore, previous exposure to vaccinating parasites appears to downregulate Th1 and IL-10

cytokine expression upon challenge infection, with the result that challenge control animals display slightly higher levels of cytokine mRNA than previously primed mice. Although this may appear surprising, the profiles detected in control mice do not seem capable of inducing resistance. Indeed the factors characteristic of irradiated cercariae responsible for altering the LN responses to a Th1 dominated profile remain unknown, but the altered kinetics of migration may play a role in induction of protection. The high expression of IFNγ in the LNs mirrors the high expression of this cytokine in the lungs upon subsequent parasite arrival in this organ (Mountford *et al.,* 1992, see also Chapter Four). It has been shown that the development of a predominantly Th1 population in the LN, and the recruitment of this schistosome-specific population to the lungs, where vaccinating parasites terminate migration, are two major stages in the induction of immunity. The presence of IFNγ producing cells in the lungs is vital to the protection mechanism (Smythies *et al.,* 1992a, 1992b). A detailed analysis of the cytokine profiles induced in the lungs during the next stage of migration and the effector mechanism are reported in the following chapter.

CHAPTER FOUR

Responses in the Lungs Following Vaccination and Challenge of C57BL/6 Mice with Cercaria of *S. mansoni*

Introduction

It is well documented that a single vaccination of C57BL/6 mice with optimally irradiated cercariae of *Schistosoma mansoni* results in resistance to reinfection of up to 70% (Dean, 1983). Furthermore, the lungs have been identified as the major site of parasite attrition following a challenge infection (Wilson *et al.,* 1986, reviewed by Wilson and Coulson, 1989). Early studies on migration indicated that normal parasites traversing the lungs undergo a period of transition involving elongation, by rhythmic contraction and lengthening, which is thought to aid passage through the narrow capillaries (Wilson, 1987). Once through the lungs, normal parasites can continue migration to the hepatic portal system where mating and egg laying begins. However, vaccinating irradiated parasites migrate no further than the lungs (Mastin *et al.,* 1983, Mangold and Dean, 1984), and it is possible that the radiation dose disrupts neuromuscular coordination, so preventing the adaptations necessary for successful passage through the capillaries (Harrop and Wilson, 1993).

The persistence of worms in the lungs post-vaccination leads to the recruitment of a cell population with Th1 properties to that organ. A proportion of migrating parasites get trapped in the alveolar spaces, presumably by disrupting the delicate blood/air barrier as they try to traverse the capillary beds (Mastin *et al.,* 1985b). These larvae attract foci of cells, and appear unable to return to the vasculature to continue migration once in the alveoli. The majority of cellular recruitment into the lungs occurs between days 10 and 16 post-vaccination (Ratcliffe and Wilson, 1991) and consists predominantly of $CD4^+$ T cells, which are capable of producing high levels of IFNγ upon *in vitro* culture, but not IL-2 or IL-4 as detectable by T cell assay (Smythies *et al.,* 1992a, Mountford *et al.,* 1988). This cytokine profile is indicative of a short term memory/effector phenotype and, in support of this, the cell population recruited to the lungs displays high levels of CD44 and low levels of CD45RB cell-surface markers (Coulson and Wilson, 1993). A transient eosinophilia around day 21 post-vaccination accounts for 30% of the leukocyte influx (Menson and Wilson, 1989), but rapidly decreases in comparison to the $CD4^+$ population which resides in the lungs for up to 10 weeks after exposure (Aitken *et al.,* 1988). In summary,

exposure of mice to a vaccinating infection results in the recruitment to the lungs of a predominantly Th1-like, schistosome-specific, cell population, rich in short term memory cells and capable of high IFNγ secretion upon stimulation.

Following challenge, normal parasites arriving in the lungs become the target of focus formation involving the cell populations recruited there after vaccination. The cellular aggregates act as a blockade, preventing parasite exit from the lungs and hence maturation into adult worms (Coulson and Wilson, 1988, Kambara and Wilson, 1990). The immunological mechanisms responsible for focus formation have been investigated at the cytokine protein and histological level (Smythies *et al.*, 1992b). The focus of cells around each migrating parasite consists mainly of $CD4^+$ T cells and macrophages (Kambara and Wilson, 1990), and produces high levels of IFNγ at day 14 post-challenge, but with little other cytokine protein detectable and an absence of proliferation in response to IL-2 (Smythies *et al.*, 1992a). In fact the importance of IFNγ in the effector mechanism has been shown by *in vivo* ablation with anti-IFNγ antibody, the effects of which result in a 90% abrogation of resistance (Smythies *et al.*, 1992b, Sher *et al.*, 1990a). Furthermore, vaccination of IFNγ receptor gene-deleted mice gives lower, but not completely abrogated, levels of protection than observed in wild type animals (see Chapter Six, and Wilson *et al.*, 1996).

The role of toxic nitrogen intermediates, released by macrophages, in the cell-mediated effector mechanism is not clear at present. IFNγ can act to upregulate inducible nitric oxide synthase mRNA expression in macrophages (Liew *et al.*, 1990, 1991, Oswald *et al.*, 1994c), and hence the production of NO, which in turn can mediate *in vitro* cytotoxic killing of newly transformed schistosomes (James and Glavern, 1989, Oswald *et al.*, 1994a). However, this mediator appears unable to harm lung stage parasites *in vitro* (Pearce and James, 1986). In contradiction to this finding, a recent study has implicated NO as an important factor in the effector mechanism, with iNOS mRNA readily detectable at elevated levels at day 2 post-challenge in vaccinated and challenged mice, compared to challenge controls (Wynn *et al.*, 1994a). Furthermore, ablation of the effects of NO by the administration of the inhibitor aminoarginine hemisulphate (AMG) leads to a 33% reduction in protection (Wynn *et al.*, 1994a). The importance of nitric oxide in parasitic infections has

already been demonstrated by the infection of iNOS-deficient mice with *Leishmania major* (Wei *et al.,* 1995). In this model, mice normally resistant to lethal infection with *L. major* become susceptible to visceral progression of the disease, and display reduced inflammatory responses compared to wild type animals .

Recent reports on human diseases involving DTH reactions have highlighted the importance of the chemokine RANTES in Th1 responses (Rathanaswami *et al.,* 1993, Devergne *et al.,* 1994). The findings reported for humans may also be applicable to murine models of parasitic infection. This molecule has been shown to play an important role in the recruitment of memory T cells in the human model of rheumatoid arthritis, and can be produced by many different cell types including macrophages and endothelial cells (Devergne *et al.,* 1994, Rathanaswami *et al.,* 1993). The cell types responsible for RANTES production in humans are similar to those present in the lung foci formed in mice upon challenge infection (Smythies *et al.,* 1992b). In addition, the cytokines necessary for RANTES upregulation in humans, namely IFNγ, TNFα and IL-1β, are also elevated in expression following vaccination and challenge of mice with *S. mansoni* (Wynn *et al.,* 1993). Thus, based on extrapolation from human studies, the expression of murine RANTES may contribute, at least in part, to the recruitment of cells into the lungs in the irradiated vaccine model.

The following experiments were carried out to investigate further the interplay of the cytokines released after exposure to both vaccinating and challenge parasites. Whole lung tissue was analysed by RT-PCR for the presence of various cytokine mRNAs along an extensive timecourse. Furthermore, the kinetics of RANTES and iNOS mRNA expression in this model were determined. The cell types responsible for the main cytokine release during the peak recruitment following vaccination were examined by analysis of cell populations recoverable from the lung airways by broncho-alveolar lavage. In addition, lung tissue from an intravenous challenge infection was investigated to determine the kinetics of cytokine expression following the arrival of the majority of parasites in the lungs as a synchronous pulse.

Materials and Methods

Experimental Animals and Parasites

Parasites of a Puerto Rican strain of *Schistosoma mansoni* were routinely passaged through LACA or MF1 outbred mice and albino *Biomphalaria glabrata* freshwater snails. Specified pathogen free (SPF) female C57BL/6 mice weighing between 18 and 20g (B&K Universal, Grimston, Hull) were used for all experiments and housed in flow cabinet or isolator conditions to maintain SPF status throughout all exposure regimes.

Exposure Regimes

The vaccination and challenge experimental plan has been previously outlined in chapter three. The following analyses and results concentrate on the lung tissue harvested from the same two timecourses analysed for the LN responses. Whole lungs were removed from three mice per timepoint and the RNA extracted immediately, or the tissue snap frozen in liquid nitrogen and stored at -80^{o}C until required. Lung tissue was sampled at days 0, 5, 10, 14, 21 and 35 post-vaccination, and at days 5, 10 and 15 after percutaneous challenge. Ten extra mice, vaccinated at the same time as the above experiment, were sacrificed at day 21 post-vaccination to determine the lung cell populations responsible for the production of various cytokines at this timepoint. The mice were anaesthetised and the perfused lungs lavaged to obtain the airway populations. The recovered cells were then sorted as described below. The broncho alveolar lavage (BAL) was performed as previously described (by A Finlay using a method from Holt, 1979).

Additional post-challenge analysis was carried out on lung tissue removed from mice exposed to an intravenous challenge with 120 lung stage parasites derived from donor mice previously infected with 1000-2000 normal cercariae (challenge procedure carried out by Prof. R A Wilson, and Drs. L E Smythies and P S Coulson, using a technique previously described by Miller and Wilson, 1978 and Wilson and Coulson, 1986). This method allows the kinetics of cytokine expression to be investigated due to the synchronicity of parasite arrival in the lungs following injection into the exposed femoral vein. In order to make a direct comparison

between previously vaccinated and naive animals, a limited sampling program was designed to allow challenge of both groups of mice on the same day, using the same pool of parasites. The small right lobe of the lung from these animals was removed for RT-PCR analysis at days 0, 2, 4 and 12 post-challenge, either directly into guanidinium isothiocyanate solution, or snap frozen in liquid nitrogen for storage.

Resistance data for both exposure regimes was calculated from the worm burdens obtained by hepatic portal perfusion at 35 days following challenge of groups of 5 mice from each of the treatments above. Resistance was calculated from the respective worm burdens as follows : %R = [(CC-VC)/CC]x100.

Total RNA Isolation

Lungs from each of 3 mice per timepoint for the percutaneous challenge, or 5 mice for the intravenous challenge, were treated individually using an RNA extraction procedure (as described in Chapter Two). For all samples the yield was measured spectrophotometrically and confirmed, along with the intact nature of the RNA, by agarose gel electrophoresis in the presence of ethidium bromide.

Semi-Quantitative RT-PCR Detection of Cytokine mRNA

Analysis of the levels of various cytokine mRNAs between samples was carried out as previously described in chapter two. After initial trial experiments the optimum amount of total lung RNA to be used in each PCR was determined as 1μg. A fixed amount of RNA was taken from each lung sample (3 per timepoint for the percutaneous challenge, and 5 per timepoint for the i.v. experiment) and pooled to give a combined total RNA sample for RT-PCR analysis in an attempt to reduce experimental cost. This pool was assayed at three different cycle numbers, with duplicated tubes at each cycle number to give a total of 6 tubes per sample per cytokine. The cycle numbers and primer sequences used were the same as described previously (Chapter Three) with the addition of the following: IL-1β sense, GGCAACTGTTCCTGAACTCAAC and anti-sense, CTCGGAGCCTGTAGT GCAGCTG (designed from published sequence, Gray *et al.,* 1986); TNFα sense, ATGGCCCAGACCCTCACACTC and anti-sense, GTAGTCGGGGCAGCCTT GTCC (adapted from Murphy *et al.,* 1993); RANTES sense, GAAGATCTCTG

CAGCTGCCCT and anti-sense, GCTCATCTCCAAATAGTTGA (designed from published sequence, Schall *et al.,* 1992).

Both percutaneous and intravenous challenge timecourses were also analysed for the presence of inducible nitric oxide synthase (iNOS) mRNA to investigate the role of this larvicidal molecule in the effector mechanism. Lung samples from the percutaneous time course were analysed individually by RT-PCR for a single cycle number. RNA from the intravenous time course were analysed by multiple cycle number PCR. The primer sequences were designed from the published cDNA sequence (Lowenstein *et al.,* 1992) and were as follows: sense, GCAGCTCCTCAC TGGGACAGCAC, antisense, ATGAGGCAGGAGCTCCTCCAGAGG.

For all RT-PCR products the detection system used was as outlined in previous chapters. Briefly, oligonucleotide probes were end-labelled with [^{32}P-γ] ATP and hybridised with PCR product slot blotted onto nylon membrane. Washed blots were then exposed to a storage phosphor screen and scanned on a phosphorimager to obtain counts for all samples which could then be processed by linear regression. The probes specific for molecules analysed in this chapter were designed from previously published sequence data and were as follows: TNFα, ATGGCCCAGAC CCTCACACTC (Caput *et al.,* 1986); IL-1β, GAGCCCATCCTCTGTGACTCA (Gray *et al.,* 1986); RANTES, CTGCCTCGTGCCCACGTCAAG (Schall *et al.,* 1992); iNOS, CATGTGACATCGACCCGT (Lowenstein *et al.,* 1992).

Competitive PCR

Quantitative PCR analysis of a limited number of samples, representative of peak cytokine responses post-vaccination and post-challenge, was carried out as described previously (Chapter Two). Samples from day 21 following vaccination, and day 10 post-challenge, were analysed for the expression of IFNγ, IL-4, IL-10 and HPRT. Background differences in the basal levels of these cytokines in naive mice were also investigated.

Sorting and Analysis of Lung Alveolar Cell Populations

The total BAL cells recovered from each mouse were individually pelleted and resuspended in 1 ml of GMEM (Glasgow minimal essential medium) plus 10% FCS

(Globefarm, Esher, Surrey), 200 U/ml penicillin and 100 μg/ml streptomycin (Sigma Chemical Co., St Louis, MO). The separate samples were then pooled and the total cell number estimated using an improved Neubauer haemocytometer. The volume of medium was adjusted to give a concentration of 10^6 cells/ml and the cell suspension plated out in a 24 well sterile plate at 1 ml per well. The cell suspension was left to adhere for 45 minutes at 37°C and then the non-adherent populations were washed from the plate with ice-cold PBS, collected, and centifuged for 8 mins, 4°C at 800 rpm (Beckman benchtop refrigerated centrifuge). The resulting pellet was resuspended in 1% bovine serum albumin (Sigma), in phosphate buffered saline pH 7.2 (1% BSA/PBS), at a concentration of $6.25x10^7$ cell /ml.

Three aliquots of $5x10^6$ cells (80μl) were labelled with 6μl of CD4, CD8 or a combination of CD4, CD8 and B220 magnetic beads (MACS system, Miltenyi Biotec Gmbh, Germany). After incubation at 6-12 °C for 15 mins, the cells were washed and centrifuged gently to remove excess label, and then resuspended in 550μl 1% BSA/PBS. A 50 μl aliquot was taken for flow cytometric analysis and the remainder purified over a magnetic cell sorting system (MACS). Both the retained and non-retained cell populations were collected from each bead labelling, and the recovered cell number estimated by haemocytometer. Samples of 10^5 cells from each of the retained and non-retained CD4 and CD8 cell purifications were labelled with the appropriate fluorescein-labelled antibody for 30 minutes on ice, washed with phenotyping buffer (0.1% BSA in PBS) and resuspended for flow cytometric analysis to obtain values for cell enrichment.

The remaining samples, including the recovered adherent cell populations, were pelleted and the RNA isolated using a small scale RNA isolation kit (Stratagene Ltd, Cambridge, UK) following the manufacturers instructions. The resulting total RNA pellets were resuspended in 100μl of DEPC-treated water and 10μl of each subjected to RT-PCR for a set number of cycles dependent upon the cytokine under assay (HPRT, 30; IFNγ, 30; IL-4, 33; IL-5, 33; IL-10, 33; and IL-12, 30). The product was analysed by Southern blotting followed by detection with specific ^{32}P end-labelled oligonucleotide probes, and exposure to autoradiographic film and phosophor storage screen. The product detected for each cytokine in a cell population was scored on a scale of three intensities following HPRT adjustment of the phosphorimager count.

Results

Cytokine Production in the Lungs Following Percutaneous Vaccination and Challenge

The expression of cytokine mRNA in whole lung tissue following exposure to irradiated and normal cercariae was investigated using semi-quantitative RT-PCR. All points for which the error bars do not overlap are significantly different ($p \leq 0.0025$) to each other as described in chapter two. Following vaccination, IFNγ, IL-2, IL-4, IL-5, IL-10, IL-12 and IL-13 all rise steadily to a peak at day 21 post-exposure, with the majority of these cytokines reaching levels between 5 and 10 fold over the detectable baseline (Figs. 1 to 4). Exceptions to this high increase in mRNA were the inflammatory cytokines TNFα and IL-1β (Fig. 5), both of which peak at 2-3 fold over naive levels. Furthermore, IL-1β peaks earlier, at day 14, than the other cytokines tested. There appears to be no real distinction between the Th1 and Th2 cytokine profiles in the lungs following exposure to vaccinating parasites.

After challenge infection of vaccinated and naive mice, differences can be seen in the levels of Th1 and Th2 cytokine expression induced in the two experimental groups. The message levels for IFNγ, IL-2, IL-4, IL-5, IL-10, IL-13 and TNFα in previously primed (VC) animals following challenge reached a maximum at lower values than those detected post-vaccination. IL-12 and IL-1β attain similar levels to those observed at day 21 post-vaccination. IFNγ, IL-12 and IL-1β all peak at day 10 post-challenge (day 45 on Figs. 1, 4 and 5) and show elevated levels of expression over the time course studied compared to challenge control (CC) animals, which only show slight increases at day 15 (day 50 on Figures). IL-2, IL-4, IL-13 and TNFα mRNA levels continue to rise up until day 15 post-challenge in previously primed mice. In contrast, control mice do not attain the same level of IL-4 and TNFα expression seen in VC animals at the time points sampled, although the former group display elevated expression of IL-2 and IL-13 at day 15 following infection with normal parasites. Cytokine message for IL-5 and IL-10 is barely elevated above the levels detected at day 35 post-vaccination in vaccinated mice. In addition, IL-10 expression does not increase above naive values in CC mice. In contrast, IL-5 rises sharply at day 15 post-challenge in CC mice to become the only cytokine detectable

above the levels expressed in vaccinated mice. Similarly, IL-13 expression in challenge controls also rises sharply at day 15 post-challenge, but levels do not increase above those detected in vaccinated animals at this time point (Fig. 3). IL-5, IL-10, IL-12, TNFα and IL-1β expression in the challenge control mice falls below the naive levels detected at day 35 before recovering (in the case of IL-5, IL-12, IL-13 and TNFα) to increase above baseline at day 15 (Figs. 2, 3, 4 and 5). However, IL-10 mRNA levels remain below baseline throughout the post-challenge time course (Fig. 4). It is interesting to note that IL-1β shows a high basal level of expression in day 35 naive mice compared to the naive group sampled at day 0 (Fig. 5), although the reason for this remains unclear.

In summary, the inflammatory cytokines IFNγ, IL-12, TNFα and IL-1β display strong anamnestic responses post-challenge, with the mRNA levels for these cytokines reaching at least 50% of the post-vaccination values. IL-2, IL-4, IL-5 and IL-10 appear to have no anamnestic response compared to levels obtained in the lungs after vaccination. However, from day 35 onwards all cytokines in vaccinated animals reached higher levels of expression than in naive controls, with only IL-5 displaying an increased value in control animals compared to vaccinated counterparts. On the whole, the mRNA expression profiles induced upon exposure of challenge control animals to normal parasites are lower and later than those observed in previously vaccinated mice. In addition to the earlier peaks observed in primed animals, cytokines are expressed at much higher levels in vaccinated mice after challenge compared to naive mice, and are dominated by Th1 and inflammatory cytokines.

RANTES Expression in the Lung

The expression of RANTES mRNA following vaccination reaches peak levels at day 21 (Fig. 6), coincident with peak values for all the major cytokines, and known inducers of RANTES in human systems, namely IFNγ, IL-1β and TNFα (Rathanaswami *et al.*, 1993).

This chemokine also peaks after challenge, at day 10 post-exposure, before falling again by day 15 (days 45 and 50 respectively on Fig. 6). There is no increase in RANTES expression in challenge control animals, on the contrary there is a slight

decrease from baseline values. In fact the baseline value obtained from day 35 naive animals is three fold higher than that detected in day 0 naive mice. Thus, although the level of expression falls in challenge control samples from day 35 onwards until a slight recovery at day 15 post-challenge (Fig. 6), these levels are still relatively higher than RANTES expression at days 0, 5, 10 and 14 post-vaccination.

Competitive PCR

PCR in the presence of a competitive internal constuct was used to determine the relative expression levels of IFNγ, IL-4 and IL-10 mRNA in naive mice and at the time of peak responses post-vaccination (day 21), and post-challenge (day 10) (see Table 1). In naive mice IFNγ mRNA is expressed at a level 10 fold that of IL-4, whilst IL-10 is undetectable. Post-vaccination, the IFNγ mRNA level is 100 fold higher than that detected in naive samples, equating to 50 pg of plasmid DNA. IL-4 expression was detectable at 10 fold above the naive baseline, but at an overall level lower than IFNγ, equivalent to 0.5 pg of construct. A similar level of IL-10 mRNA was detected at day 21 post-vaccination. Post-challenge, IFNγ mRNA levels are lower than after vaccination at between 5 and 50 pg. IL-4 and IL-10 expression was also lower, showing a similar drop to the levels of IFNγ mRNA. The level of HPRT expression equated to 50 pg of plasmid DNA for all samples under test.

The Cytokine mRNA Profiles of Sorted Cell Populations

Magnetic bead sorting of the BAL cell population resulted in high enrichment of the $CD4^+$ population (from 46% to 92%), the $CD8^+$ population (from 12.5% to 87%), and granulocytes (from 48% to 76%). However this last sample contained 15% contamination from macrophages and 10% from T lymphocytes. The adherent cell population, purified by attachment to a sterile plate, was only enriched to approximately 50% of the recovered cells.

The cytokine production from each enriched and depleted sample is shown in Fig. 7 and summarised in Table 2. RNA isolated from the adherent population gave positive results for IFNγ, IL-4, IL-5, IL-10 and IL-12 (Lanes 1, 2 and 3 on Fig. 7, first column of Table 2). The production of IL-12, IFNγ and IL-10 by the adherent cell population is not surprising in that these cells are probably activated by the

surrounding cytokine environment. However, the production of IL-4 and IL-5 by the adherent cell population is unexpected and possibly due to the presence of contaminating T cells. The main T cell population expressing cytokines appears to be the $CD4^+$ subset, with high levels of IFNγ, IL-10 and IL-5 and low levels of IL-4 and IL-12 message detectable in this sample (lane 4). In addition, all the cytokines under test could also be detected in the CD8- and granulocyte-depleted samples (lanes 7 and 8). The $CD8^+$ cell sample does not appear responsible for major cytokine production this model, with no PCR product detected for IFNγ, IL-4, IL-5, IL-10 or IL-12 in this cell population. Granulocytic cells also test positive for all cytokines (lane 9), although contamination in this cell sample is a possibility due to only 76% enrichment. Two cytokines that this cell population appears to express above the contamination level are IFNγ and IL-12, both of these being detectable at higher levels in the granulocyte-enriched sample that flowed through the column, compared to the $CD4^+$, $CD8^+$ and B cell sample retained by the magnetic field. These cytokines can be expressed by macrophages, and a small contaminating population of these cells could account for the detection of IFNγ and IL-12 in the granulocyte sample.

Cytokine Expression in Lung Tissue Following Intravenous Challenge

The cytokine message profiles of whole lung tissue after a synchronous pulse of lung stage worms were analysed by the semi-quantitative RT-PCR method described for the percutaneous challenge. IFNγ expression is higher in previously primed mice throughout the time course. A main peak occurs in VC mice at day 4 post-challenge after rising over 2 fold from day 2, and before falling again by day 12. IFNγ expression in the lungs of CC mice barely rises over baseline values (Fig. 8). The other Th1 cytokine, IL-2, shows an increase at day 2 in VC mice followed by a fall at day 4 before increasing again at day 12. As observed for IFNγ, CC levels of IL-2 mRNA only increase slightly above the naive level at day 12 post-exposure.

The Th2 cytokines also display elevated expression in previously primed animals compared to the control group (Fig. 9). IL-4 mRNA peaks at day 4, before declining again by day 12. Expression of this cytokine in the challenge control group does not rise above baseline until later, at day 12, by which time it is slightly higher than the

IL-4 mRNA level detected in VC mice. In vaccinated animals, IL-10 message is elevated dramatically over baseline at day 2, and then falls gradually over the remainder of the time course, whereas challenge controls show very little increase above background throughout. TNFα expression in vaccinated mice rises above baseline values only at day 4 post-challenge, and is not expressed at elevated levels in CC mice at any of the time points sampled (Fig. 10). In contrast, CC mice express higher levels of IL-1β at day 2 than VC mice (Fig. 10). However, at days 4 and 12 a similar pattern of IL-1β expression is observed for both groups of mice, with levels slightly higher in the vaccinated group. In general, the cytokine message detected related well to that reported for the percutaneous exposure. The values obtained for previously vaccinated mice were higher than those in control animals for almost all timepoints. The cytokine expression observed in control animals, for the most part, remained at basal levels (Figs. 8, 9 and 10), as seen following percutaneous challenge.

Expression of Inducible NOS mRNA in Whole Lung Tissue

Elevated expression of iNOS mRNA was only detected above baseline at day 10 in lung tissue from percutaneous challenge (Fig. 11a). The RT-PCR analysis carried out on individual mice gave high variation at this time point, making the observed increase insignificant, although highly reproducible. Consequently, analysis of RNA from tissue exposed to intravenous challenge was carried out to see whether this variability was due to the staggered arrival of parasites in the lungs following percutaneous challenge. The results, obtained by semi-quantitative PCR, indicate a peak at day 4 post-challenge exposure in previously primed mice (Fig. 11b). This peak was highly significant ($p \leq 0.0025$ as calculated by 95% confidence limit overlap) over levels detected in challenge control animals, which remained at base line throughout the time course. Furthermore, values remained elevated in vaccinated and challenged mice until day 12.

Resistance Data

The levels of protection obtained in the percutaneous vaccination and challenge regime gave good agreement with previously reported data at 73% resistance. The

worm burden in vaccinated mice was 12.6 ± 1.9, compared to 46.8 ± 5.4 in the control group. The intravenous challenge timecourse induced a resistance of 43.5% which correlates with measurements obtained previously using this method (Menson *et al.,* 1989, Smythies *et al.,* 1996a) and is possibly lower than the protection observed with percutaneous challenge due to the developmental state of the injected worms.

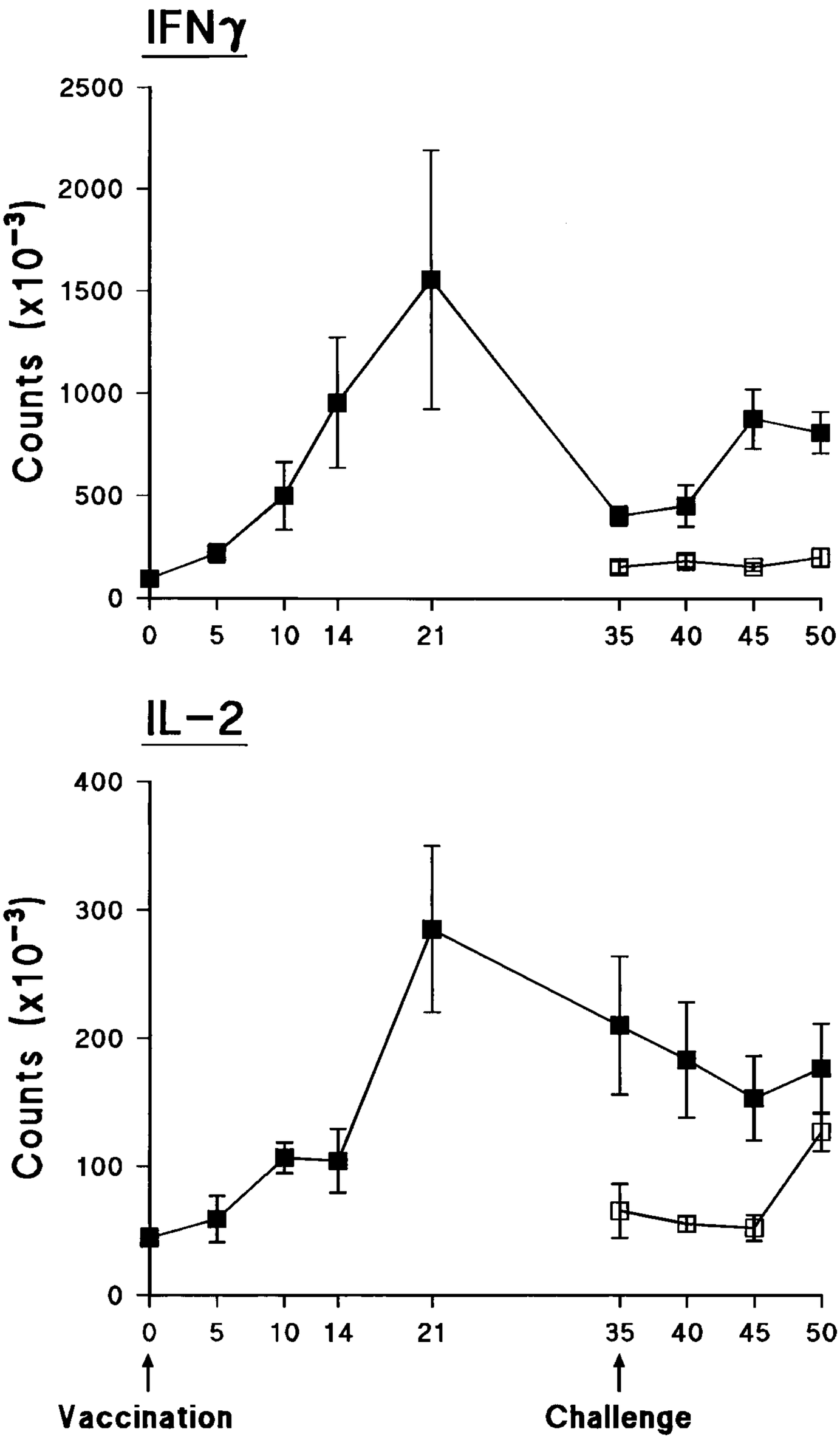


Fig. 1. The levels of Th1-type cytokine mRNA expression in whole lung tissue following percutaneous vaccination and subsequent challenge (■), or challenge alone (□). Values shown are estimated phosphorimager counts at 25 cycles derived from regression analysis as described in materials and methods

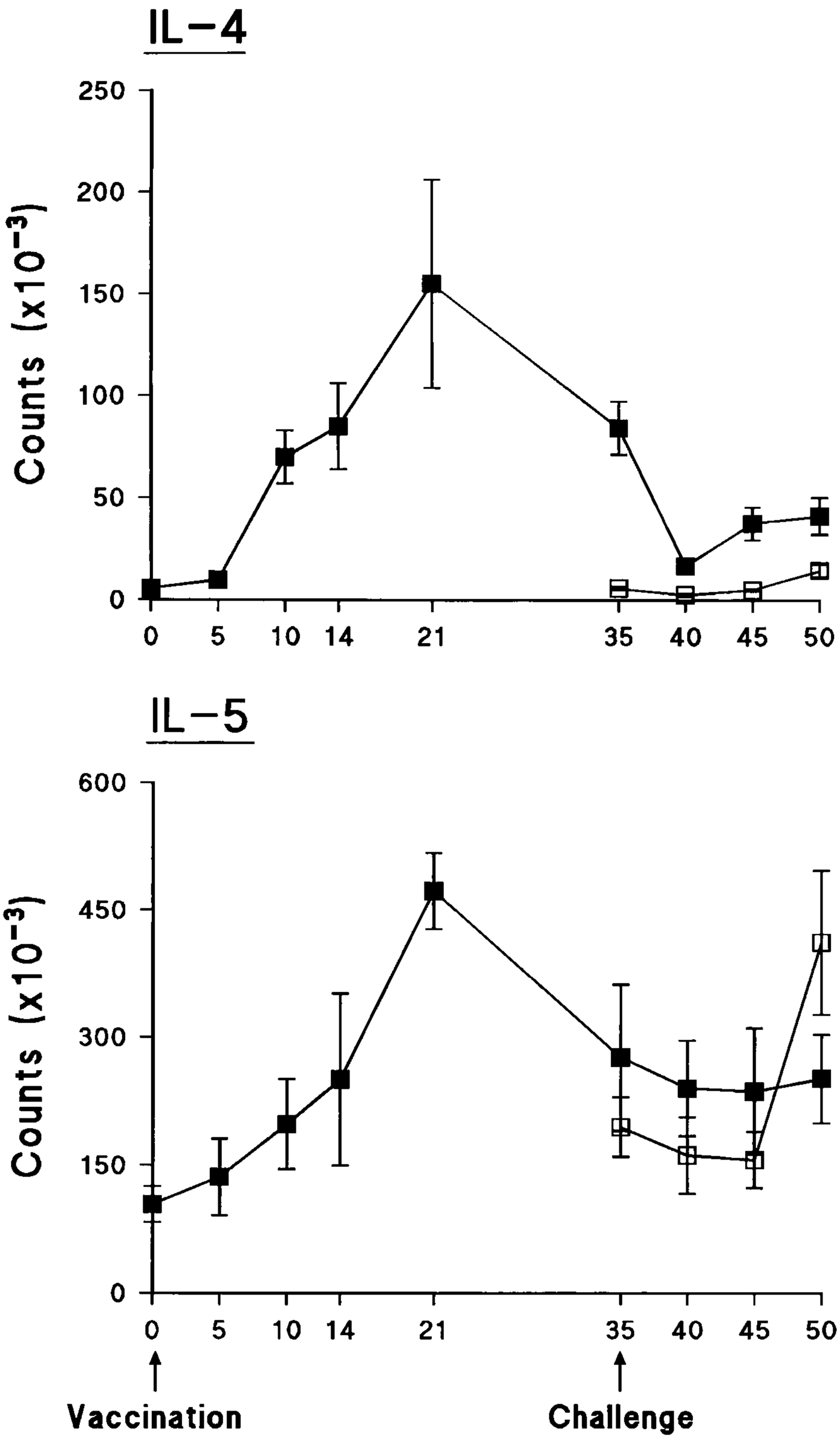


Fig. 2. The levels of Th2-type cytokine mRNA expression in whole lung tissue following percutaneous vaccination and subsequent challenge (■), or challenge alone (□). Values represented as described in Fig. 1.

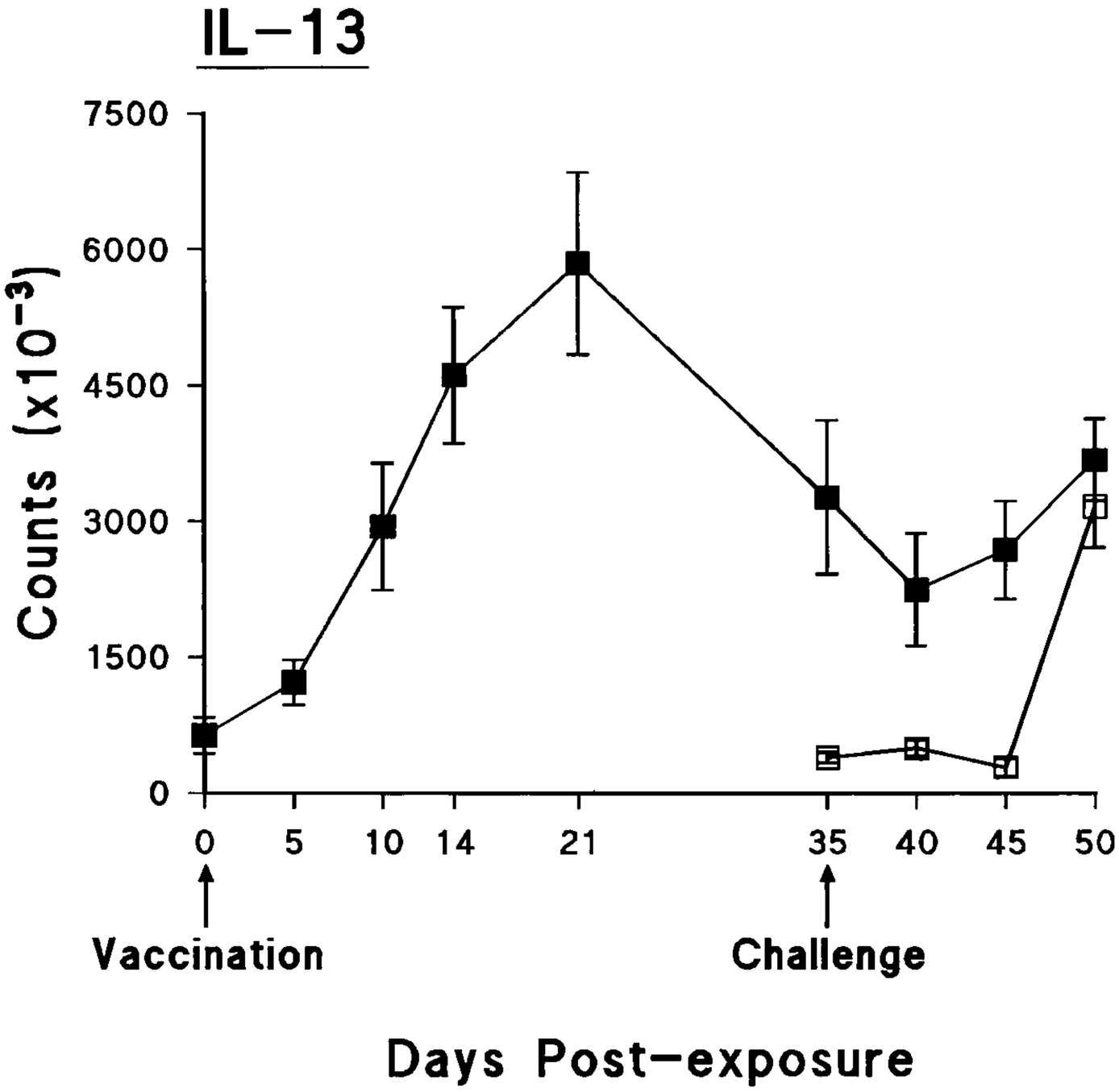


Fig. 3. The levels of IL-13 mRNA expression in whole lung tissue following percutaneous vaccination and subsequent challenge (■), or challenge alone (□) Values represented as described in Fig. 1.

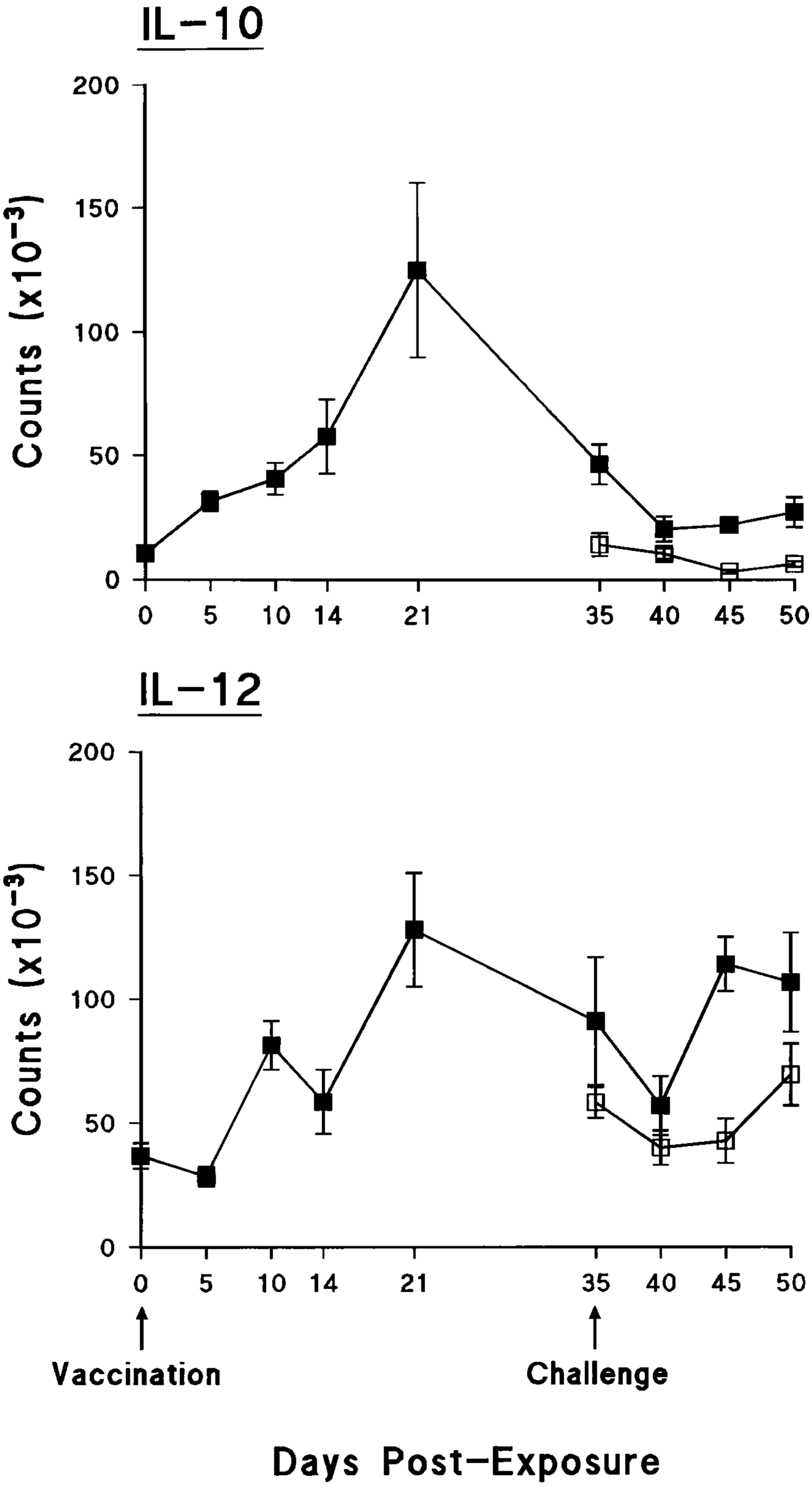


Fig. 4. The levels of regulatory cytokine mRNA expression in whole lung tissue following percutaneous vaccination and subsequent challenge (■), or challenge alone (□). Values represented as described in Fig. 1.

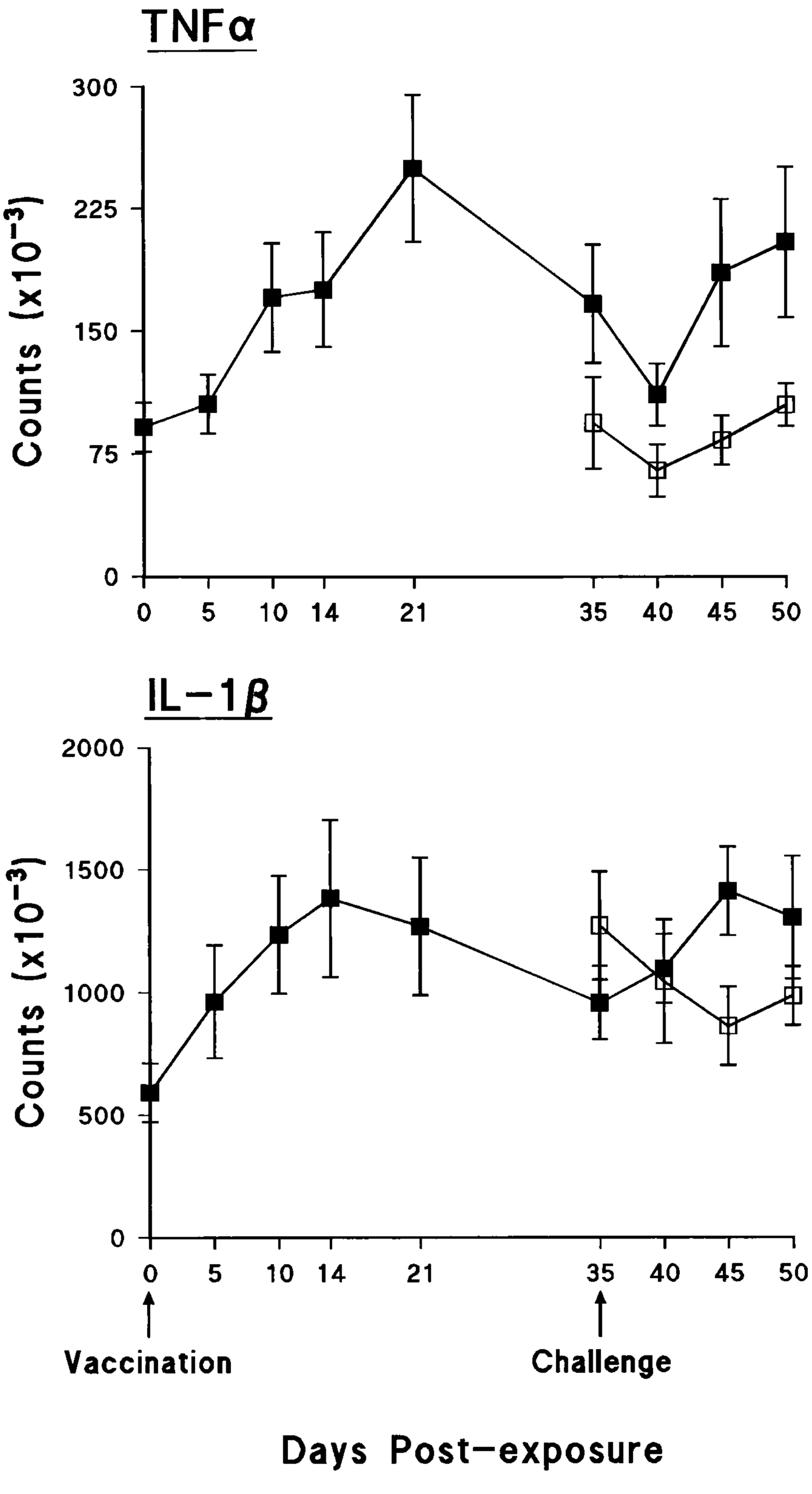


Fig. 5. The levels of inflammatory cytokine mRNA expression in whole lung tissue following percutaneous vaccination and subsequent challenge (■), or challenge alone (□). Values represented as described in Fig. 1.

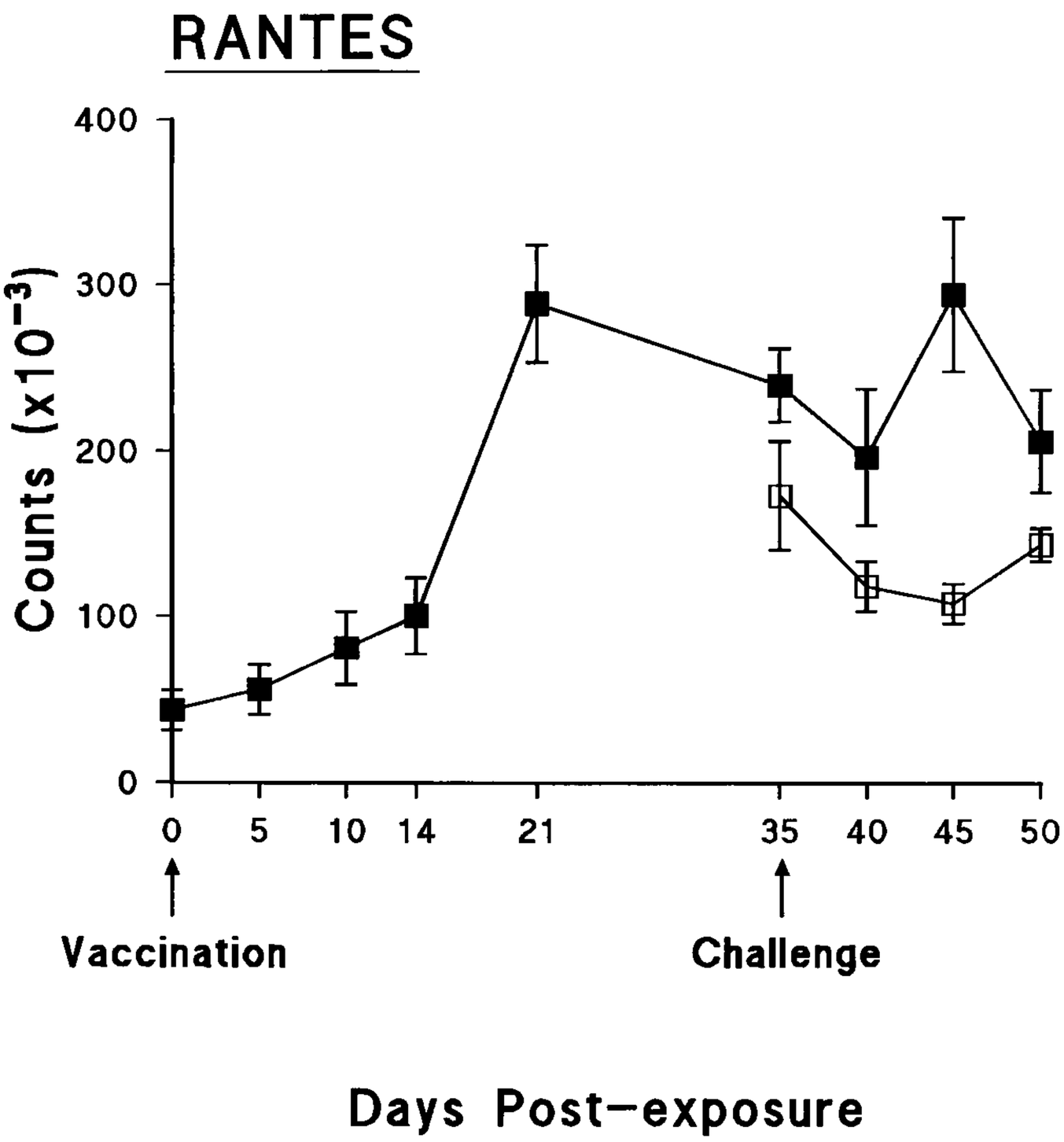


Fig. 6. The profile of RANTES mRNA expression in whole lung tissue following percutaneous vaccination and subsequent challenge (■), or challenge alone (□). Values shown are estimates of phosphorimager counts at 25 cycles, obtained from linear regression analysis of multiple cycle number PCR as described in materials and methods.

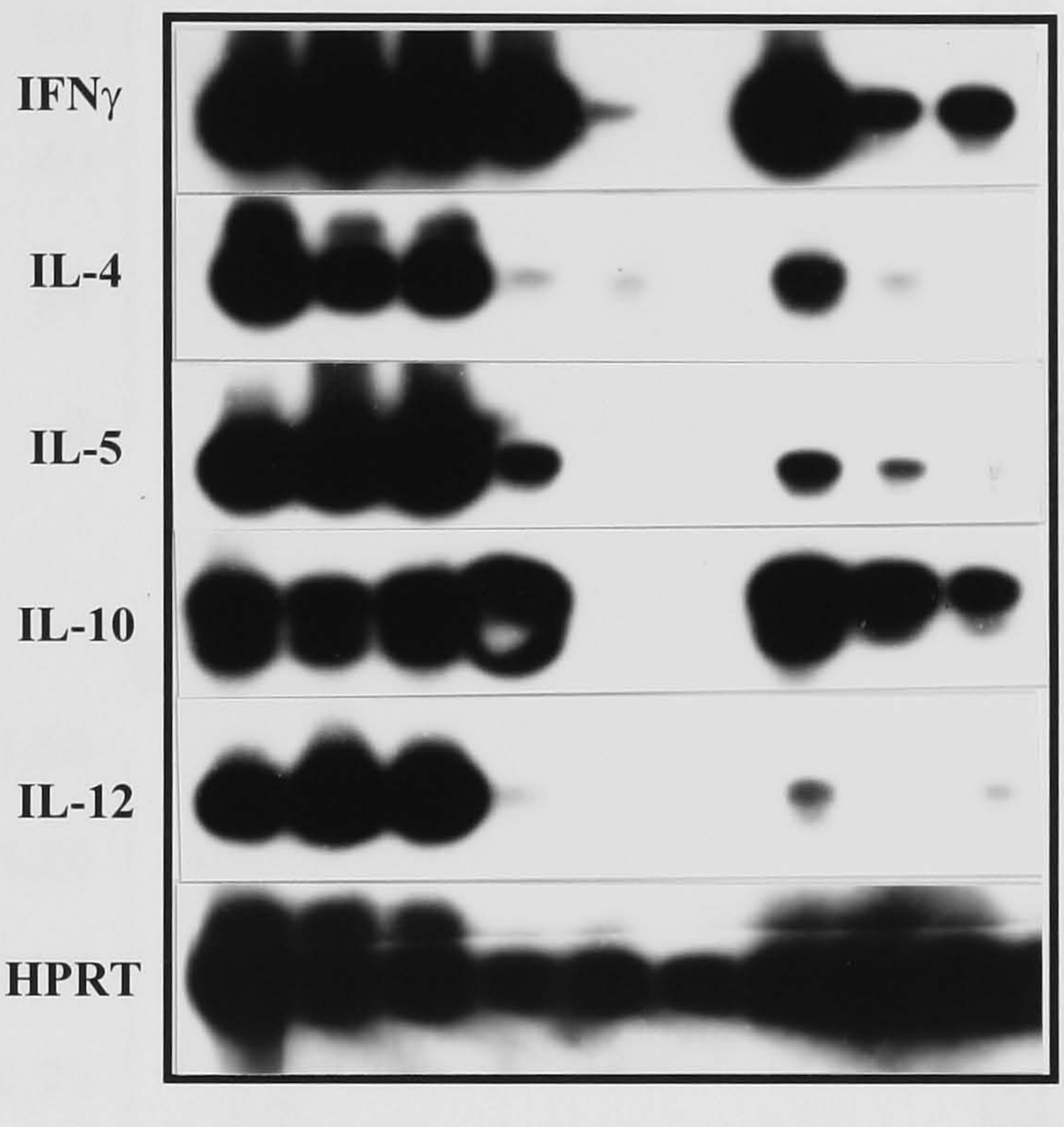


Fig. 7. Southern blot analysis RT-PCR product amplified from total RNA isolated from sorted lung cell populations, recovered by BAL, at day 21 post-vaccination. Lanes 1-9 represent: the adherent cell population (lanes 1-3); the positively selected $CD4^+$ population (lane 4); the population depleted of $CD4^+$ cells (lane 5); the positively selected $CD8^+$ population (lane 6); the population depleted of $CD8^+$ cells (lane 7); Population enriched for $CD4^+$, $CD8^+$ and B cells (lane 8); and the cell population enriched for polymorphonuclear cells (lane 9).

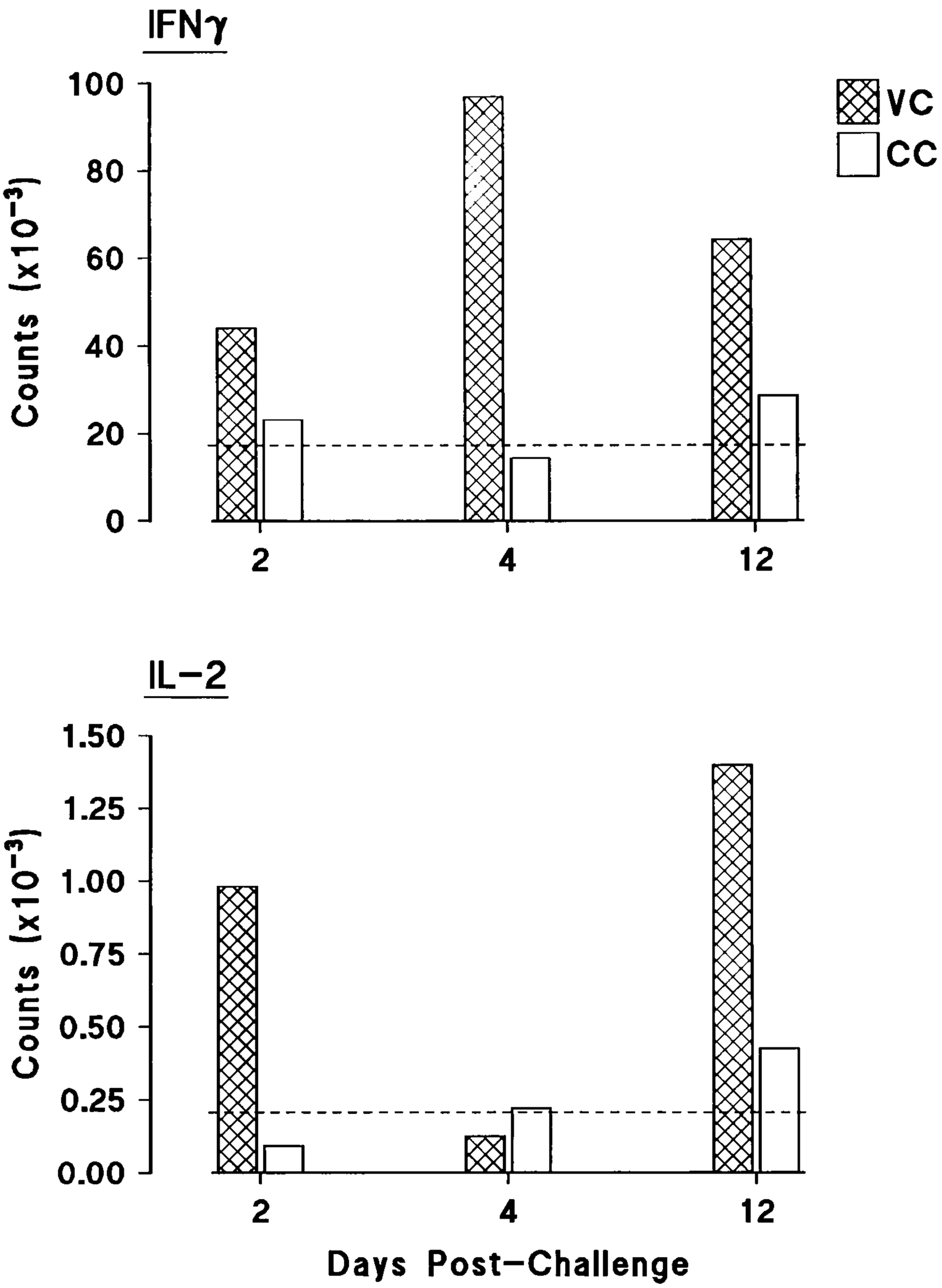


Fig. 8. The levels of Th1 cytokine mRNA expression following intravenous challenge of previously vaccinated, and naive mice, with 120 lung stage worms. Values shown are the estimated phosphorimager counts at 25 cycles derived from regression analysis of 4 data points per sample. The dashed line represents the baseline level obtained from analysis of naive animals.

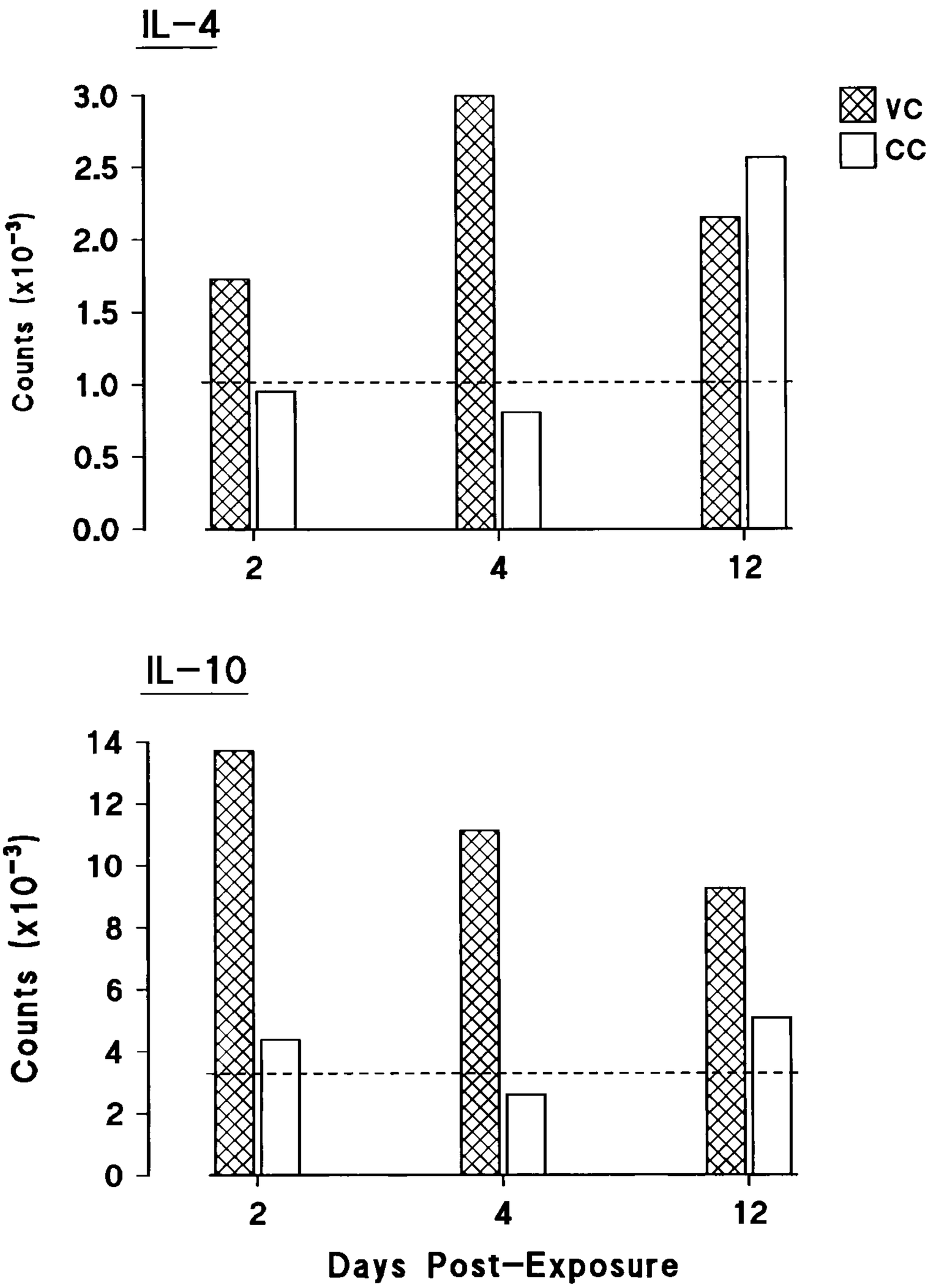


Fig. 9. The levels of Th2 cytokine mRNA expression following intravenous challenge of previously vaccinated, and naive mice, with 120 lung stage worms. Values shown are as described in Fig. 8.

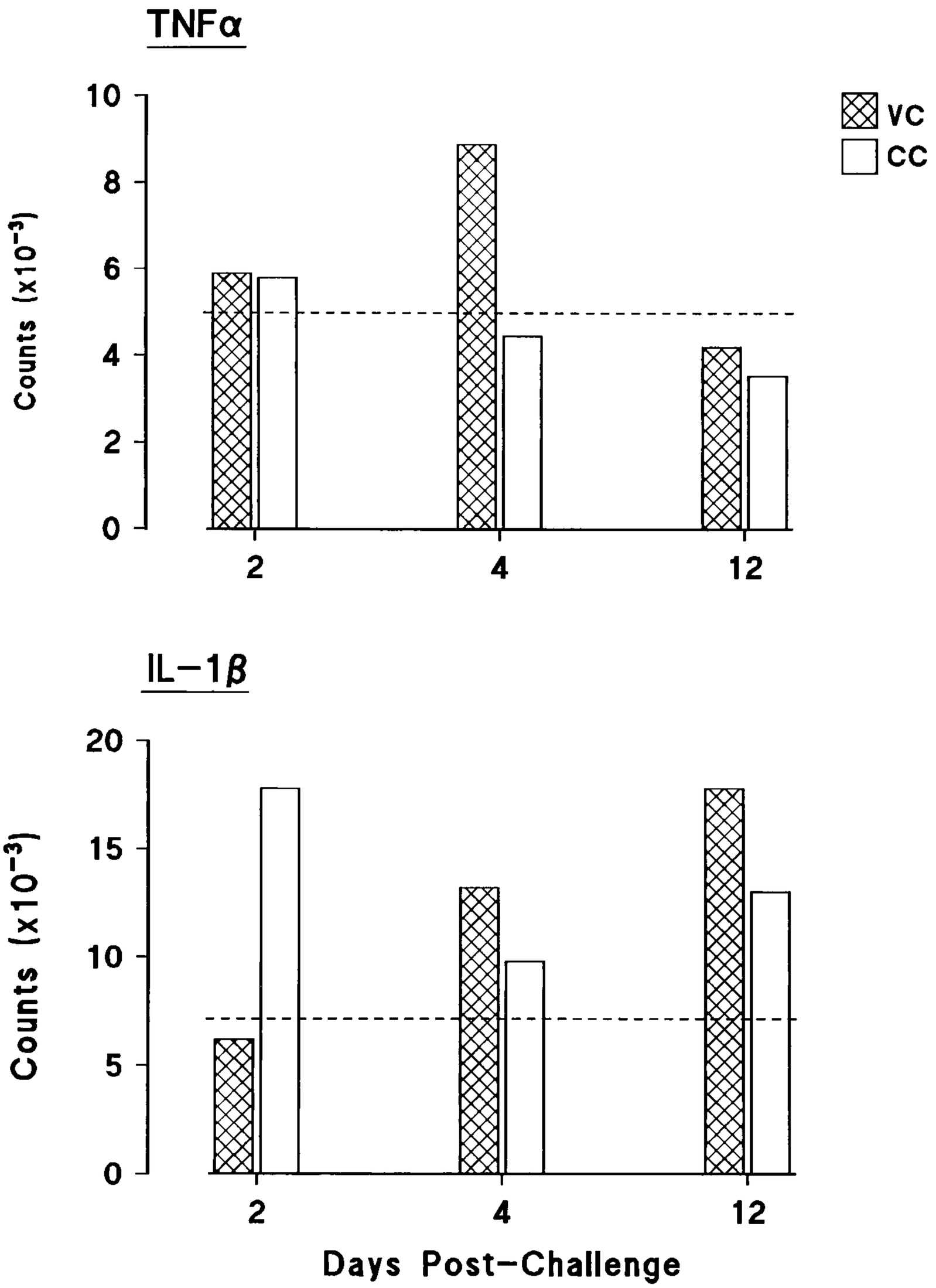


Fig. 10. The levels of inflammatory cytokine mRNA expression following intravenous challenge of previously vaccinated, and naive mice, with 120 lung stage worms. Values shown are as described in Fig. 8.

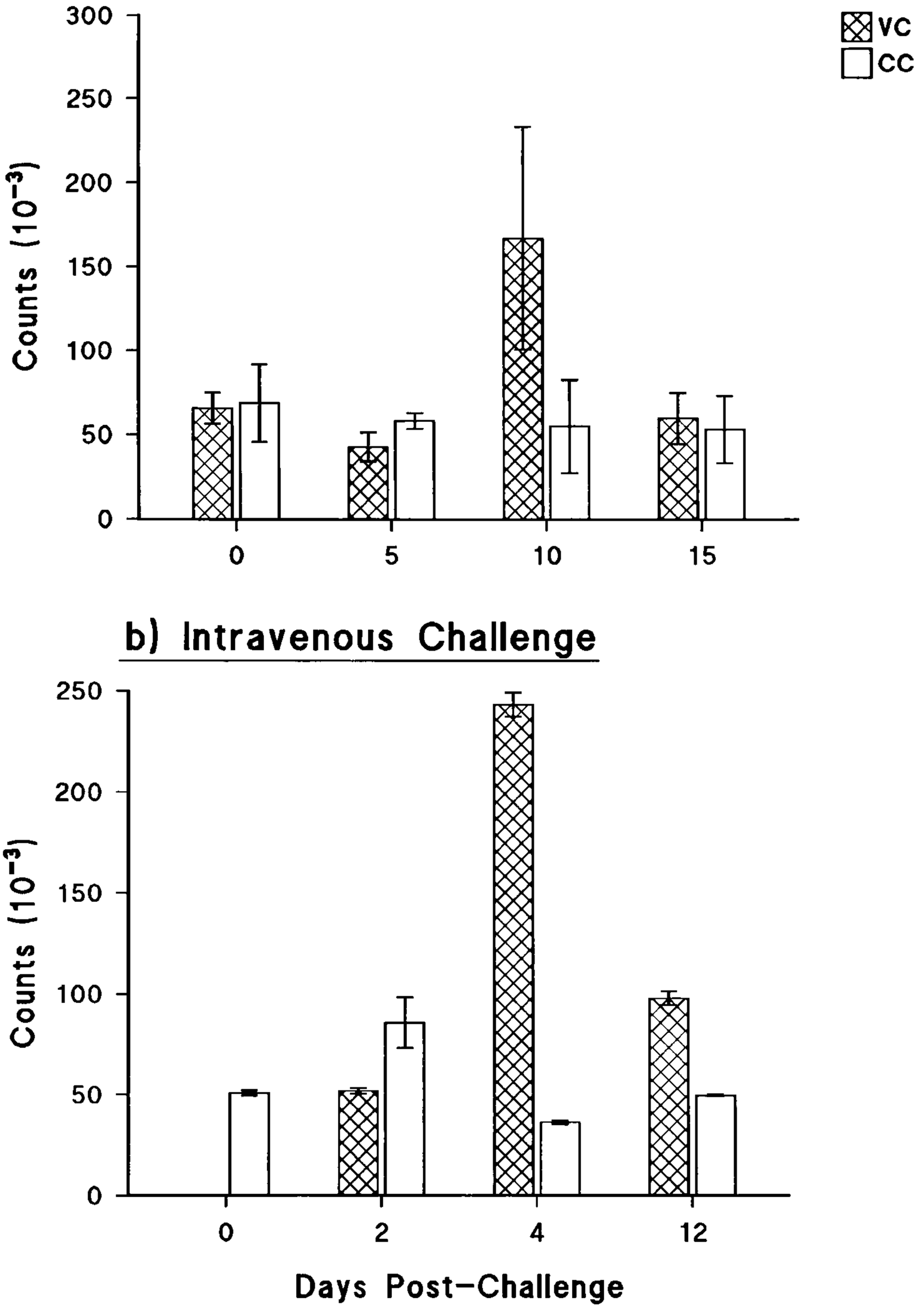


Fig. 11. The expression of iNOS in whole lung tissue following a) percutaneous challenge, and b) intravenous challenge. Values shown for a) are the mean of phosphorimager counts obtained for three mice per timepoint ± SE, there are no significant differences between samples. Values shown for b) represent estimates of phosphorimager counts at 30 cycles ± 95% confidence limits, as derived from regression analysis of 6 points per sample. Peaks at d2 CC, and days 4 and 12 VC are significantly increased over the day 0 naive sample ($p < 0.0025$).

Table. 1. The values obtained by competitive PCR analysis of naive, day 21 post-vaccination and day 10 post-challenge whole lung RNA pooled from 3 mice. The values shown represent (in pg) the amount of plasmid DNA spiked into each test PCR tube to gain a point of equivalence with the unknown sample cDNA derived from cytokine mRNA. All analysis was carried out on 1μg of total RNA and amplification for 35 cycles, with the band intensities of the separated products judged by visual inspection under UV illumination.

	IFNγ	**IL-4**	**IL-10**	**HPRT**
Day 0 Naive	0.5 pg	0.05 pg	ND	50 pg
Day 21 post-vaccination	50 pg	0.5 pg	0.5 pg	50 pg
Day 10 post-challenge	5-50 pg	0.05 pg	0.0005 pg	50 pg

ND = not detectable

Table. 2. The presence or absence of cytokine mRNA in sorted BAL cell populations as determined by RT-PCR followed by Southern blot analysis. Product was scored on a scale of 3 intensities, designated by + to +++, depending on the counts obtained by exposure to a phosphorimager screen (see below). Samples with no detectable product are marked ND.

	Adhere.	**$CD4^+$**	**$CD4^-$**	**$CD8^+$**	**$CD8^-$**	**$Poly^-$**	**$Poly^+$**
IFNγ	+++	+++	ND	ND	+++	+	++
IL-4	+++	++	++	ND	++	++	+
IL-5	+++	++	+	ND	++	+	+
IL-10	+++	+++	ND	ND	+++	++	+
IL-12	+++	++	+	ND	++	++	++

+++ > 150,000 counts

++ 50, 000 - 150,000 counts

+ 5,000 - 50,000 counts

ND < 5,000 counts

Discussion

The data presented above illustrates the cytokine profiles induced following vaccination with 500 irradiated cercariae and subsequent challenge with 200 normal parasites. The peaks observed in cytokine expression at day 21 post-vaccination correlate well with previously published data on the timing of parasite arrival in the lungs, macrophage activation, and the recruitment of leucocytes and focus formation around worms trapped in the alveolar spaces (Mastin *et al.,* 1985b, Aitken *et al.,* 1988, Menson *et al.,* 1989, Menson and Wilson, 1989).

IL-12 was the only cytokine message with an earlier, smaller peak in expression, at day 10 post-vaccination, in addition to the main peak at day 21. This cytokine has been shown to play a pivotal role in the generation and expansion of Th1 populations, both *in vitro* and *in vivo*, (Gazzinelli *et al.,* 1993, Hsieh *et al.,* 1993, Manetti *et al.,* 1993, 1994). Recent data describing the administration of IL-12 with the irradiated vaccine model demonstrated that this cytokine can increase the resistance to challenge infection (Wynn *et al.,* 1995a). Coincident with enhanced protection, a corresponding increase in IFNγ and IL-12 p40 mRNA expression and a reduction in the level of IL-4, IL-5 and IL-13 message was reported.

The exact role of IL-12 in the lungs is unclear, since it has been demonstrated that cells recovered from the lung airways post-challenge are incapable of proliferation *in vitro* (Smythies *et al.,* 1992a, Mountford *et al.,* 1992), even in response to IL-2 stimulation. This would indicate that these cells are of the memory phenotype, an observation confirmed by the analysis of the memory markers on the cell surface post-vaccination (Coulson and Wilson, 1993). It is also believed that the T helper population in the lungs is recruited after primary stimulation in the skin draining LN (Pemberton *et al.,* 1991, Pemberton and Wilson, 1995, Ratcliffe and Wilson, 1991). Thus it is difficult to envisage the role of a cytokine able to aid the development of naive cell populations in the direction of a Th1 response, in an organ already armed by an established Th1 cell-dominated population. It may be that this cytokine is released by alveolar macrophages as these cells become activated by the arrival of the first parasites in the lungs around days 7-10 (Mangold and Dean, 1984, Menson and Wilson, 1990). Alternatively, early IL-12 might stimulate the development of

any naive T cells present in the area. However, it has been shown that rIL-12 can decrease the IL-4 and IgE production upon secondary *in vitro* stimulation of human cells isolated from helminth-infected patients (King *et al.,* 1995). Thus it appears, at least in human infections, that this cytokine can have an effect on a fully differentiated response to modulate the IL-4 and related IgE production. Hence IL-12 may play a role in maintaining the Th1 response via inhibition of IL-4 and Th2 cytokine production in this murine model. *In vivo* neutralisation of IL-12 in the irradiated vaccine model might result in an increase in IL-4 message, and therefore demonstrate a regulatory role for this cytokine in the ongoing immune response.

To some degree this work supports data previously published on the kinetics and patterns of cytokine secretion in the lungs following exposure to the irradiated *S. mansoni* vaccine (Wynn *et al.,* 1994a, 1995a). However, the fold increases in cytokine expression above baseline values detected in these published reports are far in excess to those described in this chapter, and although the same cytokines are elevated, the timing is dissimilar to that reported here. In addition, the comparison between normal and vaccinating infection in the literature detects no differences in the pattern of the majority of cytokine production at the mRNA level, except for an earlier peak in IFNγ expression at day 10 post-exposure in vaccinated mice, compared to day 22 in normally infected animals (Wynn *et al.,* 1994a). This is in contrast to the strong peak of IFNγ at day 21 post-vaccination described in the above chapter, although differing exposure regimes may account for the variations in maximum cytokine expression.

In conclusion, from the data described above, and that published recently, it appears that the response generated after a vaccinating infection is mixed Th1/Th2 or Th0 in profile, at the mRNA level and not entirely polarised towards a Th1 as previously thought from cytokine protein studies (Smythies *et al.,* 1992a). However the dominance of IFNγ mRNA is confirmed by competitive template PCR, with this cytokine expressed at almost 100 fold higher levels than both IL-4 and IL-10. IFNγ message at day 21 is also expressed at approximately 100 fold that detected in naive animals. Moreover, IFNγ mRNA levels in naive mice are at least 10 fold above those of IL-4 and IL-10, making it difficult to relate the absolute amounts of cytokine message detected to the *in vivo* potency of a cytokine protein.

The discrepancies observed in the number of cytokines detectable at the mRNA level compared to previous protein analysis might reflect the greater sensitivity of PCR compared to protein assays. Alternatively, the secondary stimulation of cultured cells *in vitro* may alter the secreted cytokine profiles by masking low level expression. In addition, the analysis of protein secretion was carried out on cells recovered from the airways of vaccinated mice, whereas RT-PCR was carried out on RNA extracted from whole lung. Although BAL is one of the few ways of removing cells from the lungs without damage to cell surface molecules, many of the cell populations making up the focus may not be represented in the cells recoverable in this way. This has been confirmed by experiments using a collagenase digestion technique, which have revealed the presence of an NK cell population not previously thought to be resident in the lungs (Wilson and Coulson, unpublished observations). However, the RT-PCR analysis of RNA obtained from a total BAL population at day 21 post-vaccination has detected many of the major cytokines present in RNA isolated from whole lung tissue at this time (data not shown). Thus this method of sampling would seem to give a representative view of the cell populations trapped in the foci, even if the cell types may not be present in the same proportions as those in total lung tissue.

RT-PCR analysis of RNA derived from cell populations recovered by BAL and sorted into adherent, $CD4^+$, $CD8^+$ and granulocyte cell types indicated that most of the cytokines released during the response following vaccination are derived from the adherent/macrophage and $CD4^+$ T cell populations. High levels of IL-12, IL-10 and IFNγ mRNA are all produced by the adherent cell population. Furthermore, this sample gave positive results for all cytokines under test, but the impurity of the sample makes interpretation of this data difficult. High levels of IL-4, IL-5, IL-10 and IFNγ expression were detectable in the $CD4^+$ sample, confirming the mixed cytokine profile induced in lung tissue following vaccination. There appears to be no cytokine release from $CD8^+$ T cells in this model, an observation supported by data demonstrating that ablation of $CD8^+$ T cells with a monoclonal antibody has no effect on resistance generated (Vignali *et al.,* 1989). In contrast, CD4 ablation has previously been shown to result in loss of protection and IFNγ production (Kelly and Colley, 1988, Smythies *et al.,* 1992a). The granulocytic population appeared to have

increased expression of IFNγ and IL-12, although this may be due to contamination with monocytes, cells known to secrete these cytokines. In addition, there appears to be no IL-5 expression in the granulocyte population, contrary to human studies in which eosinophils have been shown to produce IL-5 message (Desreumaux *et al.*, 1992).

The presence of increased levels of mRNA for IFNγ, TNFα and IL-1β in the post-vaccination time course led to the investigation of RANTES mRNA, a chemokine upregulated by these inflammatory cytokines and shown to be involved in cellular recruitment in human DTH reactions. In human systems, such as rheumatoid arthritis, this chemokine plays an important role in recruitment of activated T cells and macrophages to the site of inflammation (Devergne *et al.*, 1994, Rathanaswami *et al.*, 1993). Thus RANTES can act as a mediator of DTH reactions, and is secreted by cells involved in this type of response such as macrophages, fibroblasts, endothelial cells and activated T cells (Rathanaswami *et al.*, 1993, Devergne *et al.*, 1994, Schall *et al.*, 1990). The persistence of parasites, and the release of known inducers of this chemokine such as IFNγ, IL-1β and TNFα, in the lungs parallel the expression of RANTES observed at day 21 post-vaccination. This in turn might aid the recruitment of the memory $CD4^+$ population detected in the lungs post-vaccination, a population thought to have been previously activated in the skin draining LN (Mountford *et al.*, 1992, Ratcliffe and Wilson, 1991, Coulson and Wilson, 1993). Thus this chemokine, along with other upregulated adhesion molecules such as ICAM-1 (Coulson, 1995), may account for the increase in $CD4^+$ T cells and macrophages around the trapped larvae.

There is some evidence to suggest that human RANTES can attract eosinophils *in vitro* and can express mRNA for this chemokine (Alam *et al.*, 1993, Ying *et al.*, 1996). However, recruitment may not occur *in vivo* due to the lack of this cell type in classical DTH responses. It is interesting to note that eosinophils are abundant in the lungs of vaccinated mice, peaking at day 21 (Menson *et al.*, 1989, A. Finlay unpublished observations), coincident with elevated expression of RANTES and IL-5, both of which may have a positive effect on the recruitment of this cell population. The presence of Th2 cytokines in the lungs may possibly override any inhibition that a true DTH reaction may have on RANTES attraction of eosinophils. IL-4 and IL-10

message levels are elevated following exposure to vaccinating parasites, and both these cytokines are capable of downregulating RANTES expression either directly (IL-4) or indirectly (IL-10) (Essner *et al.,* 1989, te Velde *et al.,* 1990, Devergne *et al.,* 1994). Inhibition of this chemokine may be another mechanism by which Th2 cytokine expression can control the inflammatory response.

Post-challenge, the cytokine patterns detected in this study are indicative of an anamnestic Th1 response and agree with previously published protein data implicating the importance of IFNγ in the effector mechanism (Smythies *et al.,* 1992a, 1992b, 1993). The expression of the inflammatory cytokines detected in this study peaks earlier, and at higher levels than the message associated with Th2 cells. This is consistent with the proposed effector mechanism of inflammatory focus formation around migrating parasites, blocking exit from the lungs, and hence worm maturation in the liver (Smythies *et al.,* 1993). Furthermore, animals exposed to a vaccinating infection prior to challenge produced higher levels of all cytokines than challenge control mice. Animals receiving a normal infection displayed cytokine mRNA levels barely detectable above the naive baseline, with the exception of the Th2 cytokines IL-5 and IL-13, which are elevated at day 15 post-challenge. Interestingly IL-13, an IL-4-like cytokine and good indicator of the presence of Th2 cells, also increased in expression post-challenge in primed mice, demonstrating a strong anamnestic response. This cytokine has been shown to activate NK cells to produce IFNγ early in infection (Doherty *et al.,* 1993). However, the precise role of IL-13 in this model is unclear, since its downregulatory effects on macrophage activation might inhibit the effector mechanism to some extent. It is possible that ablation of IL-4, IL-10 and IL-13 together might increase the effectiveness of the Th1 response, and thus resistance, by reducing inhibition of macrophage activation.

The abundant expression of IFNγ, IL-1β, IL-12 and TNFα post-challenge are consistent with reports detailing the activation state of macrophages during the effector response (Menson and Wilson, 1990). These cytokines are expressed at levels approaching those detected post-vaccination, and peak with the arrival of challenge parasites in the lungs (Dean *et al.,* 1984, Wilson *et al.,* 1986). This data is in agreement with previously published work demonstrating that Th1 cytokines dominate at the mRNA level in vaccinated and challenged animals (Wynn *et al.,*

1994a). Moreover, high expression of IL-12, TNFα and IL-1β following challenge has been related back to macrophage activation, and thus strong inflammatory responses (DTH) (Wynn *et al.,* 1994a).

All the cytokines required for RANTES expression in human models of disease (see above post-vaccination data), are also detected in murine lung tissue post-challenge. As found following vaccination, upregulation of this chemokine is coincident with influxes of $CD4^+$ T cells and eosinophils into the lungs (Smythies *et al.,* 1993, Aitken *et al.,* 1988). A definitive role for RANTES in cell recruitment in the effector mechanism could be investigated further by *in vivo* blocking with anti-RANTES antibodies.

Macrophage activation is a key effect of DTH reactions, and the production of toxic nitrogen intermediates as a result of stimulation can lead to killing of parasites *in vitro* and *in vivo* (Pearce and James, 1986, Liew *et al.,* 1990, 1991, Liew and Cox, 1991, Oswald *et al.,* 1994a). Expression of IFNγ, TNFα, IL-1β and IL-12 have all been associated with the upregulation of macrophage activation and subsequent NO production (Oswald *et al.,* 1994c). These cytokines are readily detected during both vaccination and challenge exposures and thus might be expected to correlate with increased expression of iNOS, the enzyme responsible for NO production. However, the analysis of iNOS mRNA following percutaneous challenge demonstrated an insignificant peak at day 10 in vaccinated animals. Further investigation of tissue samples from mice challenged intravenously indicated that there is a significant increase of iNOS message at days 4 and 12 post-exposure in primed mice but not in control animals. Recently published data have suggested the importance of NO in the effector mechanism of the irradiated *S. mansoni* vaccine (Wynn *et al.,* 1994a, Oswald *et al.,* 1994a). In these studies the elevated levels of iNOS detected post-challenge in vaccinated mice were correlated with IFNγ production, ablation of which results in abrogation of iNOS expression. In addition, ablation of NO during challenge infection lead to a 33% decrease in protection. Taken together, the above data imply that parasites trapped by inflammatory foci during migration across the lungs are restricted to a toxic microenvironment, resulting in at least some effective killing of lung stage worms (Wynn *et al.,* 1994a, Oswald *et al.,* 1994a).

However, in contradiction to the above report, a more recent experiment finds no reduction in resistance after the administration of a competitive inhibitor for iNOS (Smythies *et al.,* 1996b, manuscript in preparation). Furthermore, *in vitro* assays have been unable to demonstrate the killing capacity of NO against lung stage worms (Pearce and James, 1986, James and Glavern, 1989, Oswald *et al.,* 1994a). In addition, extraction of trapped worms from the lungs at day 17 post-challenge, the time of peak NO production reported above, and injection of these parasites into the hepatic portal system of naive recipient mice results in full maturation and development of a patent infection (Coulson and Wilson, 1988). This would imply that, at least up until day 17, the worms are unharmed by focus formation, and that the effector mechanism has a blocking action not directly harmful to the migrating parasites. In support of this, the presence of even small numbers of red blood cells in *in vitro* larvicidal assays results in complete abrogation of parasite killing, indicating that while lung parasites are intravascular it is unlikely that NO has a toxic effect (Smythies *et al.,* 1996b). It has been shown, moreover, that no further migration of schistosomula from the lungs takes place after day 17, and thus any worms trapped there will not mature. It is therefore difficult to elucidate a major role for NO in the effector mechanism which will have a direct effect on the number of worms maturing in the hepatic portal system. Apparently focus formation alone is responsible for the termination of migration in the lungs. Whether trapped parasites die by starvation or toxic compound attack is of no real consequence to the levels of resistance generated. The vaccination and challenge of iNOS-deficient mice will provide the definitive answer as to whether NO plays any role in resistance.

The investigation of the effector mechanism following a synchronous pulse of lung stage parasites permitted kinetic analysis of the cytokine expression profiles without distortion resulting from the staggered arrival of migrating parasites into the lungs. The resistance measurement of 43% obtained from this time course is somewhat lower than those routinely observed following percutaneous challenge. This might be explained by the developmental state of the injected worms. A proportion of the parasites derived from the lungs of infected mice may have already made the morphological changes required to aid passage through the narrow capillaries. Thus on arrival in the lungs after injection, these worms can rapidly

navigate the capillaries and resume migration to the hepatic portal system, accounting for the higher percentage of parasite maturation.

The patterns of Th1 and Th2 cytokines induced by intravenous challenge are similar to those observed after percutaneous challenge. However, major differences were seen in the timing of cytokine expression; IL-1β, IFNγ and TNFα all peak at day 4 post-challenge, compared to day 10 following percutaneous exposure. This can be explained by the rapid arrival of parasites into the lungs following injection into the femoral vein. Focus formation in previously vaccinated animals occurs between days 2 and 8 following parasite injection (Smythies *et al.,* 1996), whereas control animals show only a low level of infiltration at the later time point. The expression of IFNγ and TNFα may account for the upregulation of adhesion molecules such as ICAM-1 and result in recruitment during effector focus formation (Dustin *et al.,* 1986, Coulson, 1995). The recruitment of eosinophils may also be explained by the increased expression of IL-4 at day 4 post-challenge. This cytokine can upregulate VCAM-1 on endothelial cells, and VLA-4, its ligand on eosinophils, resulting in the infiltration of this cell type during focus formation (Dobrina *et al.,* 1991).

Peak expression iNOS at day 4 post-challenge coincides with IFNγ, IL-1β and TNFα mRNA elevation, cytokines which can all act as inducers of this toxic mediator. Other *in vivo* studies of intravenous challenge have described peaks of IFNγ and iNOS on days 1 and 2 post-injection respectively (Wynn *et al.,* 1994a). This very early expression was not observed in the data presented above, although a day 1 sample was not taken and there was a slight increase in IFNγ message at day 2. These discrepancies could be caused by the differences between challenge procedures. The data in this chapter is derived from the injection of 120 lung stage worms derived from donor mice, whereas the results reported above are based on the intravenous administration of 500, 24 hour cultured schistosomula derived from mechanically transformed cercariae. The arrival of so many parasites into the lungs could result in elevated responses compared to the natural migration pattern. In addition these young parasites may more reflect skin stage larvae in morphology, and thus be more susceptible to NO attack as demonstrated by *in vitro* assays (James and Glavern, 1989, Pearce and James, 1986).

To summarise, it would seem that the induction and effector mechanisms in this vaccination model are not optimised for the production of a complete Th1 response. If the residual Th2 cytokine expression could be eliminated then the effector mechanism may give higher levels of resistance. It has already been shown that IL-12 administered post-vaccination can boost the Th1 response, and partially inhibit the Th2-type cytokines, resulting in increased levels of protection (Wynn *et al.*, 1995a). However, this still only induces a resistance of 80%. By the detailed analysis of the immune responses induced by vaccination, the mechanisms required for greater protection can be determined. The importance of certain cytokines in resistance can further be investigated by the vaccination of gene-deleted mice (see Chapter Six), although more information on the interplay of immune factors is required to optimise the effects of this vaccination regime.

CHAPTER FIVE

A Comparison of the Cytokine Profiles Induced Following Vaccination of C57BL/6 and BALB/c Mice with Irradiated Cercariae of *S. mansoni*

Introduction

The impact of genetic background on the immune responses observed in murine models of parasitic infection has long been known. Many infections display dichotomy in disease progression according to host strain; *Toxoplasma gondii*, *Trichuris muris* and *Schistosoma mansoni* all show a spectrum of disease pathology dependent upon the genetic background of the murine host infected. This variation is often associated with differing cytokine expression profiles induced upon infection (Scott *et al.,* 1989, Scott and Kaufman, 1991). However, the most widely researched example of strain-specific resistance and susceptibility is *Leishmania major* infection in mice. This parasite provides a parallel model for human leishmaniasis, and the ability of some strains to resist development of the visceral form of the disease has prompted research into the immune environment necessary to control the cutaneous infection and promote self-cure. Infection of susceptible strains, such as BALB/c, with *L. major* results in disease progression and eventual death and appears associated with the production of IL-4. In contrast, C57BL/6 and C3H/HeN mice can control initial cutaneous lesions and self-heal (Heinzel *et al.,* 1989, Scott *et al.,* 1989, Scott, 1989). The ability of mice to self-cure has been found to show strong correlation with the expression of IFNγ, and inducers of the Th1 response such as IL-12 (Heinzel *et al.,* 1989, 1993a, Scharton and Scott, 1993).

The investigation into the immunological nature of resistance and susceptibility of mice exposed to *L. major* has uncovered a wealth of information on the cytokine secretion of T helper subsets, especially on the course of Th1/Th2 development. The production of Th1 cytokines by resistant strains, and Th2 cytokines by those susceptible to infection (Heinzel *et al.,* 1989), indicates that infection with the same parasite can elicit different responses from hosts of the same species but differing genetic backgrounds. This phenomenon provides an ideal model in which to study the induction of cytokine gene expression and the immune environment necessary for control or exacerbation of the disease. In addition, information leading to the ability to influence the *in vivo* development of a cell population obligatory for cure will provide a useful tool in the design of vaccine strategies.

The exact mechanisms responsible for the development of one T helper subset preferentially to the other are not clear as yet, but the involvement of different cytokines has been investigated. Indeed, it is known that IL-12, IFNγ and IL-4 play definitive roles in the development of Th1 and Th2 subsets, and hence influence the outcome of disease in *Leishmania* infection (reviewed by Scott, 1991, Scott 1993b, Reiner and Locksley, 1995). The ablation or administration of any one of these cytokines can result in an altered course of infection. Administration of IL-12 or IFNγ to susceptible mice can reduce the pathology associated with disease (Scott 1991, 1993b, Heinzel *et al.,* 1993a, 1994), and in the case of IL-12 can induce resistance to further infection (Heinzel *et al.,* 1993a, Sypek *et al.,* 1993). Ablation of IL-4 in susceptible strains has a similar effect to the administration of IL-12 (Chatelain *et al.,* 1992). In contrast, the administration of IL-4 to mice naturally resistant to *L. major* infection can lead to disease progression into visceral leishmaniasis. Similarly, ablation of IL-12 or IFNγ in these mice leads to exacerbation of pathology and an increase in the levels of Th2 cytokines (Belosevic *et al.,* 1989, Chatelain *et al.,* 1992, Sadick *et al.,* 1990, Scott, 1993b). Initial analysis of the differences observed between infected strains detected clear cytokine expression dissimilarities (Heinzel *et al.,* 1989). However, it is now apparent that most strains of mice can express similar, mixed cytokine profiles at the mRNA level during the first week of infection (Reiner *et al.,* 1994). After a period of 7 days the relevant Th1/Th2 responses then develop according to the strain, as one or other of the cytokine profiles is downregulated.

The importance of Th1 cytokines in the resistance mechanism has been related to the induction of cell-mediated responses and macrophage activation (Nathan *et al.,* 1983). Nitric oxide is a toxic intermediate used for the control of some infections, and can be released by activated macrophages in response to cytokines such as IFNγ (Oswald *et al.,* 1994a, 1994c, Liew *et al.,* 1991). The production of NO by the action of nitric oxide synthase on arginine is blocked by structural analogues such as N^G-monomethyl-l-arginine (L-NMMA), resulting in an increase in susceptibility in mice naturally resistant to leishmaniasis (Liew *et al.,* 1990, Green *et al.,* 1990). Furthermore, the generation of iNOS-deficient mice on a resistant background has demonstrated the crucial role NO plays in parasite killing in *L. major* infection (Wei

et al., 1995). These mice are completely unable to control cutaneous lesions and infection results in the development of fatal visceral pathology. Macrophage activation, and thus NO production, is down regulated by IL-4, IL-10 and IL-13 (Fiorentino *et al.,* 1991a, 1991b, Doherty *et al.,* 1993, Oswald *et al.,* 1994a, Liew *et al.,* 1991). This may account for the inability of mice with Th2 characteristics to control infection and explain the strain differences observed in leishmanial infection. It has been reported that NO has an important role in the effector mechanism against *S. mansoni* (Wynn *et al.,* 1994a, see Chapter Four). However, results are inconclusive and the effects of the inhibition of iNOS are less clear than following exposure to *Leishmania major*.

Investigations into the effects of mouse strain on the outcome of infection with *Leishmania* have highlighted the predisposition of different stains to mount specific Th1 or Th2 responses. This effect has also been shown in other models of parasitic disease as mentioned earlier. In fact, *Schistosoma mansoni* infection in different strains of mice can result in varying levels of resistance (James *et al.,* 1981, James and Sher, 1983). However, the differences in cytokine production induced in different strains have not been described. C57BL/6 mice are typically high responders, and a single vaccination with 500 irradiated cercariae routinely generates up to 75% resistance (Dean, 1983, Smythies *et al.,* 1992a, 1993). The effector mechanism which operates in the lungs is $CD4^+$ T cell-mediated and has an essential requirement for IFNγ (reviewed by Smythies *et al.,* 1993). The cytokine expression induced in less resistant mice after vaccination with irradiated cercariae may be very different from the patterns observed following exposure of highly resistant C57BL/6 mice. Thus the study of vaccination in high and low responders may highlight the cytokine profiles necessary for resistance. This chapter aims to describe the differences observed between C57BL/6 and BALB/c mice following exposure to irradiated cercariae of *S. mansoni*. Semi-quantitative RT-PCR analysis was carried out at restricted time points, estimated from previous experiments to be coincident with peak cytokine protein responses in the skin draining LN and lungs. In addition, the relative basal levels of cytokine expression between the two strains were investigated by competitive PCR.

Materials and Methods

Experimental Animals and Parasites

Parasites for the vaccination procedure were passaged as previously described (Chapter Three) through MF1 outbred mice and *Biomphalaria glabrata* snail hosts. Strains of mice known to differ in resistance to infection with other parasites, such as *L. major*, were chosen for the study (in collaboration with S. Anderson). SPF C57BL/6 and BALB/c female mice were obtained commercially (B&K Universal, Hull, UK) and maintained in isolator conditions throughout the vaccination time course.

Exposure Regimes

Mice were infected with 500 optimally irradiated cercariae percutaneously via the shaved abdomen. Unexposed control animals, and groups of 3 vaccinated mice were sacrificed at days 6, 15 and 20 post-vaccination and the lungs, and 2 of the 4 LN draining the exposure site, removed into homogenisation buffer containing RNAse inhibitor (4M guanidinium thiocyanate). Alternatively, tissue was frozen in liquid nitrogen and stored at -80^{o}C until RNA isolation. Axillary and inguinal LN were pooled from all three mice per time point for the RNA isolation procedure, whereas lung samples were treated individually. Remaining LN were removed for tissue culture and protein analysis (S. Anderson, unpublished observations).

At day 35 post-vaccination, five mice from the vaccinated group and age-matched naive controls were infected with 200 normal parasites by tail immersion in a cercarial suspension. Mice were restrained in a humane apparatus for the 30 minute duration required for maximal parasite penetration of the skin. At day 35 after challenge, the groups of mice were sacrificed and the adult worm burdens assessed for resistance measurements. Resistance was calculated from the worm burdens using the equation previously described (Chapter Three).

Total RNA Isolation

Total RNA was isolated from the pooled LN and individual lungs as previously described (Chapter Two). The yield of recovered RNA was established by

spectrophotometry at 260 nm and confirmed, along with the intact nature of the preparation, by 1% agarose gel electrophoresis in the presence of ethidium bromide.

Semi-quantitative RT-PCR Analysis of Cytokine mRNA

Total RNA isolated from each strain was analysed for the presence of message for HPRT, IFNγ, IL-4, IL-10 and IL-12 as previously described (Chapter Two). LN total RNA was diluted to a concentration of 0.25 μg/10μl, and 10μl added to each RT-PCR test. Equal quantities of lung RNA for each sample were combined in a pool to a final concentration of 1μg/10μl for each time point. Following reverse transcription, duplicates for each sample were amplified for 3 different cycle numbers at intervals of 3 cycles. The PCR primer sequences, reaction conditions and appropriate cycle numbers are described elsewhere (Chapters Three and Four).

Detection of PCR product was carried out by hybridisation of slot-blotted samples on to nylon membrane, followed by hybridisation with ^{32}P-end labelled, specific oligonucleotide probes (sequences as previously described, Chapters Three and Four). After stringent washing, membranes were exposed to a storage phosphor screen and the counts for each sample repeat obtained by analysis on a phosphorimager. The counts collected were analysed by linear regression and an accurate value for PCR product at 25 cycles derived. Estimated values were adjusted for differences in total RNA added to the RT mix by correction with a factor derived from the HPRT content of the samples.

Competitive PCR Analysis

The basal level of cytokine expression in naive lung and LN tissue from C57BL/6 and BALB/c mice was analysed by the addition of a competitive template into the PCR of test samples (see Chapter Two). Known dilutions of competitor were co-amplified with an unknown constant amount, 1μg for lung or 0.25μg for LN, of test RNA using IFNγ, IL-4, IL-10 and HPRT primers. Amplification was carried out for 35 cycles and the resulting mix of products separated by 2% agarose gel electrophoresis in the presence of ethidium bromide. The point at which the sample and competitive templates produced products of equal band intensity was judged as

the dilution of construct plasmid DNA with an equal concentration to the cDNA derived from the test RNA sample.

Results

Events in the Skin-Draining LN Following Vaccination

Cytokine profiles detected in the LN following exposure of C57BL/6 and BALB/c mice to vaccinating parasites are in general very similar for Th1-related cytokine mRNA. However, differences are seen with respect to Th2 cytokine expression. IFNγ mRNA is detected at equivalent values for both BALB/c and C57BL/6 mice at all time points except for day 0, the naive level (Fig. 1). This was much higher in C57BL/6 mice, and thus levels after day 0 are below the baseline expression detected in naive mice for this strain. IL-12 follows a similar expression profile in each group, with values dropping below baseline and not increasing over the sampled time course. This fall in LN cytokine expression following vaccination resembles that reported previously (Chapter Three).

The Th2 cytokines, IL-4 and IL-10, show greater variation between strains than the IFNγ and IL-12 expression decribed above (Fig. 2). Although the basal level of IL-4 mRNA is higher in BALB/c, compared to C57BL/6 LN tissue, both strains show a peak of IL-4 expression at day 6 post-vaccination. However, the peak in C57BL/6 mice is half that observed in BALB/c animals. In addition the levels seen in BALB/c mice remain elevated throughout the time course and do not return to baseline as rapidly as those in C57BL/6 mice. Perhaps surprisingly, given the disposition of BALB/c mice to produce Th2 cytokines after *Leishmania* infections, IL-10 mRNA levels are higher in C57BL/6 LN tissue over the whole time course. Expression of IL-10 mRNA in BALB/c LNs is much lower in comparison, with values rarely rising above the naive baseline detected at day 0.

Events in the Lung Following Vaccination

With the exception of IL-12, both C57BL/6 and BALB/c mice display elevated expression of all cytokines assayed at days 15 and 20 post-vaccination (Figs. 3 and 4). The Th1-related cytokines IFNγ and IL-12 are expressed at higher levels in C57BL/6 mice compared to BALB/c animals, especially IL-12 which does not rise above baseline in the latter group. The levels of IL-4 mRNA are similar between

strains at all time points, with increases of almost four fold above respective naive values at days 15 and 20 post-vaccination. Expression of IL-10 is higher in C57BL/6 compared to BALB/c mice, in agreement with the LN data described above. At peak expression, IL-10 mRNA levels in the lungs of C57BL/6 mice were 3 fold greater than those of BALB/c samples. This reflects more closely data obtained for IFNγ expression than that for IL-4, another Th2 cytokine.

Competitive PCR

Basal IFNγ mRNA expression in the lungs of C57BL/6 mice shows a marked difference to the levels detected in BALB/c mice, at 0.5 pg compared to 0.005 pg (Table 1). This hundred fold difference is not reflected in the semi-quantitative PCR data shown above, which describes only an 2-3 fold difference between the two strains. The IFNγ level in BALB/c LN was undetectable in this assay. IL-4 message is approximately 10 fold higher in all tissues samples for BALB/c mice (0.5 pg) compared to C57BL/6 animals (0.05 pg). The values obtained for HPRT indicate that equal amounts of starting material were added into each RT-PCR test.

Resistance Data

Resistance measurements obtained for the C57BL/6 group were of the level routinely obtained for this strain (see Chapters Three and Four). Worm burdens of: 130.5 ± 7.3 for the CC group, and 49.4 ± 5.08 for the VC group, gave a resistance value of 62%. BALB/c mice displayed similar worm burdens to infected C57BL/6 animals in the CC group (133.3 ± 3.19). However, an increased level of maturation in the VC animals (worm burden 72.2 ± 7.7) resulted in a lower resistance, at 45.8%. These values agree with previously published and unpublished observations (James *et al.*, 1981, James and Sher, 1983, S. Anderson unpublished observations).

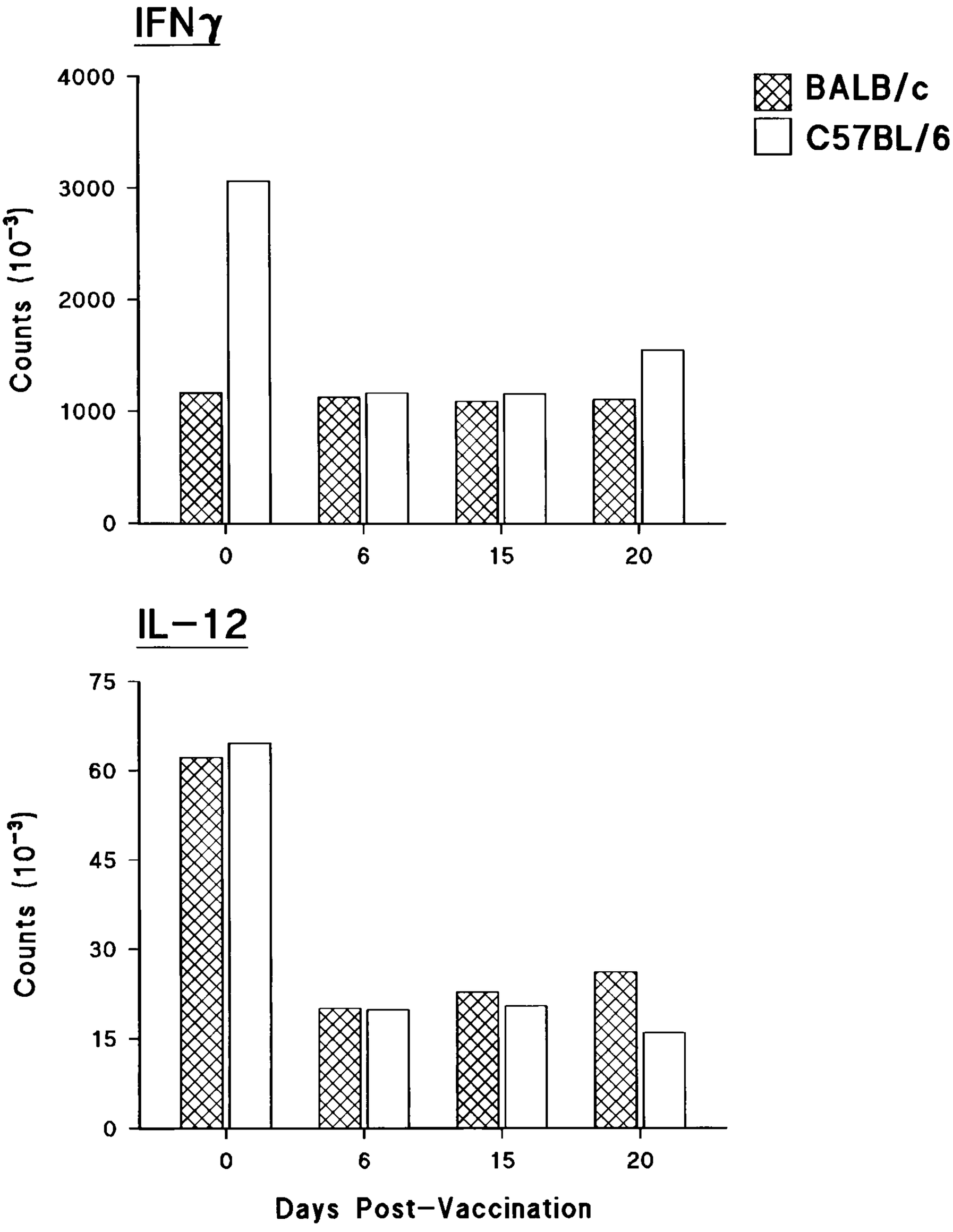


Fig. 1. Expression of IFNγ and IL-12 in the axillary and inguinal LN of C57BL/6 and BALB/c mice following exposure to 500 radiation-attenuated cercariae of *S. mansoni*. Values shown are estimates at 25 cycles derived from regression analysis performed on six values from each RNA sample. Correction for variation in HPRT mRNA levels has been applied.

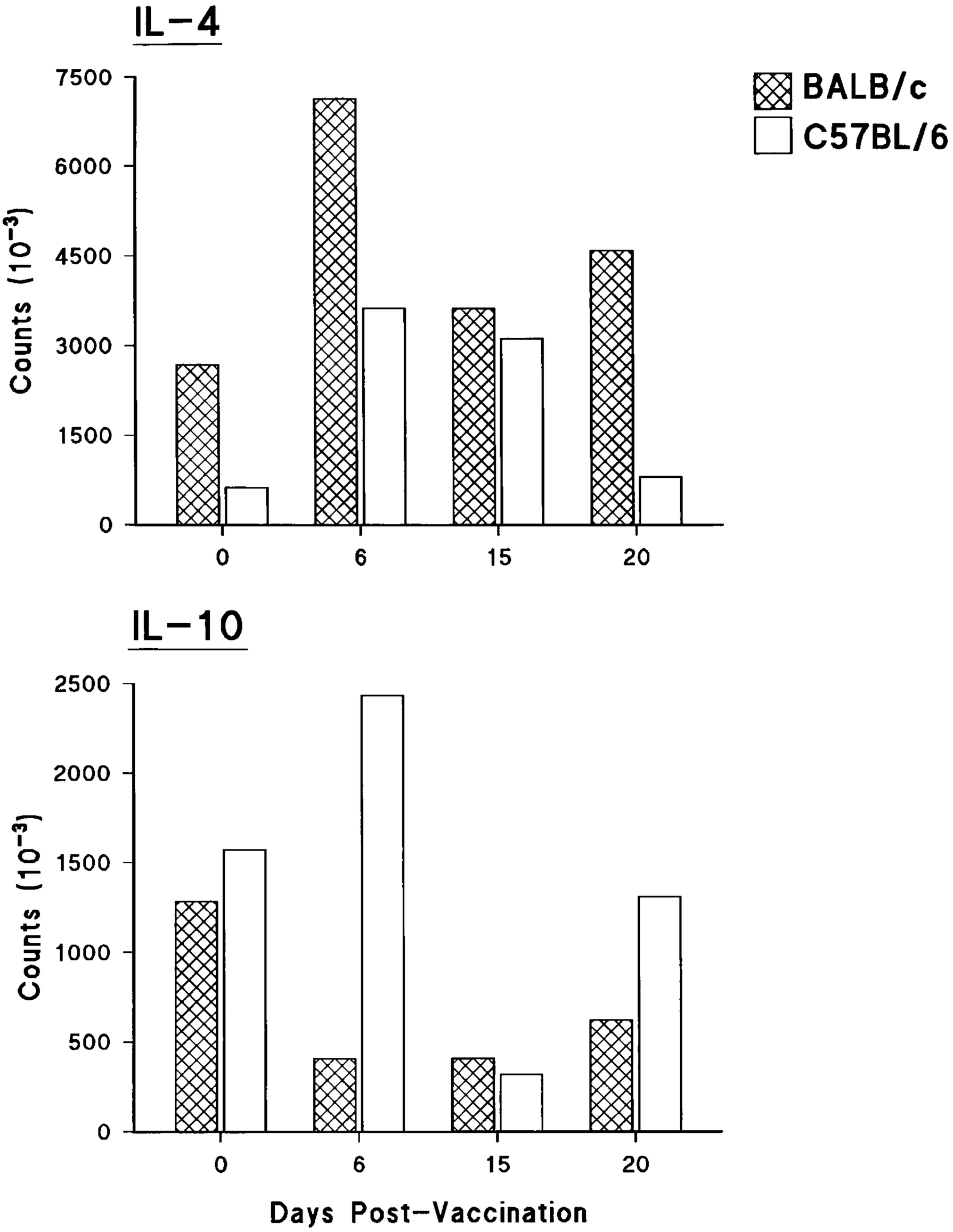


Fig. 2. Expression of IL-4 and IL-10 mRNA in the axillary and inguinal LN of C57BL/6 and BALB/c mice following exposure to 500 radiation attenuated cercariae of *S. mansoni*. Values depicted as described for Fig.1.

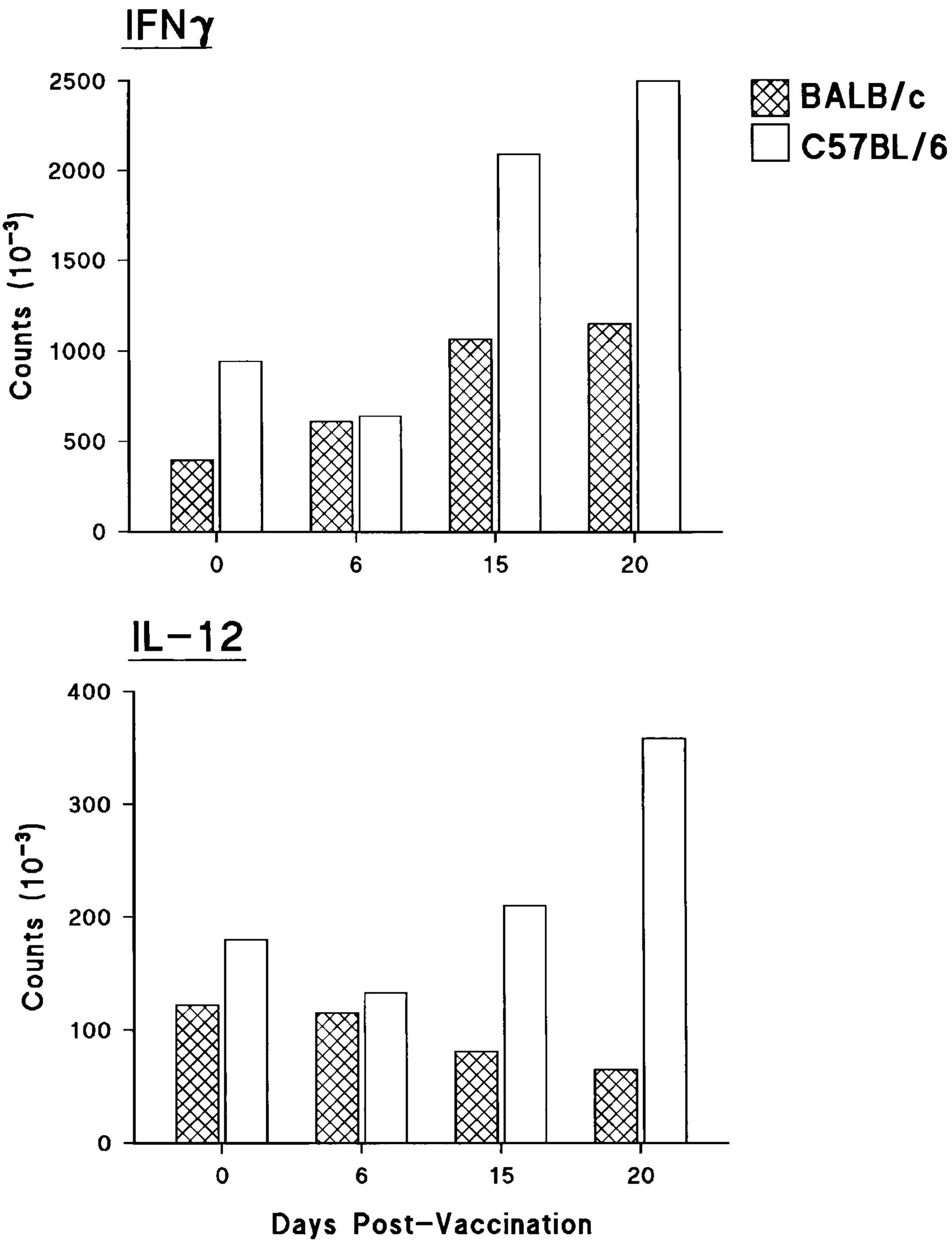


Fig. 3. Expression of IFNγ and IL-12 mRNA in whole lung tissue of C57BL/6 and BALB/c mice following exposure to 500 radiation attenuated cercariae of *S. mansoni*. Values depicted as described for Fig.1.

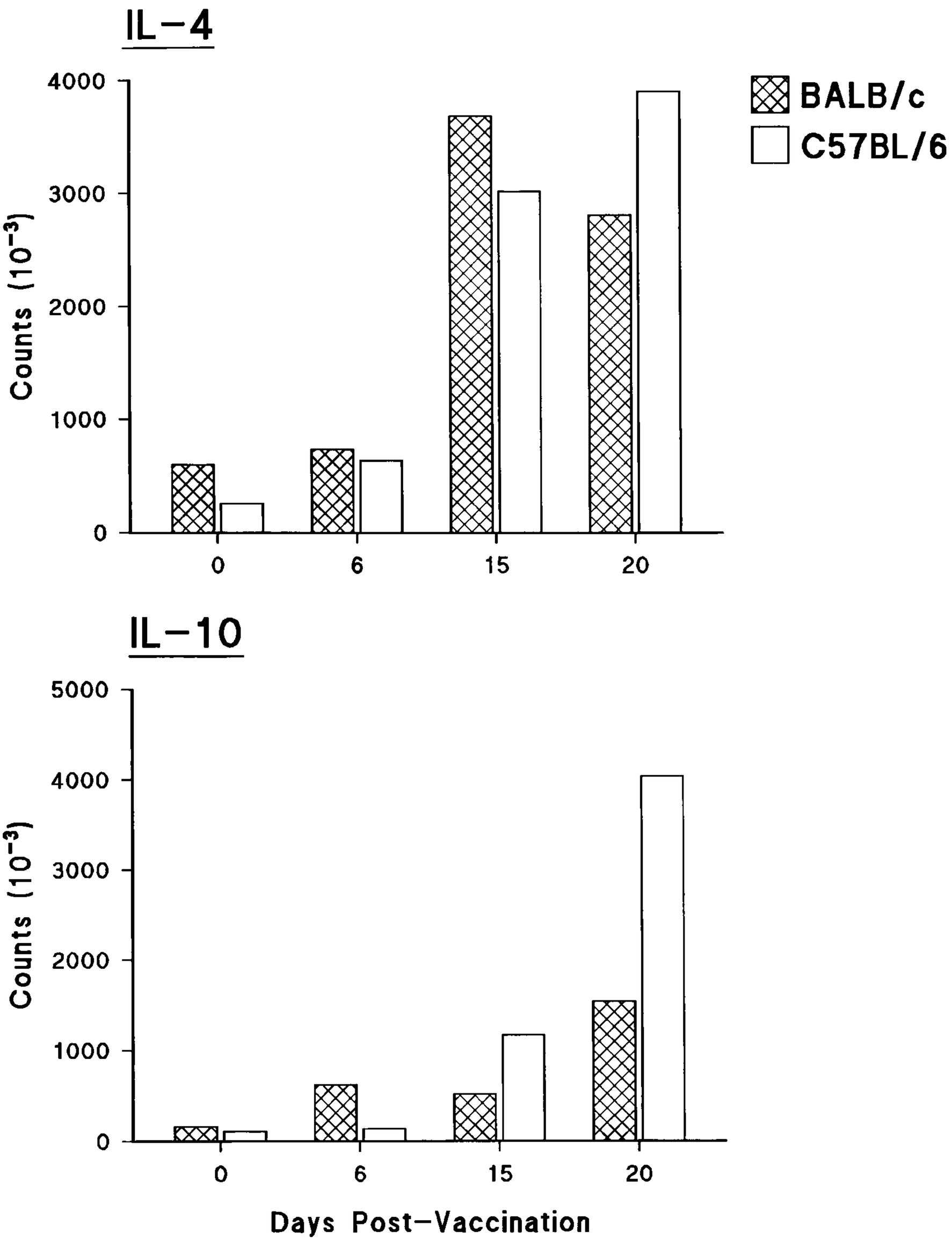


Fig. 4. Expression of IL-4 and IL-10 mRNA in whole lung tissue of C57BL/6 and BALB/c mice following exposure to 500 radiation attenuated cercariae of *S. mansoni*. Values depicted as described for Fig.1.

Table 1. Cytokine expression levels detected in the lungs and skin draining LN of naive C57BL/6 and BALB/c mice. Values shown are the amount of plasmid, in pg, added to constant amounts of sample cDNA to achieve a point of equivalence after PCR amplification. All samples were tested for HPRT values, and no differences observed.

	IFNγ	**IL-4**	**HPRT**
BALB/c naive LN	ND	0.5 pg	50 pg
C57BL/6 naive LN	0.5 pg	0.5 pg	50 pg
BALB/c naive lung	0.005 pg	0.05 pg	50 pg
C57BL/6 naive lung	0.5 pg	0.05 pg	50 pg

ND = not detected

Discussion

Cytokine expression in the LN tissue of C57BL/6 mice reflects profiles obtained with vaccination time courses previously described (Chapter Three). The cytokine mRNA levels are similar for both IL-12 and IFNγ, in that values obtained at day 0 are higher than any others across the time course. The exception to this is the basal level of IFNγ expression in BALB/c mice, which is no higher than the expression at following time points. This drop in cytokine message following vaccination may be due to the inhibition of gene transcription to below baseline levels. However, a more plausible explanation is the presence of an increasing number of B cells in the nodes after day 7 (Constant *et al.,* 1990, Constant and Wilson, 1992). As described in chapter three, the accumulation of this cell type in the LN reduces the T:B cell ratio from 4:1 to 1:1 (Constant *et al.,* 1990), thus in effect diluting the proportion of T cell-derived mRNA represented in the 0.25μg of total RNA analysed at each time point.

IFNγ expression is higher in the axillary and inguinal LN of naive C57BL/6 animals compared with BALB/c mice. This is in agreement with previously published data indicating the IFNγ disposition of the C57BL/6 strain, and hence the natural default development of Th1 cell populations (Hsieh *et al.,* 1995, Reiner *et al.,* 1994, Morris *et al.,* 1992). In contrast, IL-4 expression is higher in BALB/c mice compared to C57BL/6, again in agreement with published data, displaying a natural tendency of this strain to polarise toward Th2 cytokine production (Reiner *et al.,* 1994). The data obtained by competitive PCR supports these findings, suggesting that even in the unstimulated mouse, a pre-determined tendency exists towards Th1 or Th2 cytokine production.

IL-10, the other Th2 cytokine analysed, shows only slight differences in expression between the LNs of both strains. C57BL/6 mice display slightly higher levels of IL-10 compared to BALB/c animals, even though the same pattern of decline in expression below naive values seen for IFNγ and IL-12 is also observed here. Previous work (see Chapter Three) has demonstrated early peaks of these cytokine mRNAs in the LNs of C57BL/6 animals following vaccination, before the expression levels fall below baseline at between days 5 and 10. Thus the time points

analysed here may sample the cytokine profiles on the downward trend of an earlier peak. A repeat time course involving an earlier sampling program could address this problem.

The dominance of IFNγ and IL-12 in C57BL/6 mice following vaccination with irradiated cercariae may account for the increased resistances observed upon challenge of this strain. BALB/c mice are capable of IFNγ secretion, but to a lesser extent than the more resistant group, and show no increases in IL-12 levels above baseline in either LN or lung tissue. The data presented above correlates well with similar studies carried out on *Leishmania major* infection in these strains of mice (Reiner *et al.,* 1994). This published data demonstrates that both susceptible BALB/c, and resistant C57BL/6, animals can produce IFNγ and IL-12 early in the induction mechanism. However, in susceptible strains, the synthesis of these cytokines is dramatically reduced after approximately 5 days, and the expression of IL-4 begins to dominate (Reiner *et al.,* 1994, Reiner and Locksley, 1995). In contrast, following infection of resistant mice, IL-4 mRNA is downregulated. A similar pattern of differential cytokine production is observed during an *S. mansoni* vaccinating infection. IFNγ and IL-12 are the predominant cytokines expressed by cell populations in the lungs of C57BL/6 mice. Although IL-4 mRNA is detectable at ten fold above the baseline values determined for these mice, it is still present at an overall lower level than IFNγ, due to the higher naive levels and four fold increase in the latter cytokine. Of course, this does not account for the potency of the different cytokine proteins, and low expression of IL-4 may in fact be sufficient to induce a Th2 response above any inhibitory levels of IFNγ. Thus, as deduced in chapter four, C57BL/6 mice appear to mount a mixed Th1/Th2 cytokine profile after vaccination, with a tendency towards a Th1-like response. BALB/c mice express levels of IL-4 message similar to those detected in C57BL/6 mice. However, in contrast to the latter strain, BALB/c mice do not express high levels of IFNγ and IL-12. Thus the IL-4 production in these mice, although similar in intensity to that observed in C57BL/6 animals, represents a higher IL-4:IFNγ ratio and hence a more polarised Th2 response.

It has been suggested that a genetic defect in BALB/c mice may be responsible for the abundant production of IL-4 upon infection (Reiner and Locksley, 1995). The

predisposition of BALB/c mice to mount a Th2 response segregates with the $CD4^+$ T cell and is not associated with the APC. Furthermore, the inability of IL-12 to downregulate the expression of IL-4 might be due to the lack of suppression of IL-4 transcription in $CD4^+$ T cells from these animals. Recent data has indicated that T cells from BALB/c mice become unresponsive to the effects of IL-12 after the first week of infection (Güler *et al.,* 1996, Szabo *et al.,* 1995) and this may account for the prominent development of Th2 profiles in this strain.

To summarise, it would seem that the genetic background of some strains of mice affects the ability to respond to the irradiated vaccine, and that this ability is closely linked to cytokine production. The polarisation of the T helper responses in C57BL/6 and BALB/c mice after exposure to the irradiated *S. mansoni* vaccine is not as clear cut as that seen during *L. major* infection. This observation is not completely unexpected in that infection of these strains with *Leishmania* results in disease pathology at opposite ends of the spectrum, perhaps indicating that cytokine expression might exhibit similar diversity. However, following vaccination and infection with *S. mansoni*, both strains display partial resistance in comparison to normal infection. The differences in cytokine levels detected between the strains are also less extreme, both strains mounting a mixed Th1/Th2 type response. Complete ablation of one or other of the T helper responses by anti-cytokine antibody administration might be expected to have a more severe effect on the resistance generated with vaccination than subtle variations in strain immunology. Alternatively, the generation and analysis of cytokine gene-deleted mice may provide the ideal system for studying the consequences of infection in the absence of the major cytokines such as IFNγ and IL-4 (see Chapter Six).

A more complete post-vaccination investigation, looking at very early LN responses, could clarify the expression profiles of IFNγ and IL-12 in C57BL/6 and BALB/c mice during the induction response. Furthermore, analysis of the effector mechanism in the lungs following a challenge infection, would highlight any role the differences observed post-vaccination might have in blocking parasite migration. The increased ratio of IL-4 to IFNγ mRNA expressed in the lungs of BALB/c compared to C57BL/6 mice may result in reduced inflammatory responses post-challenge, and generate a less efficient blocking mechanism than that seen in the

latter strain. Hence, examination of cytokine production and focus formation during the BALB/c effector response could provide an explanation for the lower levels of resistance observed in these mice.

CHAPTER SIX

The Cytokine Expression Profiles Induced in IFNγ-Receptor Gene-Disrupted Mice Following Exposure to the *S. mansoni* Irradiated Vaccine

Introduction

Ablation of cytokines with specific antibodies has long been used as a means of investigating the role of individual cytokines in an immune response. However, the variable efficiency of ablation *in vivo* can lead to ambiguous results which are difficult to interpret; for example, the ablation may not be 100% successful implying the cytokine is not important in the response under investigation. The advent of gene-deletion technology has allowed the devlopment of 'knockout' mice, animals exactly the same as wild type mice except for a disruption in one of the exons required for the expression of an active cytokine protein (Capecchi, 1989, Rajewski, 1992). This technology has lead to the generation of many cytokine-, or cytokine receptor-deficient mice (Kühn *et al.,* 1991, Dalton *et al.,* 1993, Huang *et al.,* 1993, Kaufmann, 1994). Thus gene-disrupted mice provide an ideal system in which to study the outcome of infection in an animal completely incapable of synthesising or responding to the cytokine of interest, and hence leaves no doubt as to the complete absence of this component in the immune response.

Gene-disrupted mice can be generated by the manipulation of embryonic stem cells to incorporate a non-functional version of the gene of interest (Capecchi, 1989). DNA constructs containing the modified intron, usually disrupted by the insertion of a neomycin gene which confers resistance to the drug G418 (Capecchi, 1989), are used to transfect embryonic stem cells in culture. The modified DNA can either recombine with the target gene exon in a homologous fashion (replacing the wild type sequence), or insert at random in the genome resulting in no disruption to the cytokine gene. Successful transfectants, which have taken up and incorporated the modified DNA, are positively selected by growth in medium containing G418. To screen out cells containing random insertion events, a negative selection process is then applied, based on the co-insertion of the herpes simplex virus thymidine kinase (HSV-tk) gene alongside the *neo* insert during non-homologous recombination. By including the HSV-tk sequence adjacent to the region of homology, cells containing homologous recombination events will lose this gene during transformation. However, random insertion will not select against the inclusion of the HSV-tk sequence, as predicted from the observation that linear DNA integrates into the

genome via the ends of the fragment (Capecchi, 1989, Thomas *et al.,* 1986), and thus these cells will be killed by the drug gancyclovir (GANC). The selected cells are then incorporated into blastocysts and implanted in foster mothers. The resulting progeny are chimeric, and those with modified cells incorporated into the germline will produce gametes homozygous for the gene-disruption which when crossed with wild type (WT) mice produce heterozygous offspring. Selective breeding of the progeny can lead to the generation of a population of mice homozygous for the gene-disruption.

Due to the drastic nature of the complete loss of a gene, some mice cannot tolerate the deletion of certain genes resulting in abortion, or death soon after birth. For example, TGFβ knockout mice can die before birth (Christ *et al.,* 1994), and IL-2 (Sadlack *et al.,* 1993) and IL-10 (Kühn *et al.,* 1993) gene-disrupted mice can die a few weeks after birth if not maintained in isolator conditions. Mice containing other cytokine gene-disruptions require careful handling and maintenance to ensure that the animals are not exposed to environmental pathogens. The development of mice with less severe deletions has lead to the study of the role of many cytokines in various infections.

IFNγ knockout mice have been generated and the immune response to various pathogens studied, such as *Mycobacterium bovis* (Bacillus Calmette-Guerin, BCG, strain, Dalton *et al.,* 1993, Cooper *et al.,* 1993). In this infection, which typically induces a Th1 response, IFNγ gene-disrupted animals display impaired production of macrophage antimicrobial agents and are killed by a sub-lethal dose of bacteria. Furthermore, exposure of these animals to *Leishmania major* results in downregulation of the protective Th1 response and increased expression of IL-4, IL-5 and IL-13 mRNA, leading to disease exacerbation and death in a strain normally resistant to this parasite (Wang *et al.,* 1994).

Development of IFNγ receptor-deficient mice (Huang *et al.,* 1993) has also provided valuable information on the importance of this cytokine for the generation of a Th1 response. Infection of IFNγ receptor knockout mice with *Listeria monocytogenes* or BCG results in death, similar to the outcome observed in IFNγ gene-disrupted mice. The susceptibility to BCG, and ability to withstand

lippopolysaccharide (LPS) induced toxic shock has been correlated with a reduced expression of TNFα in these animals (Kamijo *et al.,* 1993, Car *et al.,* 1994).

Other cytokine-deficient mice to emerge in recent years include IL-2 knockouts (Schorle *et al.,* 1991, Kündig *et al.,* 1993), although these animals do not survive well due to the development of an ulcerative colitis-like disease (Sadlack *et al.,* 1993). A similar effect is seen in IL-10-deficient mice (Kühn *et al.,* 1993), but careful husbandry and maintenance can reduce disease. IL-4 knockout mice remain healthy if housed in isolator conditions and have been shown to develop lower IgG1 and IgE responses compared to WT animals (Kühn *et al.,* 1993), correlating with much reduced Th2 cytokine production (Kopf *et al.,* 1993). More recently, the abrogation of IFNγ responses to keyhole limpet antigen, with a corresponding increase in IL-4 expression, have been demonstrated in IL-12-deficient mice (Warner *et al.,* 1995). Both the p40 and p35 subunits of this cytokine have been disrupted, with similar effects seen in each knockout.

Genetic-deletions resulting in the abrogation of a cell lineage have also provided a broader understanding of the requirements of an immune response following infection. For example, mutation of the CD4 gene has indicated that this molecule is not necessary for the development of single positive CD8 cells from the thymus. In addition a $TCR\alpha\beta^{+ve}$, $CD4^{-ve}$ cell population capable of cytokine secretion, but with resticted B cell help, also develops (Rahemtulla *et al.,* 1991). This $CD4^{-ve}$ population is able to secrete IFNγ and mediate protection against *L. major* infection (Locksley *et al.,* 1993). Mice homozygous for a deletion in the Ig μ chain develop no mature B cells, indicating the importance of this isotype splicing event in the B cell developmental pathway (Kitamura *et al.,* 1991). These mice provide a model for human patients exhibiting immunodeficiency disease resulting from an absence of functional B cells.

This chapter will describe the screening system used to derive a homozygous breeding colony of IFNγ receptor-deficient mice (Huang *et al.,* 1993). Groups of these mice were subsequently vaccinated and challenged with cercaria of *Schistosoma mansoni* to investigate the effect on resistance of the inability to respond to IFNγ during the induction and effector responses. Vaccination of C57BL/6 mice routinely results in the generation of up to 75% resistance (Dean,

1983, Smythies *et al.,* 1992a, 1992b, 1993). The protection relies heavily on the induction of a Th1 cytokine producing $CD4^+$ cell population in the LN draining the exposure site, and the recruitment of this population to the lungs (Smythies *et al.,* 1992a, 1992b, Mountford *et al.,* 1992). Upon challenge infection, the schistosome-specific $CD4^+$ population forms inflammatory foci around migrating parasites which block larval exit from the lungs (reviewed by Smythies *et al.,* 1993). This effector mechanism is dependent on the production of IFNγ, as demonstrated by the ablation of this cytokine with *in vivo* administration of anti-IFNγ antibody (Smythies *et al.,* 1992b, Sher *et al.,* 1990a) which results in the abrogation of up to 90% resistance.

The mechanisms involved in focus formation are not fully understood as yet. Through the use of gene-disrupted mice, the roles of individual cytokines in the protective response can be analysed without the doubt involved in *in vivo* anti-cytokine antibody ablations. Initial results from the vaccination of IFNγ-$R^{-/-}$ mice confirm that IFNγ plays an important role in the generation of resistance in the irradiated schistosome vaccine model. The technology is now in place to breed and test animals of various deficiencies to pinpoint the conditions necessary for effective protection against infection with *S. mansoni*.

Materials and Methods

Mice and Parasites

Breeding pairs of homozygous IFNγ receptor-deficient (IFNγ-$R^{-/-}$) and wild type (WT) 129 Sv/Ev mice were obtained from the University of Zurich (Prof. M Aguet, Huang *et al.,* 1993) and bred and maintained in isolator conditions throughout all procedures. The breeding stock and all progeny were screened by DNA extraction of 2cm tail snips followed by PCR as described below.

Specified pathogen free C57BL/6 mice were purchased from B&K Universal and maintained in isolator conditions throughout all experiments. All infected groups were exposed to a Puerto Rican strain of *S. mansoni* obtained by routine passage through albino *Biomphalaria glabrata* snails and MF1 outbred mice.

Genetic Screening of Gene-Disrupted Mice

After initial unsuccessful attempts at DNA amplification from peripheral blood samples obtained by tail bleeding, genomic DNA was isolated from tail snips excised under anaesthesia (method modified from a protocol kindly provided by R Kühn, University of Cologne, Germany). Tail tissue was digested in buffer (50 mM TrisHCl pH8, 100 mM EDTA pH8, 100 mM NaCl, 1% SDS) containing 0.4 mg of proteinase K (Sigma) at 55^{o}C overnight. The DNA was then extracted with phenol:chloroform at a 1:1 ratio by gentle mixing for 10 minutes. The aqueous layer was removed to a clean tube with a cut pipette tip, to prevent DNA shearing, and the DNA precipitated by the addition of 0.2 volumes of 10M ammonium acetate and 2 volumes of 100% ethanol. The resulting stringy precipitate was collected and washed in 70% alcohol, pelleted and dried at room temperature and resuspended in 300 µl of TE (Tris-EDTA pH 7.5). A 1-2 µl aliquot of a 1:100 dilution of this stock DNA was used in each screening PCR.

Amplification of test DNA was carried out using primers designed to span the neomycin insert in the appropriate exon. For detection of the IFNγ-R gene, primers were designed to span exon 5 as follows: 5', AGATCCTACATACGAAACAT; 3', TTTCTGTCATCATGGAAAGGAGGG (modified from Huang *et al.,* 1993,). A neomycin-specific oligonucleotide, designed to act as a 3' primer in PCR for the

detection of heterozygous and homozygous DNA samples containing the disrupted exon, was designed from the published gene sequence as follows, CCTGCGTGCA ATCCATCTTG (Beck *et al.,* 1982). The PCR was optimised to generate a single product with DNA isolated from WT and homozygous mice of 189 and 500 base pairs respectively. The different sized PCR products obtained by PCR in the presence of the cytokine primer pair and the neomycin 3' primer could be used to distinguish wild type, heterozygous and homozygous mice.

Products were separated by 2% agarose gel electrophoresis in the presence of ethidium bromide and the gel viewed and photographed under UV illumination. For reactions yielding ambiguous results, the PCR was separated into IFNγ receptor 3' and 5', and IFNγ receptor 5' and neomycin 3' amplifications for clarity.

Exposure Regimes

Various vaccination and challenge exposure time courses were carried out on IFNγ- $R^{-/-}$ mice bred in house. These mice were generated on the 129 Sv/Ev background, a sub-strain related to the 129/Ola strain shown previously to be susceptible to portal shunting. 129/Ola mice display a seemingly resistant phenotype to infection with *S. mansoni*, with parasites accumulating in the lungs instead of the portal system (Wilson *et al.,* 1983, Coulson and Wilson, 1989, Wilson, 1990). Before vaccination, the WT 129Sv/Ev mice were analysed for portal shunts by the injection of microspheres and found to be free of this defect (carried out by Prof. Wilson and Dr. Coulson) and were therefore suitable for the study of the irradiated vaccine. Three experimental timecourses were designed (in collaboration with Dr L Smythies, Dr P Coulson and S Anderson) to analyse different stages of the induction and effector responses. Due to the small numbers of animals available from breeding stock at any one time, each time course concentrated on sampling at limited time points. Thus separate exposure regimes sampled lung and skin draining LN tissues at day 16 post-vaccination, and lung tissue at day 14 post-challenge. An extended time course further analysed the induction response at days 5 and 20 post-vaccination, and day 14 post-challenge. At days 5 and 16 post-vaccination both IFNγ-$R^{-/-}$ and WT 129 mice were sampled. At days 20 post-vaccination and 14 post-

challenge IFNγR$^{-/-}$ 129, corresponding WT, and C57BL/6 mice were analysed in parallel.

At day 35 post-vaccination five mice from each experimental group were challenged percutaneously with 200 normal cercariae on the tail. Five weeks after challenge infection portal perfusion was performed on groups of vaccinated and corresponding naive mice to determine worm burdens and the resistance calculated.

RT-PCR Analysis of Cytokine Message Generated upon Exposure of IFNγ-R$^{-/-}$ Mice

At each time point groups of 5 mice were sacrificed and the total RNA isolated separately from lung tissue or as a pool for LN samples. RT-PCR analysis was carried out for a single cycle number using the basic protocol as previously described (Chapter Two). Each individual RNA preparation was tested for the following cytokines (at a previously determined appropriate cycle number): HPRT (25), IFNγ (27), IL-4 (27), IL-10 (30), and IL-12 (30). The primer sequences and cycling temperatures were as previously described (Chapters Two and Three). PCR products were slot or Southern blotted and hybridised with short oligonucleotide probes end-labelled with ^{32}γP-ATP. The radioactivity associated with each sample was quantified by exposure to a storage phosphor screen and scanning by phosphorimager. Values obtained for the lung tissue of each mouse were plotted as a mean ± SE for the group.

Results

Screening of Gene-Disrupted Mice

A typical agarose gel depicting the products from a genotyping PCR of IFNγ-$R^{-/-}$ breeding stocks is shown in Fig. 1. From initial protocols of DNA isolation, it was deduced that the PCR reaction with three primers was extremely sensitive to contaminating inhibitors, such as iron from the haemoglobin in blood DNA isolations. The preparation of genomic DNA by tail digestion provided a clean source of test sample which yielded more reproducible PCR products.

The PCR was designed to yield products of two sizes, with a possible longer, third product obtainable under certain reaction conditions. The smallest product of 189 base pairs corresponded to amplification of the WT gene for the IFNγ-R with the 5' and 3' receptor primers. A product of approximately 450 base pairs was obtained from DNA containing the neomycin insert using the 5' receptor and 3' neomycin primers. In some reactions (not shown) containing the receptor 5' and 3' primers a large product of over 1000 bases was observed, corresponding to amplification of DNA containing the neomycin insert. However, this product was not produced in a reproducible fashion, hence the introduction of the neomycin 3' primer, the screening having been initially designed to comprise of PCR with the receptor gene primers only. The presence of only the 189 base pair product was taken to represent WT DNA. The detection of both the smallest and 450 base pair, and possibly the 1000 base pair, products indicated a sample of heterozygous nature. Samples displaying only the 450 and 1000 base pair products were classed as homozygous for the gene-deletion. In this way, many samples could be screened for the presence of gene-deletions with a single PCR test. Samples producing ambiguous results were analysed by separate PCR reactions for the 5' and 3' IFNγ receptor, or 5' IFNγ receptor and 3' neomycin primer pairs.

Cytokine Profiles in LN Tissue Following Vaccination of IFNγ-$R^{-/-}$ Mice

At day 5 following vaccination, IFNγ mRNA levels in the WT LN are approximately 3 fold higher than in knockout animals (Fig. 2). An even greater difference is seen with IL-12 message, with WT mice displaying product at almost 6

times the level detected for knockout (KO) mice. For the Th2 cytokine IL-4, the differences are smaller in comparison to the Th1 cytokines, WT IL-4 mRNA levels only showing an increase of 2 fold over those observed in RNA from gene-disrupted mice (Fig. 3). This is perhaps in contrast to the expected patterns, where the lack of IFNγ might cause an increase in the levels of Th2 cytokine mRNA. Unfortunately, no naive LN tissue was available during this vaccination and thus comparisons can only be made between the two test samples

At day 16 post-vaccination, IFNγ mRNA levels are much higher in LN tissue from WT mice than expression in knockout animals (Fig. 4). However, IL-12 expression is very similar between the two groups, indicating that IFNγ may not be required for production of IL-12. Although the Th2 cytokines, IL-4 and IL-10 (Fig. 5), show higher levels of expression in WT mice, compared to the knockout group, the fold differences seen are not as great as those detected at day 5 post-vaccination. As above, the lack of LN naive tissue available in this study makes interpretation of these results difficult.

Cytokine Profiles in Lung Tissue Following Vaccination and Challenge of IFNγ-R$^{-/-}$ Mice

Lung tissue sampled at day 5 post-vaccination shows little variation between the two genetic bacgrounds, with IFNγ, IL-4, IL-10 and IL-12 mRNA levels displaying no significant differences at this time point (Figs. 2 and 3). Furthermore, expression levels were low compared to those observed in LN tissue, and no higher than the naive background. Naive values were different in knockout and WT animals for IFNγ, but similar for IL-4, IL-10 and IL-12. By day 16 post-vaccination, the levels of cytokine expression in the lungs show marked differences between knockout and WT animals (Figs. 4 and 5, $p \leq 0.05$ in all cases except IL-4 which is n.s). In fact IFNγ, IL-12, IL-4 and IL-10 are all expressed in WT mice at between 6 and 10 fold the level detected in gene-disrupted animals.

Lung tissue extracted at day 20 post-vaccination for WT, knockout and C57BL/6 groups of mice allowed the comparison of cytokine expression between genetically different groups of animals, one of which was known to be highly resistant to further infection. IFNγ mRNA levels were similar for 129 WT and knockout mice (Fig. 6),

however, C57BL/6 mice displayed IFNγ levels significantly higher ($p \leq 0.05$) than both 129 groups. This pattern was also observed with IL-4 and IL-12 expression, with knockout mice showing slightly higher levels of IL-4 than 129 WT animals. IL-10 expression was similar in all groups tested.

Analysis of lung tissue at day 14 post-challenge demonstrated major differences in the cytokine expression in WT, knockout and C57BL/6 mice during the effector response (Figs. 7 and 8). IFNγ mRNA levels were significantly different between all groups, with C57BL/6 mice expressing this cytokine at levels three fold higher than those seen in 129 WT mice. IFNγ expression in knockout mice was under one tenth of the amount detected in C57BL/6 animals, and one third that of WT mice ($p \leq 0.001$ between all groups). The other Th1-related cytokines, IL-12 and TNFα were both expressed in a similar pattern; the differences between C57BL/6 and both 129 groups were not as great ($p \leq 0.05$), and there was no significant difference between 129 WT and knockout groups. The naive level of expression for the Th1-related cytokines were higher in C57BL/6 tissue than in samples from WT and KO mice.

The patterns of Th2 cytokine expression were less predictable, with C57BL/6 mice expressing levels of IL-4 and IL-10 equivalent to knockout animals, which might have been expected to show elevated Th2 profiles. IL-4 expression was significantly lower in 129 WT mice than in C57BL/6 and 129 IFNγ-$R^{-/-}$ animals ($p \leq 0.05$), whereas IL-10 levels were not significantly different in any group. IL-5 mRNA expression in both 129 groups was approximately 2 fold higher than that observed in C57BL/6 mice ($p \leq 0.05$), with no differences between the 129 strain WT and knockout animals. In addition, the naive level of IL-5 expression was higher in KO mice, although basal levels of IL-4 and IL-10 mRNA were similar in all groups. The patterns of expression for Th1 and Th2 cytokines were repeated in a second experiment (Fig. 9), with the exception of IL-4 mRNA levels in C57BL/6 animals which were significantly lower ($p \leq 0.05$) than in 129 IFNγ$R^{-/-}$ mice.

Resistance Data

The worm burdens, obtained by portal perfusion of the three types of mice at day 35 post-challenge, were used to calculate resistance values. The WT 129 animals, a strain not routinely used as a host for *S. mansoni*, displayed resistances of 53% and

46% in two experiments (Wilson *et al.*, 1996). This was lower than the values obtained for C57BL/6 mice at 69%. In contrast, the resistance levels induced in the IFNγ-R knockout mice were 20% and 24% in two experiments, an abrogation of 62% and 48% respectively compared to WT values.

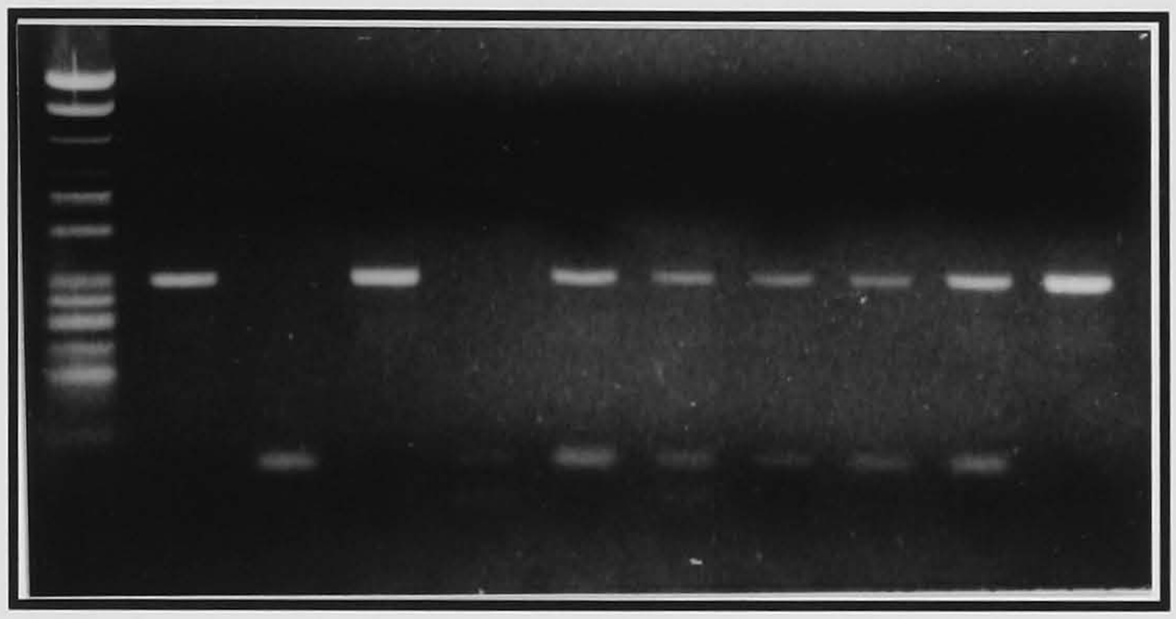


Fig. 1. A typical agarose gel, viewed and photographed under UV illumination, depicting the different band sizes observed following PCR screening of WT, heterozygous and homozygous mice with a disruption in the IFNγR gene. Band sizes are expressed in base pairs (bp).

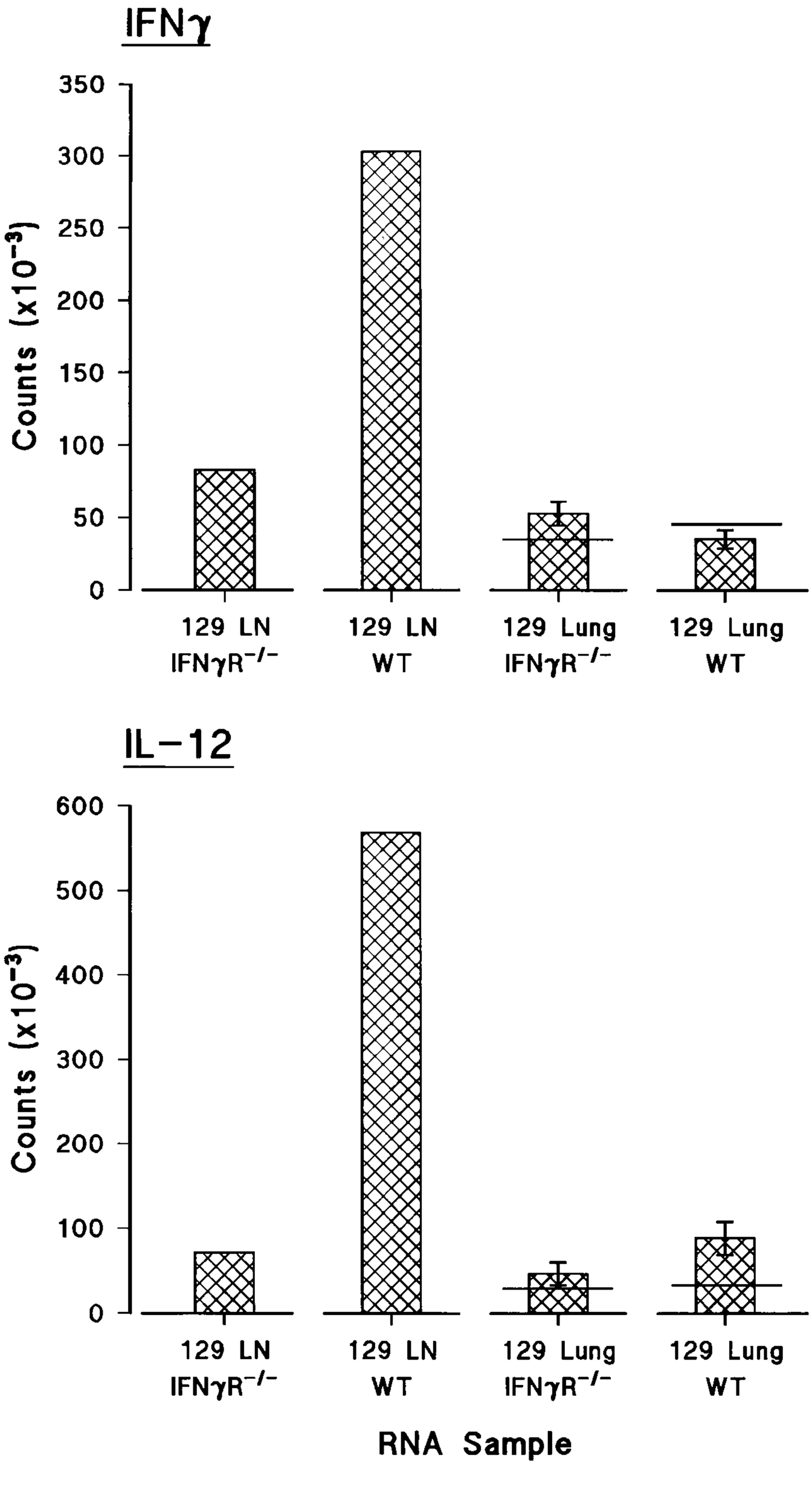


Fig. 2. Th1-related cytokine PCR product derived from total LN and lung RNA from 129 WT and IFNγ receptor gene-disrupted mice at day 5 post-vaccination . Values shown represent a pool of RNA for the LN, whereas lung RNA was analysed individually for each animal and depicted as a mean of five mice ± SE. Naive values for the lung data are indicated by a line. Values have been adjusted for variations in HPRT expression.

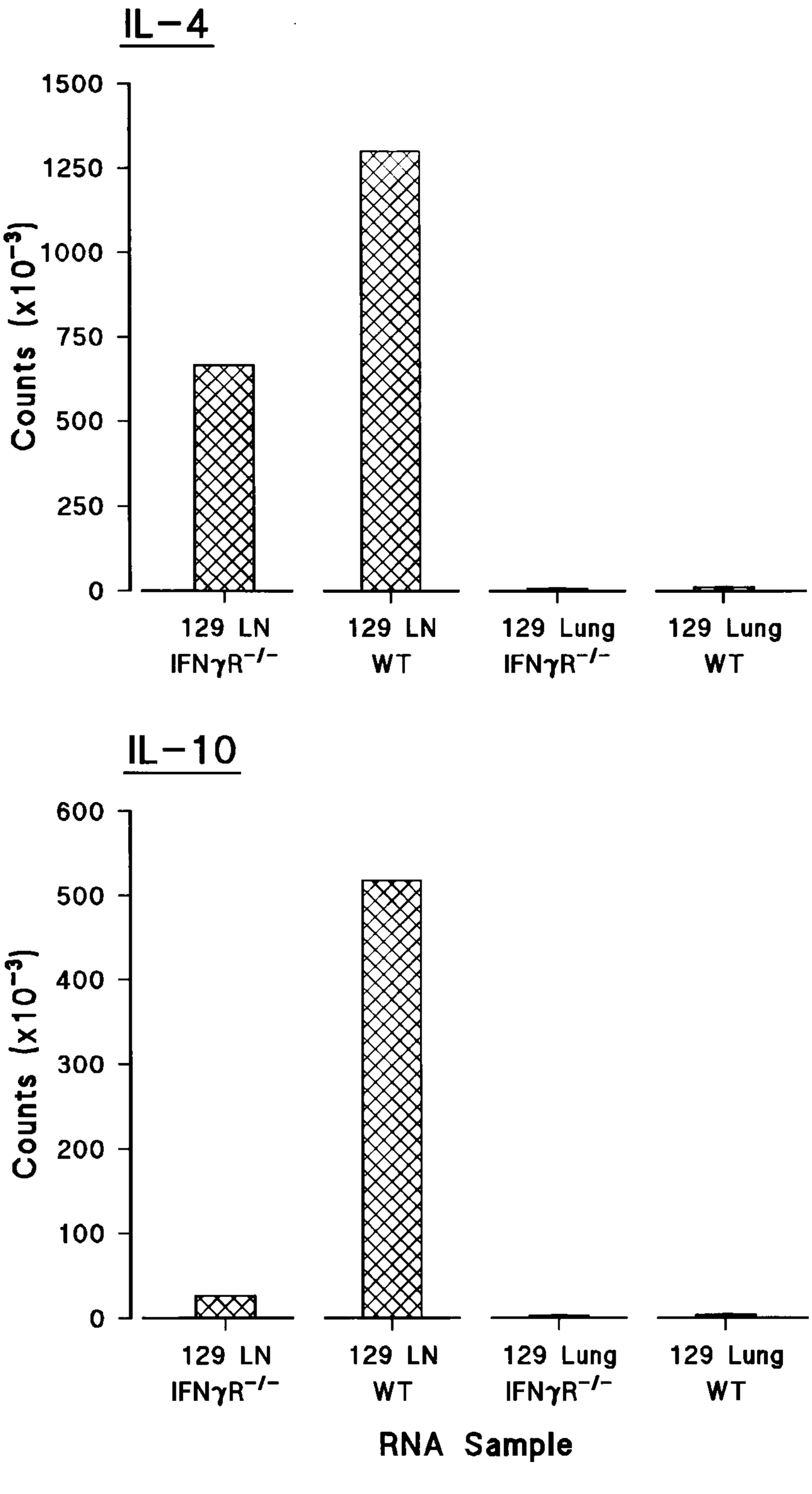


Fig. 3. Th2 cytokine PCR product derived from total LN and lung RNA from 129 WT and IFNγ R gene-disrupted mice at day 5 post-vaccination. Values depicted as in Fig. 2.

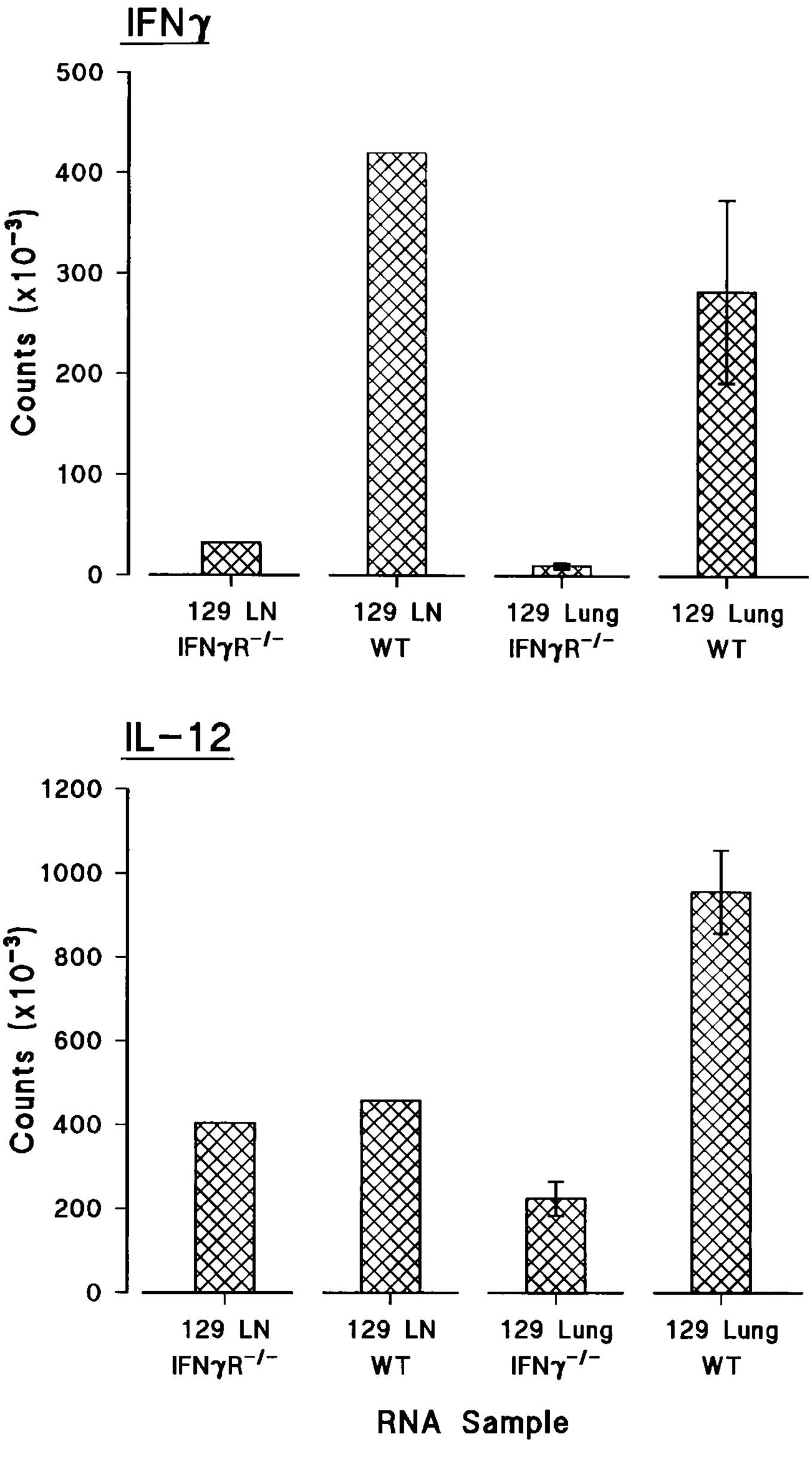


Fig. 4. Th1-related cytokine PCR product derived from total LN and lung RNA from 129 WT and IFNγ R gene-disrupted mice at day 16 post-vaccination. Values depicted as in Fig.2.

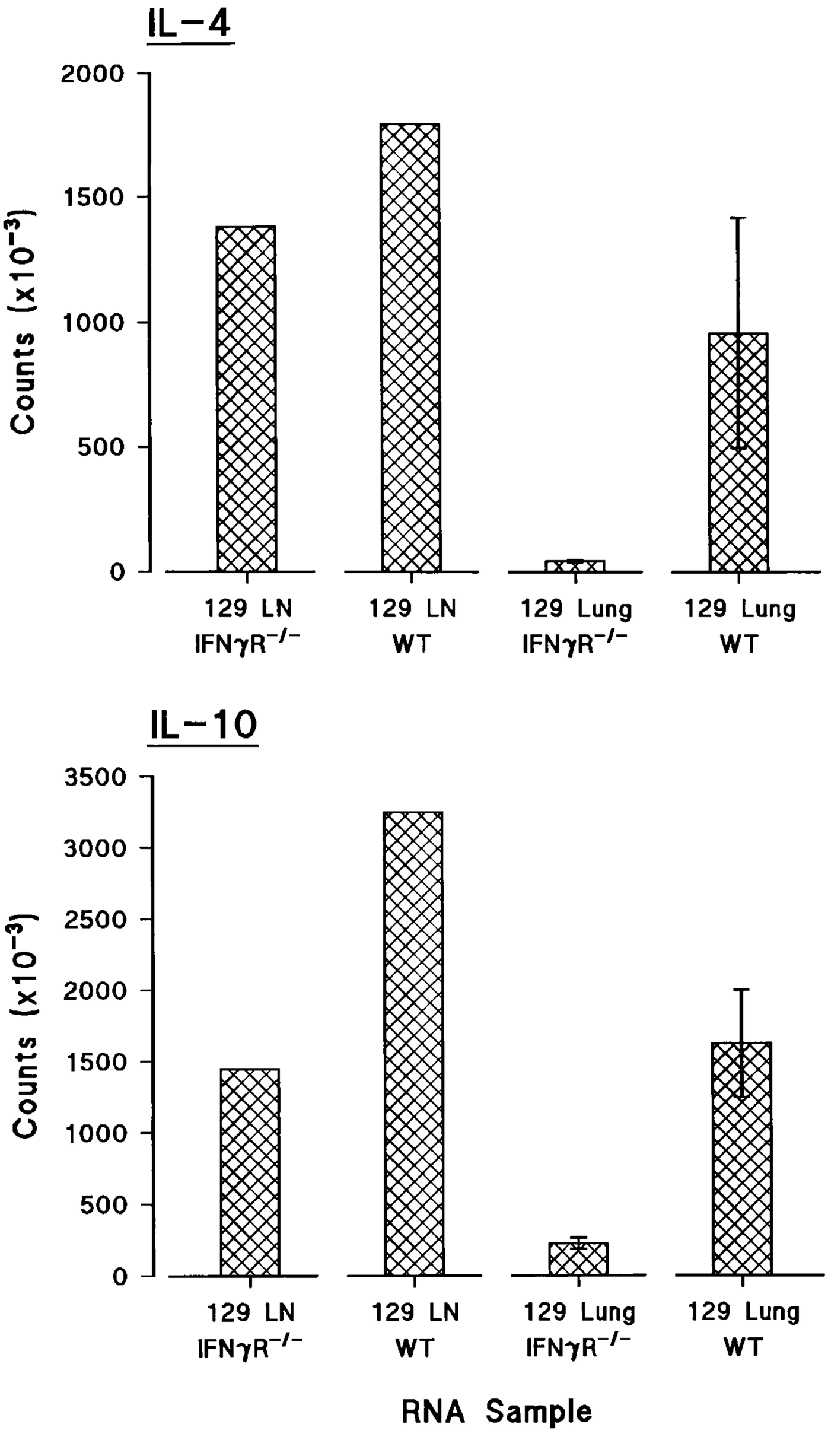


Fig. 5. Th2 cytokine PCR product derived from total LN and lung RNA from 129 WT and IFNγ R gene-disrupted mice at day 16 post-vaccination. Values depicted as in Fig. 2.

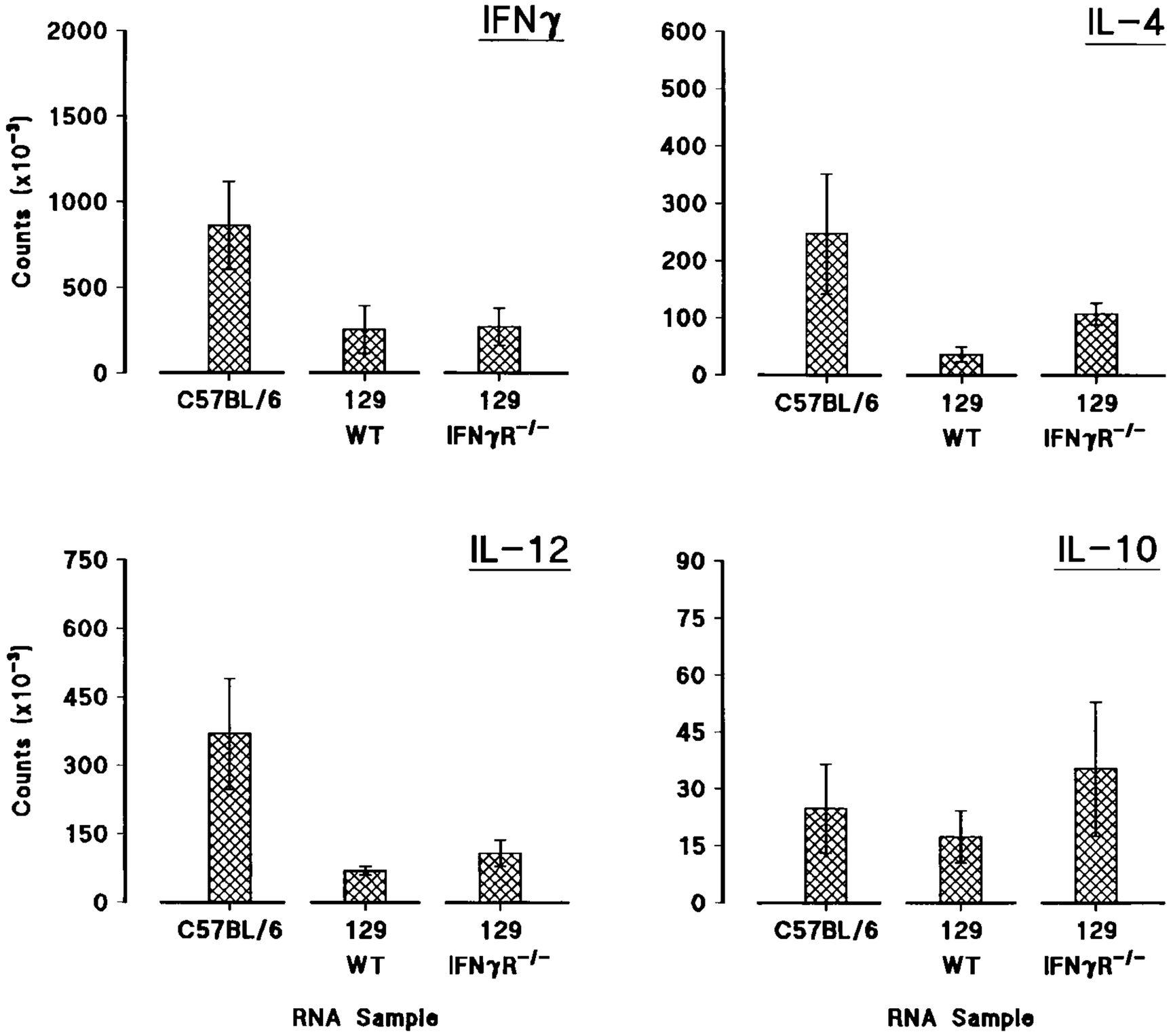


Fig. 6. Cytokine PCR product derived from total lung RNA from C57BL/6, 129 WT and 129 IFNγR gene-disrupted mice at day 20 post-infection. Values depicted as in Fig. 2.

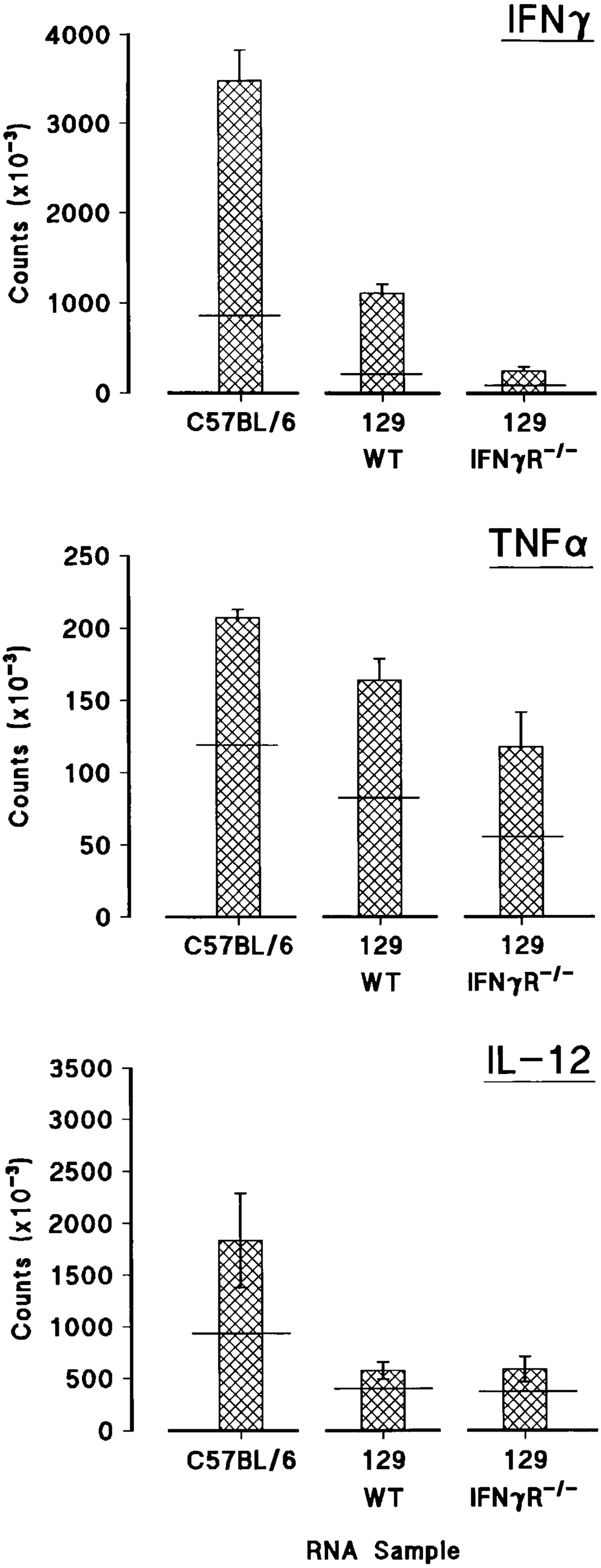


Fig. 7. Th1 cytokine PCR product derived from C57BL/6, 129WT and 129 IFNγR $^{-/-}$ whole lung RNA at day 14 post-challenge. Values depicted as in Fig. 2.

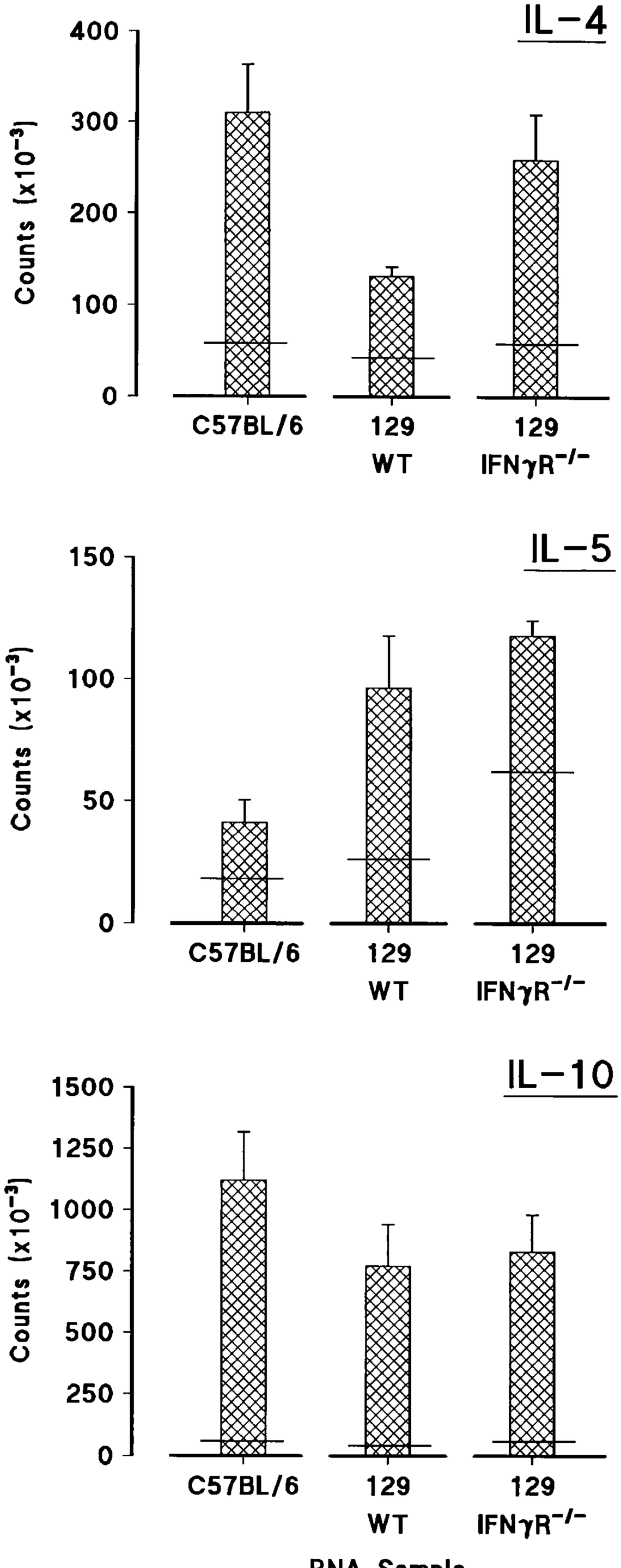


Fig. 8. Th2 cytokine PCR product derived from C57BL/6, 129WT and 129 IFNγR $^{-/-}$ whole lung RNA at day 14 post-challenge. Values depicted as in Fig. 2.

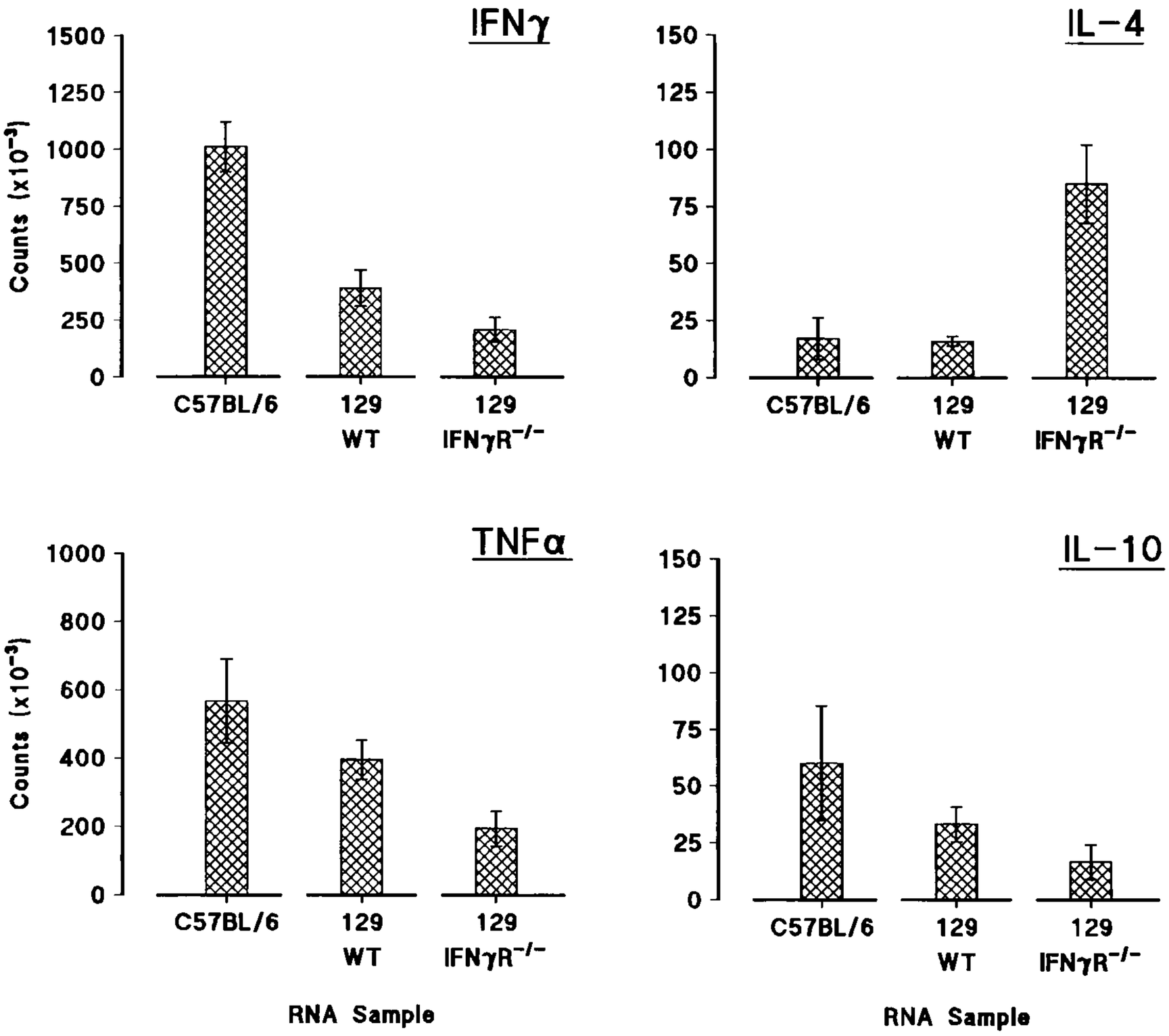


Fig. 9. Cytokine PCR product derived from C57BL/6, 129 WT and 129 IFNγR$^{-/-}$ whole lung tissue at day 14 post-challenge. Values shown are from a repeat time course and are depicted as in Fig. 2.

Discussion

The methods outlined above for the genotyping of mice containing a disrupted cytokine receptor gene provide a rapid and reliable way of distinguishing WT, heterozygous and homozygous mice without the need for Southern blotting and probe labelling. In addition, this method allows simultaneous screening of large numbers of samples. Isolation of DNA by tail snip results in the extraction of enough material for multiple test reactions, should the need arise. Initial attempts at DNA extraction from whole blood resulted in inefficient PCR, even after resin purification, presumably due to the presence of iron from the haem group in red blood cells. This PCR method is now in routine use for the monitoring of knockout colonies both at B&K Universal and the University of York.

The vaccination of IFNγ-R gene-disrupted mice with irradiated cercariae of *S. mansoni* would be expected to result in a much reduced level of resistance. This expectation, based on previous evidence demonstrating the abundance of IFNγ during the protective effector mechanism, and the dramatic effects on protection of ablating this cytokine *in vivo* (Smythies *et al.,* 1992a, 1992b), proved partially true. The 55% abrogation of resistance is lower than anticipated from anti-IFNγ antibody ablation experiments which resulted in a 90% reduction (Smythies *et al.,* 1992b). There are several possible explanations for this difference. The knockout mice are based on a 129 background, a strain not previously studied in relation to the protection levels generated after exposure to the irradiated vaccine. The resistance data of 50% obtained for WT mice from these experiments would tend to indicate that this strain is not a high responder, unlike the C57BL/6 strain more routinely used. Thus the effector mechanism is not working at optimal efficiency in 129/Sv/Ev mice, an observation confirmed by the lower IFNγ expression level detected in comparison to vaccinated and challenged C57BL/6 animals. Bearing this in mind, it might be reasonable to assume that a lack of IFNγ receptors in animals which do not typically produce high levels of this cytokine might have less of an effect than ablation of this cytokine in mice undergoing a more polarised Th1 response.

A second explanation for the moderate reduction in resistance involves the concept of immune redundancy. Acute ablation of a cytokine with antibodies at a specific

point in time allows the animal no time to adapt to the new, imposed conditions. Therefore, the sudden loss of the activity of a cytokine may have a dramatic effect on the immune response. Mice harbouring a gene-disruption may adapt to the lack of one cytokine by compensating with the activity of another, possibly one usually redundant in WT animals. The overall effects of the gene-disruption would thus be reduced, and the change in the outcome of infection not as drastic as expected. The IFNγ-$R^{-/-}$ mice vaccinated in these experiments still mount an immune response upon challenge infection, albeit one resulting in lower levels of protection. Thus the mice are not completely immuno-compromised by the loss of IFNγ receptors, but mount a less effective protection mechanism. The cytokine data discussed below support this notion of a substitute immune response consisting of predominantly Th2 cytokine expression.

Cytokine expression in the LN of knockout and WT mice following vaccination reflects the differing genetic status of the two groups of mice. IFNγ expression is very low in receptor-disrupted mice compared to WT animals at both days 5 and 16 post-vaccination. It is perhaps surprising that IFNγ is detectable at the mRNA level, an observation confirmed at the protein level (Wilson *et al.,* 1996), although there is no evidence to suggest that these animals cannot upregulate IFNγ expression in response to IL-12. Indeed, IL-12 is eight fold higher in WT than knockout mice at day 5, but at day 16 both groups express similar levels of this cytokine. This may be indicative of an early role for IL-12 in the vaccination response, possibly involving a positive feedback loop for IFNγ in early IL-12 induction. The other cytokines analysed in LN tissue at day 16 post-vaccination also show a reduction in any differences observed between knockout and WT animals at day 5. This is probably due to the transient nature of parasite residence in the skin-draining LN following vaccination, most of the migrating larvae having moved on to the lungs by day 16 (Mountford *et al.,* 1988). Thus increases seen in WT animals may be falling by day 16, although it should be noted that due to the lack of a naive baseline for the day 16 time point it is impossible to determine whether cytokine expression in knockout mice is increasing, or whether expression in WT animals is decreasing, with time. On the whole, the cytokine message levels in the WT LN are higher than those detected in tissue from knockout mice at both days 5 and 16 post-vaccination.

Cytokine profiles induced in the lungs at day 5 following vaccination are expressed minimally above baseline, and at similar levels in both WT and knockout mice. This observation is explained by the absence of the bulk of migrating schistosomula in this organ at this early time point (Mountford *et al.,* 1988). By day 16 a difference in IFNγ mRNA levels can be detected in the lungs, WT mice expressing 10 fold the amount detected in gene-disrupted animals. Higher levels of expression for IL-4, IL-10 and IL-12 are also detected in WT, compared to knockout, mice. There appears to be little difference in T helper cell polarisation between WT and knockout groups, but rather a general suppression of cytokine expression in the knockout animals at day 16.

By day 20 post-vaccination, responses have been upregulated in the lungs of knockout mice, with both IL-4 and IL-12 showing higher levels of expression in gene-disrupted animals than in 129 WT mice. However, the apparent increase in IL-12 mRNA is not significant, unlike that for IL-4 message. Significantly higher levels of IL-12 and IFNγ expression were detected in C57BL/6 lung tissue compared to both the 129 groups, with the Th2 cytokines IL-4 and IL-10 expressed at comparable levels to those observed in the latter strain. Thus both C57BL/6 and 129 strain mice appear to mount a mixed Th1/Th2 type response post-vaccination, but with the C57BL/6 group showing higher levels of IFNγ expression. Indeed, it is the C57BL/6 strain which mounts the highest cytokine response at day 20 post-vaccination, correlating with the induction of high levels of immunity.

Cytokine expression in the lung during the effector mechanism is much more polarised in nature than that observed post-vaccination. Resistant C57BL/6 mice express high levels of IFNγ, TNFα and IL-12 message, cytokines typically associated with a Th1-biased response. However, knockout mice show a more Th2-type response, with higher levels of IL-5 mRNA, and lower levels of IFNγ, TNFα and IL-12 expression than C57BL/6 mice. Reduced levels of TNFα in IFNγR$^{-/-}$ mice have been reported previously following BCG infection (Kamijo *et al.,* 1993, Car *et al.,* 1994). WT 129 mice have an intermediate cytokine profile, aptly demonstrating the intermediate resistance measurements obtained for these animals. It is interesting to note that IL-10 and IL-4 levels are equally high in C57BL/6 and knockout mice in post-challenge lung tissue. Although this might not be expected from the traditional

Th1/Th2 dogma, the presence of IL-4 and IL-10 mRNA in lung tissue expressing high levels of IFNγ message has been demonstrated previously (Chapter Four), and indicates the incomplete polarisation of the Th1 response in this model. This similarity is not repeated at the protein level, with C57BL/6 mice displaying IL-4 and IL-10 levels much lower than those observed in both knockout and WT 129 mice (Wilson *et al.,* 1996). The discrepancy may be due to the sensitivity of the RNA versus the protein assays. Alternatively, differences may be induced by *in vitro* culture of isolated BAL cells washed from the airways, compared to detection of mRNA extracted from whole lung tissue. Furthermore, analysis of released cytokine protein in culture supernatants does not account for any protein that is captured and utilised by the cells during the *in vitro* stimulation period.

A repeat of the day 14 time point gave similar data to that obtained above. Although the level of IL-10 mRNA detected in this second exposure of C57BL/6 animals was much higher than that detected in knockout mice, large variations between individuals made this result non-significant. In addition, IL-4 levels in the knockout mice were significantly higher than those detected in 129 WT and C57BL/6 animals, unlike the profile determined during the first time course.

In conclusion, vaccinated IFNγ-$R^{-/-}$ mice mount a Th2-type response when challenged with normal cercariae, unlike C57BL/6 animals which display tendencies to polarise towards Th1 cytokine production over the challenge period. The effects of the inability to respond to IFNγ, to all intents and purposes a complete abrogation of IFNγ, are not as severe as might be expected from previous anti-IFNγ antibody ablation studies (Smythies *et al.,* 1992b), as discussed above with relation to resistance. However, in disagreement with the above data, infection of IFNγ$R^{-/-}$ with *Leishmania major* results in the induction of an IFNγ, Th1 response with no detectable increases in IL-4 expression (Swihart *et al.,* 1994). These differences in cytokine expression between the two parasitic models may be due to the high efficiency of *L. major* in inducing the development of a Th1 population (Swihart *et al.,* 1994, Wang *et al.,* 1994).

A more polarised change in T helper response has been detected in IFNγ knockout mice following introduction of *Schistosoma mansoni* eggs into the lungs, or infection with *L. major* (Wynn *et al.,* 1995b and Wang *et al.,* 1994 respectively). Infection of

IFNγ$^{-/-}$ mice with *L. major* leads to progression of disease in a normally resistant strain, correlating closely with the development of a Th2 reponse (Wang *et al.*, 1994). In addition to increased Th2 cytokine expression, infection of IFNγ$^{-/-}$ mice with *S. mansoni* eggs results in the production of IL-12, indicating that IFNγ is not a prerequisite for the induction of this cytokine in these knockout animals. This result contributes to the debate as to whether IL-12 requires low level IFNγ expression to initiate production, which then acts in a positive feedback loop to induce higher levels of IFNγ release (Trinchieri and Scott, 1994, Trinchieri, 1993, Scott, 1993b, Hsieh *et al.*, 1993). Further ablation studies with anti-cytokine antibodies might reveal the mechanisms responsible for the induction of IL-12 in the absence of IFNγ.

The reduction in resistance observed following exposure of IFNγ-R$^{-/-}$ mice to the irradiated schistosome vaccine appears to be due to structural differences observed in granulomatous lesions around migrating parasite traversing the lungs (Wilson *et al.*, 1996). In C57BL/6 mice the effector response comprises of focus formation, rich in CD4^{+} T cells and macrophages, which blocks parasite exit from the lungs, and hence prevents worm maturation in the liver (reviewed by Smythies *et al.*, 1993). Knockout mice develop elevated pulmonary eosinophilia (Wilson *et al.*, 1996), presumably due to increased levels of IL-5 expression. This cytokine can act as a potent upregulator of VCAM, a ligand for VLA-4 found on eosinophils (Dobrina *et al.*, 1991). The foci which develop in the lungs of knockout mice are loose in nature and contain high numbers of eosinophils, but are apparently ineffective at preventing parasite migration. Furthermore, the lack of cohesion between the cells of these modified foci may be due to reduced levels of the adhesion molecule ICAM-1 detected on CD4^{+} T cells isolated from the lungs of IFNγ-R$^{-/-}$ mice (Coulson, 1995). Similar loose granuloma formation is seen after infection of these mice with BCG, which results in the ineffective containment of this bacterium (Kamijo *et al.*, 1993, Cooper *et al.*, 1993).

The use of knockout mice in the investigation of murine models of parasitic infection provides a valuable tool for analysis of an immune response lacking a certain component. As demonstrated in the above experiments, vaccination and challenge of IFNγ-R$^{-/-}$ mice with *S. mansoni* results in a partial loss of resistance and a shift in the cytokine expression profiles compared to 129 WT and C57BL/6

animals. However, these data also highlight the ability of IFNγ-$R^{-/-}$ mice to adapt to the lack of this cytokine receptor. Thus, future experiments should always be conducted in parallel with anti-cytokine antibody ablations in WT mice. This should ensure that any effects observed in the gene-disrupted animals are related to the missing cytokine, and not due to the instigation of a redundant mechanism. In addition, the back-crossing of the 129 IFNγR knockout mice onto a C57BL/6 background would allow a more precise comparison with the established model. Finally, the continued investigation of mice deficient in other cytokines and cytokine receptors should allow the cytokines necessary for protection to be established.

CHAPTER SEVEN

Concluding Discussion

For the last three decades research into schistosomiasis has centred on various methods of invoking resistance in animal models. The most successful protocol so far is based on percutaneous exposure to radiation-attenuated cercariae, an approach now used routinely to generate resistance to challenge infection in the mouse. Following initial experiments optimising vaccination strategy, more recent research has focused on the immune responses generated during vaccination, and the subsequent effector mechanism against challenge parasites. The ultimate goal of this research is the definition of the induction and effector mechanisms responsible for protection with a view to developing a human vaccine.

The rationale behind the experiments described in this thesis was the characterisation of the cytokines expressed during induction, and execution of the protective response. It is now generally accepted that the slower migration of irradiated cercariae through the LN during vaccination results in the stimulation of a Th1-biased cell population. Upon parasite arrival in the lungs, the schistosome-specific $CD4^+$ population is recruited to this organ and resides there for at least the following ten weeks. The lungs are thus armed against the arrival of normal parasites from any further infection. The effector mechanism consists of focal inflammation around parasites in the lung capillaries and prevents larval exit from this organ (reviewed by Smythies *et al.*, 1993). The results described in the previous chapters encompass the determination of the cytokine profiles expressed during both the induction and effector responses.

The method chosen for cytokine analysis was RT-PCR, a sensitive technique which allows the detection of cytokine mRNA without *in vitro* stimulation. Thus the experiments investigate the events taking place *in vivo* at time of sacrifice, and are not subject to artifacts in the way that cytokine protein analysis can be when isolated cells undergo secondary stimulation in culture. This type of analysis provides a different viewpoint to that obtained from protein detection and each method has its own range of advantages and pitfalls. Disadvantages of RT-PCR include the lack of true quantitation, and the fact that although RT-PCR provides an incredibly sensitive detection system for dissecting cytokine expression, mRNA is not the active form of a cytokine and thus message presence does not necessarily relate to *in vivo* protein release and an immunological response. However, the majority of cytokine messages

are thought to be inducible and unstable, hence mRNA should be a good indicator of protein production. This has been confirmed in part by the similarity between the results described in this thesis and previously published work detailing cytokine release upon secondary stimulation of LN and BAL cells *in vitro* (Pemberton *et al.*, 1991, Smythies *et al.*, 1992a, 1996a). A further advantage is that many cytokines for which protein assay reagents were unobtainable at the time of assay can be analysed by RT-PCR, for example IL-12, IL-13 and RANTES, since cDNA sequences for primer design are more readily available. Described below is a summary of the immune responses exhibited by vaccinated and challenged mice with reference to the cytokine data reported in this thesis, followed by suggestions for further research and possible vaccine strategies.

The Induction Response

Previous studies have demonstrated the importance of the generation of a Th1 cytokine-producing cell population in the LN of vaccinated C57BL/6 mice in order to attain resistance. Furthermore, culture of broncho-alveolar lavage cells from vaccinated mice results in the secretion of cytokine profiles similar to those detected upon culture of LN cells. Thus it seems that the population stimulated to expand in the LN is recruited to the lungs upon parasite arrival in this organ. The data presented in chapters three and four support these earlier findings describing cytokine secretion profiles from LN and lung cells in culture.

The production of IFNγ in the sdLN after vaccination has been demonstrated at the protein level by *in vitro* cell culture. Data presented in chapter three confirms that this occurs *in vivo* with high levels of IFNγ expression detected at day 2 after exposure. In addition, IL-2, and the Th1-inducing cytokine IL-12, both exhibit upregulated expression at this time, coincident with the arrival of the first migrating larvae in the LN. Although Th2 cytokines such as IL-4 and IL-10 are also detectable, peak levels of these messages occur at later time points compared to Th1 cytokine expression. These profiles indicate that the Th1 cell population may develop and expand rapidly upon the arrival of parasite antigen in the LN, with a slight Th2 response developing later, delayed possibly as a consequence of the high levels of Th1 cytokine expression at the earlier time points. The decreases observed in the

expression of some cytokines, such as IL-10, IL-12 and IL-13, in the LN to levels well below naive baselines are inexplicable at present. It is possible that a dilution effect of specific cytokine mRNA occurs as a consequence of an influx of B cells and their associated mRNA. Although, analysis of Ig μ chain mRNA failed to show this, decreasing levels of CD4 mRNA tend to indicate that a dilution of T cells does occur following vaccination. Alternatively, basal expression of these cytokines may be turned off by downregulatory factors such as TGFβ; indeed the involvement of inhibitory cytokines in this model is an area requiring further investigation.

The immunological factors responsible for the preferential induction and expansion of a Th1 population by irradiated larvae remain unclear. Antigen load, APC-type, the cytokine environment and costimulatory molecules present at the time of primary stimulation have all been implicated in the development of T helper subsets (reviewed by Mosmann and Sad, 1996, Reiner and Locksley, 1995). One of the major advantages of the attenuated *S. mansoni* vaccine is the self-delivery of parasite antigens, as a consequence of migration, to a site where primary T cell stimulation and expansion can occur. Thus, although the exact mechanisms required to invoke a Th1 response are not yet understood, it is known that the LN play an important role in the induction of immunity.

It is well documented that a cellular population bearing Th1 characteristics is recruited to the lungs upon the arrival of vaccinating parasites (reviewed by Smythies *et al.,* 1993). In order to mount a rapid, efficient response against challenge parasites as they arrive in the lungs, the cells resident there require a phenotype indicative of previous, specific activation. These criteria are met by the $CD4^+$ T cells recoverable by BAL following vaccination. Upon secondary stimulation with parasite antigen *in vitro*, these cells can secrete higher levels of IFNγ than splenocytes from the same individual, demonstrating the recall nature of the response (Smythies *et al.,* 1992a). Furthermore, $CD4^+$ cells recovered from the airways bear markers associated with the short term memory phenotype (Coulson and Wilson, 1993). Thus previous studies have defined the Th1 cytokine-secretion phenotype of these cells, with profiles consisting predominantly of IFNγ production with some IL-3 and little IL-4.

Building upon established data, RT-PCR analysis of whole lung tissue following vaccination revealed a more mixed cytokine response with both Th1 and Th2

cytokines readily detectable and peaking in unison (Chapter Four). The discrepancies observed between this and previously published reports are probably due to the differing sensitivity levels of the protein assays and the PCR-based technique used in this study. The mixed response peaks at day 21 post-vaccination for all cytokines under test, corresponding to maximum focus formation around vaccinating schistosomula trapped in the lungs (Mastin *et al.,* 1985a). Analysis of RNA from individual cell populations recoverable by BAL and sorted by magnetic beading indicates that the majority of cytokine expression takes place in the $CD4^+$ T cell population. However, alveolar macrophages also contribute to the detectable levels of IFNγ, IL-12 and IL-10 messages.

The mechanisms by which the cell population induced in the sdLN is recruited to, and becomes resident in, the lungs are still not understood. However, the initial signal is undoubtedly the arrival and persistence of irradiated parasites in this organ. Presumably antigens from the larvae are able to stimulate the release of specific cytokines required for the upregulation of adhesion molecules on the lung endothelium. Additionally, chemokines, such as RANTES, may play a role in this recruitment. Research into human inflammatory disease has demonstrated a role for RANTES in DTH responses due to its ability to recruit memory T cells and monocytes (Rathanaswami *et al.,* 1993). Furthermore, this chemokine can attract human eosinophils *in vitro*, possibly offering an explanation for the high level of eosinophilia observed in the foci formed around migrating parasites trapped in the lungs of vaccinated mice. RANTES expression is upregulated by IFNγ, IL-1β and TNFα, typical inflammatory cytokines which are all present in the lung tissue of vaccinated mice. RT-PCR analysis with RANTES-specific primers has demonstrated that this molecule is upregulated in the lungs at the time of peak cytokine responses. Thus RANTES may play a role in recruitment to the lungs of the $CD4^+$ T cell population generated in the LN, an hypothesis easily investigated by the *in vivo* administration of anti-RANTES antibodies following vaccination.

Investigation of the induction mechanism in genetically different strains of mice can also aid in the understanding of the protective immune response required for resistance. This is especially relevant when the strains are known to exhibit different levels of resistance upon exposure to the same parasite, presumably via the

generation of distinct immune responses. Analysis of the cytokine profiles expressed in lung and LN tissue from BALB/c and C57BL/6 mice following vaccination with irradiated cercariae of *S. mansoni* implied a correlation between Th2 expression and lower resistance levels (Chapter Five). Vaccination of these two strains resulted in a similar, albeit less extreme, polarisation of the T helper population than that described for *Leishmania major* (Heinzel *et al.,* 1989, Scott *et al.,* 1989, Reiner and Locksley, 1995). The correlation of lower resistance levels with Th2 cytokine expression was again demonstrated by the vaccination of mice lacking expression of the IFNγ receptor gene; 129 IFNγR-knockout mice exhibited a much reduced level of protection compared to C57BL/6 and 129 WT mice. Furthermore, following vaccination these animals expressed lower levels of IFNγ and IL-12 mRNA in lung and LN tissue than WT mice. This altered cytokine expression can be related to the protection obtained following challenge infection of knockout and control mice (see below).

The Effector Response

The effector mechanism in mice exposed to the attenuated cercariae vaccine may be successful because it operates in a central organ through which all parasites must pass before systemic distribution. Similarly, trafficking lymphocytes also pass through the lungs on their way around the body, and thus this organ is ideally situated for cell recruitment. Consequently, cells resident in the lungs from vaccination can attack migrating challenge larvae in a relatively confined space. Furthermore, parasites must remain in the alveolar capillaries for some days to undergo transformation into lung stage worms in order to continue migration. Hence parasites are, in effect, a sitting target for the immune response at this time.

Analysis of the cytokine profiles induced in the sdLN following challenge has highlighted the lack of importance of secondary stimulation in this organ. As normal parasites pass through the LN of previously primed mice after a challenge infection Th1 cytokine expression is much reduced in comparison to the levels detected post-vaccination; in contrast Th2 cytokine expression is drastically increased. This suppression of any anamnestic IFNγ response has been reported at the protein level (Pemberton and Wilson, 1995) and may reflect anergy of a select cell population.

This concept is intriguing, and the mechanism by which Th1 cells are rendered unresponsive, possibly by the expression of inhibitory cytokines such as TGFβ or costimulatory factors, warrants further investigation.

Upon investigation of the cytokine profiles induced in the lungs after challenge it was found that more polarised Th1 expression could be detected compared to that observed in the induction mechanism (Chapter Four). This is in agreement with published data detailing the dominance of IFNγ in the effector response. In contrast to the major Th1 and inflammatory cytokine mRNAs, Th2 message levels did not approach those attained post-vaccination. This resulted in an overall increase in the ratio of Th1:Th2 cytokine expression, and hence a shift towards Th1 polarisation.

As observed post-vaccination, the cytokines required for RANTES expression are also upregulated following challenge. The cytokine profiles detected are typical of a DTH inflammation, and as described above, RANTES has been shown to play a role in cell recruitment during this type of response. However, previous studies have implied that no further cell recruitment is required after challenge to mediate protection; whole body irradiation prior to challenge has no effect on resistance (Aitken *et al.,* 1987, Vignali *et al.,* 1988). Although no additional cell population is necessary for the effector mechanism to operate, some reports describe a secondary cellular infiltration into the lungs post-challenge, and RANTES may play a part in this event.

It has previously been shown, by the administration of anti-IFNγ antibody during the challenge period, that IFNγ plays a pivotal role in the effector mechanism (Smythies *et al.,* 1992b). This treatment results in a 90% abrogation of resistance, possibly due to the inability of mice to form foci efficient at preventing migration. The generation of IFNγ receptor-deficient mice has permitted further investigation of the effector response in the absence of this cytokine (Wilson *et al.,* 1996). By vaccination and challenge of mice harbouring a disrupted IFNγ receptor gene, it was hoped to clarify whether the remaining 10% immunity in anti-IFNγ antibody-treated mice was due to suboptimal ablation, or resistance via another mechanism. Although cytokine mRNA analysis of lung tissue from IFNγR$^{-/-}$ animals demonstrated higher IFNγ profiles than expected, an increase in Th2 expression was observed (Chapter Six). In addition, resistance levels were reduced to 20% in IFNγR$^{-/-}$ mice, but not

completely ablated implying the presence of a normally redundant mechanism which can afford limited protection in the absence of the IFNγ receptor. Furthermore, the IFNγR$^{-/-}$ mice were generated on a 129/Sv/Ev genetic background, and WT animals of this type were found to exhibit higher levels of Th2 expression, and protective responses 20% lower, than those achieved after vaccination of C57BL/6 mice. Thus, comparisons of vaccination and challenge in gene-disrupted animals based on this intermediate-responder strain with the more commonly used high-responder C57BL/6 strain are limited.

In conclusion, it appears that expression of Th2 cytokines in the absence of high levels of IFNγ can result in lower levels of protection. Suggestions for future work to reduce the levels of Th2 cytokine expression during the effector mechanism, and possible implications for vaccine design are discussed below.

Vaccination Strategies

The discovery of IFNγ-inducing cytokines such as IL-12 and the recently isolated IGIF (IFNγ-inducing factor, Okamura *et al.,* 1995), has resulted in much excitement in the field of vaccine research. The incorporation of these cytokines into vaccination regimes may boost the IFNγ response, and hence resistance to many infections requiring a Th1-based response for cure: for example leishmaniasis, schistosomiasis and leprosy. Although IL-12 administered with the irradiated cercariae vaccine can increase resistance to 80% (Wynn *et al.,* 1995a), this is only a 10% improvement on the values of 70-75% more routinely obtained with parasites alone. Coincident with this small increase in protection, a reduction in the Th2 cytokine expression levels was observed. However, no increase in the already high Th1 cytokine expression could be induced, implying that Th1 responses are already maximal and that residual Th2 activity might be responsible for the sub-optimal protection obtained in this model.

When considered together, results from the vaccination of 129 WT mice described above and the IL-12 immunisation protocols, would seem to indicate that the presence of even low level Th2 cell activity can reduce protection. Thus, research into potential vaccines might benefit from investigation into strategies for reducing the residual Th2 cytokine expression, with a view to promoting higher resistance

levels. This might be achieved by the study of the irradiated vaccine in IL-4 and IL-10 gene-disrupted mice, or the combined ablation of these cytokines using anti-cytokine antibodies or double-knockouts. Furthermore, analysis of the effector responses in low- and mid-responder strains of mice, such as BALB/c, might highlight the effects that any differences seen in cytokine expression post-vaccination have on protection. In this way, the vital cytokines and cellular components of a protective immune response can be defined and applied to vaccine design.

Future vaccines for immunising against *Schistosoma mansoni* infection may include the combined administration of a protective antigen and therapeutic cytokine to direct the development of a preferred T helper response. This rationale has been tested to some extent by the vaccination of mice with IL-12 and *S. mansoni* eggs (Wynn *et al.,* 1995c). Administration of these two factors results not in a reduction in worm and egg burdens, but a significant decrease in the level of tissue fibrosis observed in the liver. Thus this vaccine protects against the pathology of the disease rather than infection. Although not ideal, since egg and worm burdens can still increase in the host and hence no reductions in parasite transmission can occur, a vaccine to target pathology may provide an alternative strategy to reduce the development of debilitating disease. As with all therapeutic cytokine applications, the toxicity of IL-12 must be thoroughly examined before administration to the human population. In addition, predisposition of a population towards a Th1 response in areas where many infections are dependent upon the generation of a Th2 response, for example gut nematode infestations, may result in increasing severity of another endemic pathogen (Curry *et al.,* 1995).

It is still not known which *Schistosoma mansoni* antigens trigger the protective Th1 response in the LN and lungs of vaccinated mice. Investigations into potential protective antigens have been underway for many years, but have failed as yet to isolate a strong vaccine candidate able to promote similar levels of protection to those seen with the live attenuated vaccine (Richter *et al.,* 1995). A cocktail of antigens and cytokines might be required to invoke the same intensity of response generated with irradiated cercariae. In addition, any potential vaccine based on the current model must be targetted to a site where antigen presentation to $CD4^+$ T cells is optimal to promote expansion of a Th1 cytokine-secreting population, followed by

recruitment of this cell population to the lungs to arm against migrating parasites. This requirement may provide problems in administration of a vaccine, although a possible solution may lie in intradermal injection of antigen and cytokine followed by nasal or oral inhalation via an emulsion of the same mix. Alternatively, the antigen and cytokine might be administered with an adjuvent such as an immune stimulatory complex (ISCOMS). These complexes have been shown to prime a wide range of humoral and cell mediated effector immune mechanisms *in vivo* (Maloy *et al.,* 1995), and administration of antigen via ISCOMS can act to promote both a local and systemic response.

Future Work

There are still many questions to be answered with regards to the immune mechanisms acting in the irradiated vaccine model, and their application to the development of a human vaccine. The continued research into the immune response generated during the induction and effector mechanisms following exposure to *S. mansoni* cercariae will highlight the immunological parameters essential for resistance. In addition, an explanation for the sub-optimal levels of protection obtained with the irradiated vaccine at present must be found in order to elevate immunity. The scope for future work is therefore extensive.

Analysis of the cytokine production from sorted LN cell populations will help elucidate which cell types are responsible for the cytokine production leading to induction of the Th1 population. Furthermore, analysis of the LN response following challenge might highlight potential downregulatory mechanisms of the Th1 cytokine production observed in primed mice post-challenge. One such mechanism may be stimulation in the absence of the CD28/B7 ligand pair, which has been shown to anergise Th1 cell clones (Gajewski *et al.,* 1994, Jenkins, 1994, Croft and Swain, 1995). Alternatively, interaction with a certain APC or costimulatory molecule such as B7-2, which is constitutively expressed on B cells and reportedly results in the development and expansion of the Th2 subset (Hathcock *et al.,* 1994, Kuchroo *et al.,* 1995), may be responsible for the high expression of IL-4 post-challenge. Further studies on the role of costimulatory and APC factors in the induction mechanism may lead to the ability to manipulate the differentiation of a response towards the

Th1 phenotype. Knowledge of this kind would be of great importance in many vaccination strategies aimed at parasitic infection.

Vaccination of various cytokine gene-disrupted mice may provide an increased understanding of the protective response by permitting analysis of the effector mechanism in the complete absence of certain key cytokines. Furthermore, selective breeding of the gene-disruption on to a C57BL/6 background will allow more accurate comparisons between the established model and infection in the absence of a given cytokine or receptor. In addition, antibody ablation of the cytokine under analysis should be carried out in parallel to confirm that results obtained in knockout mice are due solely to the loss of that cytokine, and not to redundant mechanisms altering the *in vivo* pattern normally seen in wild type mice. Further studies are required in this area to clarify the key cytokines involved in the generation of optimal resistance.

Final Summary

The results described in these chapters support the current Th1-based theory of protection in the irradiated vaccine model. The development of an RT-PCR-based technique has allowed the true *in vivo* cytokine mRNA profiles to be appraised during both induction and protection. Research carried out on lung and LN tissue following vaccination and challenge has uncovered the presence of a mixed cytokine profile during induction, with the dominance of Th1-type cytokines in the effector mechanism. Now that the baseline cytokine mRNA levels for this model have been established, the foundations are in place for manipulation of the system in an attempt to increase resistance. Further research will advance the understanding of the immune components necessary for optimal protection. This knowledge may provide a significant step towards the development of a successful vaccine, the ultimate goal of schistosome research.

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