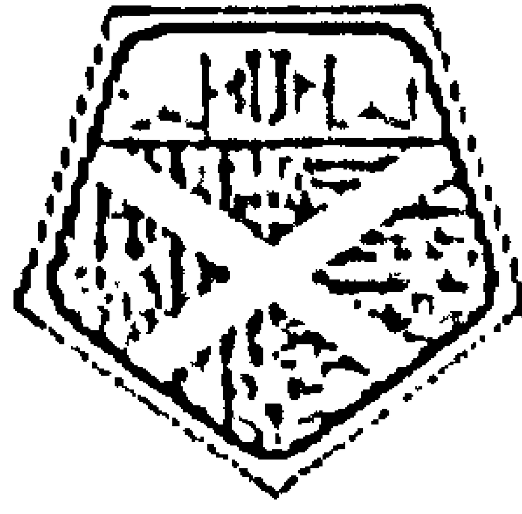

**University of Strathclyde**

Department of Immunology

# Factors influencing the development of protective and pathological responses to *Trichinella spiralis* infection

By

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A thesis presented in fulfillment of the requirements

for the degree of Doctor of Philosophy

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## Contents

Title Page i

Acknowledgements iii

Contents iv

List of Figures xii

List of Abbreviations xviii

Abstract xxi

**1 General Introduction** **1**

*1.1 Introduction* *2*

*1.2 Potential effects of GI nematodes on unrelated immune responses* *5*

*1.3 Vaccination against helminthiasis* *7*

*1.4 Trichinosis* *8*

*1.4.1 Epidemiology* *8*

*1.4.2 Life cycle* *9*

*1.5 Gastrointestinal pathology associated with* T. spiralis *infection.* *11*

*1.6 The Immune response to GI nematode infection.* *15*

*1.6.1 Introduction* *15*

*1.6.2 T helper 2 responses* *16*

*1.6.3 Costimulation by B7-1, B7-2 and CD28* *18*

*1.6.4 Novel costimulatory molecules* *19*

1.6.4.1 OX40 protein and OX40 ligand 19

1.6.4.2 4-1BB protein and 4-1BB ligand 20

1.6.4.3 Inducible costimulator protein 21

1.6.4.4 Heat-stable antigen 21

*1.6.5 Mast cells* *22*

*1.6.6 Eosinophils* *23*

*1.6.7 Goblet cells* *24*

*1.6.8 'Weep and Sweep' theory of expulsion* *26*

*1.6.9 Antibody responses* *27*

*1.7 Aims and experimental design* *28*

**2 Materials and Methods.** **30**

*2.1 Animals.* *31*

*2.2 Parasites.* *32*

*2.3 Preparation of parasite antigen.* *33*

*2.4 Anti-ICOS antibody treatment.* *33*

*2.5 OX40 and OX40 ligand fusion protein treatment.* *33*

*2.6 PAR-2 activating peptide treatment.* *34*

*2.7 The collection of sera samples.* *34*

*2.8 Assessment of enteropathy.* *34*

*2. 9 Enumeration of mucosal mast cells.* *35*

*2.10 Mucosal mast cell protease-1 ELISA.* *36*

*2.11* *Cytokine production* ex vivo. *37*

*2.12* *Measurement of cytokine levels in cell culture supernatants.* *38*

*2.13* *Measurement of total IgE.* *40*

*2.14* *Measurement of parasite specific IgG1 and IgG2a.* *41*

*2.15* *Statistical analysis.* *42*

**3 The role of IL-4, IL-13 and IL-4Rα in the development of protective and pathological responses to *T. spiralis*.** **43**

*3.1* *Introduction* *44*

*3.2* *Results* *52*

*3.2.1* *Expulsion of* T. spiralis *is significantly delayed in the absence of IL-4, IL-13 and IL-4Rα.* *52*

*3.2.2* *Reduced enteropathy in the absence of IL-13 but not IL-4.* *56*

*3.2.3* *Mast cell numbers and serum mMCP-1 titres are reduced in the absence of IL-4 but not IL-13.* *65*

*3.2.4* *IFN-γ responses were increased in the absence of IL-4 in BALB/c mice but not in C57BL/6 mice.* *72*

*3.2.5* *Serum total IgE and antigen specific IgG1 titres are significantly reduced in the absence of IL-4, IL-13 and IL-4Rα.* *81*

3.2.6 *Expulsion of* T. spiralis *is not significantly effected by the absence of the IL-4Rα from cells of the macrophage/ neutrophil lineage* 90

3.2.7 *Villus atrophy following* T. spiralis *is increased in the absence of signalling through the IL-4Rα in cells of the macrophage/ neutrophil lineage.* 94

3.2.8 *The development of mastocytosis is not effected by the deficiency in IL-4Rα in cells of the macrophage /neutrophil lineage during infection with* T. spiralis. 100

3.2.9 *Cytokine production by MLN cells stimulated ex-vivo with TAg was not altered by deficiency in IL-4Rα from cells of the macrophage /neutrophil lineage.* 103

3.2.10 *Antigen specific IgG1 levels in the sera were reduced by deficiency in IL-4Rα from cells of the macrophage /neutrophil lineage* 107

3.3 *Discussion* 111

**4 The role of co-stimulatory signals in protective and pathological immune responses induced by infection with *Trichinella spiralis*.** **120**

4.1 *Introduction* 121

4.2 *Results* 127

| | | |
|---|---|---|
| *4.2.1* | *Blocking ICOS has no effect on intestinal worm burdens but reduces muscle larvae loads.* | *127* |
| *4.2.2* | *Blocking ICOS protects against intestinal pathology.* | *131* |
| *4.2.3* | *Serum mMCP-1 but not mastocytosis was increased by anti-ICOS treatment.* | *135* |
| *4.2.4* | *Anti-ICOS antibody treatment reduces IL-4 and IL-5 but increases IFN-γ, IL-10 and IL-13 secretion by MLN cells following infection with* T. spiralis. | *138* |
| *4.2.5* | *Serum antibody levels are not significantly affected by anti-ICOS treatment.* | *141* |
| *4.2.6* | *OX40-Ig and OX40L-Ig treatment does not effect the establishment in, or expulsion from the small intestine of adult worms or the accumulation of muscle larvae.* | *144* |
| *4.2.7* | *OX40-Ig or OX40L-Ig treatment does not significantly alter the development of villus atrophy or crypt hyperplasia but OX40-Ig treatment delays the reduction oedema at day 14 p.i.* | *148* |
| *4.2.8* | *OX40-Ig treatment significantly enhances* T. spiralis *induced mucosal mastocytosis and but does not effect serum mMCP-1 levels.* | *152* |

4.2.9 *OX40L-Ig treatment enhances IL-4, IL-13 and IL-18 but not IFN-γ secretion by mesenteric lymph node cells stimulated ex-vivo with TAg.* *155*

4.2.10 *OX40L-Ig treatment increases total IgE but does not effect TAg specific IgG1 or IgG2a titres.* *158*

4.3 *Discussion* *161*

**5 The role of transmembrane TNFα,soluble TNFα and LTα in the development of enteropathy and protective responses following infection with *Trichinella spiralis*.** **174**

5.1 *Introduction* *175*

5.2 *Results.* *182*

5.2.1 *Expulsion of adult* T. spiralis *is delayed in the absence of soluble TNFα and LTα.* *182*

5.2.2 *Enteropathy is enhanced in the absence of soluble TNFα and LTα.* *186*

5.2.3 *Mucosal mastocytosis is significantly reduced in the absence of soluble TNFα and LTα.* *190*

5.2.4 *In the absence of soluble TNFα and LTα the production of IL-4 is reduced but production of IL-13 and IFNγ are unaffected.* *193*

5.2.5 *Total serum IgE is significantly reduced in the absence of soluble TNFα and LTα.* 196

5.3 *Discussion.* 200

**6 Protease activated receptor-2 plays a role in the development of enteropathy but not in the expulsion of *T. spiralis* from the small intestine.** **208**

6.1 *Introduction* 209

6.2 *Results* 216

6.2.1 T. spiralis *expulsion from the small intestine and the accumulation of muscle larvae is unaltered in PAR-2 -/- mice compared to wild type controls.* 216

6.2.2 T. spiralis *induced villus atrophy is reduced while crypt hyperplasia is in creased in PAR2-/- mice compared to wild type controls.* 219

6.2.3 *Mastocytosis developed normally in PAR2-/- mice compared to wild type mice during infection however degranulation, as indicated by serum mMCP-1 levels, was increased.* 225

6.2.4 *Cytokine production by MLN cells stimulated ex-vivo with TAg is not altered in PAR2-/- mice compared to wild type.* 228

6.2.5 *Total IgE and TAg specific IgG1 levels in the sera of PAR-2-/- mice are reduced compared to wild type mice at day 16 p.i.* 231

6.2.6 T. spiralis *expulsion from the small intestine is not affected by PAR-2 agonist treatment.* 234

6.2.7 *PAR-2 Agonist treatment does not alter the development of enteropathy.* 237

6.2.8 *The development of peak mastocytosis is accelerated by ASKH-95 treatment but degranulation is not significantly altered by agonist treatment.* 243

6.2.9 *Cytokine production 14 dpi is not significantly affected by PAR-2 agonist treatment.* 247

6.2.10 *Total IgE and TAg specific IgG1 and IgG2a responses are not significantly different in PAR-2 agonist treated mice compared to scramble peptide treated or untreated controls.* 250

6.3 *Discussion.* 253

**7 General Discussion.** **261**

Bibliography 272

Appendix 322

## List of Figures

FIGURE 1.1 The life cycle of *T. spiralis* 10

FIGURE 1.2 The enteropathy associated with the intestinal phase of *T. spiralis* infection. 12

FIGURE 3.1 IL-4Rα, IL-13Rα1 and IL-13Rα2 complexes. 46

FIGURE 3.2 The role of host strain, IL-4, IL-13 and IL-4Rα in the expulsion of *T. spiralis* from the intestine. 54

FIGURE 3.3 The role of host strain, IL-4, IL-13 and IL-4Rα in the development of enteropathy. 59

FIGURE 3.4 The role of host strain, IL-4, IL-13 and IL-4Rα in the increase in proliferation in crypt epithelium following infection with *T. spiralis*. 61

FIGURE 3.5 The role of host strain, IL-4, IL-13 and IL-4Rα in the intestinal oedema following infection with *T. spiralis*. 63

FIGURE 3.6 The role of host strain, IL-4, IL-13 and IL-4Rα in the development of mucosal mastocytosis following infection with *T. spiralis*. 68

FIGURE 3.7 The role of host strain, IL-4, IL-13 and IL-4Rα in the mast cell degranulation following infection with *T. spiralis*. 70

FIGURE 3.8 Role of host strain, IL-4, IL-13 and IL-4Rα on mesenteric lymph node cell IL-4 secretion following infection with *T. spiralis*. 75

FIGURE 3.9 Role of host strain, IL-4, IL-13 and IL-4Rα in ex vivo mesenteric lymph node cell IL-13 secretion following infection with *T. spiralis*. 77

FIGURE 3.10 Role of host strain, IL-4, IL-13 and IL-4Rα on mesenteric lymph node cell IFNγ secretion following infection with *T. spiralis* 79

FIGURE 3.11 The role of host strain, IL-4, IL-13 and IL-4Rα in total IgE responses following infection with *T. spiralis*. 84

FIGURE 3.12 The role of host strain, IL-4, IL-13 and IL-4Rα in Antigen specific IgG1 responses following infection with *T. spiralis*. 86

FIGURE 3.13 The role of host strain, IL-4, IL-13 and IL-4Rα in Antigen specific IgG2a responses following infection with *T. spiralis*. 88

FIGURE 3.14 The role of IL-4Rα signalling to macrophages and neutrophils in the expulsion of *T. spiralis* from the small intestine and in the accumulation of muscle larvae. 92

FIGURE 3.15 The role of IL-4Rα signalling to macrophages and neutrophils in the development of enteropathy following infection with *T. spiralis*. 96

FIGURE 3.16 The role of IL-4Rα signalling to cells of the neutrophil/macrophage lineage in the development of intestinal oedema following infection with *T. spiralis*. 98

FIGURE 3.17 The role of IL-4Rα signalling to macrophages and neutrophils in the development of mastocytosis and mast cell degranulation following infection with *T. spiralis.* 101

FIGURE 3.18 The role of IL-4Rα signalling to macrophages and neutrophils in the development of mesenteric lymph node cytokine responses. 105

FIGURE 3.19 The role of IL-4Rα signalling to macrophages and neutrophils in the development of IgE, IgG1 and IgG2a responses to *T. spiralis* infection. 109

FIGURE 4.1 The effect of anti-ICOS treatment on the expulsion of *T. spiralis* from the intestine and the accumulation of muscle larvae. 129

FIGURE 4.2 The effect of anti-ICOS treatment on the development of enteropathy following infection with *T. spiralis.* 133

FIGURE 4.3 The effect of anti-ICOS treatment on the development of mucosal mastocytosis and mast cell degranulation following *T. spiralis.* 136

FIGURE 4.4 The effect of anti-ICOS treatment on MLN cytokine secretion following infection with *T. spiralis.* 139

FIGURE 4.5 The effect of anti-ICOS treatment on antibody responses following infection with *T. spiralis.* 142

FIGURE 4.6 The effect of OX40-Ig and OX40L-Ig treatment on the expulsion of *T. spiralis* from the intestine and the accumulation of muscle larvae. 146

| | | |
|---|---|---|
| FIGURE 4.7 | The effect of OX40-Ig and OX40L-Ig treatment on the development of enteropathy following infection with *T. spiralis.* | 150 |
| FIGURE 4.8 | The effect of OX40-Ig and OX40L-Ig treatment on the development of mucosal mastocytosis and mast cell degranulation following *T. spiralis.* | 153 |
| FIGURE 4.9 | The effect of OX40-Ig and OX40L-Ig treatment on MLN cytokine secretion following infection with *T. spiralis.* | 156 |
| FIGURE 4.10 | The effect of OX40-Ig and OX40L-Ig treatment on antibody responses following infection with *T. spiralis.* | 159 |
| FIGURE 5.1 | The role of LTα, sTNFα and tmTNFα in the expulsion of *T. spiralis* from the intestine and the accumulation of muscle larvae. | 184 |
| FIGURE 5.2 | The role of LTα, sTNFα and tmTNFα in the development of enteropathy following infection with *T. spiralis.* | 188 |
| FIGURE 5.3 | The role of LTα, sTNFα and tmTNFα in the development of mucosal mastocytosis following infection with *T. spiralis.* | 191 |
| FIGURE 5.4 | Role of LTα, sTNFα and tmTNFα on spleen cell cytokine secretion following infection with *T. spiralis.* | 194 |
| FIGURE 5.5 | Role of LTα, sTNFα, tmTNFα in antibody responses following infection with *T. spiralis.* | 198 |
| FIGURE 6.1 | Novel method of activation of PAR-2 by serine proteases. | 210 |
| FIGURE 6.2 | The activation of PAR-2 by agonist peptides in the absence of proteolytic cleavage. | 212 |

FIGURE 6.3 Effect of PAR-2 deficiency on the expulsion of *T. spiralis* from the intestine and the accumulation of muscle larvae. 217

FIGURE 6.4 The role of PAR-2 in the development of intestinal oedema following infection with *T. spiralis*. 221

FIGURE 6.5 The role of PAR-2 in the development of enteropathy following infection with *T. spiralis*. 223

FIGURE 6.6 The role of PAR-2 in the development of mucosal mastocytosis and on serum mMCP-1 titres following infection with *T. spiralis*. 226

FIGURE 6.7 The role of PAR-2 in the secretion of the cytokines IL-4, IL-13, and IFNγ from MLN cells stimulated *ex vivo* with TAg. 229

FIGURE 6.8 The role of PAR-2 in the development of antibody responses following infection with *T. spiralis*. 232

FIGURE 6.9 The effect of treatment with PAR-2 agonists on the expulsion of *T. spiralis* from the small intestine. 235

FIGURE 6.10 Effect of PAR-2 agonist treatment on the development of enteropathy following infection with *T. spiralis*. 239

FIGURE 6.11 The effect of PAR-2 agonist treatment on the development of oedema following infection with *T. spiralis*. 241

FIGURE 6.12 The effect of PAR-2 agonist treatment on the development of mucosal mastocytosis and on serum mMCP-1 titres following infection with *T. spiralis*. 245

FIGURE 6.13 The role of PAR-2 in the secretion of the cytokines IL-4, IL-13, and IFNγ from MLN cells stimulated *ex vivo* with TAg. 248

FIGURE 6.14 The effect of PAR-2 agonist treatment on the development of antibody responses following infection with *T. spiralis.* 251

FIGURE 6.15 Possible role for PAR-2 in the development of enteropathy following infection with *T. spiralis.* 258

FIGURE 7.1 Overview of the interaction of various factors involved in the development of enteropathy and the expulsion of *T. spiralis.* 269

## Abbreviations List

| | |
|---|---|
| -/- | Deficient |
| ALUM | Aluminium hydroxide gel |
| APC | Antigen presenting cell |
| ASKH-95 | 2-furoyl- Leu-Ile-Gly-Lys-Val |
| B7-RP | B7-related protein |
| CD | Cluster of differentiation |
| CHO | Chinese hamster ovary |
| ConA | Concanavalin A |
| Crelox | Macrophage/neutrophil lineage specific IL-4Rα -/- mice |
| CSF | Cerebro-spinal fluid |
| CTL | Cytotoxic lymphocyte |
| CTLA | Cytotoxic lymphocyte antigen |
| DC | Dendritic cell |
| EAE | Experimental allergic encephalomyelitis |
| ELISA | Enzyme linked immunosorbent assay |
| FCS | Foetal calf serum |
| GALT | Gut associated lymphoid tissue |
| GvHD | Graft versus host disease |
| HRP | Horse radish peroxidase |
| i.p. | Intraperitoneal |

| | |
|---|---|
| i.v. | Intravenous |
| IBD | Inflammatory bowel disease |
| ICOS | Inducible costimulator protein |
| IFN-γ | Interferon-gamma |
| Ig | Immunoglobin |
| IL- | Interleukin- |
| iNOS | Inducible nitric oxide synthase |
| L | Ligand |
| LPS | Lipopolysaccaride |
| LRGILS-$NH_2$ | Leu-Arg-Gly-Ile-Leu-Ser-amide |
| LT | Lymphotoxin |
| MHC | Major histocompatability complex |
| MLN | Mesenteric lymph node |
| mMCP | Mucosal mast cell protease |
| NK | Natural killer |
| OD | Optical density |
| p.i. | Post infection |
| PAR | Protease activated receptor |
| PBS | Phosphate buffered saline |
| R | Receptor |
| SCF | Stem cell factor |
| SCID | Severe combined immunodeficiency |

| | |
|---|---|
| SEM | Standard error of the mean |
| SLIGRL-$NH_2$ | Ser-Leu-Ile-Gly-Arg-Leu-amide |
| Stat | Signal transducer and activator of transcription |
| TACE | Tumour necrosis factor alpha converting enzyme |
| TAg | *Trichinella* antigen |
| TFF | Trefoil factor family |
| tg | Transgenic |
| Th | T helper |
| tm | Transmembrane |
| TMB | 3,3' 5,5' -Tetramethylbenzidine |
| TNBS | 2,4,6-trinitrobenzene-sulphonic acid |
| TNF | Tumour necrosis factor |
| VCU | Villus crypt unit |

**Abstract.**

This thesis confirms the importance of Th2 responses in the development of protective and pathological responses to *T. spiralis* infection and shows that the role of IL-4 in the development of enteropathy depends on host mouse strain. Conversely a role in Th2 cytokine signalling to cells of the macrophage/neutrophil lineage in limiting enteropathy was also demonstrated. This thesis also demonstrated that co-stimulation via ICOS and OX40 can modulate the development of Th2 responses and the development of *T. spiralis* induced enteropathy and mastocytosis. The failure of TNFα/LTα -/- mice to expel *T. spiralis* from the small intestine despite developing a more severe enteropathy, this suggests that TNFα or LTα may play important protective roles in both expulsion and in limiting enteropathy. TNFα/LTα -/- mice also failed to develop mucosal mastocytosis suggesting that enteropathy in these mice is mast cell independent. It was also shown that signalling via the novel receptor PAR-2 plays a role in the development of enteropathy but not in the expulsion of *T. spiralis*, and was not required for the development of mucosal mastocytosis but enhanced mast cell degranulation. Thus, in conclusion this thesis provides further evidence that the expulsion of *T. spiralis* is not a direct result of the development of enteropathy and that different mechanisms are responsible for parasite expulsion and the development of enteropathy.

# Chapter One

## General Introduction

# 1 General Introduction.

## *1.1 Introduction*

Gastrointestinal nematodes pose a significant health problem in the developing world, with an estimated one third of the world's population infected (Crompton, 1999). The four most important species of gastrointestinal (GI) nematodes infecting humans are *Ascaris lumbricoides, Trichuris trichuria* and the hookworms *Ancylostoma duodenale* and *Necator americanis.* Many GI nematodes do not cause overt pathology in the majority of those infected, although it is evident that the greater the intensity of infection harboured by a host or population the greater the degree of pathology likely to be present. The pathological consequences of nematode infection depend on the site in the gut that the nematode inhabits the activity of the worm in the gut and on the migration of the juvenile worms throughout the host's body. *A. lumbricoides*, for example, is a large (20cm long) lumen dwelling parasite of humans. Although this parasite does not actively damage the intestine, it is due to its size, the commonest cause of gastrointestinal obstruction of children in the developing world. *A. lumbricoides* juveniles released from ingested eggs migrate through the body and break through the alveolar epithelia in the lungs where this helminth may cause an asthma-like syndrome. The hookworms, *N. americanis* and *A. duodenal*e are also lumen dwelling, however these worms feed on blood by grazing on the mucosa and releasing anti-coagulants. Women and children, because of their relatively high iron requirements, or those with low iron diets may suffer from anaemia if heavily infected (Crompton and Whitehead, 1993). *T. trichiura* is

a tissue-penetrating nematode that dwells in the caecum, the associated local inflammatory reaction results in damage to the intestinal muscles, which, in severe cases may lead to rectal prolapse.

Some studies have shown evidence that infection with nematodes has a detrimental effect on the development of infected children. One study compared the performance of children in tests of memory, and who where heavily or moderately infected with *T. trichiura* with those who where not. This study showed correlation between infection and poor performance in the tests (Sakiti *et al.*, 1999). However, it is difficult in studies of type to be sure that the relationship between nematode infections and poor test performances is a causal one or rather a relationship between the factors that predispose a child to helminth infections and poor performance in tests.

GI nematodes are also a considerable veterinary problem, again particularly in the developing world but also in developed countries, this is partly due to environmental factors and financial factors. Infection with GI nematodes has been shown to have a detrimental effect nutritional state of infected animals; for example, the treatment of yearling cattle with the anti-helminthic, ivermectin has been shown to result in increased weight gain over a grazing season compared to untreated animals (Mertz *et al.*, 2005). The nematode parasites of animals however are not only an issue of animal welfare and husbandry may also be important zoonotic infections such as *Trichinella spiralis*, which readily infects humans and can be fatal and *Toxocara canis*, a common parasite of domestic dogs and cats, the infection of humans by larvae of this nematode can lead to

larva migrans occularis, which may result in blindness or sight impairment particularly in children.

Despite a variety of drug-based control programs, GI nematodes have proved difficult to eradicate from an endemic population, whether animal or human. Although effective drug treatments are available, there is no method currently available to prevent re-infection, which readily occurs in endemic areas (Albonico *et al.*, 1995; Guyatt, 1999). The astounding population stability exhibited by parasitic nematodes is a result of the high fecundity of these parasites. The over-dispersed nature of parasite distribution within the host population means that one individual missed by a control program is capable of re-infecting the entire population (Schad and Anderson, 1985). As a result GI nematode infection control requires regular drug treatment and therefore is a significant drain on the resources of developing countries. The regular repeated use of any drug in a community may potentially lead to the development of resistance to the drug in the parasite population, this phenomena is readily observed in malaria and in bacterial infections such as *Staphylococcus aureus*, and although more rarely seen, nematode resistance to drug treatment has been observed in *Haemonchus* infection of cattle in central Argentina (Anziani *et al.*, 2004).

### *1.2 Potential effects of GI nematode infection on unrelated immune responses.*

Helminth infections induce a typically T helper 2-type dominated immune response characterised by eosinophilia, mastocytosis, IgE, IgG1, elevated Interleukin (IL)-4, IL-5, IL-13, IL-9, IL-3, and reduced IL-12, IFN-γ and plasma IgG2a (Cooper *et al.*, 2000). This strongly induced bias of the immune responses may affect the outcome of other immune responses. Understanding the effects of concurrent nematode infections on unrelated aspects of the immune response is important for the control of disease by vaccination in areas where nematodes are endemic at high levels.

Nematode infections may also affect the outcome of diseases common in the developing world such as HIV, tuberculosis and malaria. For example a study in areas endemic for both *Ascaris lumbricoides* and *Plasmodium falicparum* showed a correlation between *A. lumbricoides* infection and reduced risk of suffering the potentially fatal cerebral malaria (Nacher *et al.*, 2000). However, the influence of GI nematodes on the immune system is unlikely to be entirely beneficial; many protective responses such as those to the *Leishmania major* or to viral infections are Th1 mediated. The high levels of Th2 cytokines stimulated by helminth infections may therefore inhibit these protective immune responses. This may prove important for the efficacy of vaccinations and in the outcome of infections that stimulate protective Th1 responses in areas endemic for GI nematodes. A variety of *in vivo* studies have suggested that infections with GI nematodes may significantly alter the immune response. For example *Nippostrongylus*

*brasiliensis* infection in mice has been shown to break an already established tolerance to the unrelated antigen *Stappylococcus* endotoxin B (Rocken *et al.*, 1992). Liwski *et al* (1992) showed that allografts from BALB/c into C57BL/6 mice survived longer in mice infected with *Nippostronglus brasiliensis* compared to uninfected animals. The prolonged graft survival appears to be due to the development of a predominantly Th2 response to graft antigens (Liwski *et al.*, 2000).

Nematodes infections may also form a part of the environmental factors that affect the development of autoimmune disease. In many cases autoimmune diseases have a Th1 profile, which may be down regulated by Th2 cytokines. However, some diseases are caused by a Th2 response against self-antigens. For example, a group studying autoimmune responses to an ovarian self-peptide, which normally results in IFNγ production, found that neonatal exposure to the rodent pinworm *Syphhacia obvelata,* induced a Th2 response and eosinophilic inflammation of the ovary (Agersborg *et al.*, 2001). Cooper *et al.* (1999) conducted a study to examine the effect of infection with the filarial nematode *Onchocerca volvulus* on the host immune response to tetanus toxoid vaccination. Although the overall findings of this study did not suggest that concurrent nematode infection significantly reduced the protective responses, they did find a significant difference in the immune response of those with heavy infections compared to lightly or uninfected individuals (Cooper *et al.*, 1999). Coelemic fluid from *A. lumbricoides* (ABF) has been shown to modulate a DTH response in mice immunised against ovalbumin with Freund's complete adjuvant (FCA) (Boitelle *et al.*, 2003).

### *1.3 Vaccination against helminthiasis*

Currently there are no vaccines against human helminthiasis, although in animals irradiated live infective juvenile worms have been used to successfully prevent infection by organisms such as *Dictyocaulus viviparous* (Jarrett *et al.*, 1960; Johnson *et al.*, 2003) and whole parasite homogenates have been shown to provide protection against parasites in experimental rodent infections (Jacobs *et al.*, 1999). There are however a variety of reasons that make similar vaccines inappropriate for use in humans. For a vaccine to be used in humans it must be safe; the risks of the irradiation of larvae failing to prevent virulence or the larvae reverting back to its virulent state on inoculation are too great while allergic reactions or other adverse effects from the complex mixtures of molecules present in parasite homogenates are too hazardous. As these infections are generally most common in the developing world the vaccine must firstly be cheap to produce and to deliver, in many of these countries the infrastructure does not exist to provide a sustainable cold chain from production to delivery to patients. Nematode infections not only affect humans but also animals and as such they have financial consequences for the agricultural industry. For example, the cost of reduced growth in infected animals and the cost of regular drug treatment to control infections. However, for mass vaccination to be viable in farming, the vaccine must be reliable; easy to administer and store and relatively cheap. In order to produce any vaccine against nematodes it is

necessary to gain a better understanding of the pathological and protective processes involved in the naturally occurring immune responses (Maizels *et al.*, 1999).

### *1.4 Trichinosis.*

#### *1.4.1 Epidemiology.*

Trichinosis is the disease caused by infection with nematodes of the genus *Trichinellae*: there are a number of species in this genus, which have been found in a wide range of host organisms including mammals, reptiles and birds. In mammals the most important are *Trichinella spiralis, T. nativa, T.* T-5, *T.* T-6 (temperate, sylvatic) and *Trichinella pseudospiralis*. In Europe the major natural reservoir of infection is the red fox, *Vulpes vulpes* (Rafter *et al.*, 2005) and is also commonly found in rats which are thought to be important sources of infection on pig farms (Mikkonen *et al.*, 2005), although this nematode will parasitize virtually any mammal, particularly wild carnivores and omnivores or domestic animals given contaminated food.

*T. spiralis* is a cosmopolitan parasite and is endemic in many countries worldwide, and as such is one of the few helminth parasites still to pose a human health risk in Europe and North America. Most human infections are associated with the ingestion of raw or undercooked meats particularly pork. Most infections in pigs are believed to result from undercooked or raw meat in the pig feed or from rats, which are ubiquitous on pig farms. Pigs housed in close quarters are likely to spread the infection among them by biting one

another, particularly each other's tails. Within the EU rules exist to prevent the spread of *T. spiralis* to humans via the food chain. However, these rules regarding meat inspection are not always strictly followed and human infections do occur. It is estimated that $590 million are spent annually to control this infection in the European domestic pig population (Pozio, 1998). In recent years a number of human outbreaks have occurred from the ingestion of infected horse meat, which is now becoming the major source of human infection in Europe, and as a result similar control mechanisms are being brought into effect for horse meat as have been used for pork.

### *1.4.2 Life cycle*

The life cycle of this parasite involves no intermediate host or free-living egg of larval stage, both male and female adult worms dwell in the small intestine of the mammalian host. The worms live within the mucosal layer, burrowing directly through the cells. The female worm is viviparous, producing first stage larvae (L1) rather than eggs. L1 are released into the gut lumen, from where they penetrate the mucosa and migrate into the host tissues; the L1 enters a muscle cell which under direction of helminth products becomes a nurse cell whose function is to support the L1 larvae until such a time as it is able to infect the next host. Infection of a new host occurs when another mammal ingests infected tissue. The action of host gastrointestinal acids and proteases release the larvae from its nurse cell, stimulate the juvenile to moult and become the adult worm in the small intestine of its new host. (Figure 1.1)

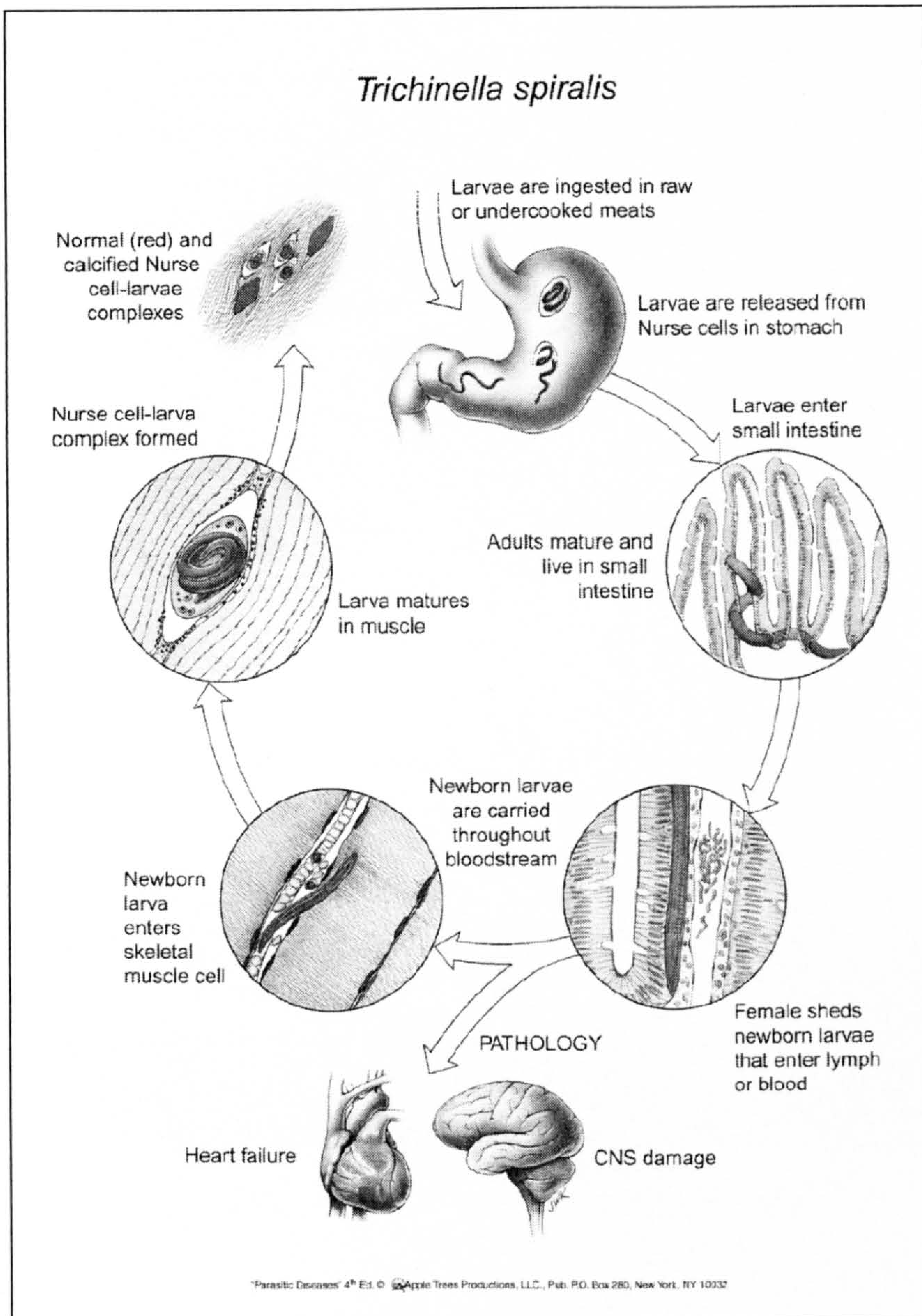
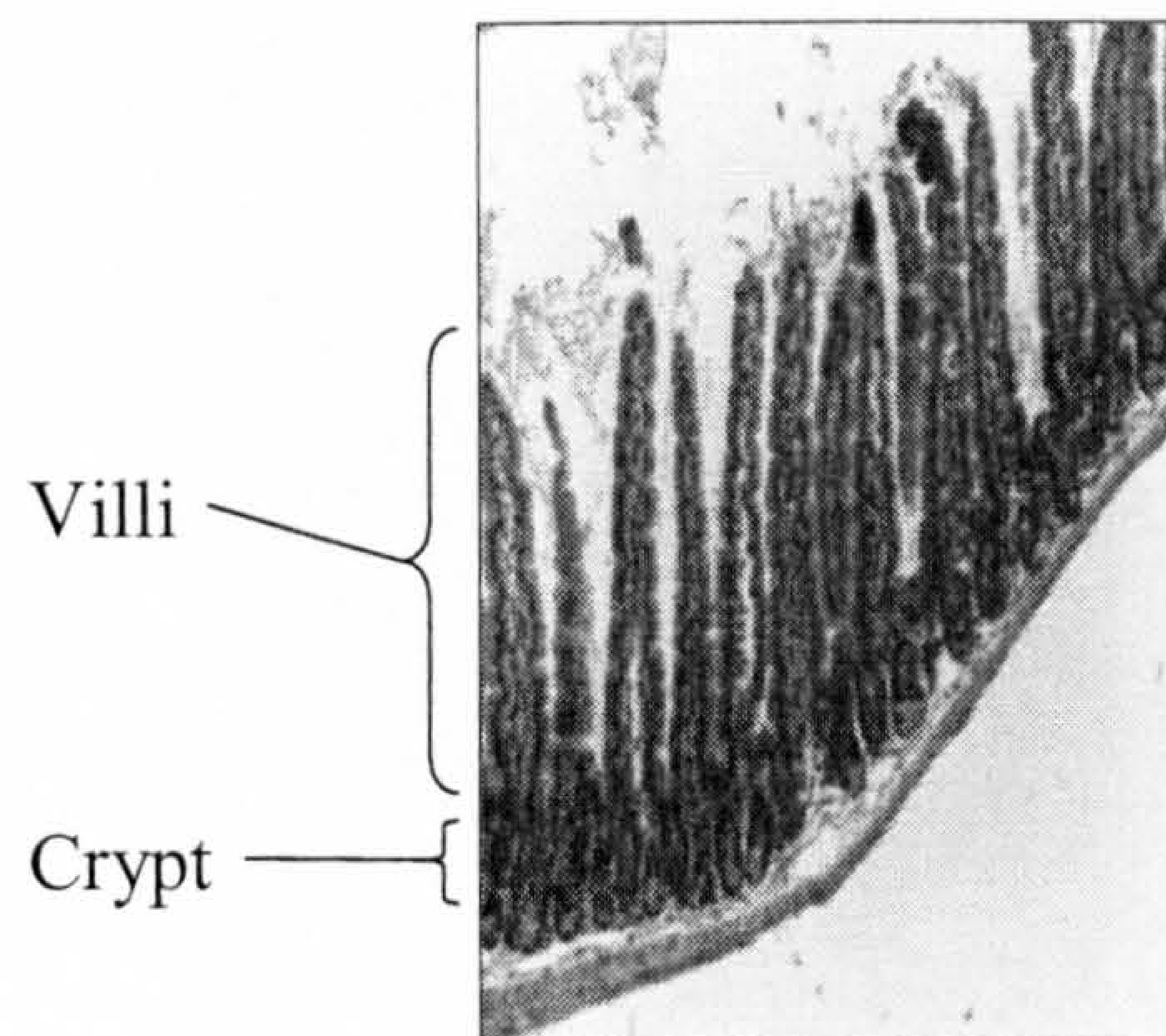



FIGURE 1.1: The life cycle of *T. spiralis* (reproduced from 'Parasitic Diseases' 4th Ed. © Apple Tree Productions, LLC, Pub. P.O. Box 280, New York, NY 10032).

## *1.5 Gastrointestinal pathology associated with* T. spiralis *infection.*

*T. spiralis* infection typically results in disruption of the normal structure of the small intestine, characterised by villus atrophy and crypt hyperplasia. This disruption of the epithelial architecture is seen in a variety of nematode infections and other pathological conditions of the intestinal tract. In the healthy small intestine, cells are constantly sloughing off the ends of the villi and are replaced by dividing cells in the crypts. As the cells move up the villus-crypt unit they differentiate to become specialised absorptive cells. However, in a variety of pathological conditions, the rate at which the cells at the end of the villi are lost increases and exceeds the rate at which the crypt cells can replace them. Undifferentiated cells build up in the crypt leading to hyperplasia, while the villi become shortened, villus atrophy (Figure1.2A and B).

**A) Uninfected**




**B) Infected**

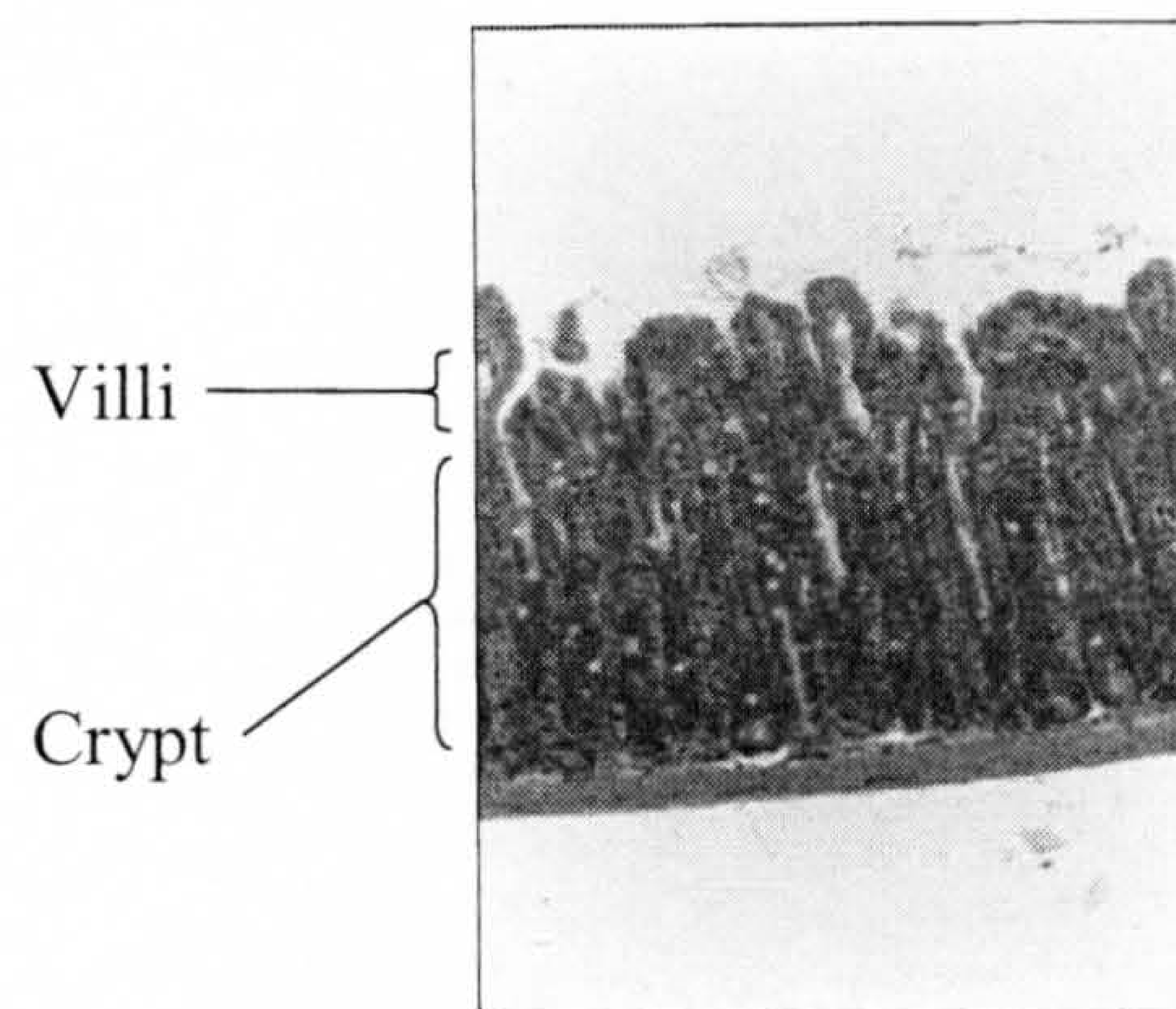



FIGURE 1.2: The enteropathy associated with the intestinal phase of *T. spiralis* infection. Haematoxylin and eosin stained sections of jejunum from uninfected (A) and infected (day 6) (B) mice, showing villus atrophy and crypt hyperplasia.

This alteration in gut architecture leads to a reduction and eventual loss of the small intestine's ability to absorb nutrients from the lumen. In severe cases this may lead to weight loss and diarrhoea; *T. spiralis* infected experimental rodents are seen to progressively lose body weight.

The integrity of the gut epithelia as a barrier between the internal environment of the host and the external environment also breaks down; this increase in permeability of the gut epithelium allows increased access to the parasites by host immune effector mechanisms such as antibodies, cytokines, complement, and lymphocytes. This increase in permeability may also lead to an increase in leakage of bacterial products such as LPS, from commensal bacteria in the intestine, into the systemic circulation of the host that may result in liver damage and a generalised inflammatory reaction. *T. spiralis* is a tissue-penetrating helminth and therefore may directly carry bacteria on its surface from the intestine lumen into tissues. This has been shown in a rather extreme form in pigs infected with *Ascaris suum* where juveniles where shown to carry bacteria into host tissues causing liver abscesses and pneumonia (Adedeji *et al.*, 1989).

At a cellular level nematode infections result in an increase in the number of goblet cells -the mucus producing cells, in the gut epithelia. It has been demonstrated that along with an increase in the number of goblet cells and the quantity of mucus produced there is a difference in the composition of this mucus between *Trichostrongylus colubriformis* infected and uninfected guinea pigs (Manjili *et al.*, 1998). There is also a massive infiltration of immune cells such as eosinophils and mast cells into the lamina propria.

Mast cells express IgE receptors on their surfaces, cross-linking of these receptors by antigen bound IgE causes degranulation, releasing a wide variety of inflammatory mediators and cell growth factors which further up regulating the inflammatory response (Burd *et al.*, 1989). An important role for mast cells in the development of enteropathy following infection with *T. spiralis* has previously been shown using the mast cell deficient WWv mouse which fails to develop enteropathy, furthermore mice deficient in mucosal mast cell protease (mMCP)-1 also fail to develop enteropathy following infection with *T. spiralis* (Lawrence *et al.*, 2004).

The development of enteropathy has been suggested to be a mechanism for the expulsion of gastrointestinal parasites by altering the intestinal environment and removing the parasites niche, furthermore it has been demonstrated that mouse strains resistant to chronic infection with *T. muris* show increased epithelial cell turn over, which is a factor in the development of villus atrophy and crypt hyperplasia, earlier in infection than mice susceptible to chronic infection (Cliffe *et al.*, 2005). However time course studies in wild type mice infected with *T. spiralis* suggest that expulsion and pathology may be unrelated. The pathology as measured by crypt hyperplasia and villus atrophy is already beginning to resolve before the main phase of worm expulsion (Lawrence *et al.*, 1998).

## *1.6 The immune response to GI nematode infection.*

### *1.6.1 Introduction.*

Although it has been long recognised that the immune system mounts a vigorous response against gastrointestinal nematodes, it was believed that the immune system was ineffective against helminth parasites.

The eventual loss of the parasite from the host was almost entirely attributed to the death of the helminth from old age rather than any active response by the host. Animal models and *in vitro* experiments have however shown that the immune response is indeed capable of affecting the longevity of the helminth infection. NIH in bred mice infected with *T. spiralis* typically expel their parasites after approximately two weeks, however, on a second exposure to the same species of parasite, worm expulsion begins on the first day of infection (Wakelin and Lloyd, 1976). This type of response, with a slow primary response and a rapid secondary response is typical of immune reactions. The delayed expulsion of *T. spiralis* in congenically athymic (nude), thymectomised, and cyclosporin A treated mice has confirmed the T cell dependent nature of immunity to helminth parasites (Garside *et al.*, 1992; Manson-Smith *et al.*, 1979).

### *1.6.2 T helper 2 responses*

Studies of inbred rodent strains who differ in their susceptibility to helminth infections such as those performed by Else *et al* (1993) on *Trichuris muris* infections in mice have shown that resistance to infection - that is, the ability to rapidly expel the parasites - correlates with the production of a largely Th2 cytokine profile, while susceptibility correlates with higher Th1 cytokine levels in response to infection (Else *et al.*, 1993). However, similar studies using *T. spiralis* infection have shown that, while infection was associated with the production of Th2 cytokines and the relative absence of Th1 responses, and no correlation between the magnitude of the Th2 responses and expulsion were observed (Grencis *et al.* 1991).

Further work using experimental animals deficient for particular cytokines have demonstrated the Th2 nature of both the protective and pathological aspects of this immune response. IL-4 is considered as one of the cardinal regulators of the Th2 response, necessary for B-cell class switching to IgE and IgG1. IL-4 deficient (-/-) mice show a diminished Th2 response to *T. spiralis* infections, delayed expulsion and exhibit reduced gastrointestinal pathology (Lawrence *et al.*, 1998). Expulsion in IL-4 -/- mice is believed to be dependent on an IL-13 mechanism. Interestingly a role for NK cell derived IL-13 has been demonstrated in SCID mice, which are unable to expel, but develop comparable enteropathy to wild type mice, and show large numbers of IL-13$^{+}$ CD49$^{+}$ NK cells in the lamina propria. The enteropathy in SCID mice was also inhibited by treatment with exogenous IL-13R2, a soluble high affinity decoy receptor for IL-13

(McDermott *et al.*, 2005). Both IL-4 and IL-13 signal through the IL-4 receptor α chain and IL-4Rα -/- mice are unable to expel the parasite (Bancroft, 2000).

Interestingly, Tumour Necrosis Factor Receptor 1 (TNFR1) deficient mice are able to expel the parasite in a similar manner to wild type mice but show a significantly reduced pathology. This expulsion but no pathology phenotype is also seen in inducible nitric oxide synthase (iNOS) deficient animals. TNFα is known to up-regulate iNOS, so pathology has been attributed to TNFα activation of iNOS, resulting in an increase in nitric oxide (NO) levels in the gut tissues; it is this NO that increases the rate of cell death at the end of the villi resulting in their atrophy. Crypt hyperplasia is simply a result of the gut trying to maintain its structure and repair the damage (Lawrence *et al.*, 2000).

NO is generally associated with Type I responses such as that to *Toxoplasma gondii* where TNFα up-regulation as a result of IFNγ secretion produces a protective NO production (via iNOS) in infected macrophages (Oswald *et al.*, 1994). Here type 2 cytokines fail to induce NO synthesis in infected macrophages and allows the continued survival of the protozoan. So how in an immune response where type 2 cytokines are very high and IFN-γ levels are low is TNFα synthesis stimulated resulting in the pathology. IFN-γ -/- mice show identical pathology and expulsion profiles to wild type mice so TNFα secretion is not stimulated in this situation by IFN-γ but by another route, presumably IL-4 (Lawrence *et al.*, 1998).

The pathology caused by *T. spiralis* infection resembles the pathology found in inflammatory bowel diseases (IBD). *In vivo* mouse models and *in vitro* work done on cells removed human patients implicate Th1 responses in the aetiology of this type of pathology and it was thought that in GI nematode infections an imbalance between Th1 and Th2 responses may be the cause of the pathology (Powrie *et al.*, 1994). However, the work described above shows that the pathology is caused by the Th2 response to nematode infections, and does not involve IFN-γ, which is elevated in IBD patients. However the TNFα stimulated pathology in nematode infections may provide a link between these seemingly unrelated conditions.

It is interesting to note that the protective and pathological aspects of the immune response differ between nematode species. For example, TNFα the major mediator of pathology in *T. spiralis* has been shown to be an important protective factor in the IL-13 mediated expulsion of *Trichuria muris,* TNFR -/- mice fail to expel *T. muris,* producing an increased Th1 response to the worm implicating TNFα in the generation of the Th2 response under these circumstances (Artis *et al.*, 1999).

### *1.6.3 Costimulation by B7-1, B7-2 and CD28*

B7-1, B7-2 and their receptor CD28 are the most extensively studied. At the most simplistic level B7-1 has been shown to stimulate the generation of a Th1 response while B7-2 stimulates a Th2 response. The blockage of B7-2–CD28 interactions with

monoclonal antibodies, in *T. spiralis* infected mice reduces the Th2 response to this parasite, as measured by specific IgE, IL-4 and IL-5 (Wang *et al.*, 2000). In the absence of CD28 (CD28 KO mice) both Th1 and Th2 responses to many antigens are absent, for example vaccinia virus, *Leishmania major* and *Heligomosomoides polygyrus*.

However in other models Th1 and Th2 responses, although reduced are not absent, suggesting the presence of CD28 independent mechanisms of T cell activation.

### *1.6.4 Novel costimulatory molecules*

A variety of elements may be responsible, including the innate factors such as the activation of APCs via pattern recognition receptors leading to prolonged APC survival and enhanced antigen processing and presentation. In addition to CD28 a number of other accessory molecules are capable of stimulating the specific T cell response for example, are OX40, 4-1BB, heat-stable antigen (HAS), and ICOS.

#### 1.6.4.1 OX40 protein and OX40 ligand

OX40 is a member of the tumour necrosis factor receptor (TNFR) super-family that has only been discovered relatively recently and is implicated in the generation of Th2 responses. In *Leishmania* infections where Th1 responses are protective, wild-type BALB/c mice generate a Th2 response so are susceptible to chronic infection; in these

mice treatment with anti-OX40L monoclonal antibodies, reduced the susceptibility of these mice to chronic infection (Akiba *et al.*, 2000). OX40 stimulation leads to the generation of a Th2 response. Mice transgenic for the CD28 competitive inhibitor cytotoxic lymphocyte associated molecule-4 IgG1 fusion protein (CTLA-4 Ig) show reduced expression of OX 40 and IL-4 (Walker *et al.*, 1999). OX40 also plays a role in the migration of CD4+ T cells into the germinal centres and therefore in T-B cell interactions, via up regulation of the chemokine receptor Blr-1 (Flynn *et al.*, 1998).

#### 1.6.4.2 4-1BB protein and 4-1BB ligand

4-1BB like OX40 is a member of the TNFR super-family, 4-1BB is expressed on CD4+ and CD8+ T cells. Its ligand 4-1BBL has been found on activated B cells, macrophages, cultured dendritic cells and several B lymphomas. Antibodies against 4-1BB have been shown to be sufficient to co-stimulate T cells pre-activated via their TCR in the absence of CD28, resulting in high levels of 4-1BB expression but little IL-2 up regulation. However studies using APC expressing the 4-1BBL show that the interaction of 4-1BB with its ligand leads to the production of IL-2 and IL-4 by the T cell, so 4-1BB is a potential stimulator of Th2 differentiation. Other work suggests that anti 4-1BB antibodies preferentially stimulates CD8+ T Cells to proliferate and IFNγ production (Shuford *et al.*, 1997).

1.6.4.3 Inducible costimulator protein

Inducible costimulator protein (ICOS) is a surface molecule expressed specifically by activated Th2 cells, and although ICOS is a CD28 homologue it does not bind to B7-1 or B7-2, but binds to B7 related protein (B7-RP)-1. A role for both ICOS and CD 28 in Th1 and Th2 responses has been shown by infecting wild type or CD28 -/- mice with either lymphocytic choriomeningitis virus (LCMV), vesicular stromal virus (VSV) or the *N. brasiliensis*, with or without anti-ICOS monoclonal antibody treatment. These experiments show that where CD28 is not essential, ICOS plays a role in the generation of T helper responses but not in cytotoxic T lymphocyte (CTL) responses.

1.6.4.4 Heat-stable antigen

CD28 deficient mice show impaired germinal centre formation and antibody responses to certain antigens; however, IgG and IgA responses to infectious agents such as LCMV, VSV and parasite infections are largely unaffected. HSA KO animals show unimpaired antibody responses, CD28/HSA double knockout animals, however, show normal IgM responses but reduced IgG and IgA responses, indicating an important role for HSA in CD28 independent antibody class switching

### *1.6.5 Mast cells.*

In mice mast cells may divided in to two distinct subsets the connective tissue mast cells (CTMC) and the mucosal mast cells (MMC). CTMCs are located in tissues such as the skin and it has been shown that their granules contain predominantly mMCP-5 while MMC are found in the intestinal and respiratory mucosa and their granules predominantly contain mMCP-1. CTMCs are continually present in the connective tissues of the skin and in the peritoneum while MMC are only present in small numbers in normal mucosa but may be dramatically up regulated following infection or inflammation. Approximately 14 days following infection with *T. spiralis* a pronounced mucosal mastocytosis can be observed. (Behnke *et al.*, 1994), and this response is attenuated in thymectomised mice, suggesting T cell dependency (Brown *et al.*, 1981). *N. brasiliensis* expulsion is delayed in the mast cell deficient W/Wv mouse (Crowle and Reed, 1981). Mastocytosis is reduced and expulsion of *T. spiralis* or *N. brasiliensis* is delayed in mice co-infected with the GI nematode *Heligosomoides polygyrus* (Dehlawi *et al.*, 1987). Mastocytosis has been shown to be stimulated by IL-9. Studies of mice infected with *T. spiralis* overproducing IL-9 resulted in an intense mucosal mastocytosis and high serum mucosal Mast Cell Protease-1 (mMCP-1). This elevated mastocytosis and degranulation was associated with the rapid expulsion of *T. spiralis* (Faulkner *et al.*, 1997). The c-kit ligand is involved in the development of mastocytosis (Maurer *et al.*, 1998), and *T. spiralis* induced mastocytosis is suppressed by treatment with anti-c-kit antibodies results in the inability to expel the parasite (Grencis *et al.*, 1993). The

expulsion of *T. spiralis* is also delayed in mice lacking mMCP-1 (Knight *et al.*, 2000). Whilst being important in the expulsion of *T. spiralis* it is also thought that mast cells may play a role in the development of enteropathy. Enteropathy has been shown to be reduced in the mast cell deficient W/Wv mouse and in mice deficient in mMCP-1(Lawrence *et al.*, 2004). In addition to mMCP-1, mast cells are a source of TNFα, NO and IL-4 (Bischoff *et al.*, 1999; Bradding *et al.*, 1993; Furuta *et al.*, 1997; Gordon and Galli, 1990), which have been shown to be involved in the development of enteropathy following *T. spiralis* infection (Lawrence *et al.*, 1998; Lawrence *et al.*, 2000).

### *1.6.6 Eosinophils*

IL-5 is the signal for the proliferation and differentiation of myeloid progenitor cells to eosinophils, which are strongly up regulated in nematode infections. The role of eosinophils in helminth infections has been widely studied and *in vitro* killing of larvae has been shown but the *in vivo* evidence is more uncertain. Anti-IL-5 antibody treated mice shown a significant fall in the numbers of eosinophils, however no differences were observed in the duration of a primary infection with *T. spiralis* compared to untreated controls (Herndon and Kayes, 1992). IL-5 -/- mice, which fail to produce eosinophilia in response to infection, show a reduced protective response to challenge infections. Eosinophilia may therefore be important in the rapid expulsion of nematodes seen in a challenge infection rather than in the induction of immunity in a primary infection (Vallance *et al.*, 2000). Eosinophilia as part of the innate response may also be

particularly important in preventing opportunistic parasitism of a non-natural host. For example, primary infection with *Strongyloides ratti* in rats - the natural host - results in little inflammation. However, primary infection of mice, a non-natural host, results in marked inflammation and eosinophil infiltration, leading to parasite death (Meeusen and Balic, 2000).

### *1.6.7 Goblet cells*

Concurrent with *T. spiralis* infection there is a goblet cell hyperplasia in the intestinal epithelium and, an increase in the production of mucous (Ishikawa *et al.*, 1997). Goblet cell hyperplasia, the increase in mucin quantity and changes in composition that accompany helminth infection are attenuated by anti-CD4+ antibody treatment (Khan *et al.*, 1995), suggesting that goblet cell hyperplasia is mediated by the immune response. Furthermore, animals undergoing a primary nematode infection develop goblet cell hyperplasia slower than immune animals. This distinction between primary and secondary infections is a cardinal feature of the immune response.

As mentioned above guinea pigs infected with *Trichostrongylus colubriformis* also develop goblet cell hyperplasia. Interestingly, guinea pigs that expel worms more rapidly showed an earlier peak in goblet hyperplasia and more rapid alteration in the mucin composition than slow responder guinea pigs (Manjili *et al.*, 1998). Alterations in the sugar moieties of mucins in the intestines of *Nippostrongylus brasiliensis* infected

rats has been observed. These alterations have been shown to occur more rapidly when worms are transplanted into immune compared to non-immune rats (Ishikawa *et al.*, 1993). Mucins produced by goblet cells during nematode infections may play a role in expulsion by preventing dislodged worms from reattaching to the gut epithelia. During *T. spiralis* infection mucins may help to prevent the larvae penetrating the gut, thus limiting the muscle burden developed, however the precise role of mucins during *T. spiralis* infection is unclear.

Goblet cells also produce a group of proteins known as trefoil peptides or factors that are up regulated during intestinal inflammation (Tomita *et al.*, 1995). Exogenous trefoil peptides have been shown to protect against ethanol-indomethacin induced gastric injury (Babyatsky *et al.*, 1996). The expression of trefoil factor family-3 (TFF-3) has been shown to be stimulated by IL-4 and IL-13 (Durual *et al.*, 2002). As IL-4 and IL-13 production are important factors during nematode infection, the stimulation of TFF-3 by these cytokines and the capacity of exogenous trefoil factors to protect against epithelial injury suggests that trefoil factors have a protective role during nematode infections, including *T. spiralis*. However, the precise role played by trefoil factors in the development of nematode induced enteropathy is not yet clear.

### *1.6.8 "Weep and Sweep" theory of expulsion*

Once immune mediated expulsion had been accepted it was theorised that the pathological consequences of infection where a necessary part of the expulsion of the helminth. The alteration in the gastro-intestinal epithelium produced an environment that was no longer suitable for the parasite. Following nematode infection there is an accumulation of fluid in the intestinal lumen is observed, this is associated with increased mucosal permeability (McDermott *et al.*, 2003) and alterations in epithelial cell function (Madden *et al.*, 2002). In addition to luminal fluid accumulation, neuro-muscular dysfunction also develops, which is characterised by hyper-contractility of the intestinal smooth muscle (Khan *et al.*, 2001; Zhao *et al.*, 2003) and increased gastrointestinal transit (Farmer, 1981). These parameters describe the "weep and sweep" theory of nematode expulsion from the intestine, where by the intestines "weep" mucous and fluid which is "swept" away by increased peristaltic activity.

Interestingly these "weep and sweep" responses, although seemingly non-specific, have been shown to be dependent on Th2 responses. Primary infection with either *T. spiralis* or *N. brasiliensis* and secondary infections with *H. polygyrus* have been shown to decrease epithelial resistance, indicating increased permeability and to reduce glucose dependent sodium absorption from the intestinal lumen, this results increased fluid retention in the intestine leading to oedema. Both increased permeability and reduced sodium absorption are absent in the absence of STAT6 signalling (Madden *et al.*, 2004)

and may be stimulated by exogenous IL-4 and IL-13 in the absence of nematode infection (Madden *et al.*, 2002). Both *N. brasiliensis* and *H. polygyrus* infections have been shown to increase explanted intestinal smooth muscle responses to acetylcholine compared to tissue from uninfected animals. This effect is attenuated in the absence of STAT6 and may be mimicked by treating smooth muscle tissue from naive mice with IL-4 and IL-13, demonstrating that hyper-contractilty is a Th2 mediated response (Zhao *et al.*, 2003). Furthermore the development of smooth muscle hyper-contractility in *T. spiralis* infected mice is dependent on STAT6 (Khan *et al.*, 2001), while exogenous IL-9 treatment has been shown to enhances smooth muscle contractility in *T. spiralis* infected mice and the blockade of IL-9 inhibits hyper-contractility in *T. muris* infected mice (Khan *et al.*, 2003).

### *1.6.9 Antibody responses.*

Nematode and other helminth infections stimulate significant antibody production, in particular elevated serum IgE is characteristic of helminth infection. Furthermore an association with higher IgE titres and an increased resistance to helminth infections has been demonstrated in humans (Faulkner *et al.*, 2002; Hagan *et al.*, 1991) and mice (Else *et al.*, 1993). During *T. spiralis* infection the transfer of immune serum into naïve rats has been shown to accelerate expulsion (Love *et al.*, 1976) suggesting a role for antibodies in parasite expulsion. Furthermore the nasal vaccination of mice with *Trichinella* homogenate and peptide antigens protected mice against challenge infection

and was associated with elevated IgE, IgG1 and IgA (McGuire *et al.*, 2002), while the passive transfer of a monoclonal IgA antibody recognising the cuticle of *T. britovi* muscle larvae inhibited the establishment of *T. pseudospiralis* in mice (Abbas *et al.*, 1996).

### *1.7 Aims and experimental design*

The host immune system mounts a strong Th2 response against *Trichinella spiralis* infection in the gut, ultimately leading to expulsion of the adult worms (Brown *et al.*, 1981; Garside *et al.*, 1992; Grencis *et al.*, 1991). Despite effectively eradicating the parasite, immune mediated damage to the structure and function of the small intestine is incurred during this immune response. Previous studies suggest that this pathology is largely mediated by the activity of IL-4 leading to the production of TNFα and iNOS, which ultimately induces villus atrophy and crypt hyperplasia (Lawrence *et al.*, 1998; Lawrence *et al.*, 2000). However, it is also apparent that this enteropathy is not a necessary component of the expulsion of *T. spiralis* (Lawrence *et al.*, 1998; Lawrence *et al.*, 2000). This thesis examines mouse strain specific differences in the role of IL-4 and the role of IL-13 in the development of enteropathy. Co-stimulatory signals are important in the generation of T cell responses; here we examine the role of the novel co-stimulatory molecules ICOS and OX40 in the development of responses to *T. spiralis*. It has been shown that TNFα is involved in the development of enteropathy; here we examine the role of the transmembrane TNFα and lymphotoxin α in the

development of protective and pathological responses to *T. spiralis*. Proteases have been shown to be involved in tissue remodelling and therefore in the development of pathology, in particular the mast cell tryptase mMCP-1 has been shown to be important in both pathology and parasite expulsion. Novel receptors have been identified that allow cells to respond to proteases, the protease activated receptors (PARs), and here we examine the role of PAR-2 in the development of responses to *T. spiralis*.

Dissecting the non-specific pathological response from those involved in protective responses to *T. spiralis* is important as it may inform rational vaccine design, allowing the development of a vaccine that provides protective immunity while by passing those responses that lead to the development of enteropathy. The aim of this thesis is to further examine factors influencing the development of Th2 responses to *T. spiralis* infection and the development of protection and pathology

# Chapter Two

## Materials and Methods

# 2 Materials and Methods.

## *2.1 Animals*

ICR and CD1 mice were obtained from Harlan (UK), all other mice were bred, and maintained under conventional conditions and in accordance with local and Home Office regulations in the animal facility at the University of Strathclyde (UK). All mice used were female and aged 8-12 weeks. All mice were euthanised by $CO_2$ inhalation.

IL-4 -/- mice on a BALB/c and IL-4 -/- mice on a C57BL/6 background were originally obtained from Prof. F. Brombacher, University of Cape Town (South Africa) (Noben-Trauth *et al.*, 1996; Noben-Trauth *et al.*, 1997). IL-13 -/- mice on a BALB/c background were originally obtained from Dr A. N. McKenzie, MRC Laboratory of Molecular Biology (UK). (McKenzie *et al.*, 1998). IL-4Rα -/- mice on a BALB/c background were obtained from Prof. F. Brombacher, University of Cape Town (South Africa) (Mohrs *et al.*, 1999). Macrophage/neutrophil linage specific IL-4Rα -/- mice on a BALB/c background were originally obtained from Prof. F. Brombacher, University of Cape Town (South Africa) (Herbert *et al.*, 2004). TNFα/LTα -/- mice were on a C57BL/6 background; tmTNFα transgenic (-tg) mice were on a TNFα/LTα -/- C57BL/6×129 cross background and were both originally obtained from Prof. C. Mueller, University of Bern (Switzerland) (Mueller *et al.*, 1999). PAR-2 -/- mice were on a C57BL/6 background and were obtained from Professor Robin Plevin, University of Strathclyde (UK).

## *2.2 Parasites*

*Trichinella spiralis* parasites were maintained by serial passage through wild type BALB/c, ICR or CD1 mice. *T. spiralis* muscle larvae were obtained from mice infected at least 30 days previously. Mice were killed and skins, snouts, extremities and abdominal organs removed. The carcasses were then diced and homogenised in a Kenwood blender. Tissue was then digested in at least 200ml 0.9% NaCl/ 0.5%HCl/ 0.5% Pepsin (Sigma-Aldrich, UK) solution per mouse at 37°C under agitation for 1 hour 30 minutes. Digests were filtered through a 10μm filter to remove undigested tissue and bone fragments. Larvae were allowed to settle and supernatants aspirated, larvae were washed in 0.9%NaCl solution until samples were clear. The larvae were then re-suspended in 50ml 0.9%NaCl solution and the total number of larvae were calculated by counting the number of larvae in an inverted McMaster's egg counting slide (Wakelin and Wilson, 1977). Experimental mice were infected orally as detailed with *T. spiralis* larvae in 0.2ml 0.1%Agarose/0.9%NaCl. Intestinal burdens were assessed by opening the small intestine, placing in gauze and suspending in Hank's Balanced Salt Solution (HBSS) (pH 7.4) (Sigma-Aldrich, UK) and incubating at 37°C for 3 hours to allow the migration of the adult worms out of the epithelium. The total number of adult worms were then counted under a dissecting microscope. Muscle larvae burdens were obtained by the method described above.

### 2.3 *Parasite antigen preparation.*

Extracted *T. spiralis* muscle larvae were washed in PBS (see appendix) and homogenised in a glass pestle and mortar at 4°C. Homogenates were centrifuged at 4000rpm and the supernatant filter sterilised. Protein concentration was determined by Coomassie assay (Pierce, USA) in comparison with a bovine serum albumin standard (Sigma-Aldrich, UK). Sterile antigen was stored at –20°C until use.

### 2.4 *Anti-ICOS antibody treatment.*

Wild type BALB/c mice where injected with 100μg monoclonal antibody 128A, or a control antibody (matched isotype of irrelevant specificity) i.p. on the day prior to infection and on the third day post infection . MAb 128A is a rat anti-mouse ICOS that blocks ICOS from binding to ICOSL (Ozkaynak *et al.*, 2001). 128A and the isotype control were kind gifts from Dr. A. J. Coyle, Millennium Pharmaceuticals.

### 2.5 *OX40 and OX40 ligand fusion Protein treatment.*

Wild type BALB/c mice were injected i.p. with 100μg of OX40 and OX40 ligand (OX40L) fusion proteins in 0.2ml sterile PBS on day 2 and 5 post infection. OX40 and OX40L fusion proteins were obtained from Xenova (Cambridge, UK).

### *2.6 PAR-2 activating peptide treatment.*

Wild type C57/BL6 mice were treated with 30μg each SLIGRL-$NH_2$, ASKH95 and the scramble peptide LRGILS-$NH_2$ in 0.2ml sterile PBS i.p. daily from day -1 to 16 p.i. The PAR-2 agonist peptides, SLIGRL-$NH_2$, ASKH-95 and LRGILS-$NH_2$ were obtained from Kowa Ltd (Japan)

### *2.7 The collection of sera samples.*

Blood samples where collected from mice uninfected and infected mice by cardiac puncture; the blood was incubated overnight at 4°C to allow clotting to occur. The samples were centrifuged at 1300 rpm (Hereus BioFuge) and the sera removed and stored at -20°C until use.

### *2.8 Assessment of enteropathy.*

Intestinal pathology was assessed in a sample of jejunum taken from approximately 10cm below the pyloric sphincter muscle. The sample was opened longitudinally and placed on a square of cardboard. The tissue was fixed overnight in 5ml Clarke's Fixative (see appendix), and then stored in 70% ethanol. Prior to use the tissue was re-hydrated by incubating for 10 minutes in 10ml 50% ethanol, followed by 20ml tap water. The tissue was then permeablised in 5ml 1M HCl (see appendix) at 60°C for 7.5 minutes and

washed for 10 minutes, 3 times in 20 ml tap water. The sample was then removed from the cardboard backing and stained with Schiff's reagent (Sigma-Aldrich, UK) for 30 minutes and washed in tap water. Sections one villi thick were cut under a dissecting microscope and placed on a slide in 45% Acetic Acid (see appendix). Lengths of 10 villi and 10 crypts measured using an eyepiece micrometer (Leica Microscopes) and lengths in μm were calculated. The sample was then gently crushed and the number of mitotic figures for 10 villus/crypt units was counted at x400 on a Leica light microscope. The mean villus, and crypt lengths and the mean number of mitotic figures/ villi-crypt unit were calculated for each mouse.

### *2.9 Enumeration of mucosal mast cells.*

Samples of jejunum, approximately 4cm in length, were opened longitudinally and wound luminal side outermost around a cocktail stick. The tissue was then fixed in Carnoy's fixative (see appendix) for 3-4 hours and transferred into 70% ethanol (see appendix). The cocktail stick was removed and samples placed in histocasettes and transferred through ascending grades of ethanol (50%, 70%, 90%, and 100% (twice) ethanol) for 30 minutes each and cleared in 50/50 Ethanol/Histoclear (National Diagnostics, UK) followed by 100% histoclear, twice for 45 minutes each. The tissue was then impregnated with paraffin wax with a melting point of 56°C (Fisher Scientific, UK), twice for 1 hour and embedded into wax blocks. Sections 5μm thick were cut on a

microtome (ThermoElectron, UK) and mounted on superfrost glass slides (ThermoElectron, UK) and dried for 48 hours at 37°C.

For the staining of mast cells slides were heated for 1 hour at 56°C and then re-hydrated through 100% Histoclear (twice), 50/40 Histoclear/Ethanol and descending grades of ethanol (100% (twice), 90%, 70%, and 50% ethanol) into water for 5 minutes each. Re-hydrated slides where stained overnight in 0.5% Toluidine blue (pH0.3) (see appendix) for the visualisation of mast cells, then rinsed in 0.7M HCl and counterstained for 2 minutes with Nuclear Fast Red (Vector Ltd, UK). The slides were then rinsed in running tap water to remove excess stain. The slides were then dehydrated and cover slips were mounted on the sections with Histomount (National Diagnostics, UK) and allowed to set.

Mucosal mast cells where counted under a light microscope at x200 magnification in 20 villus-crypt units (VCU) and the data expressed as the number of mast cells per VCU.

*2.10 Mucosal mast cell protease-1 ELISA.*

96 well ELISA plates (Greiner, UK) were coated overnight at 4° C with 50µl/well of anti- mouse mMCP-1 antibody (Moredun Laboratories, UK) at 2µg/ml in 0.1M $Na_2CO_3/NaHCO_3$ buffer (pH9.6). Plates were then washed three times in PBS/0.05% Tween 20 (see appendix) and then blocked with 200µl/well of PBS/10%FCS for 2 hrs at

37°C. Plates were then washed three times in PBS/0.05% Tween 20. Serum samples were diluted 1 in 2000 in PBS/10%FCS and 50µl was added to wells, in triplicate. Seventy-five-µl/well Recombinant murine mMCP-1 (Moredun Laboratories, UK) was made up in PBS/10%FCS at 40ng/ml and added to wells in triplicate, doubling dilutions were made down the plate in PBS/10%FCS to give a final volume of 50µl/well. Plates were incubated at 37°C for 1hr. Plates were then washed three times in PBS/0.05% Tween 20. Fifty-µl/well biotinylated anti-mouse mMCP-1 antibody (Moredun Laboratories, UK) at 2µg/ml in PBS/10%FCS was added and incubated for 1hr at 37°C. Plates were then washed three times in PBS/0.05% Tween 20 and dried. Streptavidin-horse radish peroxidase conjugate (PharMingen, BDBiosciences, UK) was diluted 1 in 1500 in PBS/10%FCS and 50µl added to each well and incubated at 37°C for 1 hr. Plates where then washed three times in PBS/0.05% Tween 20. Fifty-µl/well of TMB substrate (BDBiosciences, UK) was added to each well and the colour allowed to develop in the dark at room temperature; the reaction was stopped with 50µl/well of 0.4M $H_2SO_4$. Plates where read on a Spectromax™ microplate reader at 540/650nm

*2.11 Cytokine production* ex vivo.

Single cell suspensions of mesenteric lymph node were prepared in RPMI 1640 (GIBCO-BRL, UK) supplemented with 10% FCS, 5mM L- glutamine, 100U/ml penicillin, 1µg/ml streptomycin, 1.25µg/ml Amphotericin B, (all GIBCO-BRL, UK)

25mM HEPES and 0.05mM 2 Mercaptoethanol (SIGMA, UK), by forcing through nitex (Cadisch Precision Meshes, UK) using a syringe plunger. Cell numbers and viability was assessed using by diluting a sample of cells 1 in 10 in 0.2% Trypan Blue (see appendix) and counting the number of viable cells in a neubaur improved haemocytometer.

$1.5\times10^7$ cells in 1.5ml singly or $1\times10^6$ cells in 0.2ml in triplicate in supplemented RPMI media were incubated in the presence of *T. spiralis* muscle larval antigen (TAg) (prepared as described above) at 50μg/ml. For 1.5ml cultures cells were pooled within groups whilst for 200μl cultures cells from individual animals were cultured alone unless insufficient cell numbers were obtained. After 24 hours cell cultures were centrifuged at 350g and the supernatants transferred into clean eppendorfs, for 1.5ml cultures or into clean low binding 96 well plates for 200μl cultures. Supernatants were stored at -20°C until use.

*2.12 Measurement of cytokine levels in cell culture supernatants.*

Cytokine concentrations in cell culture supernatants were measured by sandwich ELISA using antibody pairs; recombinant standards; and Streptavidin-Horse Radish Peroxidase (HRP) conjugates purchased from BioSource (for IL-4, IL-5 IL-10 and IFNγ); PharMingen (for IL-9); and R&D Systems (for IL-13)

96 well ELISA plates (Greiner, UK) where coated overnight with 50µl/well of anti-mouse IL-4; IL-10; IFNγ at 1.25µg/ml; IL-9 at 2µg/ml in 0.05M $Na_2CO_3/NaHCO_3$ buffer (pH9.4); IL-13 at 2µg/ml in PBS (pH7.4), at 4°C for IL-4, IL-10, IFNγ and IL-9 and at room temperature for IL-13. Plates were then washed three times in PBS/0.05% Tween 20 (Sigma Aldrich, UK), dried, and blocked with 200µl/well PBS/10%FCS for 2 hours at 37°C. Plates were then washed three times in PBS/0.05% Tween 20 and dried. 50µl of cell culture supernatants were added to the 96 well. Recombinant murine protein standards corresponding to the appropriate cytokine were made up in PBS/10%FCS as follows: IL-4 at 1000pg/ml; IL-10 at 4000pg/ml; IFNγ at 2000pg/ml; IL-9 at 5000pg/ml and IL-13 at 4000pg/ml and were added to plates in triplicate, doubling dilutions were made down the plate in PBS/10%FCS giving a final volume of 50µl/well. Plates were incubated at 37°C for 1hour and 30 minutes. Plates were then washed three times in PBS/0.05% Tween 20 and dried. 50µl/well of biotinylated anti-mouse cytokine antibodies in PBS/10%FCS at the following concentrations anti-IL-4 and IL-10 at 0.125µg/ml; anti-IFNγ at 0.05µg/ml; anti-IL-9 at 0.5µg/ml and anti-IL-13 at 0.2µg/ml was added and incubated for 1hr at 37°C. Plates were then washed three times in PBS/0.05% Tween 20 and dried.

50µl of streptavidin-HRP conjugates at 0.2µg/ml for IL-4 and IL-5; 0.5µg/ml for IL-10; 0.3µg/ml for IFNγ; and 1 in 1000 dilution for IL-9 and IL-13 added to each well and incubated at 37°C for 1 hr. Plates where then washed three times in PBS/0.05% Tween 20. 50µl/well of 'SureBlue' TMB substrate was added to each well and the colour

allowed to develop in the dark at room temperature; the reaction was stopped with 50μl/well of 0.4M $H_2SO_4$. Plates where read on a Spectromax™ plate reader at 450/650nm.

*2.13 Measurement of total IgE.*

Total serum IgE was measured by ELISA using antibody pairs, standard and streptavidin-HRP purchased from PharMingen, BD Biosciences (UK). High binding 96 well ELISA plates (Greiner, UK) were coated overnight with 50μl/well of anti-mouse IgE at 2μg/ml in 0.1M $Na_2CO_3$ (pH8.2) at 4°C. Plates were then washed three times, dried and blocked with 200μl/well PBS/10%FCS for 2 hrs at 37°C. Plates were then washed three times in 0.05%Tween/PBS and dried. Sera samples where diluted: 1:100, for uninfected and 1:400, for infected mice in PBS/10%FCS and 50μl was added to the plates in triplicate. Murine IgE standard was diluted to 8μg/ml and plated out in triplicate. Doubling dilutions were made down the plate in PBS/10%FCS giving a final volume of 50μl/well. Plates were incubated at 37°C for 1hr and 30 minutes. Plates were then washed three times in 0.05%Tween/PBS and dried. 50μl/well of biotinylated anti-mouse IgE antibody in PBS/10%FCS at 2μg/ml was added and incubated for 1hr at 37°C. Plates were then washed three times in 0.05%Tween/PBS and dried. 50μl of streptavidin-HRP diluted 1:1000 in and incubated at 37°C for 45 minutes. Plates where then washed three times in 0.05%Tween/PBS and dried. 50μl/well of 'SureBlue' TMB

substrate was added to each well and the colour allowed to develop in the dark at room temperature; the reaction was stopped with 50µl/well of 0.4M $H_2SO_4$. Plates where read on a Spectromax™ plate reader at 450/650nm.

*2.14 Measurement of parasite specific IgG1 and IgG2a.*

Parasite specific IgG1 and IgG2a were measured by ELISA TAg as target antigen and HRP conjugated anti-mouse IgG1 or IgG2a purchased from Northern BioTech (UK). 96 well ELISA plates (Greiner, UK) were coated overnight with 50µl/well of TAg at 2µg/ml in 0.05M $Na_2CO_3$/$NaHCO_3$ buffer (pH9.6) at 4°C. Plates were then washed three times in PBS/0.05%Tween 20, dried and blocked with 200µl/well 10%FCS/PBS for 2 hrs at 37°C. Plates were then washed three times in PBS/0.05%Tween 20 and dried. Sera samples were diluted: 1:40 in 10%FCS/PBS and 100µl was added to the plates in triplicate 1:4 dilutions were made down the plate giving a final volume of 75µl/well. Plates were incubated at 37°C for 1hr and 30 minutes. Plates were then washed three times in PBS/0.05%Tween 20 and dried. 50µl/well of HRP conjugated anti-mouse IgG1 or IgG2a antibody diluted 1:10,000 in 10%FCS/PBS was added and incubated for 1hr at 37°C. Plates were then washed three times in PBS/0.05%Tween and dried. 50µl/well of 'SureBlue' TMB substrate was added to each well and the colour allowed to develop in the dark at room temperature; the reaction was stopped with 50µl/well of 0.4M $H_2SO_4$. Plates were read on a Spectromax plate reader at 450/650nm.

*2.15 Statistical Analysis.*

Where possible data is expressed as mean + standard error of the mean. Significance was tested using the Mann Whitney U test (StatView™) and a p value of less than or equal to 0.05 was taken to be significant.

# Chapter Three

## The role of IL-4, IL-13 and IL-4Rα in the development of protective and pathological responses to *T. spiralis*.

# 3 The role of IL-4, IL-13 and IL-4Rα in the development of protective and pathological responses to *T. spiralis*.

## *3.1 Introduction*

T helper type 2 (Th2) responses, which are characterised by the production of the cytokines Interleukin (IL)-4, IL-5 and IL-13 (Mossmann and Coffman, 1989), have been shown to be important in protective responses to helminth infections (Bancroft *et al.*, 1997; Else *et al.*, 1994; Urban *et al.*, 1995; Urban *et al.*, 1998) and the development of pathological conditions such as atopy and asthma (Cohn *et al.*, 1997; Grunewald *et al.*, 2001; Grunig *et al.*, 1998; Hayashi *et al.*, 2005). IL-4 is produced by a variety of cell types including T cells (Kondo *et al.*, 2005), eosinophils (Gessner *et al.*, 2005), mast cells (Zhao *et al.*, 2005), and basophils (Gessner *et al.*, 2005). IL-4 is a major regulator of Th2 responses, stimulating B cell proliferation (Rush and Hodgkin, 2001), antibody class switching to IgE (Hasbold *et al.*, 1998), the release of other Th2 cytokines such as IL-5, leading to the development of eosinophilia (Mochizuki *et al.*, 1998) and mastocytosis (Watanabe *et al.*, 2001).

IL-4 has been shown to be important in the development of protective responses to gastrointestinal nematode infections; exogenous IL-4 treatment has been shown to cure established *Heligomoides polygyrus* infection in wild type BALB/c mice and to reduce fecundity of *H. polygyrus* and cure *Nippostrongylus brasiliensis* in the highly

susceptible severe combined immune deficient (SCID) mouse (Urban *et al.*, 1993). In IL-4 deficient (-/-) mice expulsion of *T. spiralis* from the small intestine is delayed (Lawrence *et al.*, 1998) and in *Trichuris muris* infection IL-4 deficiency (Bancroft *et al.*, 1998) and IL-4 blockade (Else *et al.*, 1994) delays expulsion. In both these nematode infections IL-4 deficiency leads to impaired Th2 antibody responses (IgE and IgG1) (Else *et al.*, 1994; Lawrence *et al.*, 1998). IL-4 -/- mice however, retain the capacity to mount Th2 responses following immunisation with ovalbumin in aluminium hydroxide (Brewer *et al.*, 1999). This is thought to be due to the action of another cytokine, IL-13. IL-13 is homologous to IL-4, and has also been shown to be an important mediator during Th2 responses (Barner *et al.*, 1998). IL-13 mediates responses similar to those of IL-4, stimulating B cell proliferation, antibody class switching to IgE (Emson *et al.*, 1998) and eosinophilia (Li *et al.*, 1999; Nonaka *et al.*, 2004), and is produced by a similar set of cell types including T cells, eosinophils (Gessner *et al.*, 2005), and mast cells (Gessner *et al.*, 2005; Zhao *et al.*, 2005).

Both IL-4 and IL-13 bind to IL-4 receptor α chain (IL-4Rα), the ligation of this receptor has been shown to result in the phosphorylation and activation of signal transducer and activator of transcription factor 6 (STAT6), which in turn leads to the activation of GATA3. In the absence of STAT6 lymphocytes fail to proliferate in response to IL-4, and B cells fail to produce IgE (Kaplan *et al.*, 1996). *In vivo* STAT6 has been shown to be necessary for the expulsion of *T. spiralis* (Khan *et al.*, 2001) and for the development of eosinophilia, airway hyper-responsiveness and mucous hypersecretion in a murine

model of asthma (Hoshino *et al.*, 1999). Interestingly, Th2 cytokine secretion has been detected in Stat6 deficient mice in both nematode and asthma models (Hoshino *et al.*, 2004), GATA3 however has been shown to be the master regulator of Th2 cell differentiation, and is absolutely necessary for the development of Th2 responses (Seki *et al.*, 2004) (Figure 3.1).

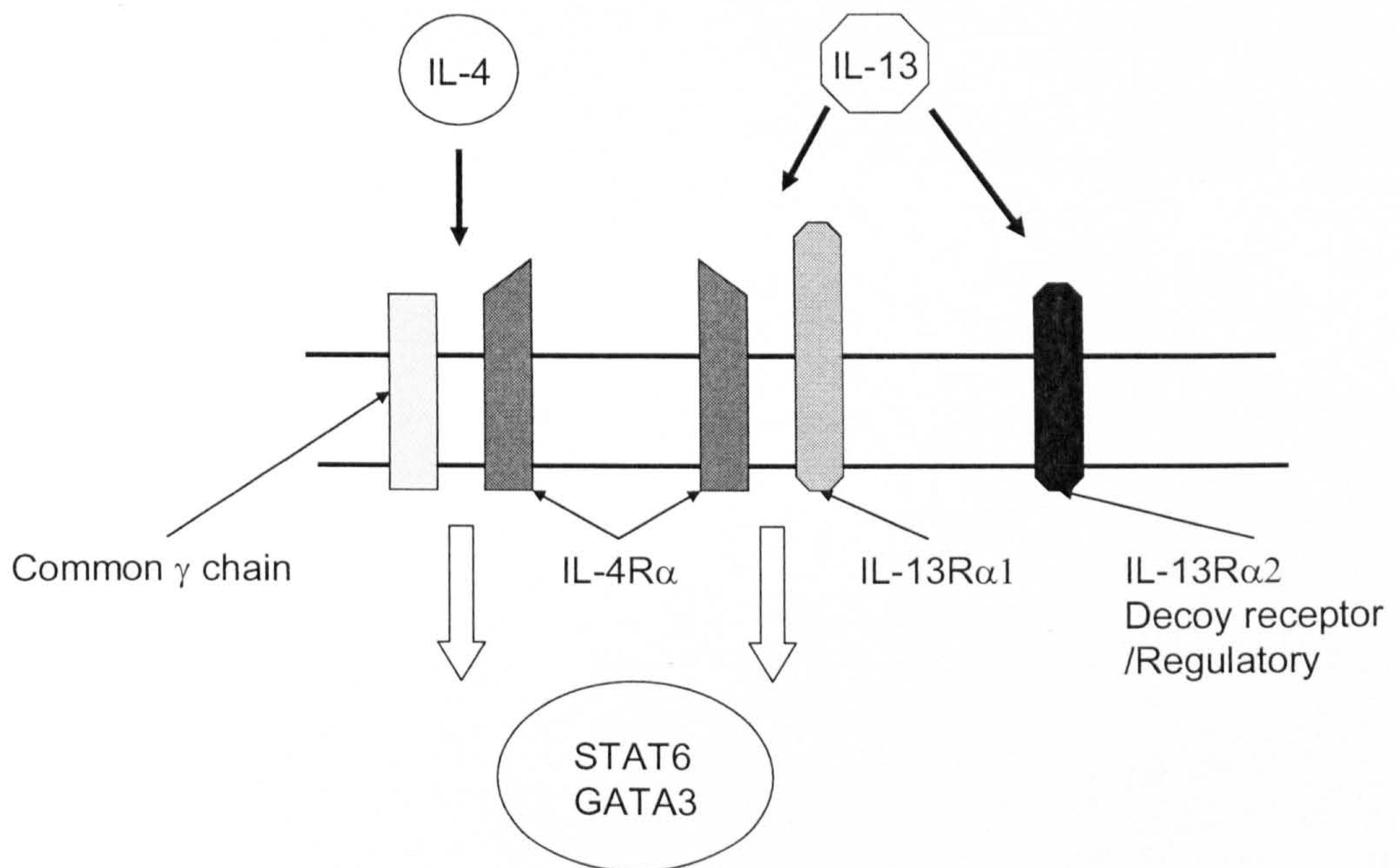



FIGURE 3.1: IL-4Rα, IL-13Rα and IL-13Rβ complexes.

Despite functional similarities to IL-4, IL-13 has been shown to mediate effects independently to IL-4. The inhalation of IL-13 has been shown to activate a set of genes in pulmonary tissue, distinct from those activated by IL-4 inhalation including eotaxin precursor, and pro-collagen (Finkelman *et al.*, 2005). A major role for IL-13 has been shown in the pathogenesis of asthma and schistosomiasis; where IL-13 has been shown to stimulate fibroblasts and lead to the development of fibrosis (Fallon *et al.*, 2000; Zhu *et al.*, 2001). Although both IL-4 and IL-13 bind the IL-4Rα, IL-13 also binds to two IL-13 receptors, IL-13Rα1 and IL-13Rα2. IL-13Rα1 is membrane bound and has low affinity for IL-13, but forms dimers with IL-4Rα to form a high affinity receptor, while IL-13Rα2 is present in both a membrane bound and a soluble form and has high affinity for IL-13 and is thought to act as a decoy receptor and therefore play a role in modulating IL-13 activity *in vivo* (Figure 3.1). Furthermore IL-13 has been shown to be internalised on binding IL-13R2 on transfected CHO cells without activating STAT6 and therefore Th2 responses (Kawakami *et al.*, 2001).

Different inbred mouse strains have been shown to develop different cytokine profiles following infection with *T. muris*. The levels of Th1 vs. Th2 cytokines correlates with degree of resistance to infection exhibited by these mice, with increased resistance observed in those strains where Th2 cytokines predominate (Else *et al.*, 1993; Else *et al.*, 1992). It has also been shown that BALB/c and C57BL/6 mice have differences in their IL-4Rα gene resulting in differences in the binding of IL-4, that correlate with the

increased ability of IL-4 to stimulate allergic responses *in vivo* when signalling via the BALB/c IL-4Rα compared to the C57BL/6 IL-4Rα (Webb *et al.*, 2004).

IL-4Rα is expressed on a wide range of cells such as T cells, B cells, epithelial cells, mast cells, neutrophils and macrophages. Neutrophils and macrophages are part of the innate immune system; neutrophils are among the first cell types to infiltrate sites of inflammation while many tissues constitutively contain macrophages. Macrophages are professional antigen presenting cells and thus help to initiate adaptive responses, by presenting antigen to T cells via MHCII and by providing co-stimulatory signals such as CD40. Macrophages also play important roles as effector cells in the defence against pathogens such as *Leishmania spp.* and *Mycobacterium tuberculosis*.

The activation of macrophages may occur by three different pathways. Firstly, classical activation, where macrophages are primed by IFNγ and respond to stimulation by TNFα, LPS or other bacterial products, to secrete IL-12, IL-6 and TNFα; up regulate MHCII and CD86 (Nathan *et al.*, 1983). Activated macrophages also produce reactive oxygen intermediates, which have been shown to play an important role in the killing of intracellular parasites such as *Leishmania* species (Diefenbach *et al.*, 1998; Greil *et al.*, 1988; Nathan and Hibbs, 1991). Secondly type 2-activation, where FcγR ligation by antibodies followed by the Toll-like receptor (TLR) stimulation or the activation of costimulatory signals such as CD40. Type 2 activated macrophages secrete TNFα and IL-6 but switch off IL-12 and secrete IL-10. These macrophages may act in an anti-

inflammatory fashion but may also drive Th2 responses via IL-10 (Anderson and Mosser, 2002). Thirdly, IL-4 stimulation results in "alternatively activated" macrophages, these cells express IL-10 and the IL-1Rα but are poor producers of reactive oxygen intermediates and are therefore unable to control intracellular pathogens. Furthermore, it has been shown that signalling to macrophages by via IL-4Rα leads to up regulation of MHCII and was necessary for the priming of mice to an inhaled antigen (Padilla *et al.*, 2005).

Neutrophils are important in protective responses to bacterial and fungal infection, and are associated with the development of the Th1 mediated pathologies such as Crohn's disease. Neutrophils are phagocytic cells with potent microbicidal activity and are among the first cell types seen infiltrating sites of inflammation. Following activation neutrophils can generate a respiratory burst leading to the production of reactive oxygen intermediates and degranulate releasing inflammatory mediators and enzymes. It has been shown that the increased susceptibility to bacterial infection following infection with FIV, a model for HIV infection, is associated with defects in neutrophil function (Kubes *et al.*, 2003). Further evidence of the importance of neutrophils in resistance to bacterial infection has been demonstrated in murine *Peusdomonas aeruginosa* infection where resistant mouse strains (C3H/HeN) rapidly increased neutrophil expression of CD11b (Jensen *et al.*, 2004), which mediates phagocytosis, the generation of the respiratory burst and degranulation, compared to susceptible mice (BALB/c). Neutrophils have also been shown to play a role in deleterious inflammatory conditions

such as rheumatoid arthritis and inflammatory bowel diseases such as Crohn's and ulcerative colitis. In these conditions neutrophils have been shown to be present in clinical samples of inflamed tissue at higher numbers than in normal tissue and show an activated phenotype (Tarlton *et al.*, 2000). A role for neutrophils has also been demonstrated in mouse models such as collagen-induced arthritis (Cannetti *et al.*, 2003) and dextran sulphate sodium (DSS) induced colitis (Ohkawara *et al.*, 2005). The depletion of neutrophils by specific anti-granulocyte antibodies has also be shown to influence the development of Th responses in mice following infection with *Legionella pneumonia*, resulting in a diminished Th1 response and an enhanced Th2 response to the bacteria (Tateda *et al.*, 2001).

Activated neutrophils secrete myeloperoxidase which has been shown to increase in intestinal tissue following infection with *T. spiralis* (Lawrence *et al.*, 1998), while neutrophilia has been observed in the blood of *T. spiralis* infected between days 3 and 20 post infection (Stewart *et al.*, 1999), and *in vitro,* both neutrophils and myeloperoxidase have been shown to be toxic to newborn *T. spiralis* larvae (Bass and Szejda, 1979; Buys *et al.*, 1984). During the intestinal phase of *T. spiralis* infection it has been shown that depletion of host neutrophils leads to a reduction in early cytokine production by epithelial cells (Stadnyk *et al.*, 2000). These data suggest that neutrophils may play a role in immune responses following infection with *T. spiralis,* while both neutrophils and macrophages appear to play a role in the development of Th2 responses. However, the precise role played by these cells during infection with *T. spiralis* is not clear. So it is of interest to evaluate the effect of signalling to neutrophils and

macrophages by IL-4 and IL-13, important regulators of Th2 responses, via their co-receptor IL-4Rα in the development of protective and pathological responses to *T. spiralis* infection.

In this study the development of protective responses, enteropathy and Th responses following infection with *T. spiralis* was examined in wild type and IL-4 -/- BALB/c and C57BL/6 mice, to evaluate the role of IL-4 in different host backgrounds. The role of IL-13 and IL-4Rα in BALB/c mice in the development of protection, enteropathy and Th responses following infection with *T. spiralis* was also examined using BALB/c IL-13 -/- and BALB/c IL-4Rα -/- mice. Additionally the role of IL-4 and IL-13 signalling via IL-4Rα to neutrophils and macrophages was examined using transgenic macrophage/neutrophil lineage specific IL-4Rα-/- mice, where IL-4Rα chain is expressed on all cells except those of the macrophage/neutrophil lineage.

### *3.2 Results.*

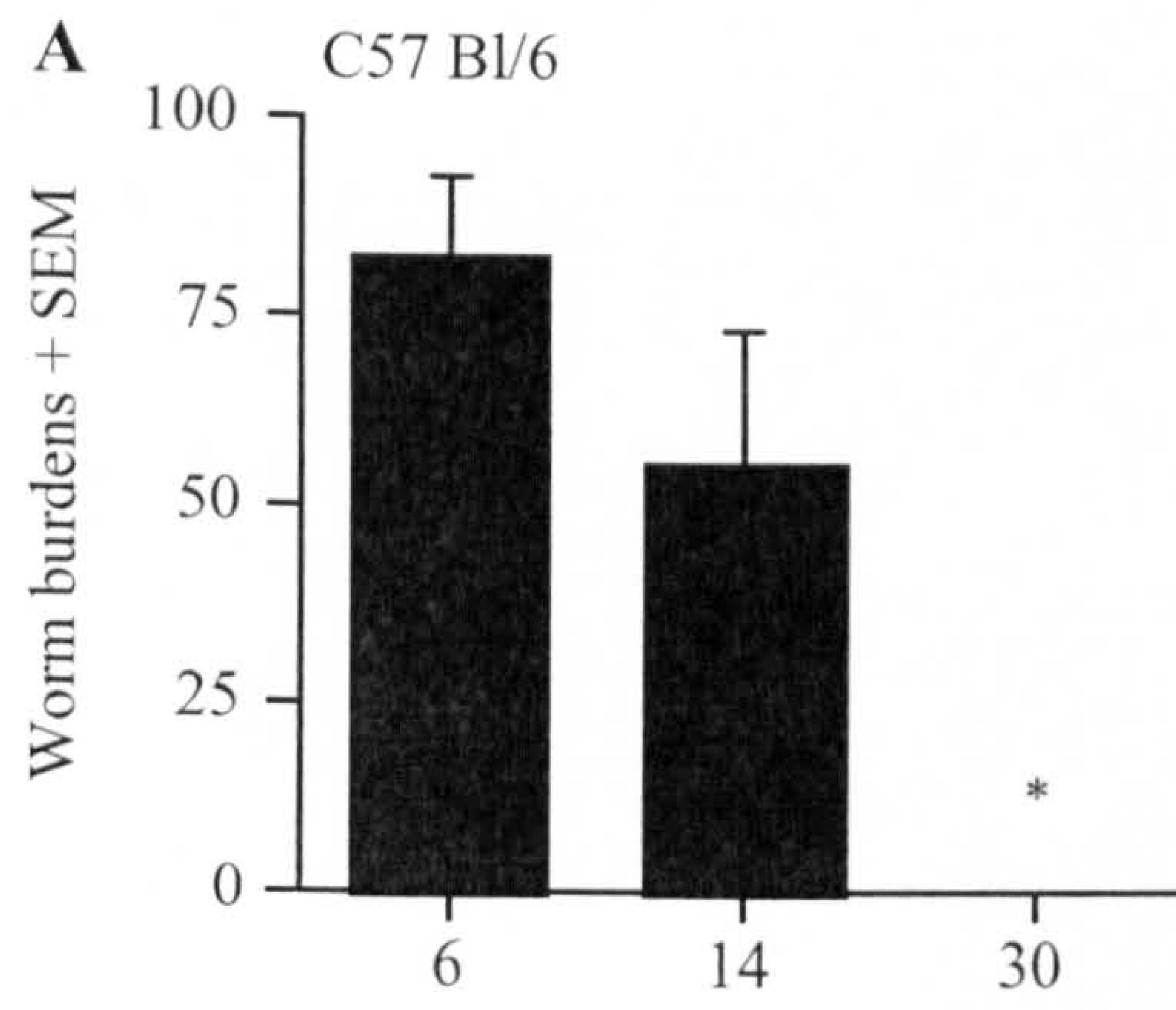
#### *3.2.1 Expulsion of* T. spiralis *is significantly delayed in the absence of IL-4, IL-13 and IL-4Rα.*

To assess the role of the Th2 cytokines in the establishment and expulsion of *T. spiralis*, wild type BALB/c, BALB/c IL-4 -/-, BALB/c IL-13 -/-, BALB/c IL-4Rα, wild type C57BL/6, and C57BL/6 IL-4 -/- were infected with 400 freshly isolated larvae. Worm establishment was measured at day 6 p.i. and worm expulsion was assessed at days 14 and 30 p.i.

No significant differences were observed between any of the strains in the establishment of *T. spiralis* in the small intestine at day 6 p.i. Wild type BALB/c mice had significantly reduced worm burdens at day 14 p.i. compared with day 6 p.i., by day 14 wild type C57BL/6 mice had not significantly reduced their worm burdens. However, worm burdens were not significantly different to wild type BALB/c mice at day 14 p.i. , and by day 30 p.i. both strains had completely expelled their parasites (Figure 3.2C and A). BALB/c IL-4 -/- (Figure 3.1D), BALB/c IL-13 -/- (Figure 3.2E), BALB/c IL-4Rα -/- (Figure 3.1F), and C57BL/6 IL-4 -/- (Figure 3.2B) mice had not significantly reduced their worm burdens at day 14 p.i. compared to day 6 p.i. and BALB/c IL-13 -/- ($p=0.009$), BALB/c IL-4Rα -/-($p=0.009$), and C57BL/6 IL-4 -/- ($p=0.009$) had significantly higher worm burdens at day 30 p.i. compared to wild type mice. BALB/c

IL-4 -/- mice still harboured some worms at day 30 p.i., however, this was highly variable and was not significantly different to wild type BALB/c mice. At day 30 p.i. BALB/c IL-4Rα -/- mice had significantly higher worm burdens than both BALB/c IL-4 -/- (p=0.09) and BALB/c IL-13 -/- mice (p=0.009), BALB/c IL-13 -/- mice also had significantly more worms than BALB/c IL-4 -/- mice at day 30 p.i. (p=0.0107). Thus the expulsion of *T. spiralis* from the small intestine appears to occur more rapidly in the BALB/c mouse compared to the C57BL/6 mouse and is dependent on Th2 responses, expulsion being significantly delayed in the absence of IL-13 in the BALB/c mouse and IL-4 in the C57BL/6 mouse. It is interesting to note the discrepancy between the role of IL-4 in BALB/c and C57BL/6 mice, IL-4 being necessary for expulsion in C57BL/6 but not in BALB/c mice.

FIGURE 3.2: The role of host strain, IL-4, IL-13 and IL-4Rα in the expulsion of *T. spiralis* from the intestine. The establishment and expulsion of *T. spiralis* was measured in wild type C57BL/6, (A), C57BL/6 IL-4 -/- (B), wild type BALB/c (C), BALB/c IL-4 -/- (D), BALB/c IL-13 -/- (E) and BALB/c IL-4Rα -/- (F) mice at day 6 and 14 p.i. The small intestine was excised and the total number of worms present was counted. Data is expressed as mean number of worms/mouse + SEM, five mice were used per group. *, represents significantly different to mice at day 6 p.i.; **, represents significantly different wild type C57BL/6 mice; §, represents significantly different to wild type BALB/c mice; †, represents significantly different to BALB/c IL-4Rα -/- mice; ‡, represents significantly different to BALB/c IL-4 -/- mice ($p<0.05$).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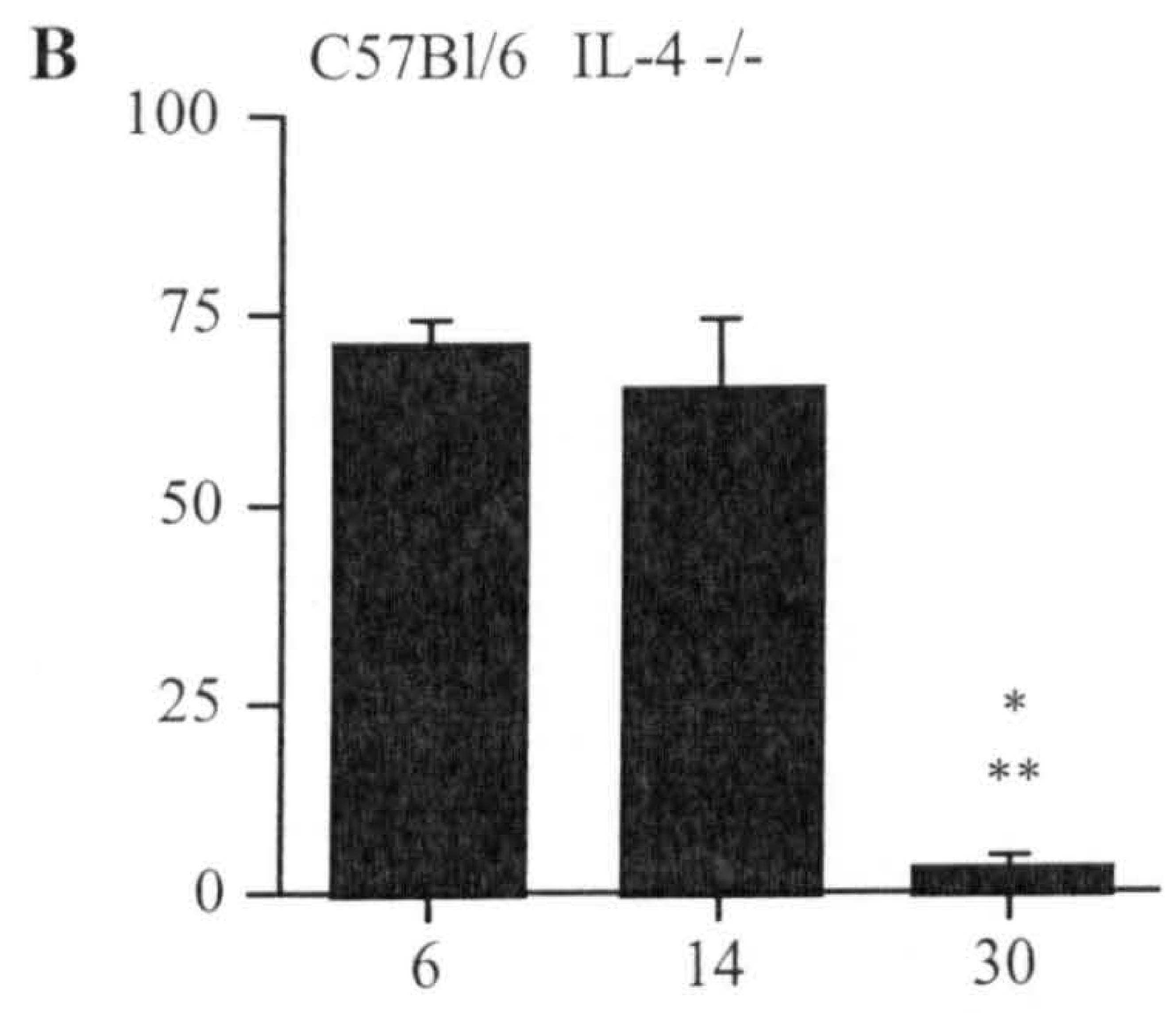
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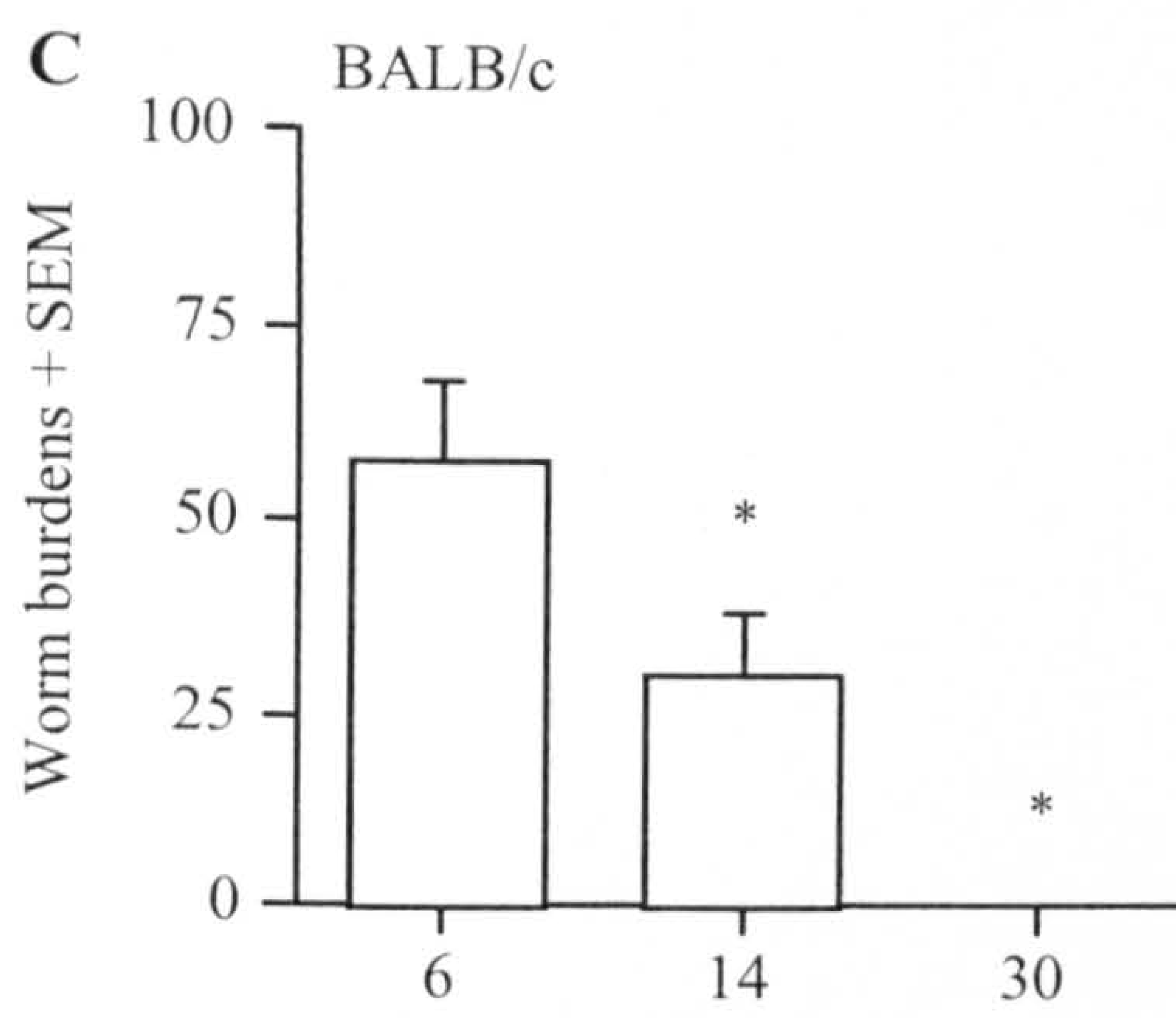
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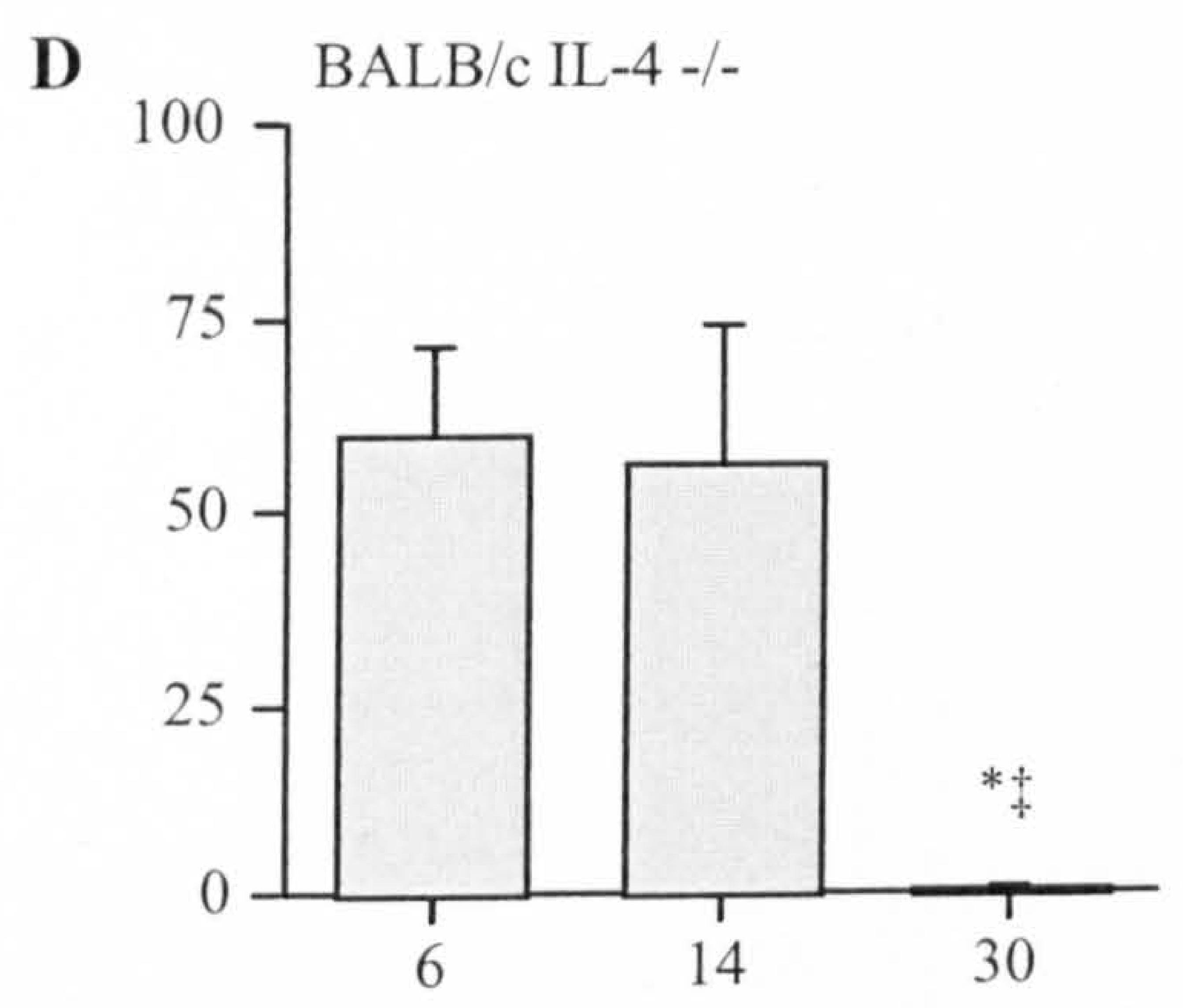
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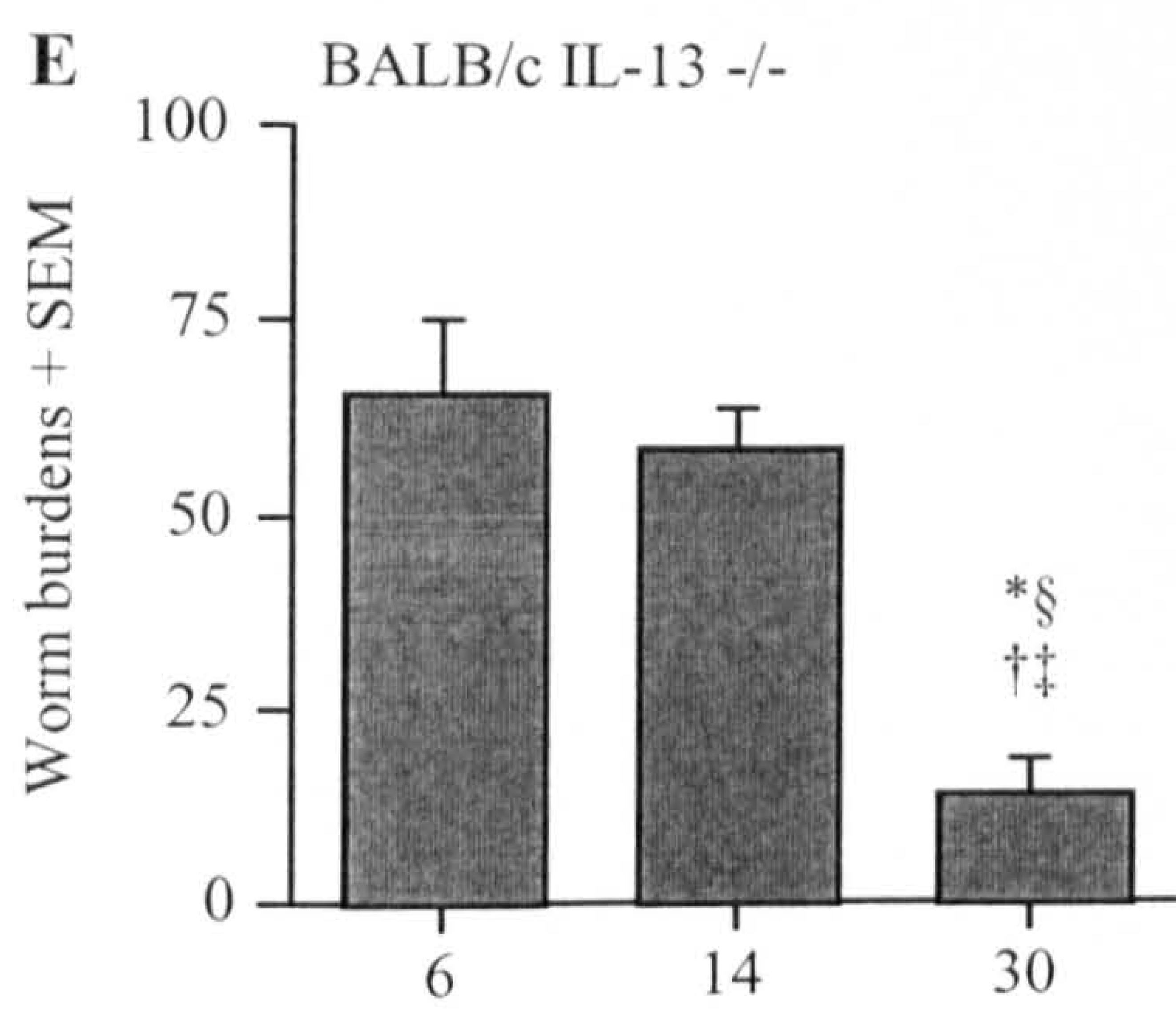
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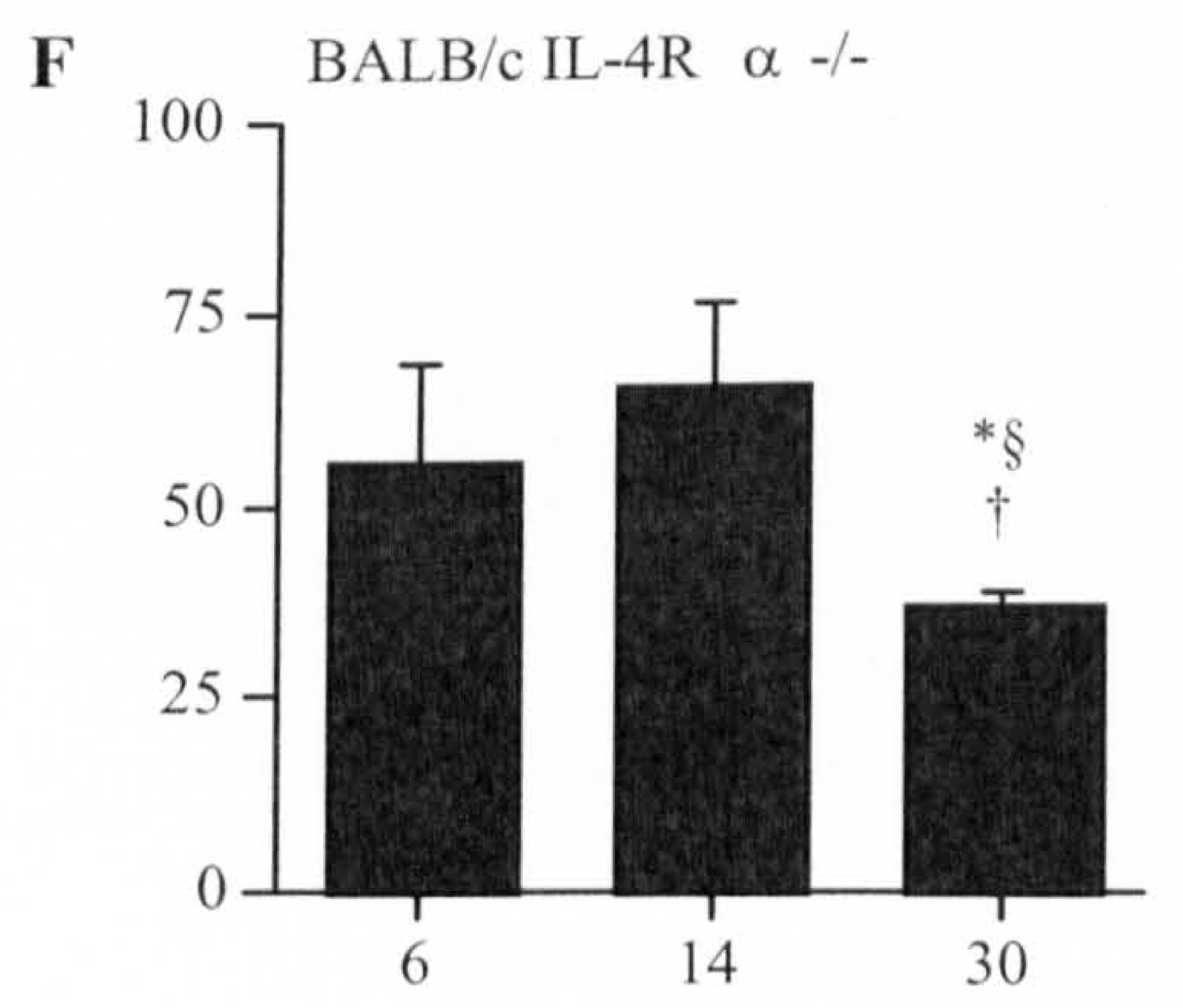
30
Days Post Infection


F
BALB/c IL-4R α -/-
100
75
50
25
0
*§
†
6
14
30
Days Post Infection

### *3.2.2 Reduced enteropathy in the absence of IL-13 but not IL-4.*

To analyse the role of Th2 responses in the development of enteropathy following infection with *T. spiralis* the development of villus atrophy and crypt hyperplasia was assessed in tissue fixed in Clarke's solution and stained with Schiff's reagent. The samples were then micro-dissected and the length of villi and crypts measured and as a measure of the proliferation in the crypts, the number of dividing cells or mitotic figures per villus-crypt unit (VCU) was counted. The development of intestinal oedema was measured by weighing the entire small intestine.

Significant villus atrophy and crypt hyperplasia had developed in both wild type BALB/c (p=0.0253 and 0.0253) (Figure 3.3C) and C57BL/6 (p=0.009 and 0.009) (Figure 3.2A) mice at day 6 p.i. compared to uninfected mice. No significant differences were observed between wild type BALB/c and C57BL/6 mice at either day 0 or 6 p.i. Villus atrophy and crypt hyperplasia had also developed in BALB/c IL-4 -/- (p=0.0143 and 0.0143) (Figure 3.3D) and C57BL/6 IL-4 -/- (p=0.0209 and 0.0209) (Figure 3.3B) mice at day 6 p.i. No significant differences were observed between BALB/c IL-4 -/- and C57BL/6 IL-4 -/- mice at either day 0 or 6 p.i. In BALB/c IL-13 -/- mice villus atrophy had not developed with villi being significantly longer in BALB/c IL-13-/- than wild type BALB/c mice at 6 dpi (p=0.009), however, crypt lengths in BALB/c IL-13 -/- mice were not significantly different to wild type BALB/c mice at day 6 p.i., and were significantly longer than that observed in uninfected BALB/c IL-13 -/- mice (p=0.009)

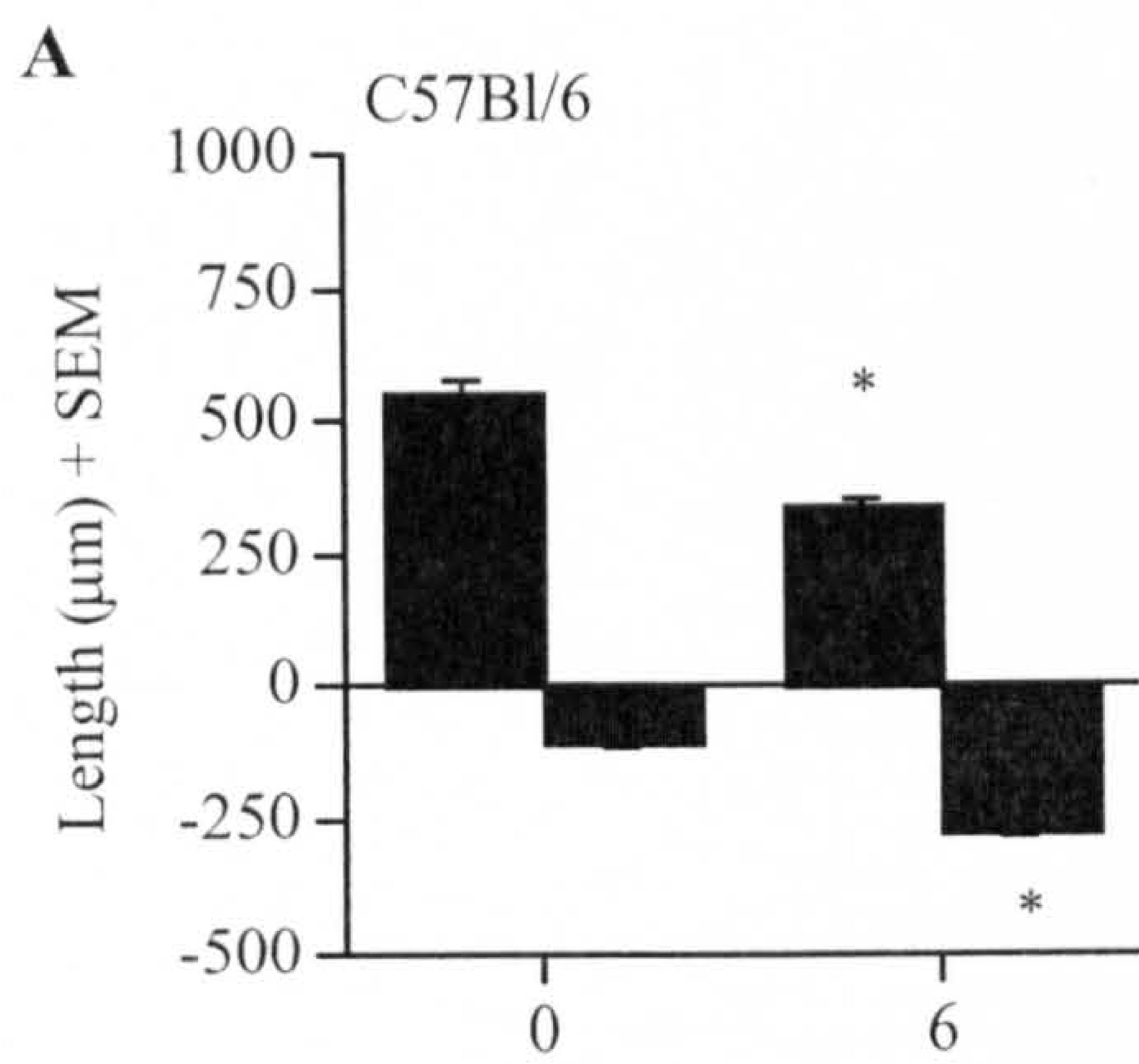
(Figure 3.3E). BALB/c IL-4Rα-/- mice had developed neither villus atrophy or crypt hyperplasia at day 6 p.i., having significantly longer villi (p=0.009) and shorter crypts (p=0.0283) than wild type BALB/c mice (Figure 3.2F). Both BALB/c IL-13 and BALB/c IL-4Rα -/- mice had significantly longer villi at day 6 p.i. than BALB/c IL-4 -/- mice (p=0.009 and 0.009). However only BALB/c IL-4Rα -/- mice had shorter crypts than BALB/c IL-4 -/- mice at day 6 p.i. (p=0.0163).

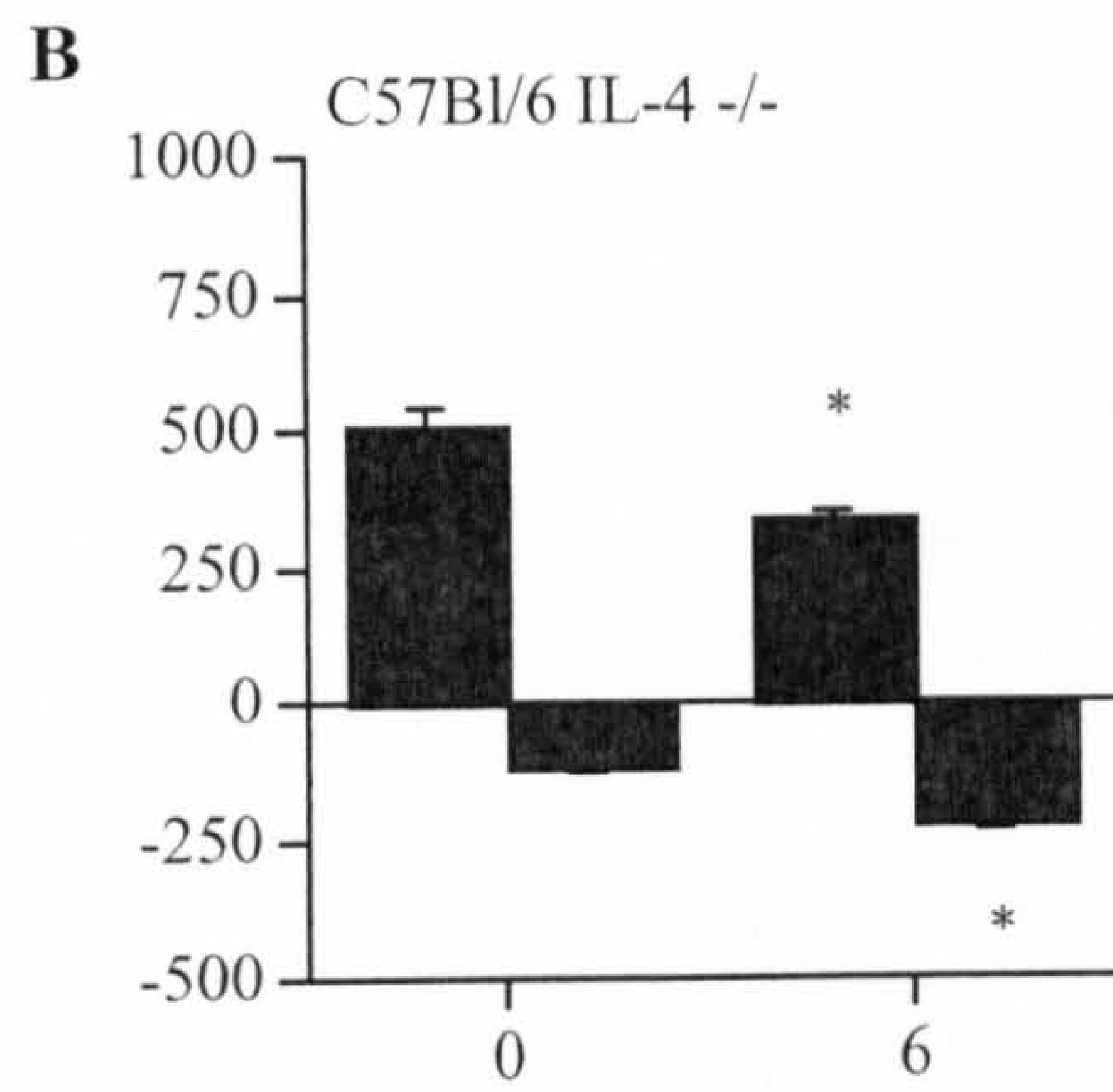
An increase in the number of mitotic figures present in the crypt epithelium was observed in wild type BALB/c (p=0.0253)(Figure 3.4C), BALB/cIL-4-/- (p=0.05)(Figure 3.4D), BALB/c IL-13-/- (p=0.0268) (Figure 3.4E), and C57BL/6 IL-4-/- (p=0.0209) (Figure 3.4B) mice. Interestingly BALB/c IL-4Rα -/- mice did not develop an increased number of mitotic figures/VCU at day 6 p.i. and had significantly fewer mitotic figures than wild type BALB/c. (p=0.0283) and BALB/c IL-4 -/- (p=0.0459) mice at day 6 p.i (Figure 3.4F). Although the increase in the number of mitotic figures observed in wild type C57BL/6 mice was not significant, the number of mitotic figures did not differ significantly from other strains at day 0 or 6 p.i. No significant differences were observed between wild type C57BL/6 and BALB/c mice or between C57BL/6 IL-4 -/- and BALB/c IL-4 -/- mice at day 0 or 6p.i. No significant differences were observed in intestinal architecture or the number of mitotic figures present in the small intestine between day 6 and 14 p.i. (data not shown)

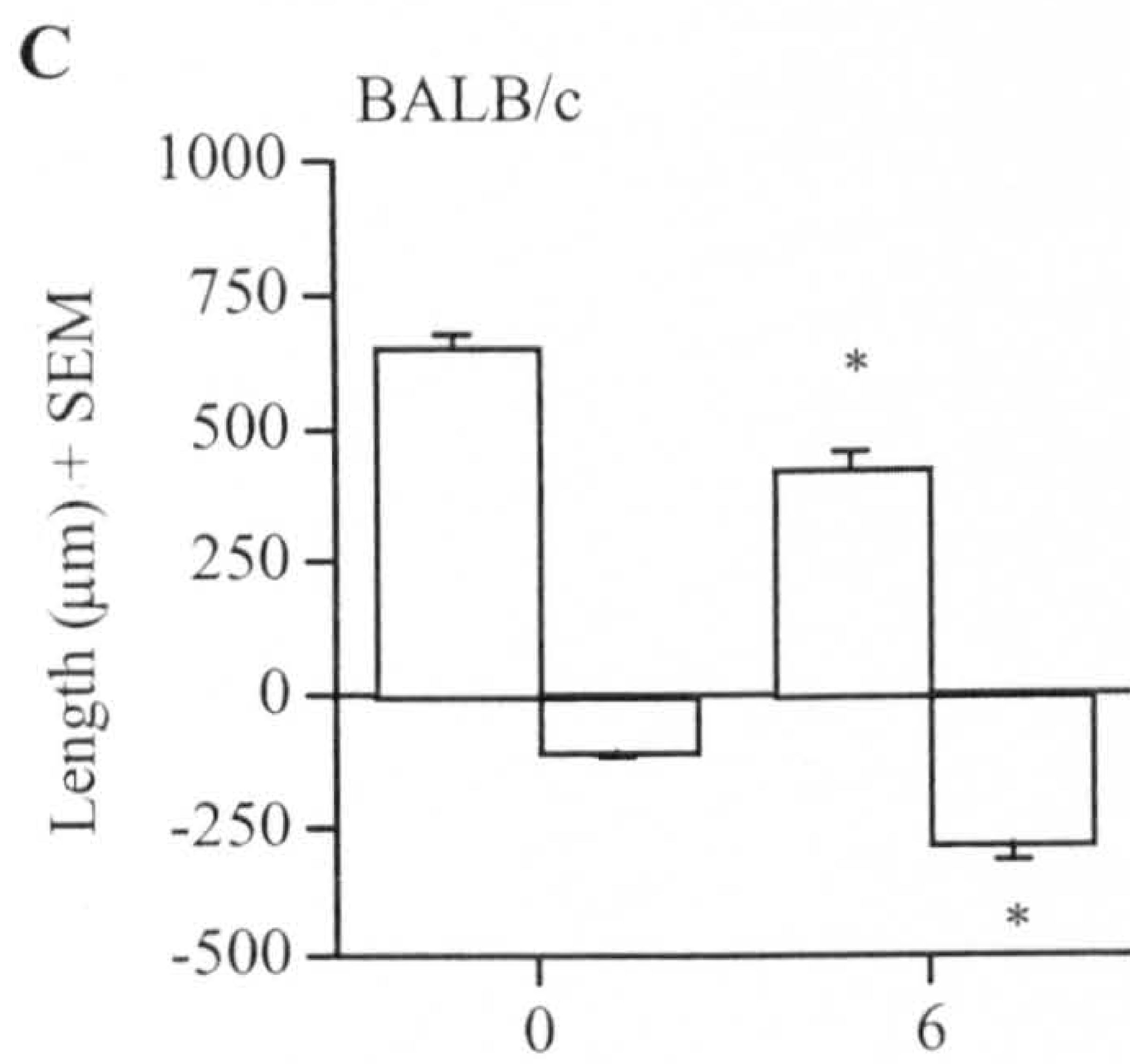
Significant oedema as shown by an increase in intestinal weight had developed in wild type BALB/c, BALB/c IL-4 -/-, and wild type C57BL/6 mice by day 6 p.i. (p=0.009, 0.009, 0.009) and was still present at day 14 p.i. (p=0.009, 0.009, 0.009)(Figure 3.5 A, C and D) compared to uninfected mice. In BALB/c IL-13 -/- mice the development of significant was delayed until day 14 p.i. (p=0.009) compared to uninfected mice (Figure 3.4E). In both BALB/c IL-4Rα -/- and C57BL/6 IL-4 -/- mice a significant increased in intestinal weight was not observed at day 6 or 14 p.i. (Figure 3.5 B and F).

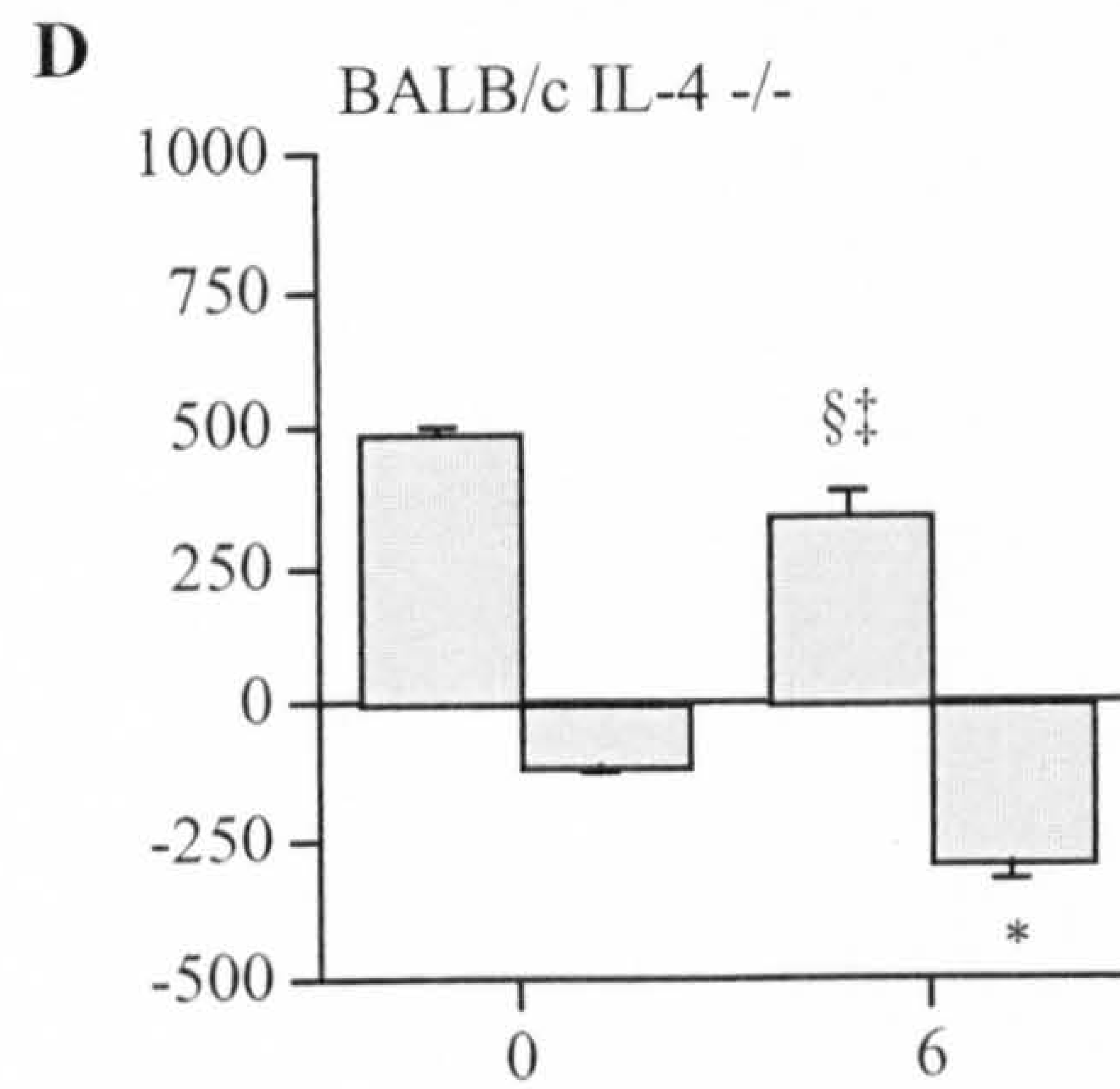
The development of villus atrophy and crypt hyperplasia was therefore significantly reduced in the absence of IL-13 and was further ameliorated in the absence IL-4Rα but not by deficiency in IL-4 alone. Intestinal oedema was delayed in the absence of IL-13 and completely absent in IL-4Rα deficient mice following infection. These data shown that IL-13 plays a key role in the development of enteropathy while IL-4 plays a more minor role.

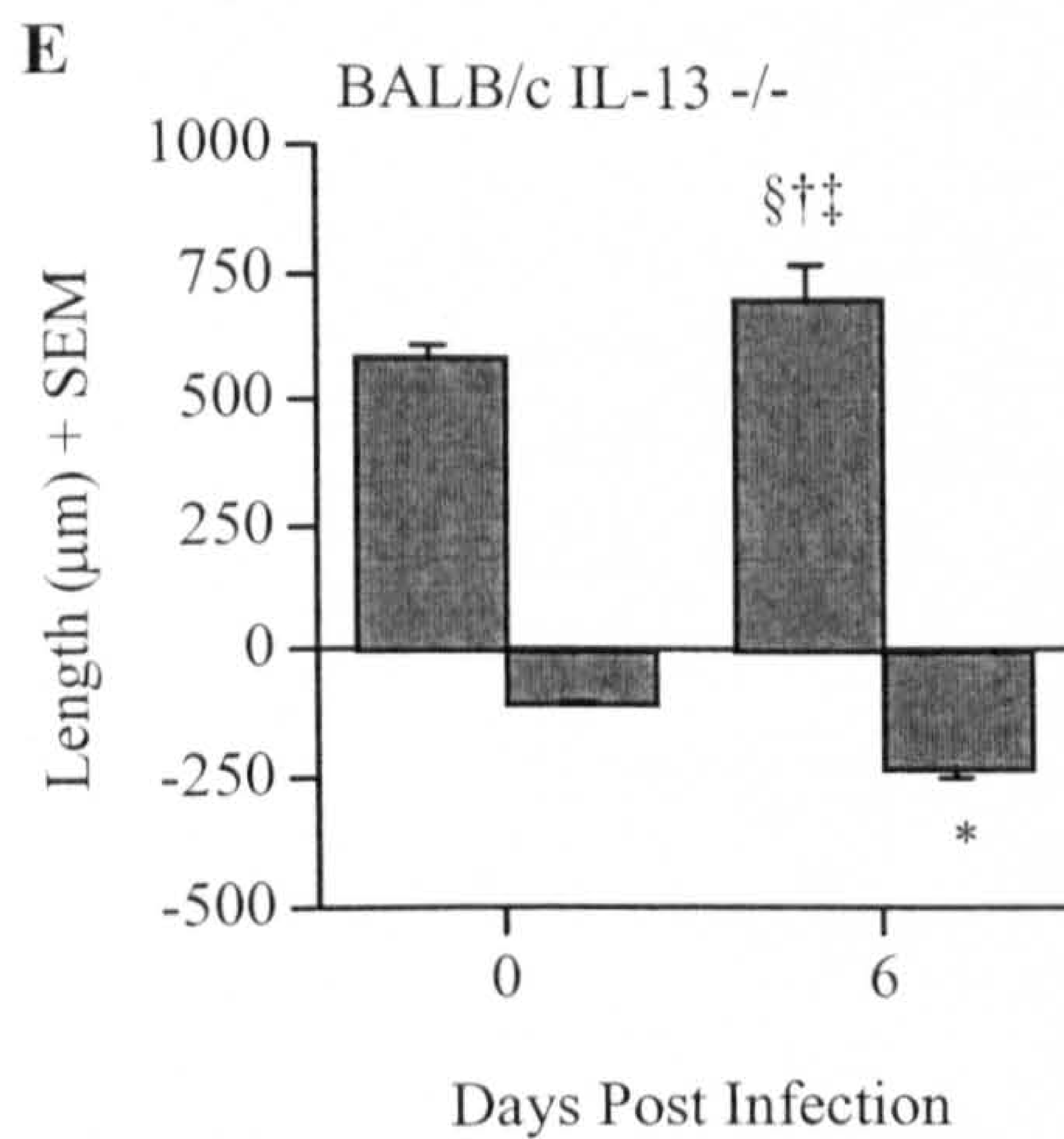
FIGURE 3.3: The role of host strain, IL-4, IL-13 and IL-4Rα in the development of enteropathy. The development of villus atrophy and crypt hyperplasia was measured in wild type C57BL/6, (A), C57BL/6 IL-4 -/- (B), wild type BALB/c (C), BALB/c IL-4 -/- (D), BALB/c IL-13 -/- (E) and BALB/c IL-4Rα -/- (F) mice at days 0 and 6 p.i. Data is expressed as mean length (μm) + SEM, five mice were used per group. *, represents significantly different to uninfected mice.; **, represents significantly different wild type C57BL/6 mice; §, represents significantly different to wild type BALB/c mice; †, represents significantly different to BALB/c IL-4 -/- mice; ‡, represents significantly different to BALB/c IL-4Rα -/- mice ($p<0.05$).


A
C57Bl/6
1000
750
500
250
0
-250
-500
Length (μm) + SEM
*
*
0
6


B
C57Bl/6 IL-4 -/-
1000
750
500
250
0
-250
-500
*
*
0
6


C
BALB/c
1000
750
500
250
0
-250
-500
Length (μm) + SEM
*
*
0
6


D
BALB/c IL-4 -/-
1000
750
500
250
0
-250
-500
§‡
*
0
6


E
BALB/c IL-13 -/-
1000
750
500
250
0
-250
-500
Length (μm) + SEM
§†‡
*
0
6
Days Post Infection

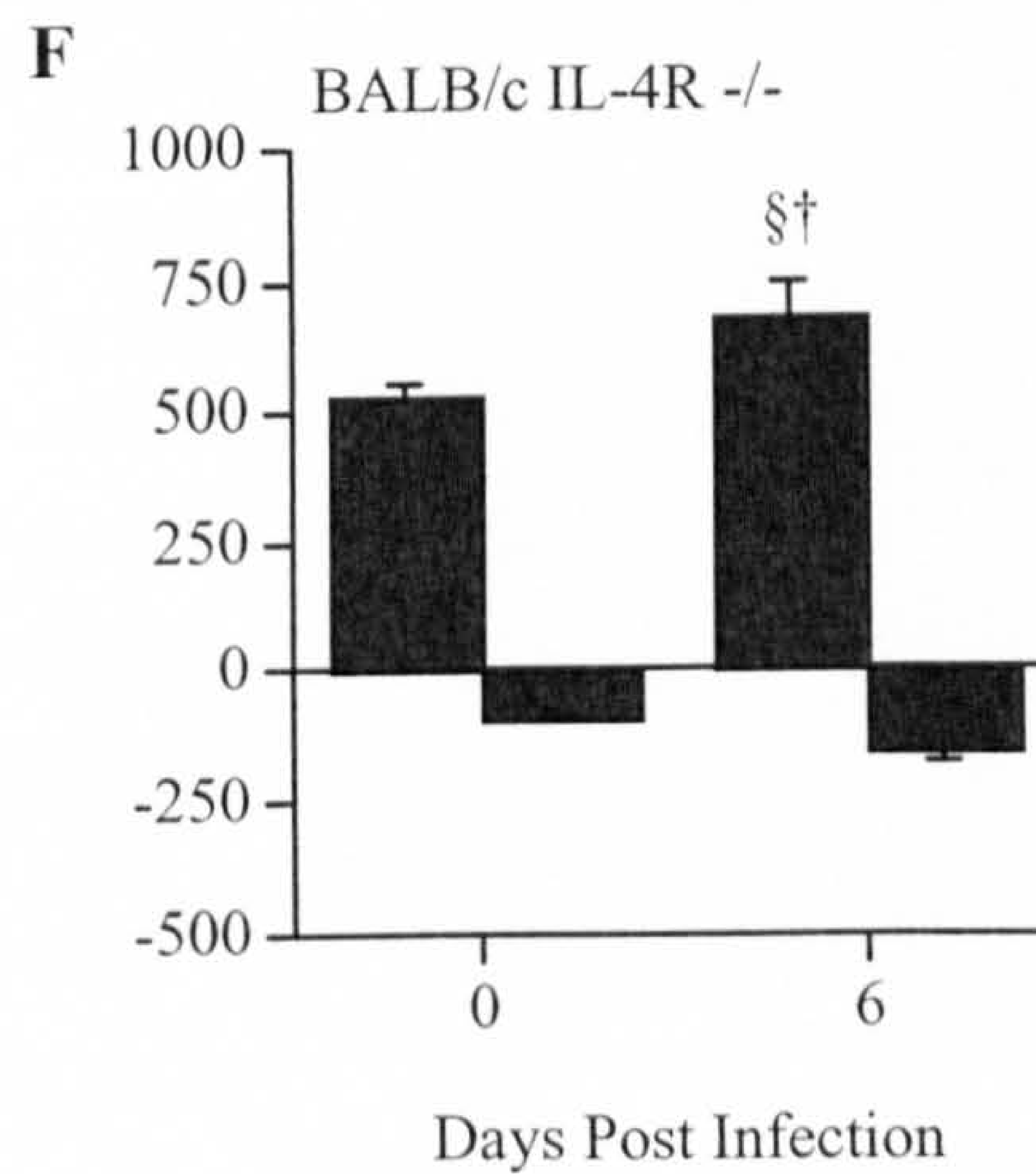
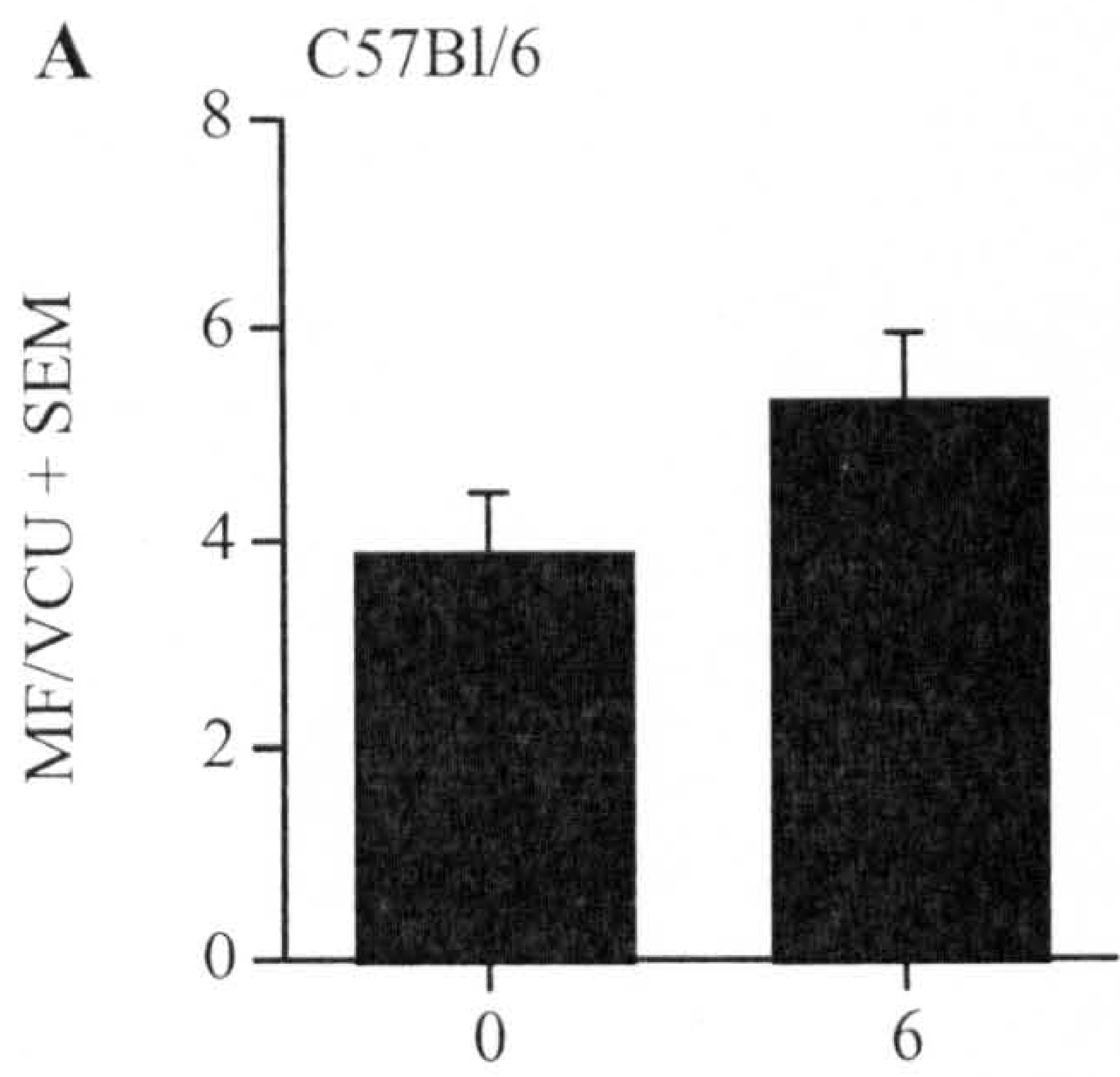
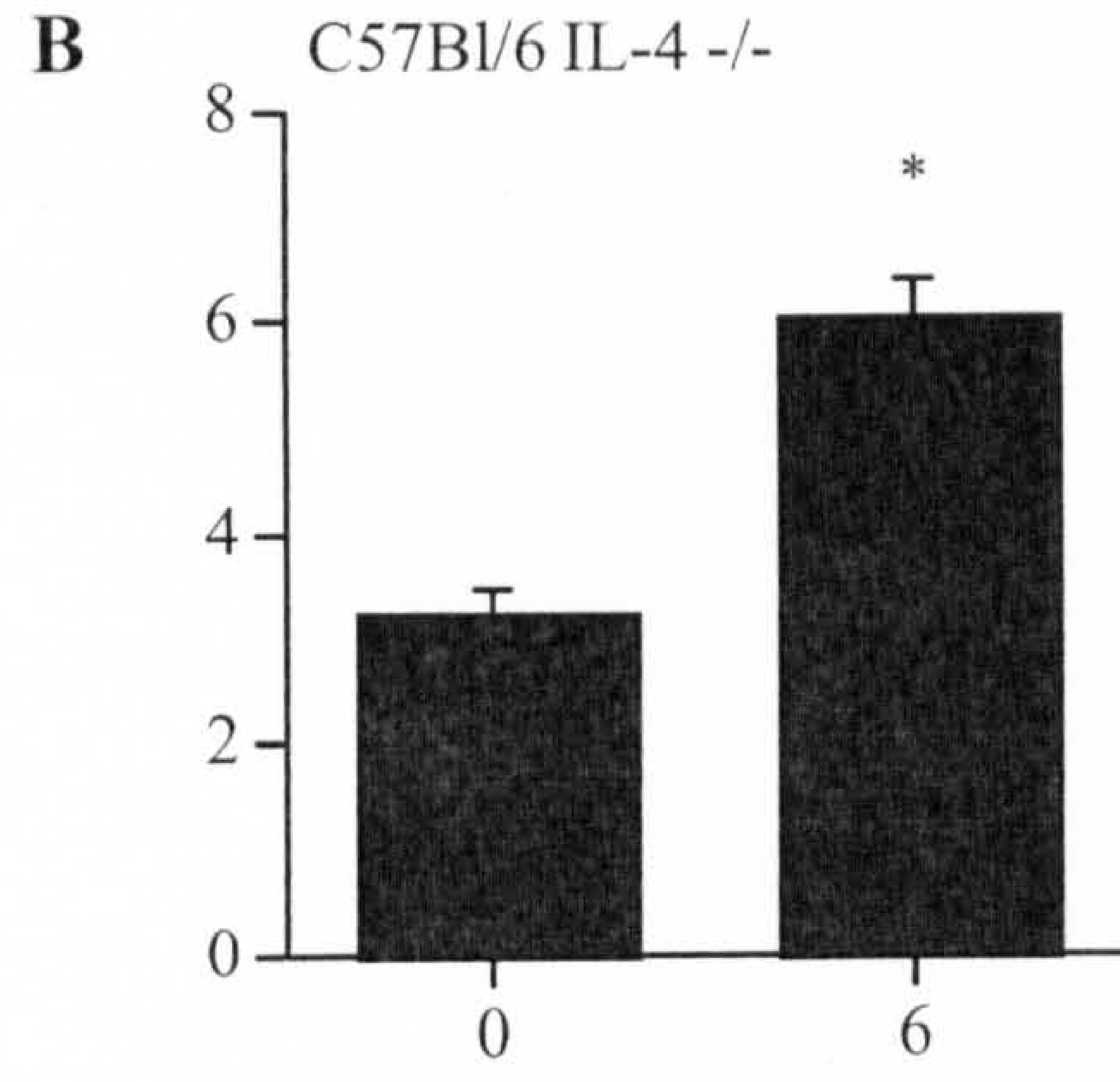
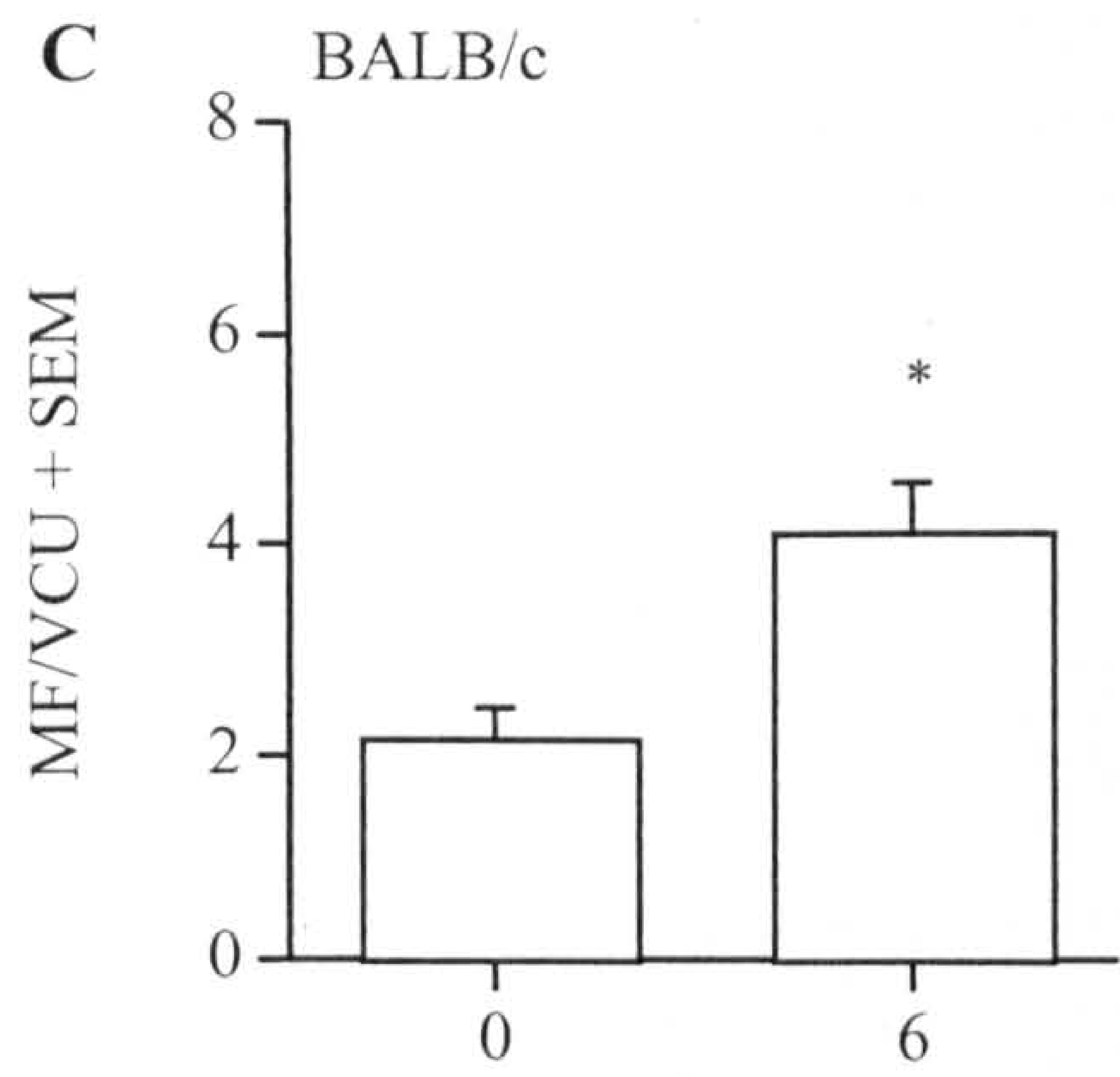
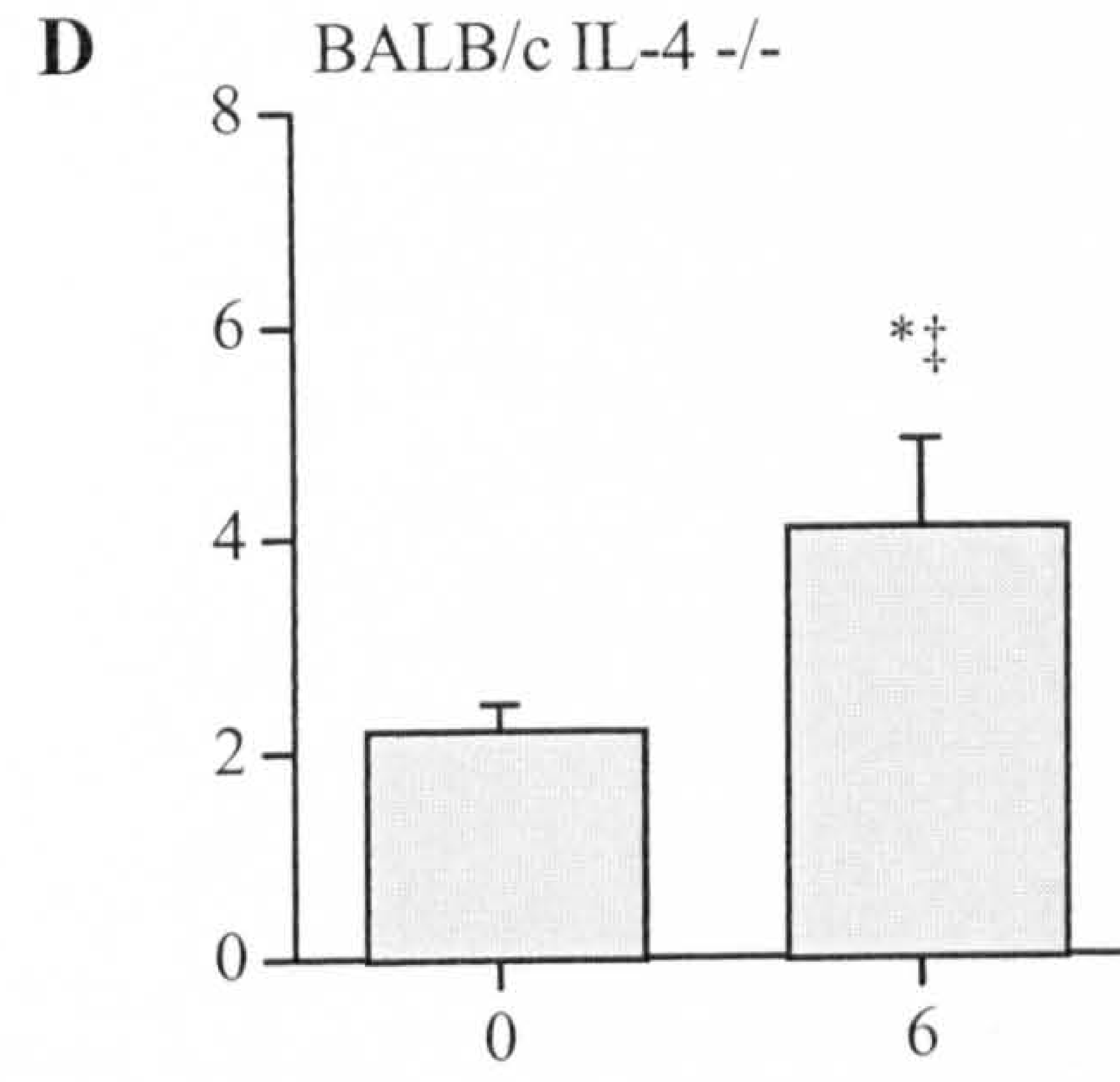

F
BALB/c IL-4R -/-
1000
750
500
250
0
-250
-500
§†
0
6
Days Post Infection

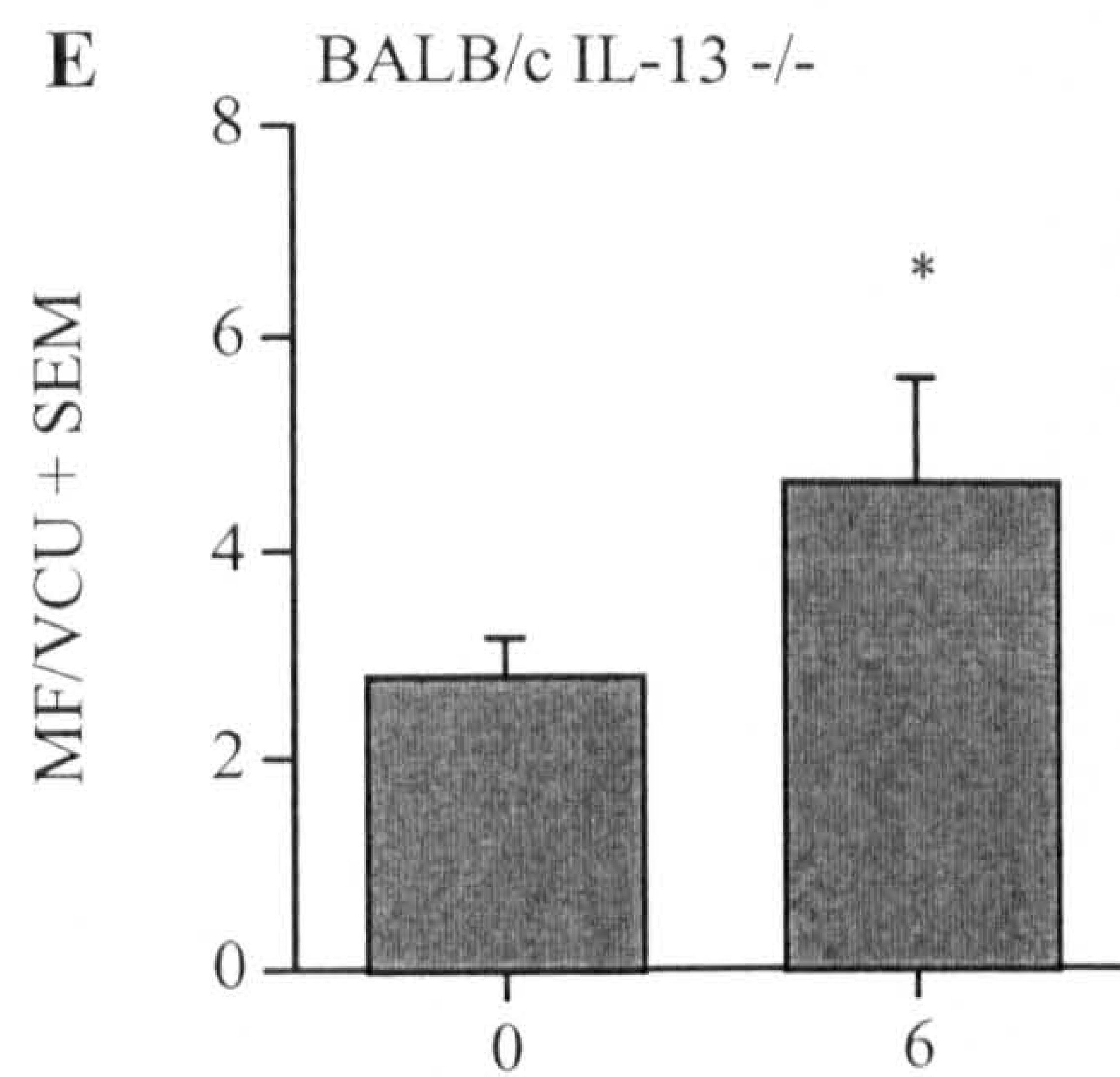
FIGURE 3.4: The role of host strain, IL-4, IL-13 and IL-4Rα in the increase in proliferation in crypt epithelium following infection with *T. spiralis*. The number of mitotic figures per crypt was determined in uninfected and infected (day 6 p.i.) wild type C57BL/6, (A), C57BL/6 IL-4 -/- (B), wild type BALB/c (C), BALB/c IL-4 -/- (D), BALB/c IL-13 -/- (E) and BALB/c IL-4Rα -/- (F) mice. Data is expressed as mean number of worms/mouse + SEM, five mice were used per group. *, represents significantly different to uninfected mice.; **, represents significantly different wild type C57BL/6 mice; §, represents significantly different to wild type BALB/c mice; †, represents significantly different to BALB/c IL-4 -/- mice; ‡, represents significantly different to BALB/c IL-4Rα -/- mice ($p<0.05$).


A
C57Bl/6
MF/VCU + SEM
8
6
4
2
0
0
6


B
C57Bl/6 IL-4 -/-
8
6
4
2
0
*
0
6


C
BALB/c
MF/VCU + SEM
8
6
4
2
0
*
0
6


D
BALB/c IL-4 -/-
8
6
4
2
0
*‡
0
6


E
BALB/c IL-13 -/-
MF/VCU + SEM
8
6
4
2
0
*
0
6
Days Post Infection

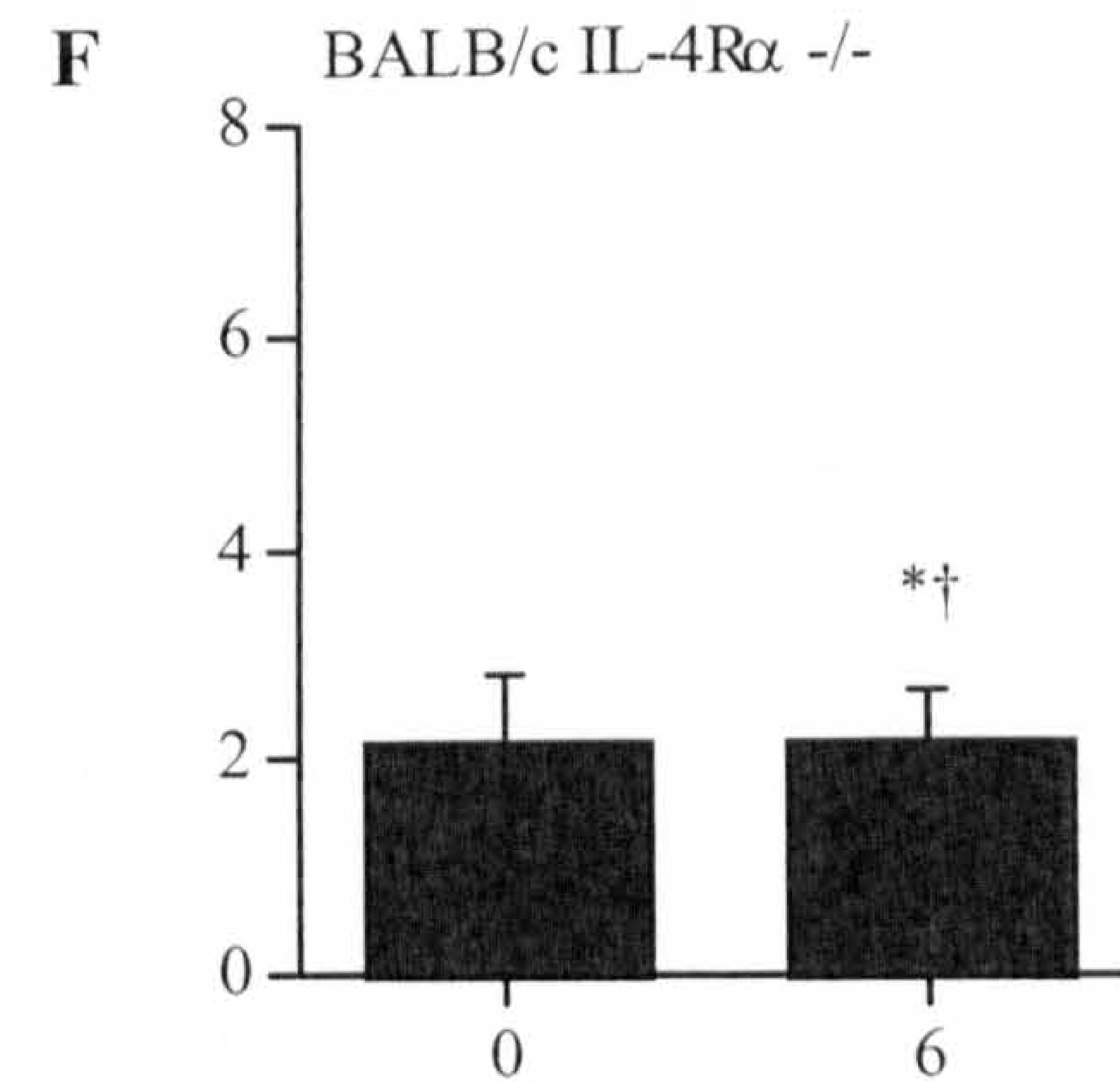
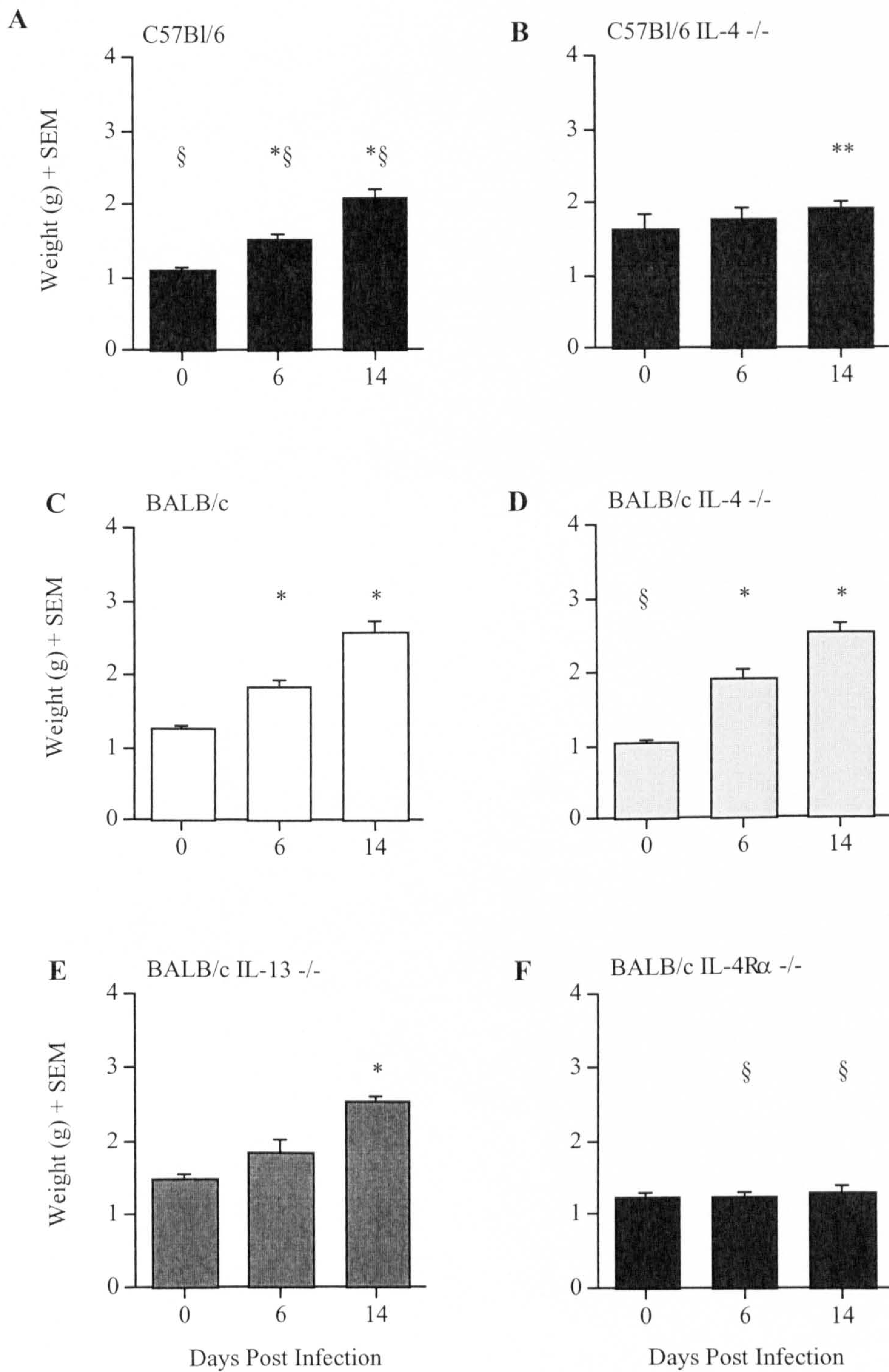

F
BALB/c IL-4Rα -/-
8
6
4
2
0
*†
0
6
Days Post Infection

FIGURE 3.5: The role of host strain, IL-4, IL-13 and IL-4Rα in the intestinal oedema following infection with *T. spiralis*. The weight of the small intestine was determine uninfected and infected (day 6 p.i.) wild type C57BL/6, (A), C57BL/6 IL-4 -/- (B), wild type BALB/c (C), BALB/c IL-4 -/- (D), BALB/c IL-13 -/- (E) and BALB/c IL-4Rα -/- (F) mice. Data is expressed as mean weight (g) + SEM, five mice were used per group *, represents significantly different to uninfected mice; **, represents significantly different wild type C57BL/6 mice; §, represents significantly different to wild type BALB/c mice. ($p<0.05$).


A
C57Bl/6
B
C57Bl/6 IL-4 -/-
C
BALB/c
D
BALB/c IL-4 -/-
E
BALB/c IL-13 -/-
F
BALB/c IL-4Rα -/-
Weight (g) + SEM
Days Post Infection
0
6
14
§
*§
**
*

### *3.2.3 Mast cell numbers and serum mMCP-1 titres are reduced in the absence of IL-4 but not IL-13.*

To assess the role of host strain, IL-4, IL-13 and IL-4Rα in the development of mucosal mastocytosis following infection with *T. spiralis*, samples of jejunum were fixed in Carnoy's solution and stained with toluidine blue to reveal mucosal mast cells. The number of mast cells per VCU was counted. The degranulation of mast cells was assessed by measuring serum titres of mMCP-1 in uninfected and infected mice (day 14 p.i.) by ELISA against a recombinant protein standard.

Significant mastocytosis was observed in wild type C57BL/6 (p=0.009), C57BL/6 IL-4 -/- mice (p=0.009) wild type BALB/c (p=0.009), BALB/c IL-4 -/- (p=0.009), BALB/c IL-13 -/- (p=0.009), and BALB/c IL-4Rα -/- (p=0.009), (Figure 3.6A-E) following infection with *T. spiralis*. No significant differences were observed between wild type BALB/c and BALB/c IL-4 -/- mice (Figure 3.6C and D). However, mastocytosis was significantly enhanced in BALB/c IL-13 -/- mice compared to wild type BALB/c at day 14 p.i. (p=0.009) and BALB/c IL-4 -/- (p=0.009) (Figure 3.6E). In contrast in BALB/c IL-4Rα -/- mice the number of mast cells was significantly lower than that observed in wild type BALB/c mice at day 14 p.i. (p=0.009) (Figure 3.6F). Interestingly the number of mast cells in C57BL/6 IL-4 -/- was significantly reduced compared to wild type C57BL/6 mice at day 14 p.i. (p=0.009) (Figure 3.6A and B) and was also reduced compared to BALB/c IL-4 -/- mice (Figure 3.6B and D). No significant differences were

observed between wild type BALB/c and C57BL/6 mice at day 0 or day 14 p.i. No significant differences were observed between strains, in the numbers of mast cells present in the mucosa of uninfected mice.

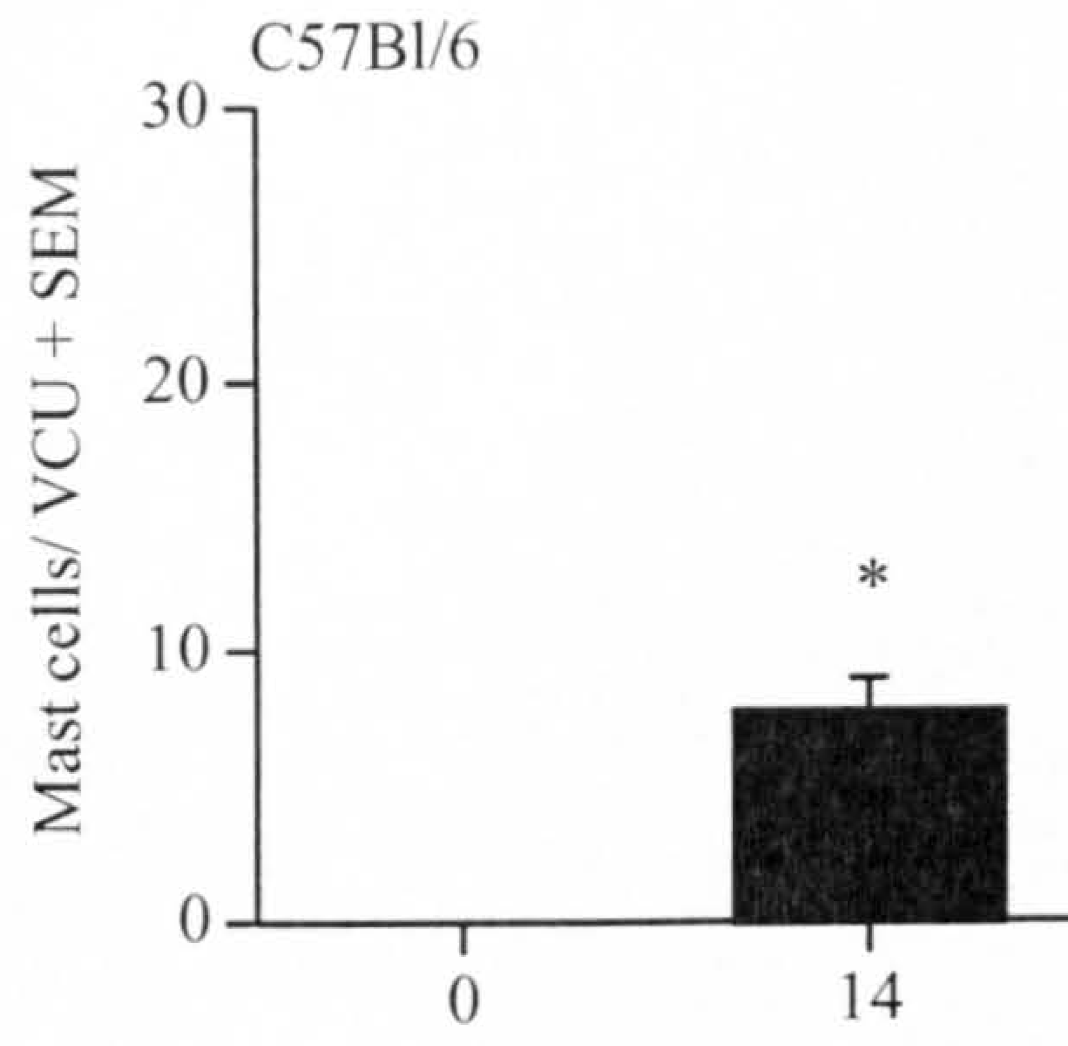
The mast cell granule protease mMCP-1 is released on mast cell activation and degranulation. mMCP-1 has also been shown to be important in the expulsion of *T. spiralis* from the small intestine (Knight *et al.*, 2000) and in the development of enteropathy (Lawrence *et al.*, 2004). Serum mMCP-1 titres were broadly consistent with mast cell numbers for all groups suggesting that mature activated mast cells were present in the small intestines. No significant differences were observed between the serum mMCP-1 titres of uninfected mice of any strain. Mast cell degranulation, as indicated by serum mMCP-1 titres, was significantly increased in wild type BALB/c (p=0.009), BALB/c IL-4 -/- (p=0.009), BALB/c IL-13 -/- (p=0.009), BALB/c IL-4Rα -/- (p=0.009), wild type C57BL/6 (p=0.009), and C57BL/6 IL-4 -/- (p=0.009) mice following infection with *T. spiralis* at day 14 p.i. (Figure 3.7A-E). Consistent with the increase in mast cell numbers observed in BALB/c IL-13 -/- mice serum mMCP-1 titres are significantly higher in BALB/c IL-13 -/- mice compared to wild type BALB/c mice at day 14 p.i. Again as observed with mast cell numbers serum mMCP-1 was significantly reduced in BALB/c IL-4Rα -/- and C57BL/6 IL-4 -/- compared to wild type BALB/c and C57BL/6 mice respectively at day 14 p.i. However in BALB/c IL-4 -/- mice serum mMCP-1 titres were significantly lower than those observed in wild type BALB/c mice (p=0.0143) suggesting that although no significant differences were observed in mast cell numbers

between wild type BALB/c and BALB/c IL-4 -/- mice mast cell degranultion is reduced in the absence of IL-4.

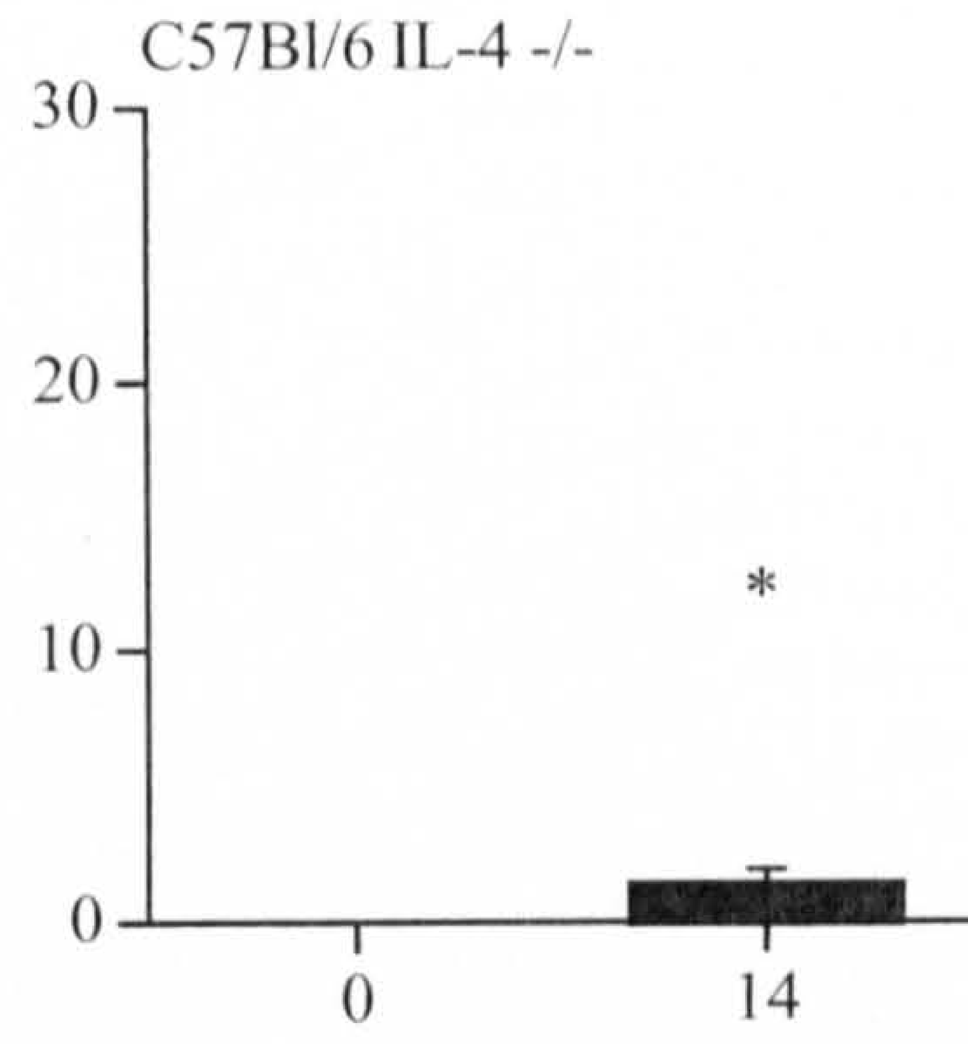
These data show that mast cell responses require IL-4 and IL-4Rα, although differences in the role of IL-4 between C57BL/6 and BALB/c mice were observed, IL-4 is required for the development of normal mucosal mast cell numbers, in C57BL/6 mouse following infection but not in the BALB/c mouse where only mMCP-1 titres were reduced in the absence of IL-4. Curiously, the absence of IL-13 appeared to enhance mast cell responses with higher numbers observed in the mucosa and an increase in serum mMCP-1 detected.

FIGURE 3.6: The role of host strain, IL-4, IL-13 and IL-4Rα in the development of mucosal mastocytosis following infection with *T. spiralis*. Carnoy's fixed jejunum from uninfected and infected (day 14 p.i) wild type C57BL/6, (A), C57BL/6 IL-4 -/- (B), wild type BALB/c (C), BALB/c IL-4 -/- (D), BALB/c IL-13 -/- (E) and BALB/c IL-4Rα -/- (F) mice were processed and stained with 0.5% toluidine blue, revealing mast cells. The numbers of mucosal mast cells were counted in 20 randomly selected villus crypt units (VCU). Data is expressed as mean number of mast cells/VCU + SEM, five mice were used per group *, represents significantly different to uninfected mice; **, represents significantly different wild type C57BL/6 mice §, represents significantly different to wild type BALB/c mice; †, represents significantly different to BALB/c IL-4 -/- mice; ‡, represents significantly different to BALB/c IL-4Rα -/- mice. ($p<0.05$).

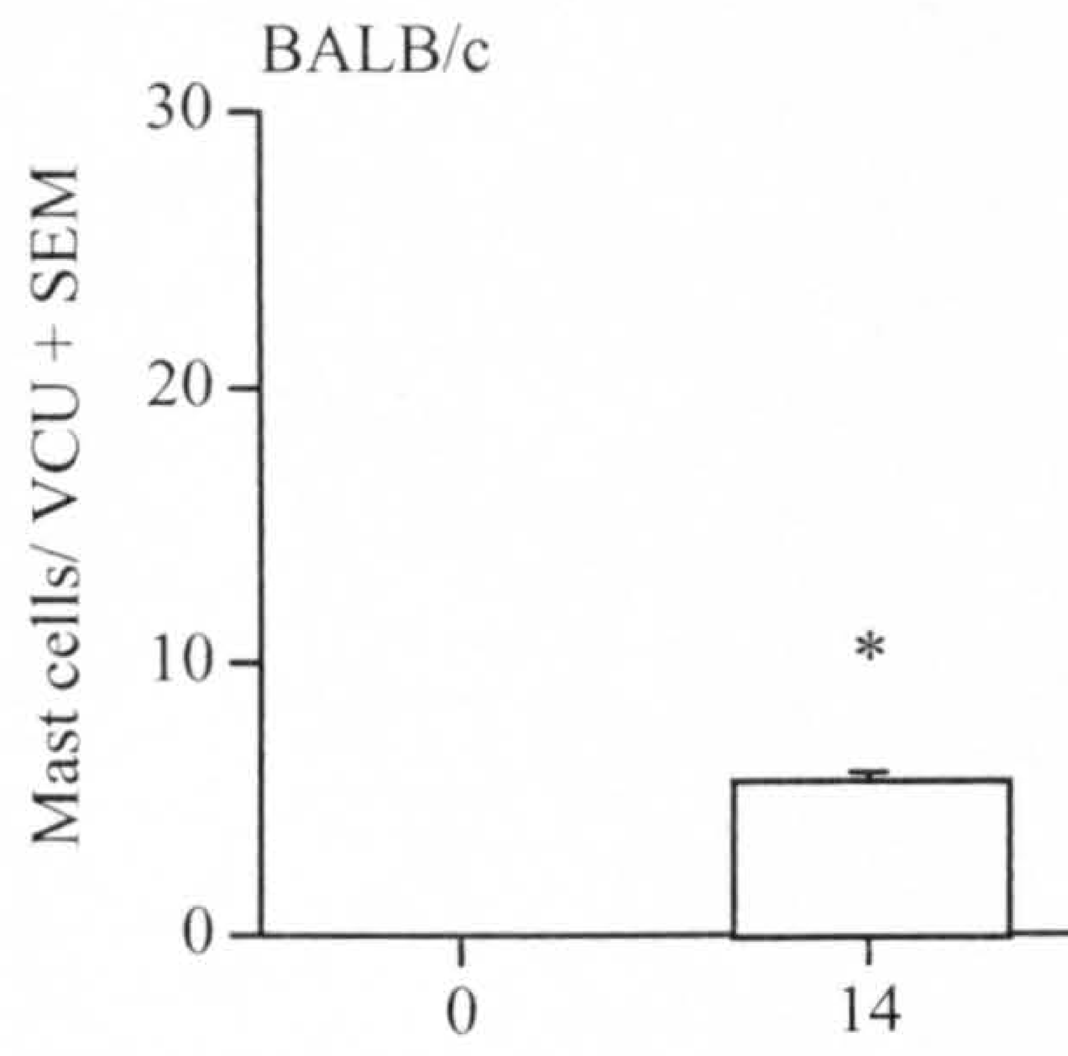
A



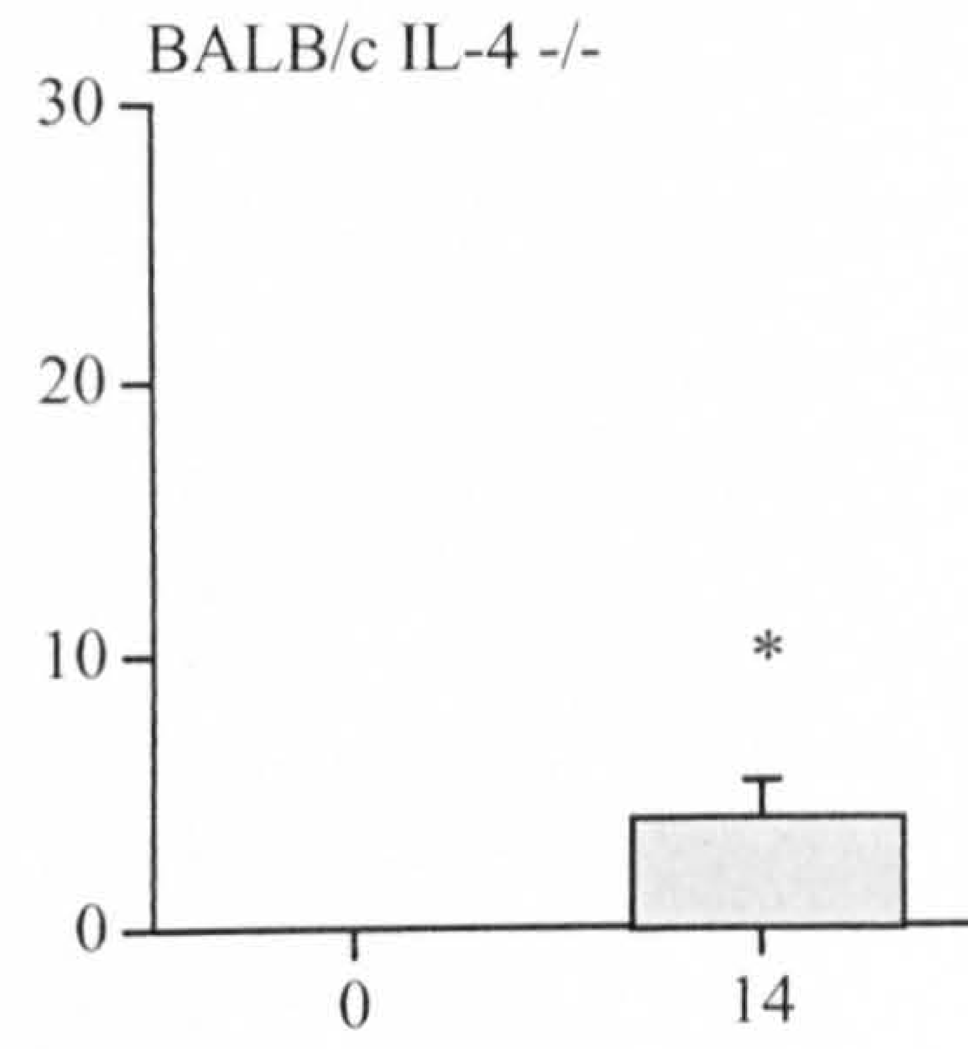

B



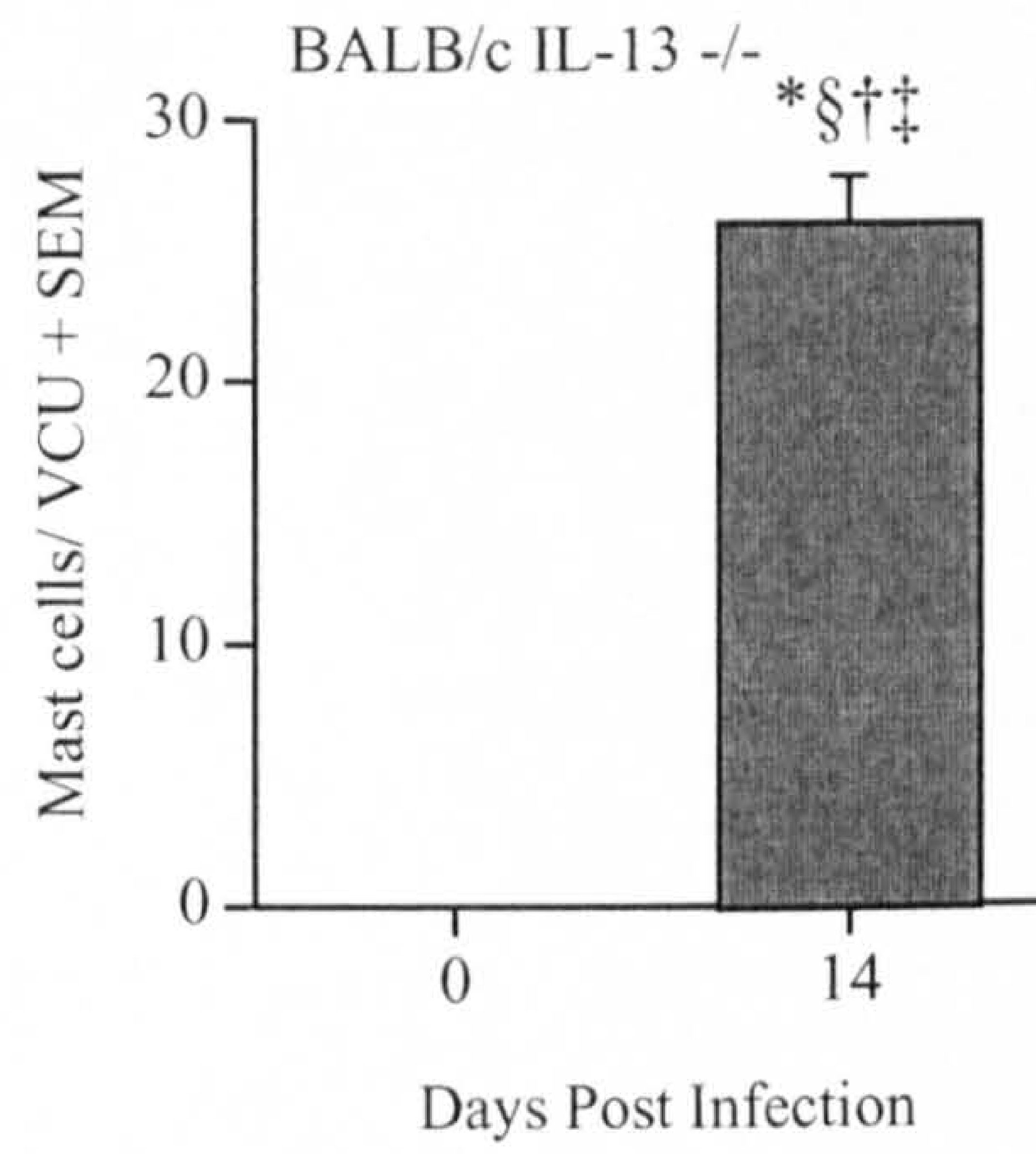

C




D




E




F

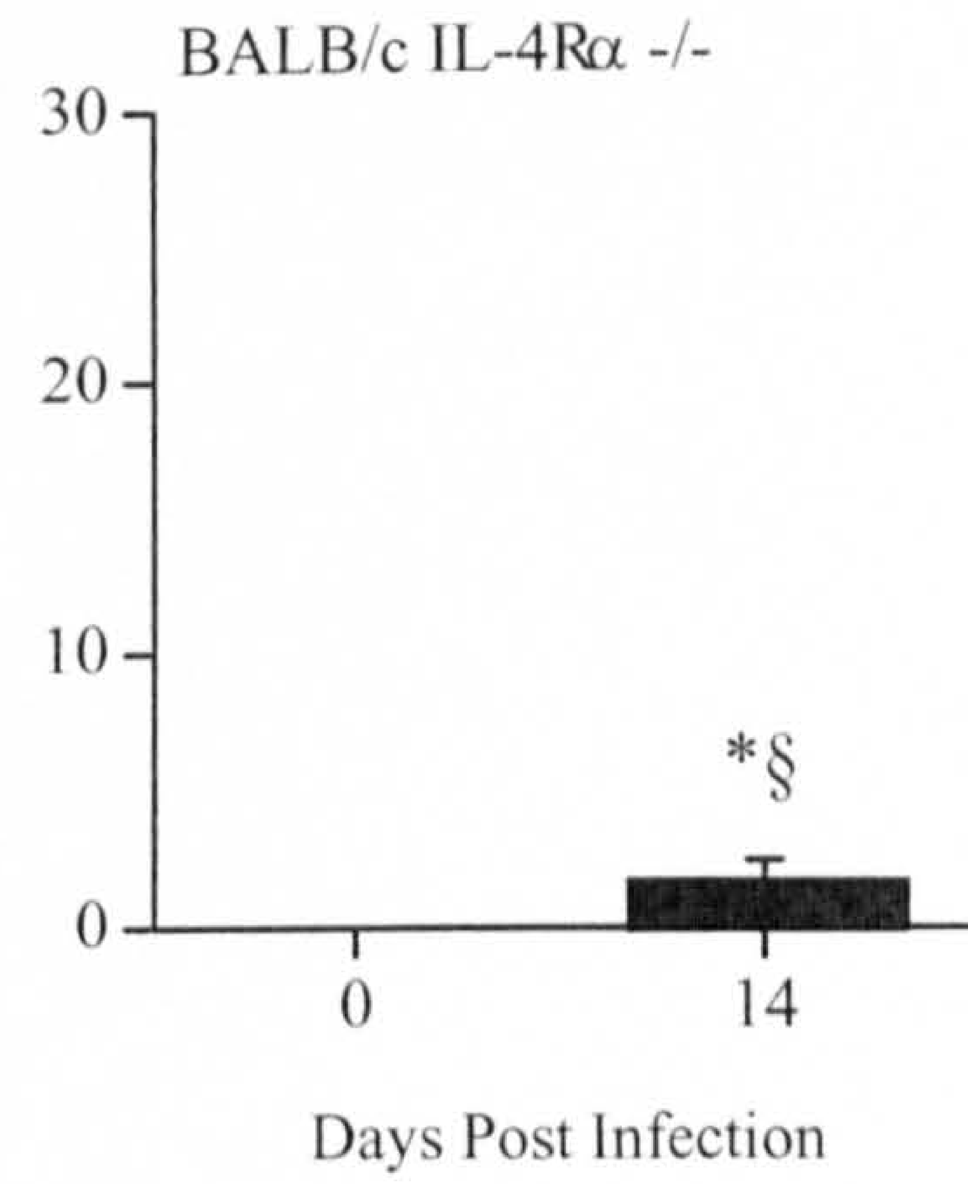
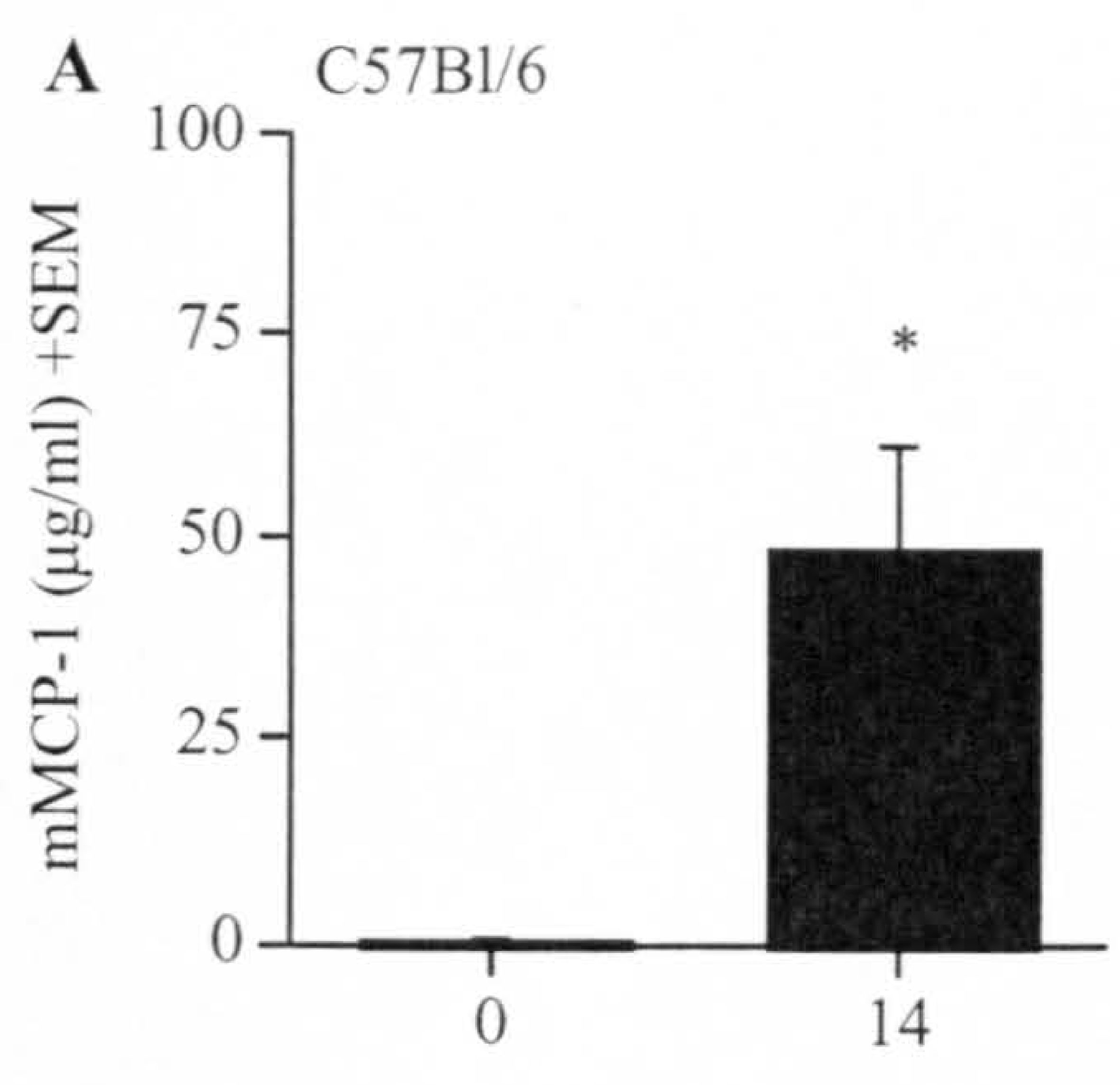
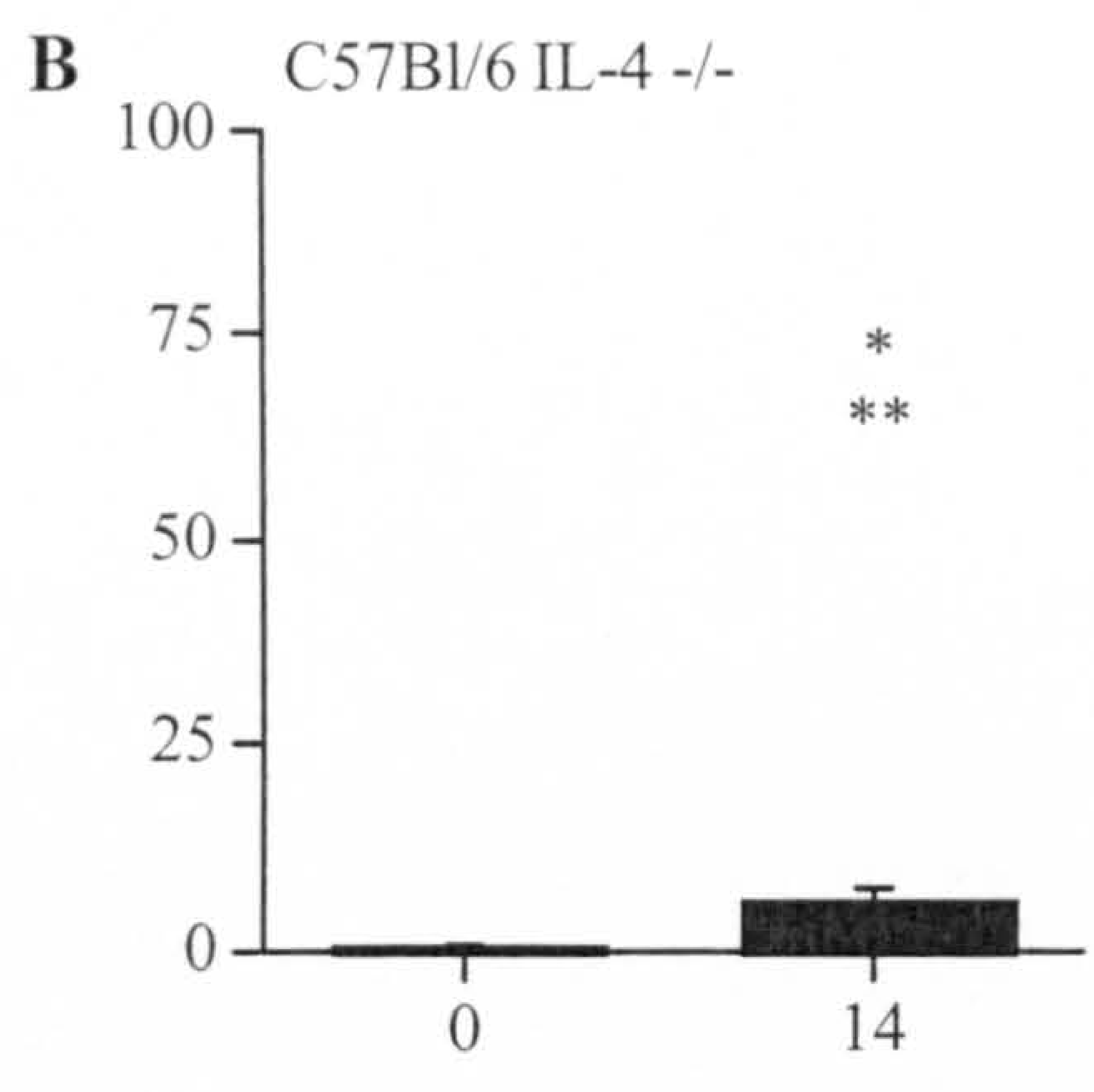
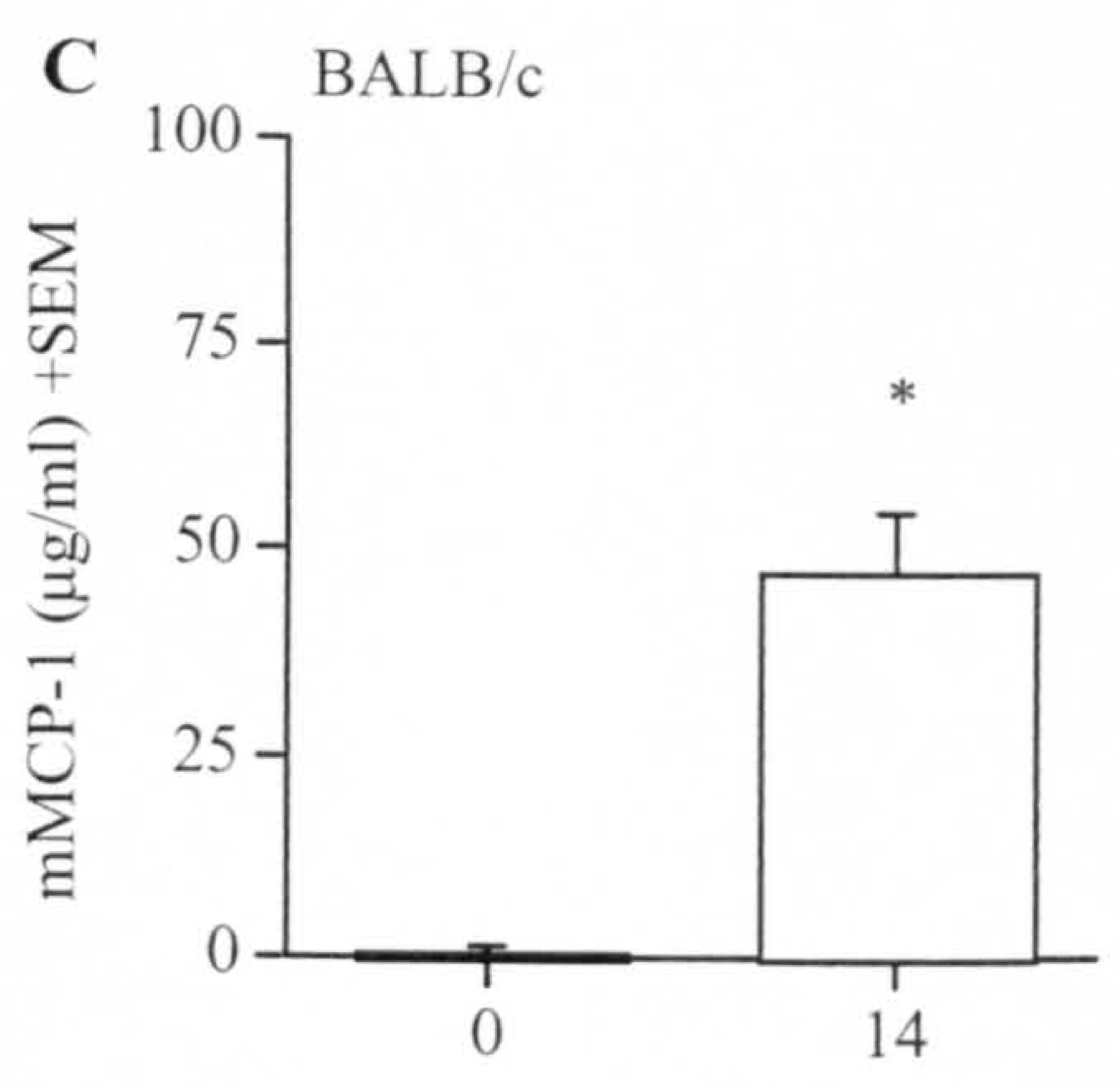
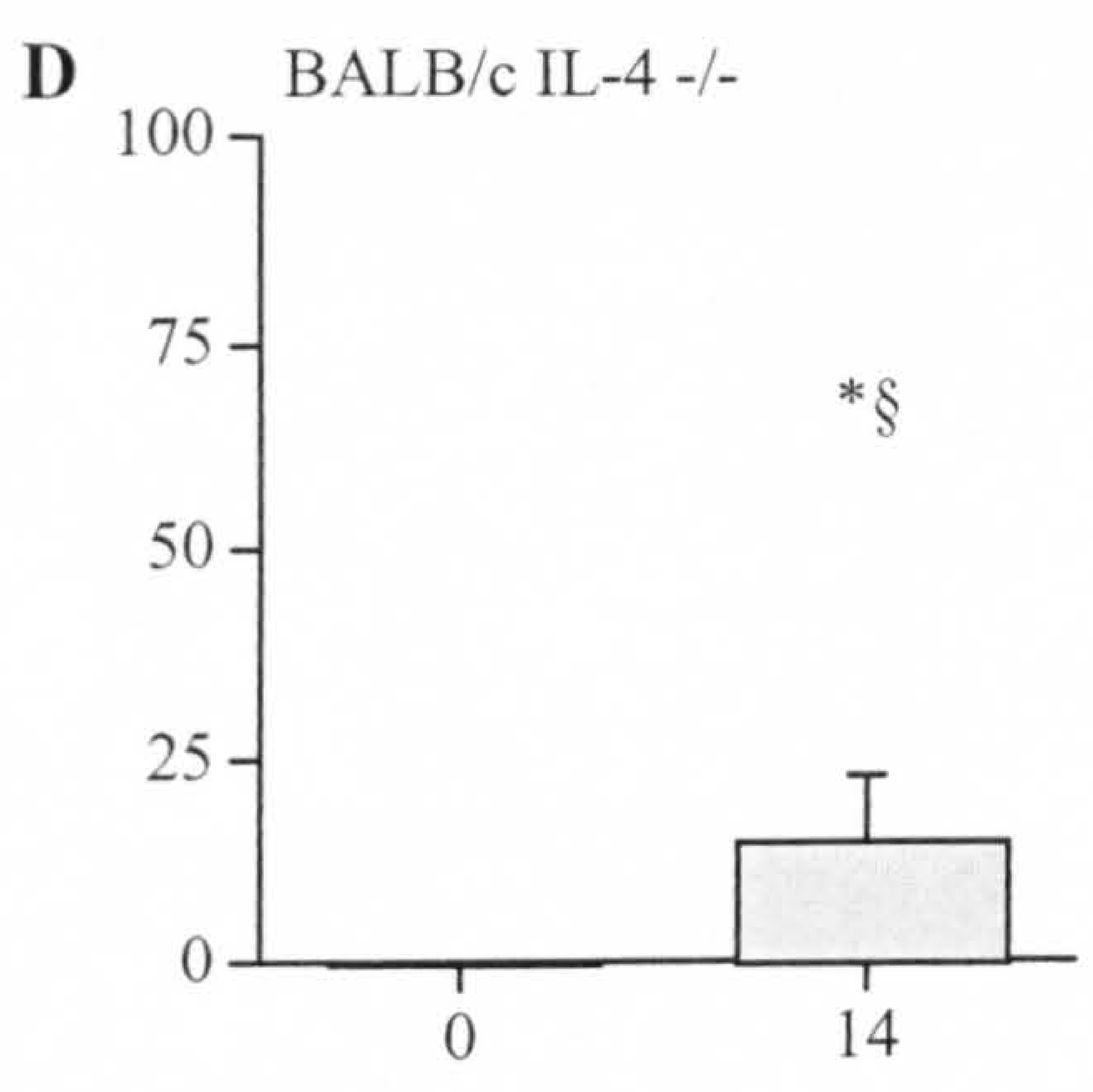


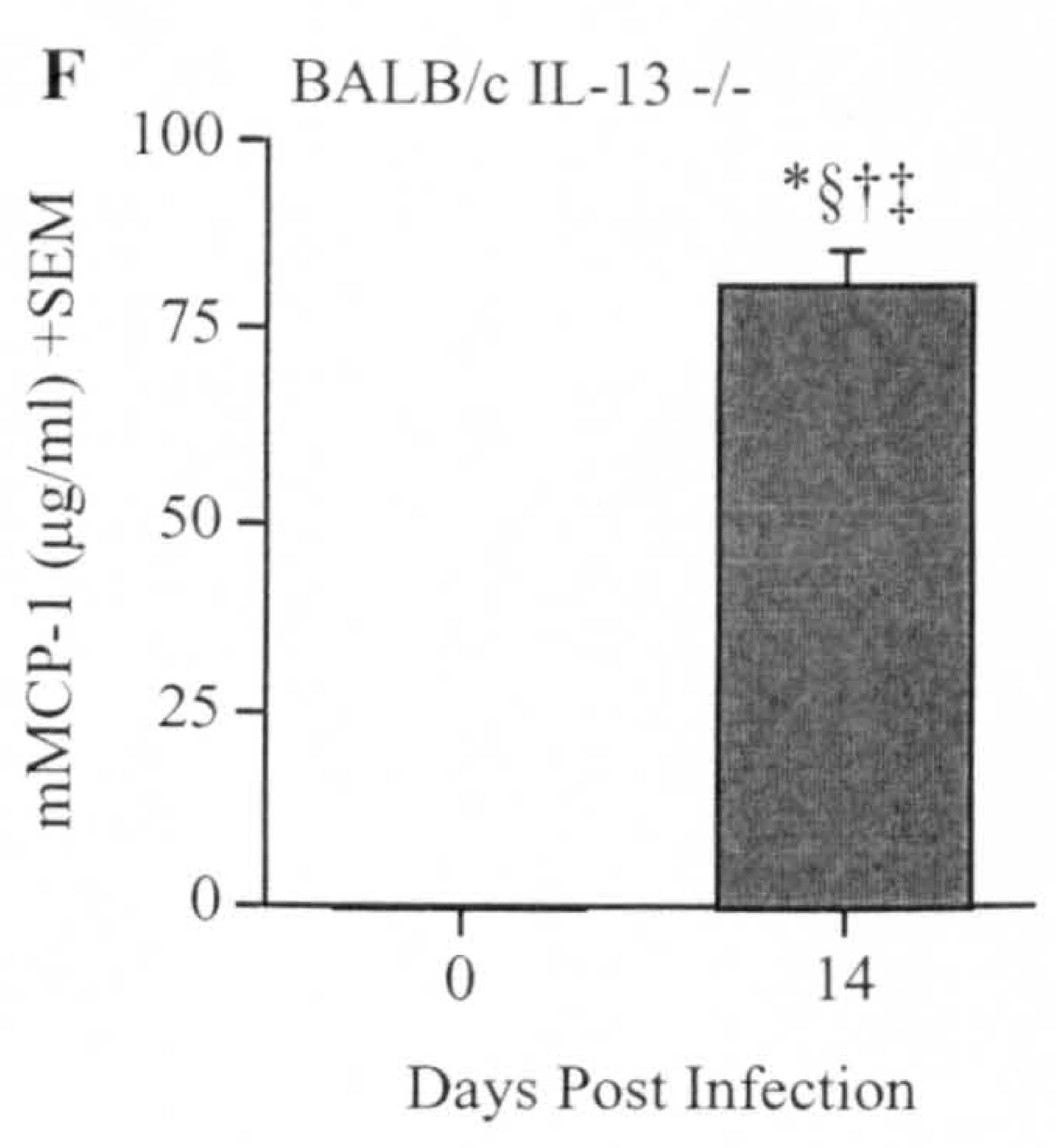
FIGURE 3.7: The role of host strain, IL-4, IL-13 and IL-4Rα in the mast cell degranulation following infection with *T. spiralis*. The activation and degranulation of mucosal mast cells in uninfected and infected (day 14 p.i) wild type C57BL/6, (A), C57BL/6 IL-4 -/- (B), wild type BALB/c (C), BALB/c IL-4 -/- (D), BALB/c IL-13 -/- (E) and BALB/c IL-4Rα -/- (F) mice was assessed by measuring serum titres of mMCP-1 by ELISA against a recombinant protein standard. Data is expressed as mean mMCP-1 concentration (μg/ml) + SEM, five mice were used per group *, represents significantly different to uninfected mice; **, represents significantly different wild type C57BL/6 mice; §, represents significantly different to wild type BALB/c mice; †, represents significantly different to BALB/c IL-4 -/- mice; ‡, represents significantly different to BALB/c IL-4Rα -/- mice ($p<0.05$).

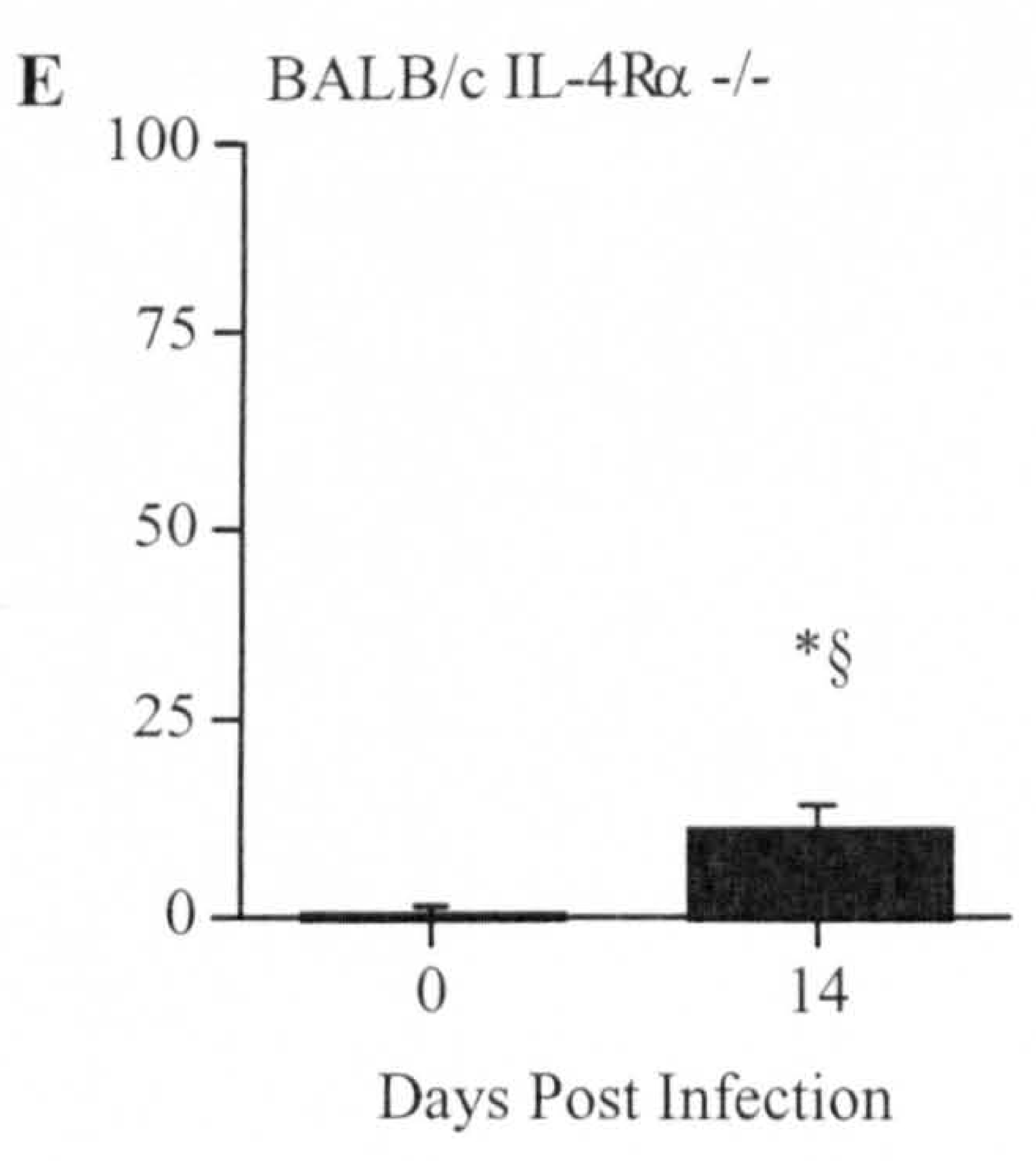

A
C57Bl/6
mMCP-1 (µg/ml) +SEM
100
75
50
25
0
*
0
14


B
C57Bl/6 IL-4 -/-
100
75
50
25
0
*
**
0
14


C
BALB/c
mMCP-1 (µg/ml) +SEM
100
75
50
25
0
*
0
14


D
BALB/c IL-4 -/-
100
75
50
25
0
*§
0
14


F
BALB/c IL-13 -/-
mMCP-1 (µg/ml) +SEM
100
75
50
25
0
*§†‡
0
14
Days Post Infection


E
BALB/c IL-4Rα -/-
100
75
50
25
0
*§
0
14
Days Post Infection

### *3.2.4 IFN-γ responses were increased in the absence of IL-4 in BALB/c mice but not in C57BL/6 mice.*

To analyse the role of IL-4, IL-13 and IL-4Rα in the development of T helper responses following infection with *T. spiralis* mesenteric lymph nodes were removed from uninfected and infected (day 6 p.i) wild type BALB/c, BALB/c IL-4 -/-, BALB/c IL-13 -/- BALB/c IL-4Rα -/-, C57BL/6 and C57BL/6 IL-4 -/- mice and cultured with *Trichinella* antigen. The secretion of the Th2 cytokines IL-4 and IL-13 and the Th1 cytokine IFN-γ into culture supernatants were measured by ELISA. As cell yields from uninfected mice were low, samples were pooled, so no statistical analysis of differences between uninfected mice and mice at day 6 p.i. were possible.

Following infection with *T. spiralis* IL-4 secretion from MLN cells taken from wild type C57BL/6 (Figure 3.7A), wild type BALB/c (Figure 3.8C), BALB/c IL-13 -/- (Figure 3.8E), and BALB/c IL-4Rα -/- (Figure 3.8F) mice compared to uninfected mice of the same strains. No IL-4 secretion was detected from C57BL/6 IL-4 -/- (Figure 3.7B) or BALB/c IL-4 -/- (Figure 3.8D) mice at either time point assessed. IL-4 secretion by MLN cells taken at day 6 p.i from BALB/c IL-4Rα -/- mice was lower than that observed for wild type BALB/c mice. Similar IL-4 levels were observed in wild type BALB/c mice and wild type C57BL/6, and BALB/c IL-13 -/- mice at day 6 p.i.

The secretion of IL-13 by MLN cells from wild type C57BL/6 (Figure 3.9A), wild type BALB/c (Figure 3.9C), and BALB/c IL-4Rα -/- (Figure 3.9F) mice at day 6 p.i. was compared to that seen in uninfected mice of the same strains. The levels of IL-13 observed in C57BL/6 IL-4 -/- mice (Figure 3.9B) at day 6 p.i. was comparable to those seen in wild type C57BL/6 mice. MLN cells from uninfected BALB/c IL-4 -/- mice secreted IL-13 at approximately the same levels as wild type BALB/c mice at day 6 p.i. and following infection no alteration in IL-13 levels was observed in BALB/c IL-4 -/- mice (Figure 3.9D). IL-13 secretion by MLN cells taken from BALB/c IL-4Rα -/- mice at day 6 p.i. although higher than from uninfected BALB/c IL-4Rα -/- mice was greatly reduced compared to wild type BALB/c mice at day 6 p.i. Only background levels of IL-13 were detected in BALB/c IL-13 -/- mice at either time point analysed (Figure 3.8E).

The secretion of IFN-γ by MLN cells from uninfected mice of all strains was low except from BALB/c IL-4 -/- mice (Figure 3.10D), were levels higher than those seen at day 6 post infection were detected. No increase in IFN-γ levels was observed following infection in wild type BALB/c mice (Figure 3.10C) and only a very small increase was observed in wild type C57BL/6 mice (Figure 3.10A). The secretion of IFN-γ by MLN cells increased following infection in C57BL/6 IL-4 -/- (Figure 3.10B), BALB/c IL-13 -/- (Figure 3.10E) and BALB/c IL-4Rα -/- (Figure 3.10F) mice. IFN-γ levels in BALB/c IL-4 -/- mice were significantly higher than those observed at day 6 p.i. in wild type BALB/c ($p=0.0143$), no significant differences in IFN-γ secretion, however, were

observed between wild type C57BL/6 and C57BL/6 IL-4 -/- mice or between wild type BALB/c and BALB/c IL-13 -/- mice at day 6 p.i.

Thus, T helper cytokine responses were not significantly different between wild type C57BL/6 and BALB/c mice, with IL-13 responses remaining intact in the absence of IL-4 in both C57BL/6 and BALB/c mice. IL-4 responses were essentially normal in the absence of IL-13 in BALB/c mice. IFN-$\gamma$, were increased in the BALB/c IL-4 -/- mice compared to the wild type but not in the C57BL/6 IL-4 -/- mice.

FIGURE 3.8: Role of host strain, IL-4, IL-13 and IL-4Rα on mesenteric lymph node cell IL-4 secretion following infection with *T. spiralis*. MLN were removed from wild type, uninfected and infected (day 6 p.i.) wild type C57BL/6 (A), C57BL/6 IL-4 -/- (B), wild type BALB/c (C), BALB/c IL-4 -/- (D), BALB/c IL-13 -/- (E), BALB/c IL-4Rα -/- (F) mice. Single cell suspensions were made; because viable cell yields were low samples were pooled for all uninfected mice only one sample was obtained except C57BL/6 IL-4 -/- mice where no viable cells were obtained. From infected wild type C57BL/6, 5 samples; C57BL/6 IL-4 -/-, 3 samples; wild type BALB/c, 4 samples; BALB/c IL-4 -/-, 2 samples; BALB/c IL-13 -/-, 3 samples, and BALB/c IL-4Rα -/-, 2 samples were obtained. Cells were cultured at $1\times10^6$ cells with 50μg/ml TAg. The secretion of the IL-4 was measured in the culture supernatants by ELISA against a recombinant standard. Data expressed mean cytokine concentration in pg/ml + SEM.

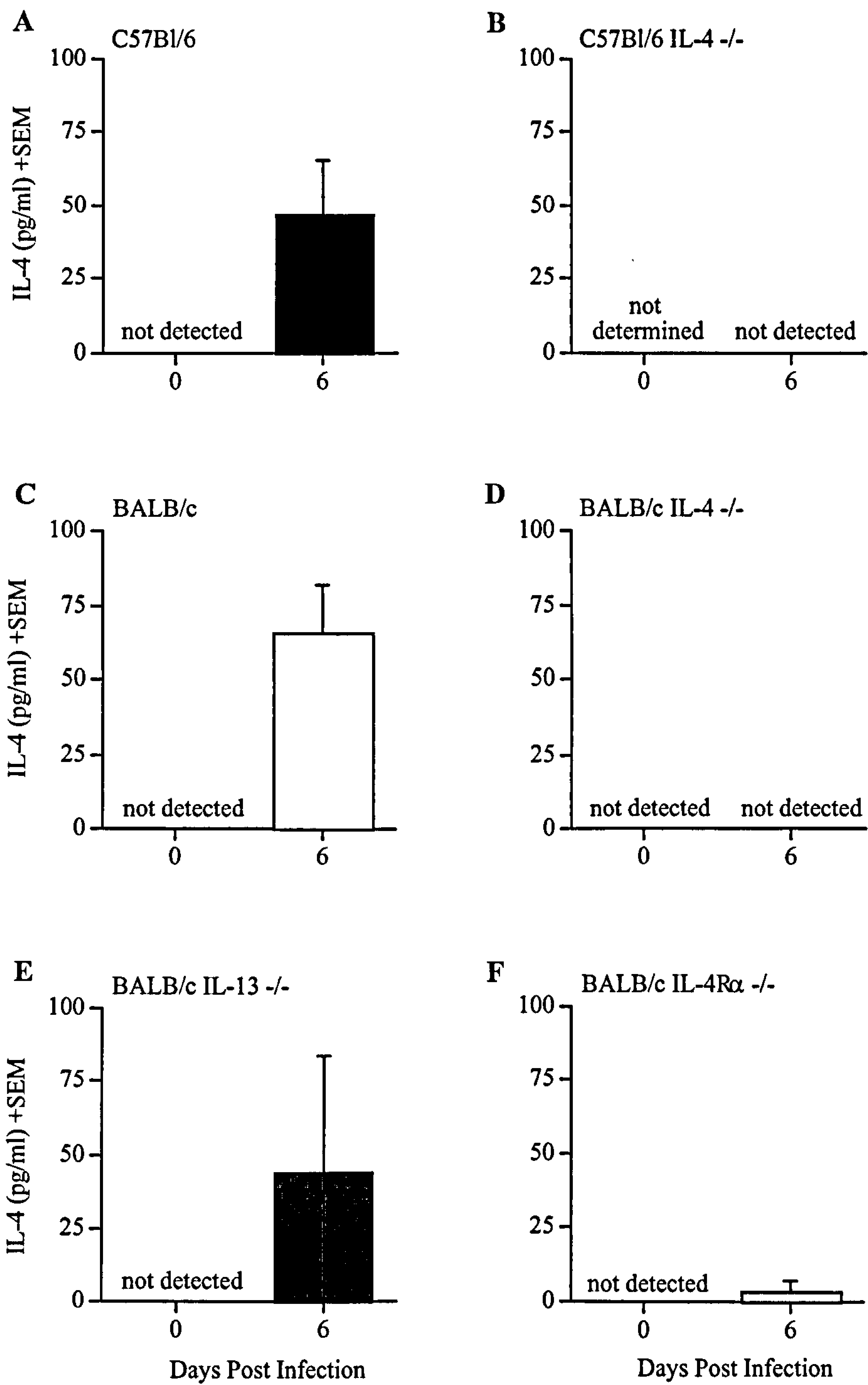
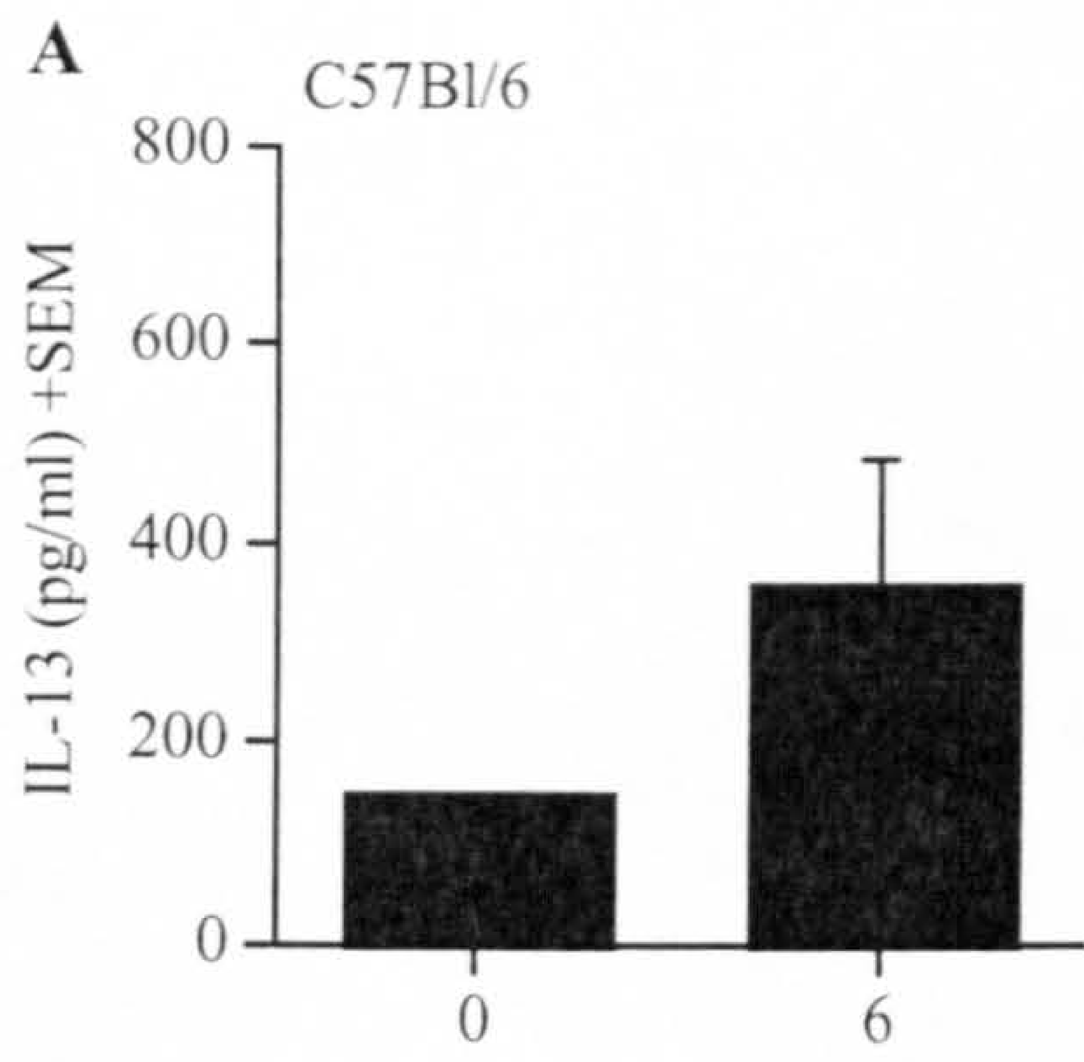
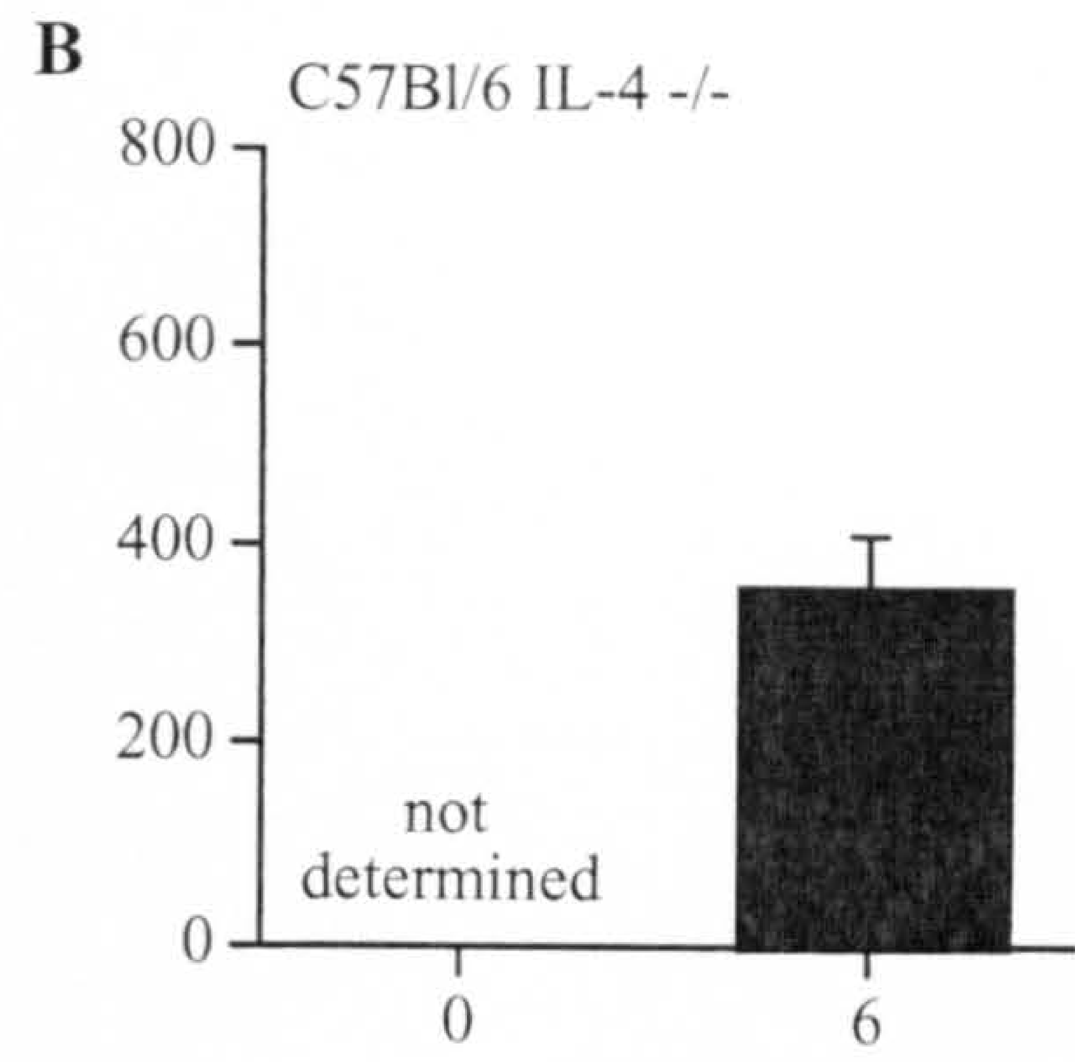
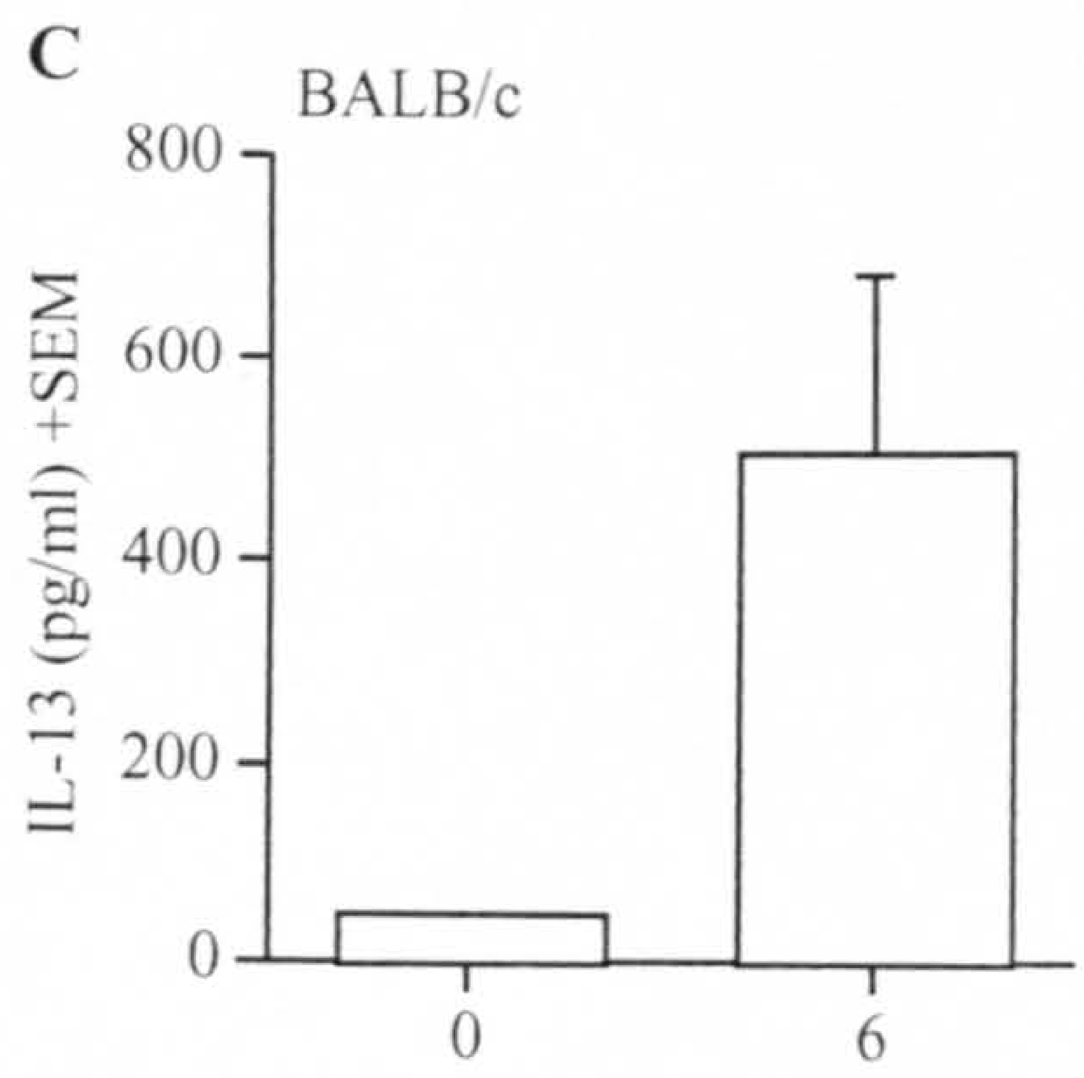
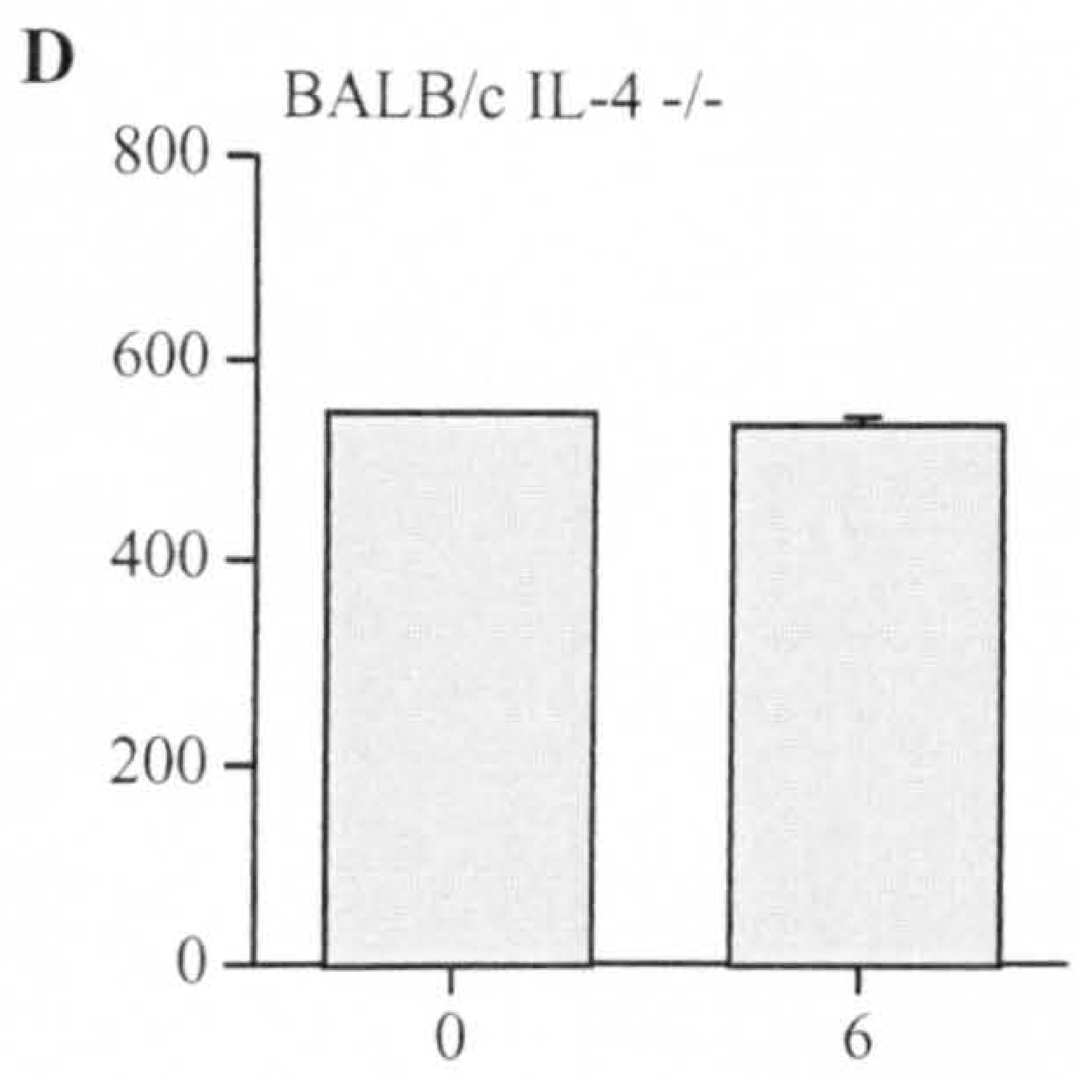

A
C57Bl/6
IL-4 (pg/ml) +SEM
100
75
50
25
0
not detected
0
6
B
C57Bl/6 IL-4 -/-
100
75
50
25
0
not determined
not detected
0
6
C
BALB/c
IL-4 (pg/ml) +SEM
100
75
50
25
0
not detected
0
6
D
BALB/c IL-4 -/-
100
75
50
25
0
not detected
not detected
0
6
E
BALB/c IL-13 -/-
IL-4 (pg/ml) +SEM
100
75
50
25
0
not detected
0
6
Days Post Infection
F
BALB/c IL-4Rα -/-
100
75
50
25
0
not detected
0
6
Days Post Infection

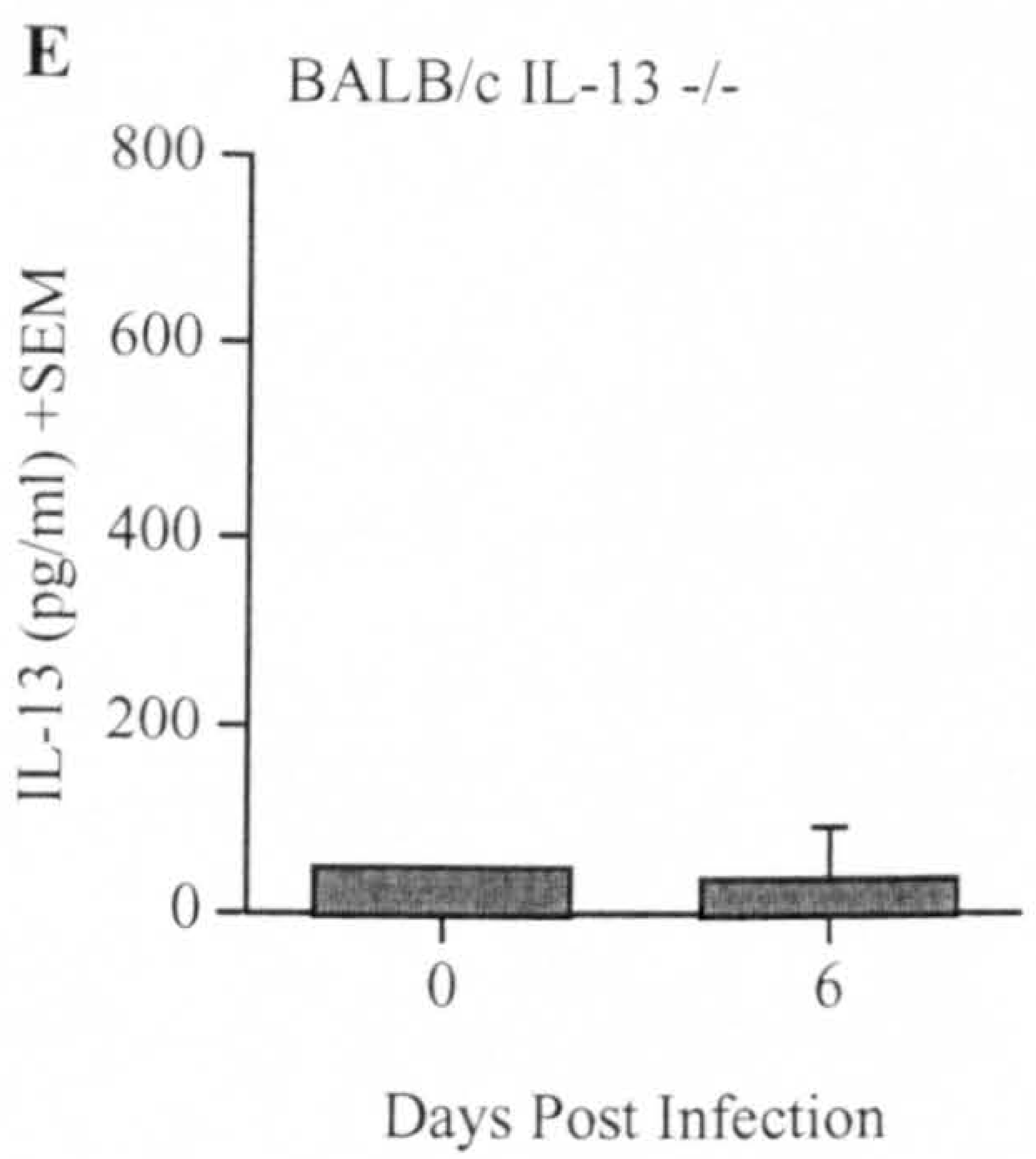
FIGURE 3.9: Role of host strain, IL-4, IL-13 and IL-4Rα in ex vivo mesenteric lymph node cell IL-13 secretion following infection with *T. spiralis*. MLN were removed from wild type, uninfected and infected (day 6 p.i.) wild type C57BL/6, (A), C57BL/6 IL-4 -/- (B), wild type BALB/c (C), BALB/c IL-4 -/- (D), BALB/c IL-13 -/- (E) and BALB/c IL-4Rα -/- (F) mice. Single cell suspensions were made; because viable cell yields were low samples were pooled for all uninfected mice only one sample was obtained except C57BL/6 IL-4 -/- mice where no viable cells were obtained. From infected wild type BALB/c, 4 samples; BALB/c IL-4 -/-, 2 samples; BALB/c IL-13 -/-, 3 samples, BALB/c IL-4Rα -/-, 2 samples; wild type C57BL/6, 5 samples and C57BL/6 IL-4 -/-, 3 samples were obtained. Cells were cultured at $1\times10^6$ cells with 50μg/ml TAg. The secretion of the IL-13 was measured in the culture supernatants by ELISA against a recombinant standard. Data expressed mean cytokine concentration in pg/ml + SEM.


A
C57Bl/6
800
600
400
200
0
IL-13 (pg/ml) +SEM
0
6


B
C57Bl/6 IL-4 -/-
800
600
400
200
0
not
determined
0
6


C
BALB/c
800
600
400
200
0
IL-13 (pg/ml) +SEM
0
6


D
BALB/c IL-4 -/-
800
600
400
200
0
0
6


E
BALB/c IL-13 -/-
800
600
400
200
0
IL-13 (pg/ml) +SEM
0
6
Days Post Infection

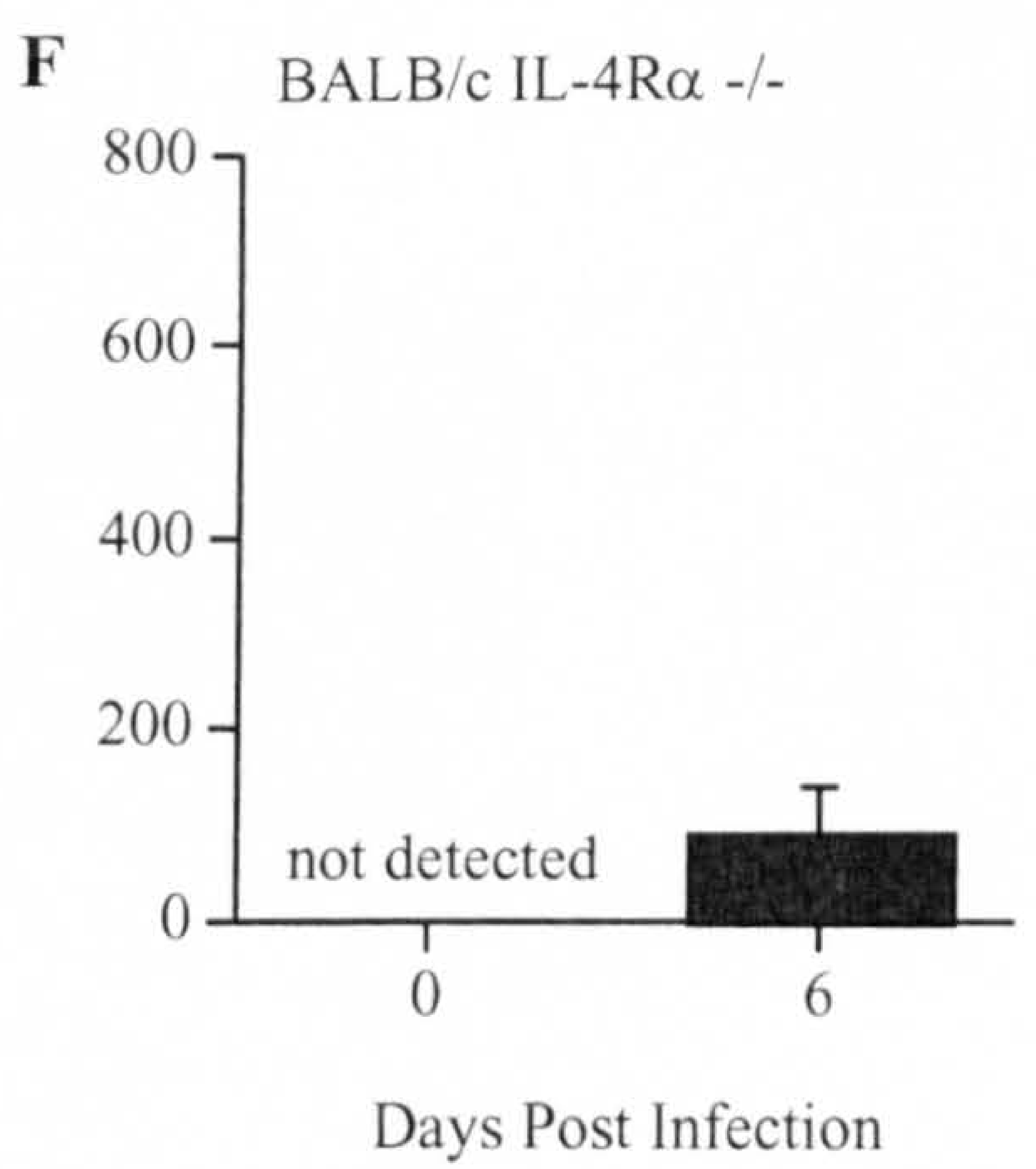
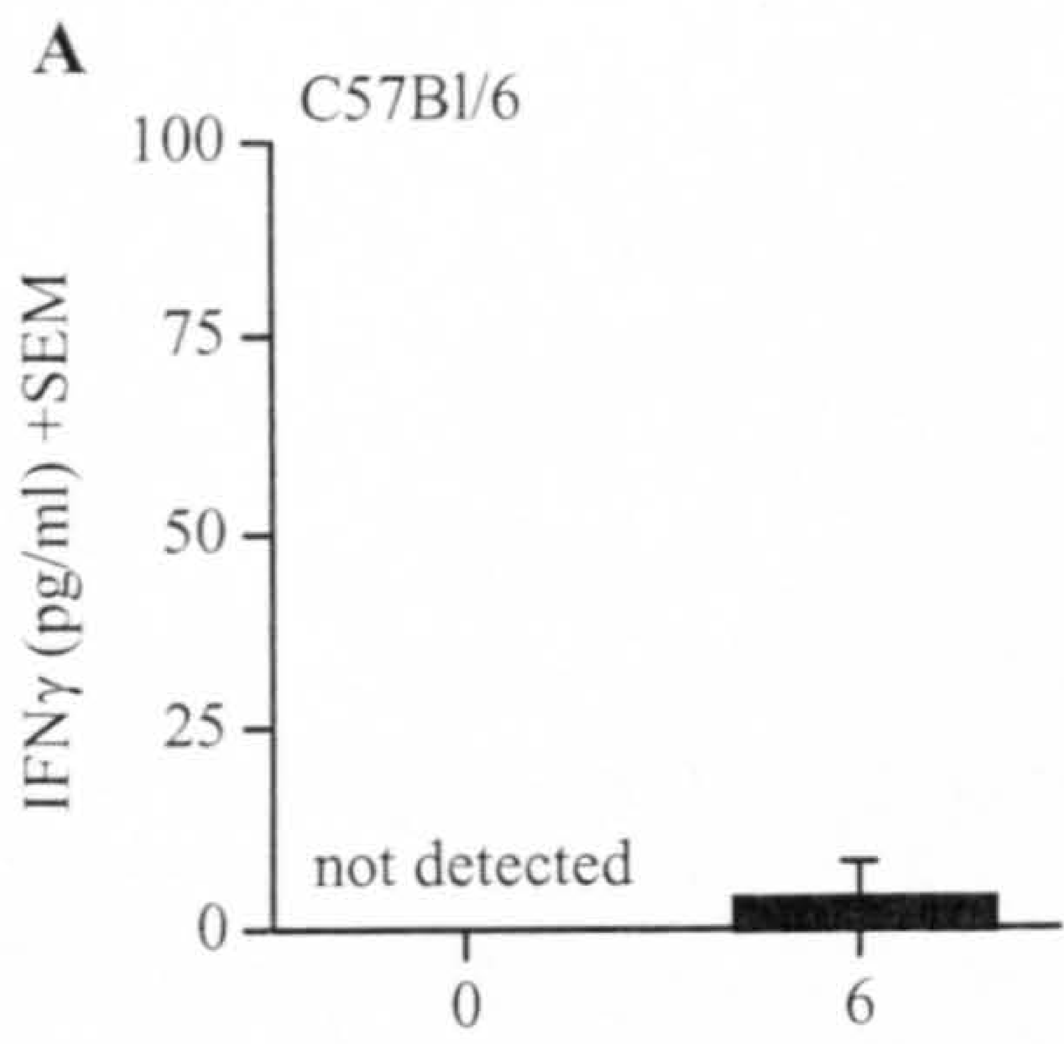
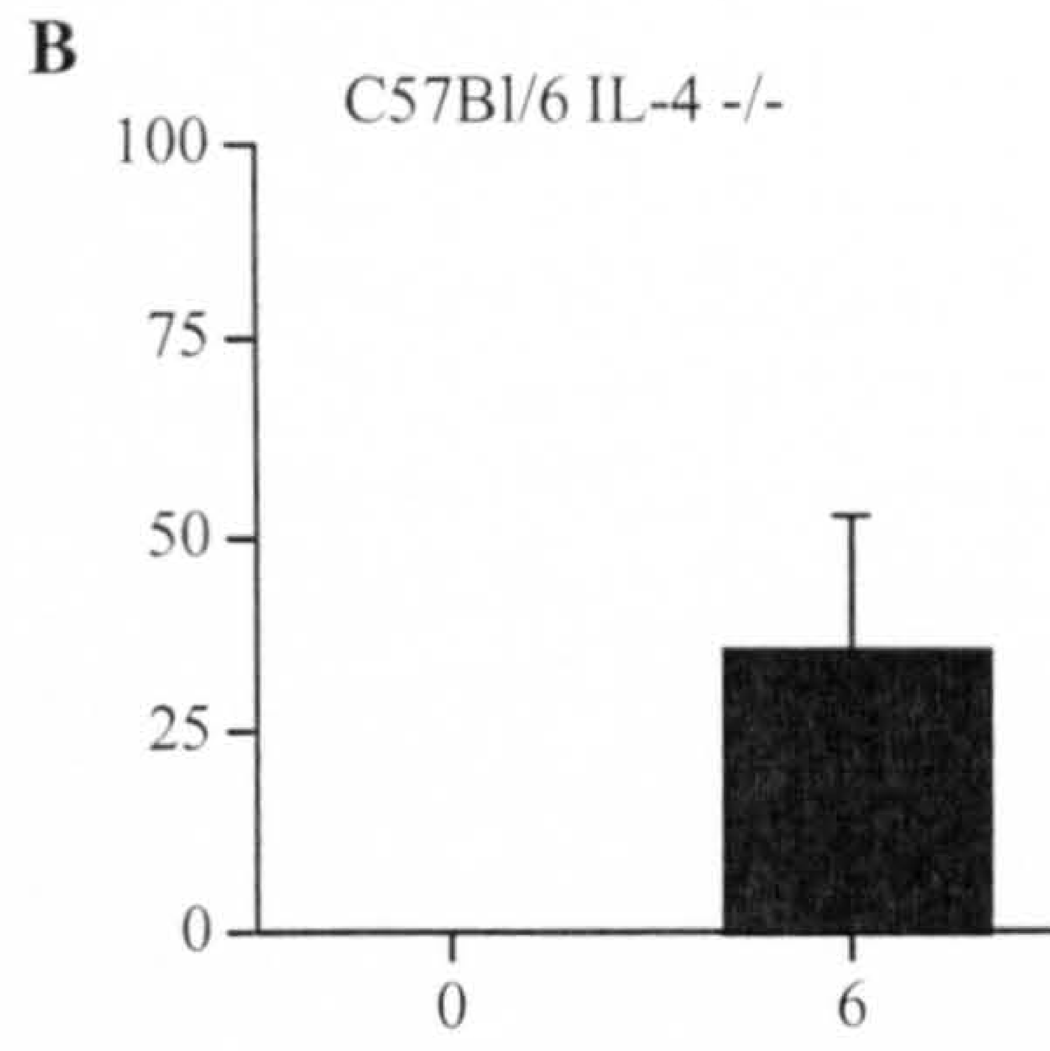
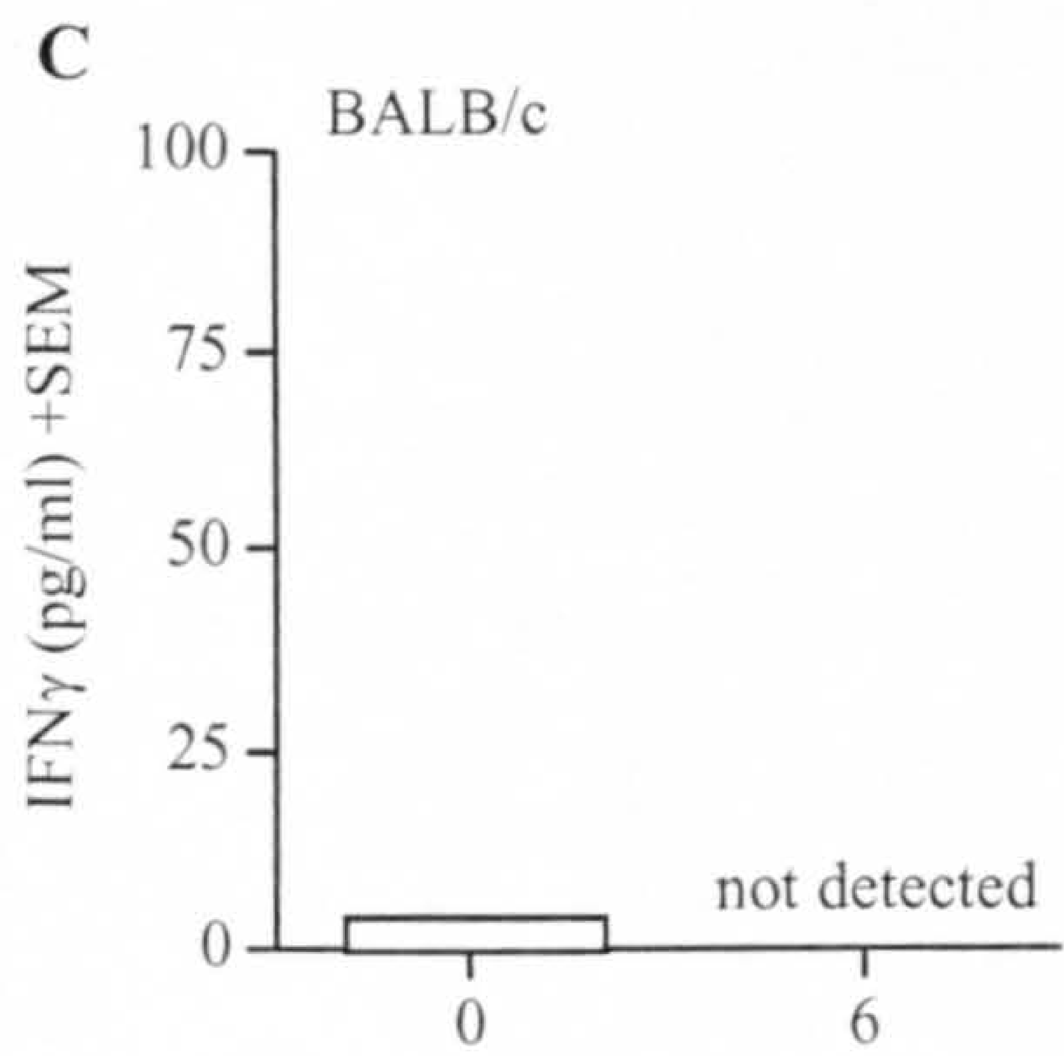
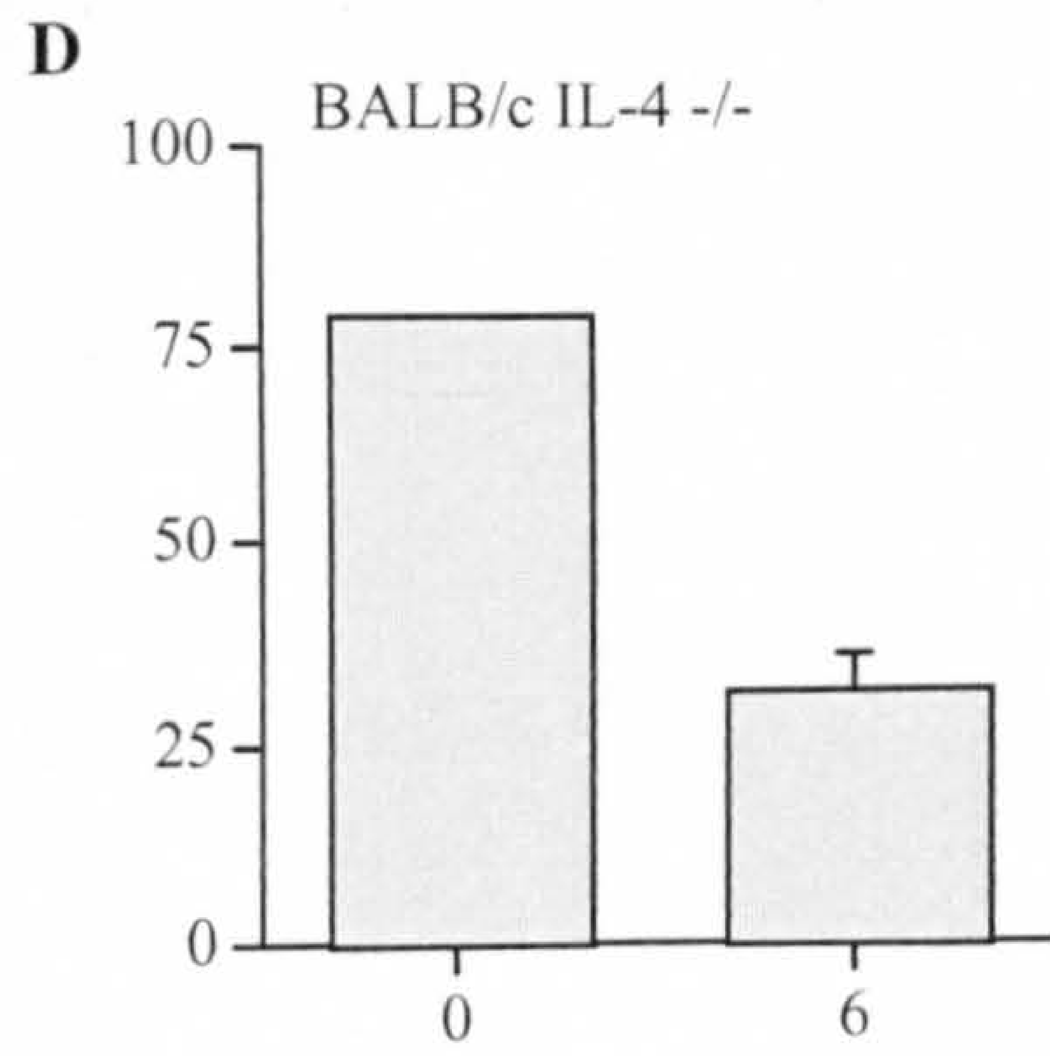

F
BALB/c IL-4Rα -/-
800
600
400
200
0
not detected
0
6
Days Post Infection

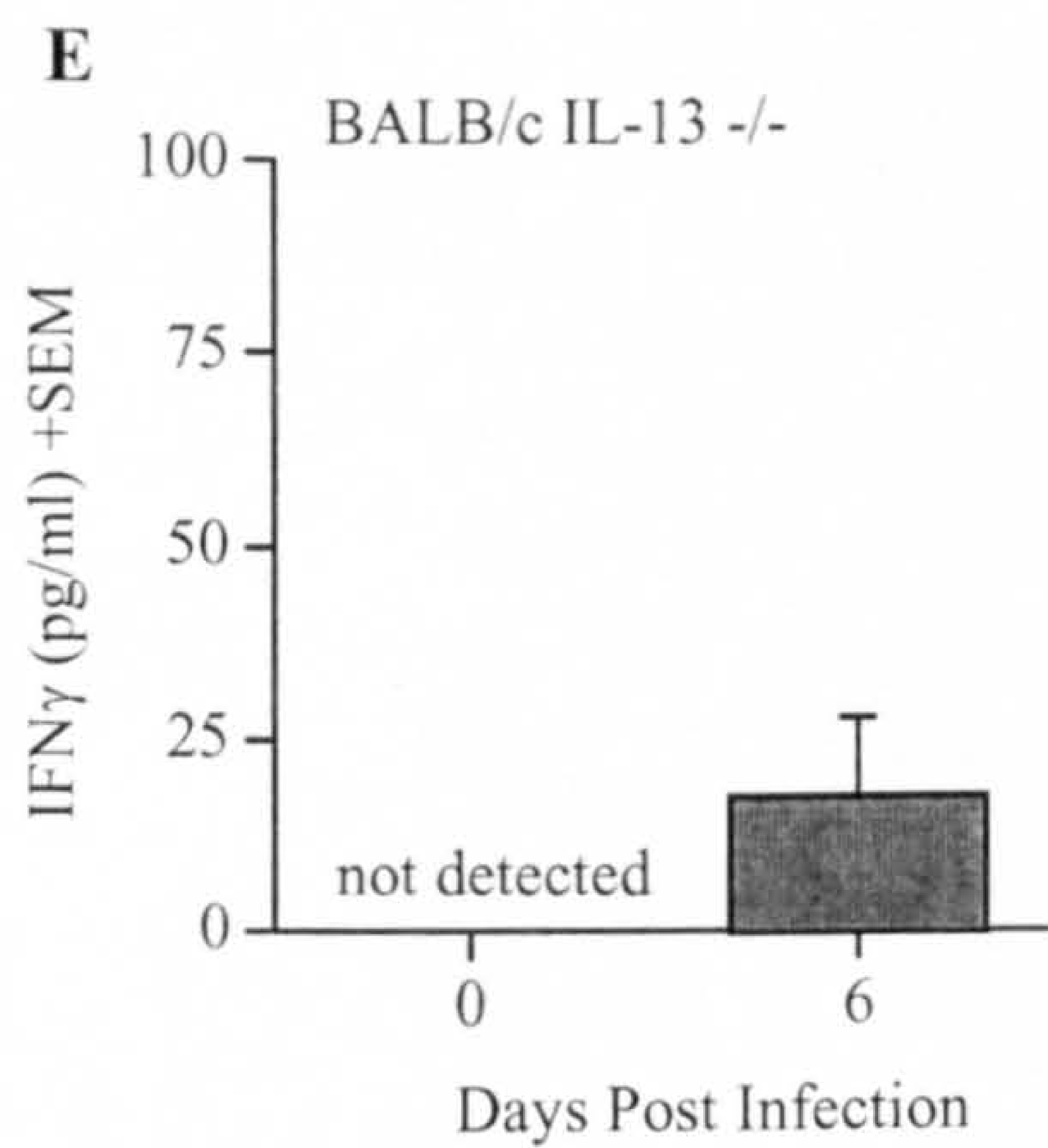
FIGURE 3.10: Role of host strain, IL-4, IL-13 and IL-4Rα on mesenteric lymph node cell IFNγ secretion following infection with *T. spiralis*. MLN were removed from wild type, uninfected and infected (day 6 p.i.) wild type C57BL/6, (A), C57BL/6 IL-4 -/- (B), wild type BALB/c (C), BALB/c IL-4 -/- (D), BALB/c IL-13 -/- (E) and BALB/c IL-4Rα -/- (F) mice. Single cell suspensions were made; because viable cell yields were low samples were pooled for all uninfected mice only one sample was obtained except C57BL/6 IL-4 -/- mice where no viable cells were obtained. From infected wild type BALB/c, 4 samples; BALB/c IL-4 -/-, 2 samples; BALB/c IL-13 -/-, 3 samples, BALB/c IL-4Rα -/-, 2 samples; wild type C57BL/6, 5 samples and C57BL/6 IL-4 -/-, 3 samples were obtained. Cells were cultured at $1\times10^6$ cells with 50μg/ml TAg. The secretion of the IFNγ was measured in the culture supernatants by ELISA against a recombinant standard. Data expressed mean cytokine concentration in pg/ml + SEM.

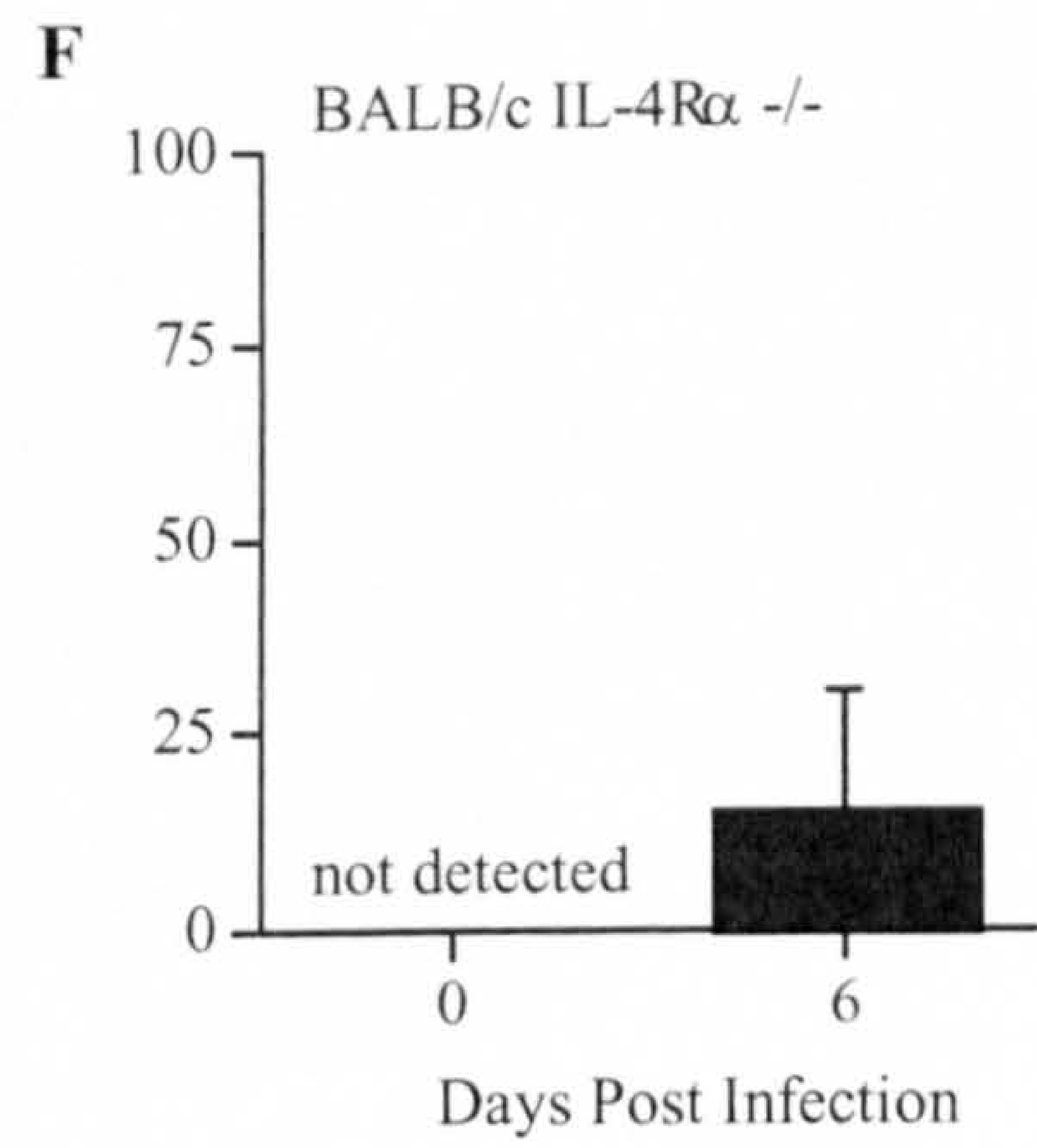

A
C57Bl/6
100
75
50
25
0
IFNγ (pg/ml) +SEM
not detected
0
6


B
C57Bl/6 IL-4 -/-
100
75
50
25
0
0
6


C
BALB/c
100
75
50
25
0
IFNγ (pg/ml) +SEM
not detected
0
6


D
BALB/c IL-4 -/-
100
75
50
25
0
0
6


E
BALB/c IL-13 -/-
100
75
50
25
0
IFNγ (pg/ml) +SEM
not detected
0
6
Days Post Infection


F
BALB/c IL-4Rα -/-
100
75
50
25
0
not detected
0
6
Days Post Infection

### 3.2.5 *Serum total IgE and antigen specific IgG1 titres are significantly reduced in the absence of IL-4, IL-13 and IL-4Rα.*

To further analyse the role of IL-4, IL-13 and IL-4Rα in the development of Th1:Th2 responses following infection with *T. spiralis* total serum IgE and antigen specific IgG1 and 2a titres were measured in uninfected and infected (day 14 p.i.) wild type C57BL/6, C57BL/6 IL-4 -/-wild type BALB/c, BALB/c IL-4 -/-, BALB/c IL-13 -/- and BALB/c IL-4Rα -/-, mice.

No significant differences in IgE were observed between uninfected mouse strains. Following infection serum IgE titres increased significantly in all stains (C57BL/6 p=0.009 (Figure 3.11A); C57BL/6 IL-4 -/- p=0.0163 (Figure 3.11B); wild type BALB/c p=0.009 (Figure 3.11C); BALB/c IL-4 -/- p=0.009 (Figure 3.11D) and BALB/c IL-13 -/- p=0.009 (Figure 3.11E)) except BALB/c IL-4Rα mice (Figure 3.11F). No significant difference in IgE titres were observed between wild type BALB/c and C57BL/6 or between BALB/c IL-4 -/- and C57BL/6 IL-4 -/- mice at day 14 p.i. However in BALB/c IL-4 -/- (p=0.009), BALB/c IL-13 -/- (p=0.009), and C57BL/6 IL-4 -/- (p=0.009) mice serum IgE was significantly lower than that observed in wild type BALB/c and C57BL/6 mice. Furthermore serum IgE titres in BALB/c IL-4Rα -/- mice were significantly lower than those observed in BALB/c IL-13 -/- mice (p=0.0086) although no significant differences were observed between IgE titres between BALB/c IL-4 -/- mice and

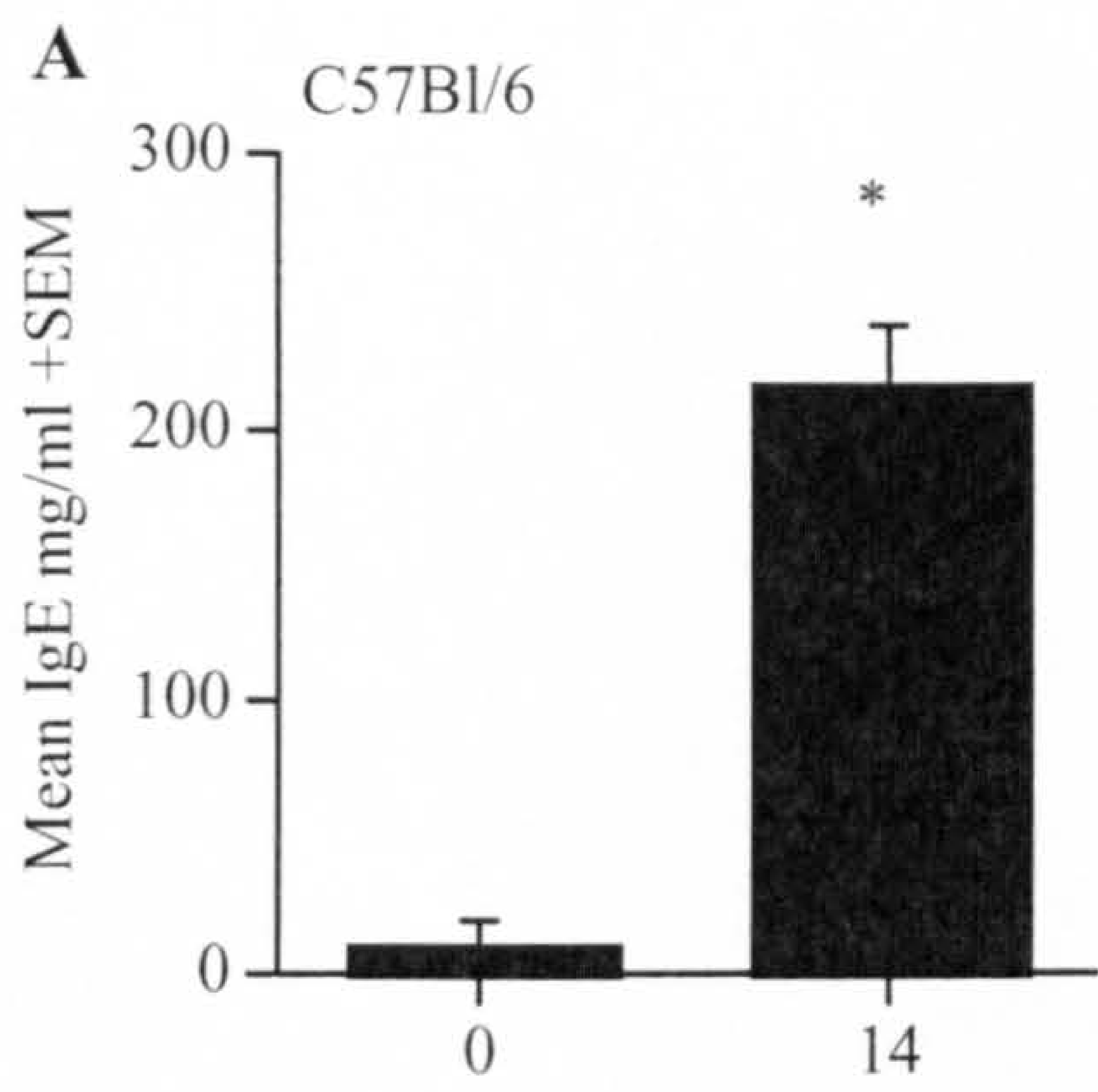
BALB/c IL-13 -/- and BALB/c IL-4Rα -/- mice at day 14 p.i. due to the higher variability in IgE titres observed in BALB/c IL-4 -/- mice.

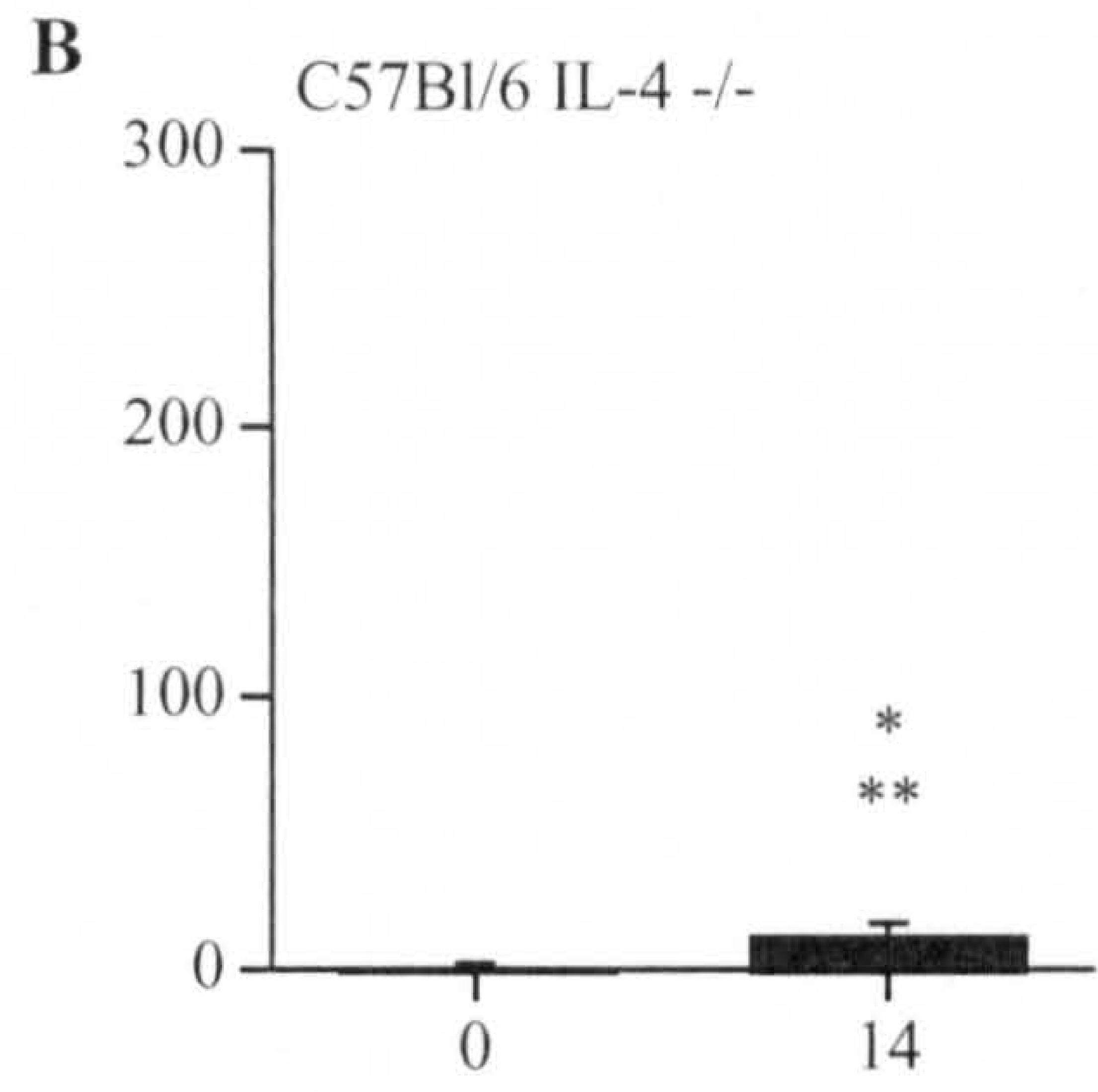
*Trichinella* specific IgG1 titres increased following infection (day 14 p.i.) in all strains (wild type C57BL/6, p=0.009 (Figure 3.12A); C57BL/6 IL-4 -/-, p=0.0143 (Figure 3.12B); wild type BALB/c, p=0.0143 (Figure 3.12C); BALB/c IL-4 -/- p=0.009 (Figure 3.12D); BALB/c IL-13 -/- p=0.009 (Figure 3.12E) and BALB/c IL-4Rα -/- p=0.0143 (Figure 3.12F)) compared to uninfected mice. No significant differences in IgG1 titres were observed between wild type BALB/c mice, wild type C57BL/6, BALB/c IL-4 -/-, BALB/c IL-13 -/- mice at day 14 p.i. Interestingly C57BL/6 IL-4 -/- mice had significantly lower IgG1 titres than wild type C57BL/6 mice at day 14 p.i. (p=0.0143), further more IgG1 titres were significantly lower in the C57BL/6 IL-4 -/- mice than in the BALB/c IL-4 -/- mice (p=0.0143). BALB/c IL-4Rα -/- mice had significantly reduced levels of IgG1 compared to wild type BALB/c, BALB/c IL-4 -/- and BALB/c IL-13 -/- mice at day 14 p.i. No differences were observed between *Trichinella* specific IgG2a titres of uninfected mice of any strain. Following infection specific IgG2a titres increased significantly in wild type C57BL/6 (p=0.0472) (Figure 3.13A), wild type BALB/c (p=0.0143) (Figure 3.13C), BALB/c IL-4 -/- (p=0.0367) (Figure 3.13D), BALB/c IL-13 -/- (p=0.009) (Figure 3.13E) and BALB/c IL-4Rα -/- mice (p=0.0143) (Figure 3.13F). No increase in IgG2a titres was observed in C57BL/6 IL-4 -/- following infection. No significant differences were observed between wild type or IL-4 -/- C57BL/6 and BALB/c mice at day 14 p.i. Interestingly although BALB/c IL-4 and IL-

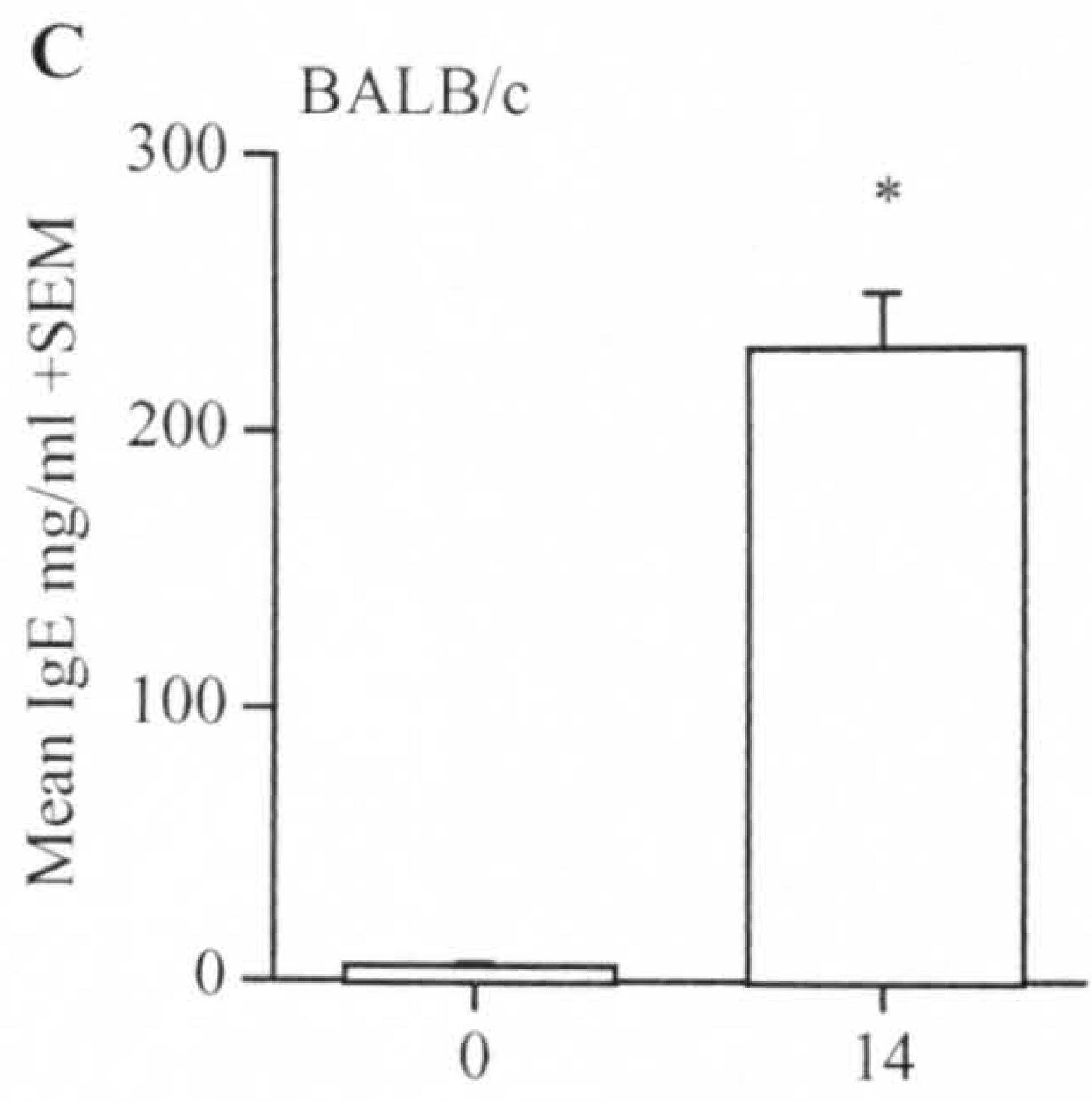
4Rα -/- mice do not differ significantly from wild type BALB/c mice, BALB/c IL-13 -/- mice have significantly higher IgG2a titres ($p=0.0275$).

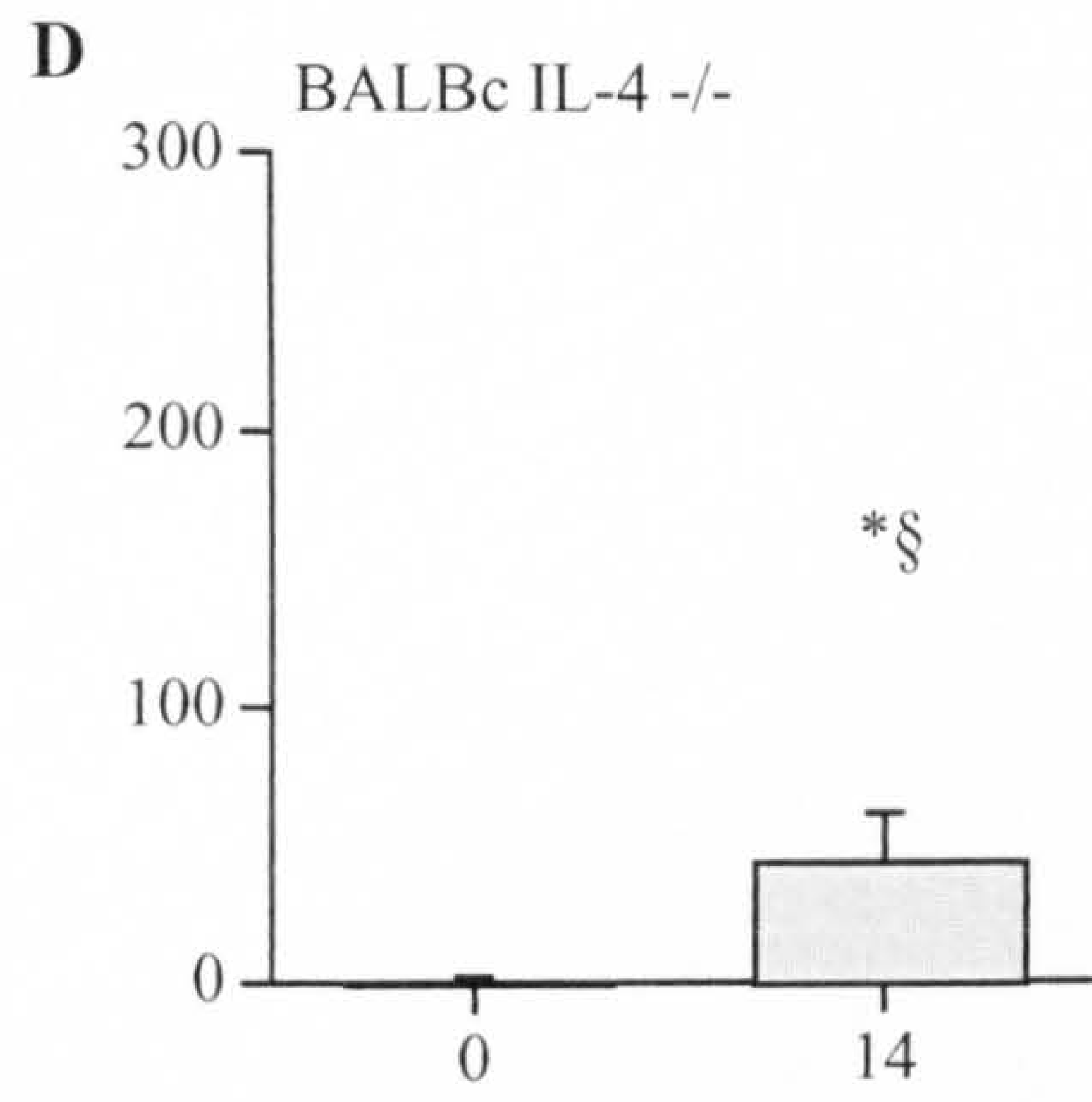
As with T helper cytokine responses, no significant differences in antibody responses were observed between wild type C57BL/6 and BALB/c mice. Although total IgE titres significantly increased in all mice following infection with *T. spiralis*, IgE levels were significantly reduced at day 14 p.i. in the absence of IL-4 (in both C57BL/6 and BALB/c mice), IL-13 and IL-4Rα, antigen specific IgG1 titres however were only reduced in the absence of IL-4 in C57BL/6 mice and in the absence of IL-4Rα in BALB/c mice. Antigen specific IgG2a responses were elevated in BALB/c IL-13 -/- mice but not in the absence of IL-4 (in either BALB/c or C57BL/6 mice) or in the absence of IL-4Rα suggesting a role for IL-13 in modulating Th1 responses.

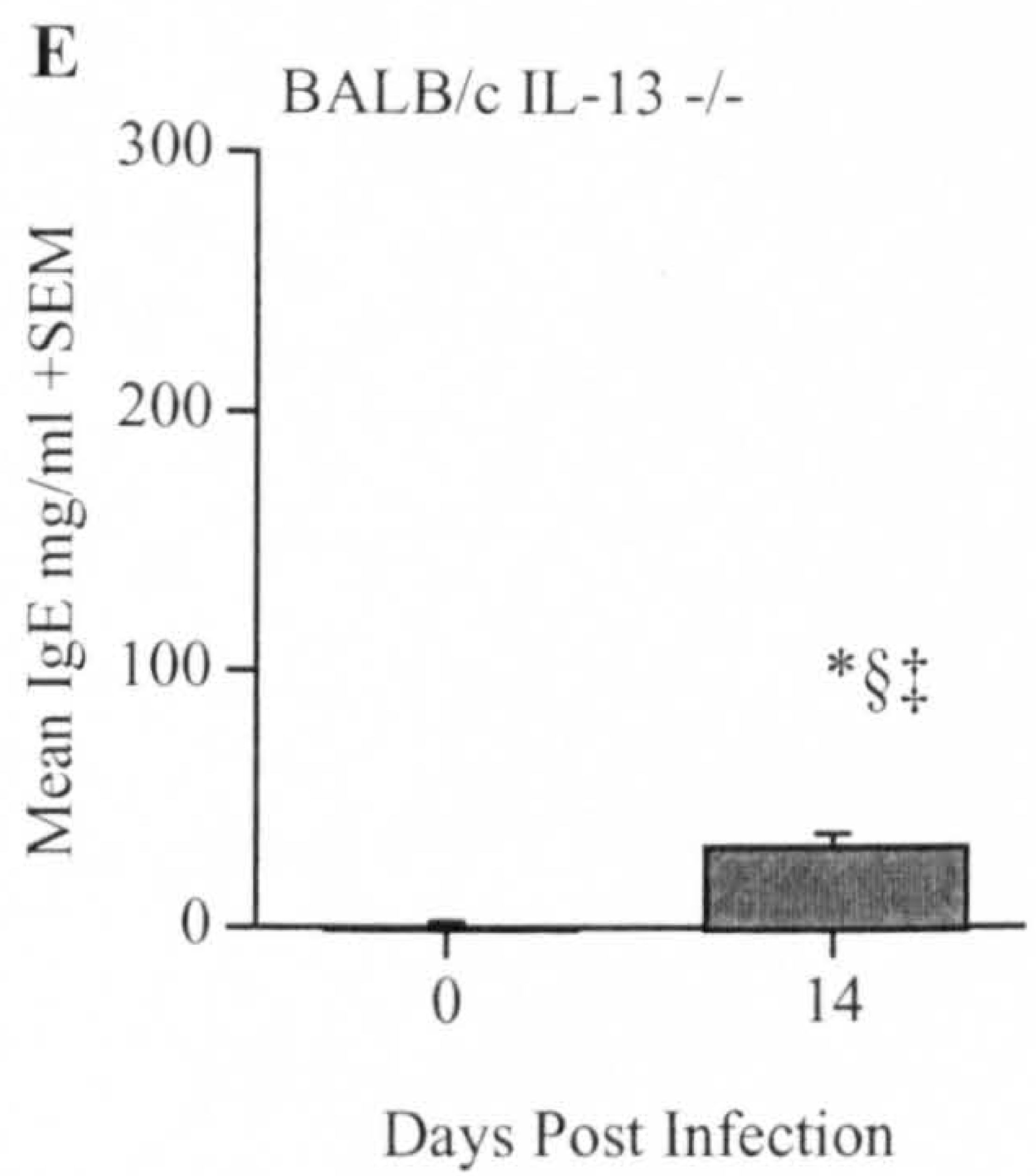
FIGURE 3.11: The role of host strain, IL-4, IL-13 and IL-4Rα in total IgE responses following infection with *T. spiralis*. Total IgE was measured in sera taken uninfected and infected (day 14 p.i.) wild type C57BL/6, (A), C57BL/6 IL-4 -/- (B), wild type BALB/c (C), BALB/c IL-4 -/- (D), BALB/c IL-13 -/- (E) and BALB/c IL-4Rα -/- (F). Total IgE titres were measured by sandwich ELISA against a purified IgE standard and were expressed as mean μg/ml + SEM. Data is expressed as mean IgE concentration (μg/ml) + SEM, five mice were used per group *, represents significantly different to uninfected mice; **, represents significantly different wild type C57BL/6 mice; §, represents significantly different to wild type BALB/c mice ($p<0.05$).


A
C57Bl/6
Mean IgE mg/ml +SEM
300
200
100
0
*
0
14


B
C57Bl/6 IL-4 -/-
300
200
100
0
*
**
0
14


C
BALB/c
Mean IgE mg/ml +SEM
300
200
100
0
*
0
14


D
BALBc IL-4 -/-
300
200
100
0
*§
0
14


E
BALB/c IL-13 -/-
Mean IgE mg/ml +SEM
300
200
100
0
*§‡
0
14
Days Post Infection

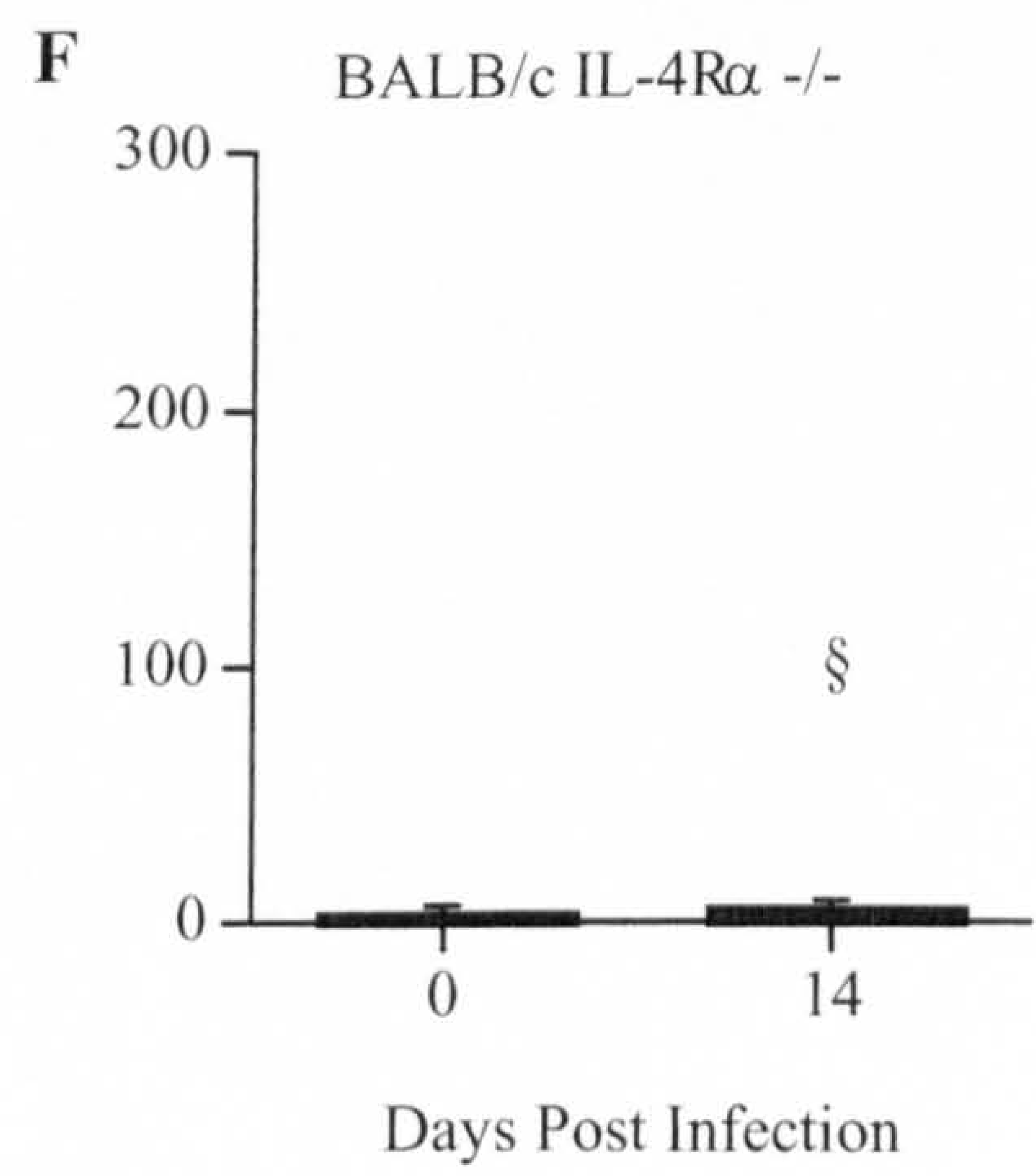
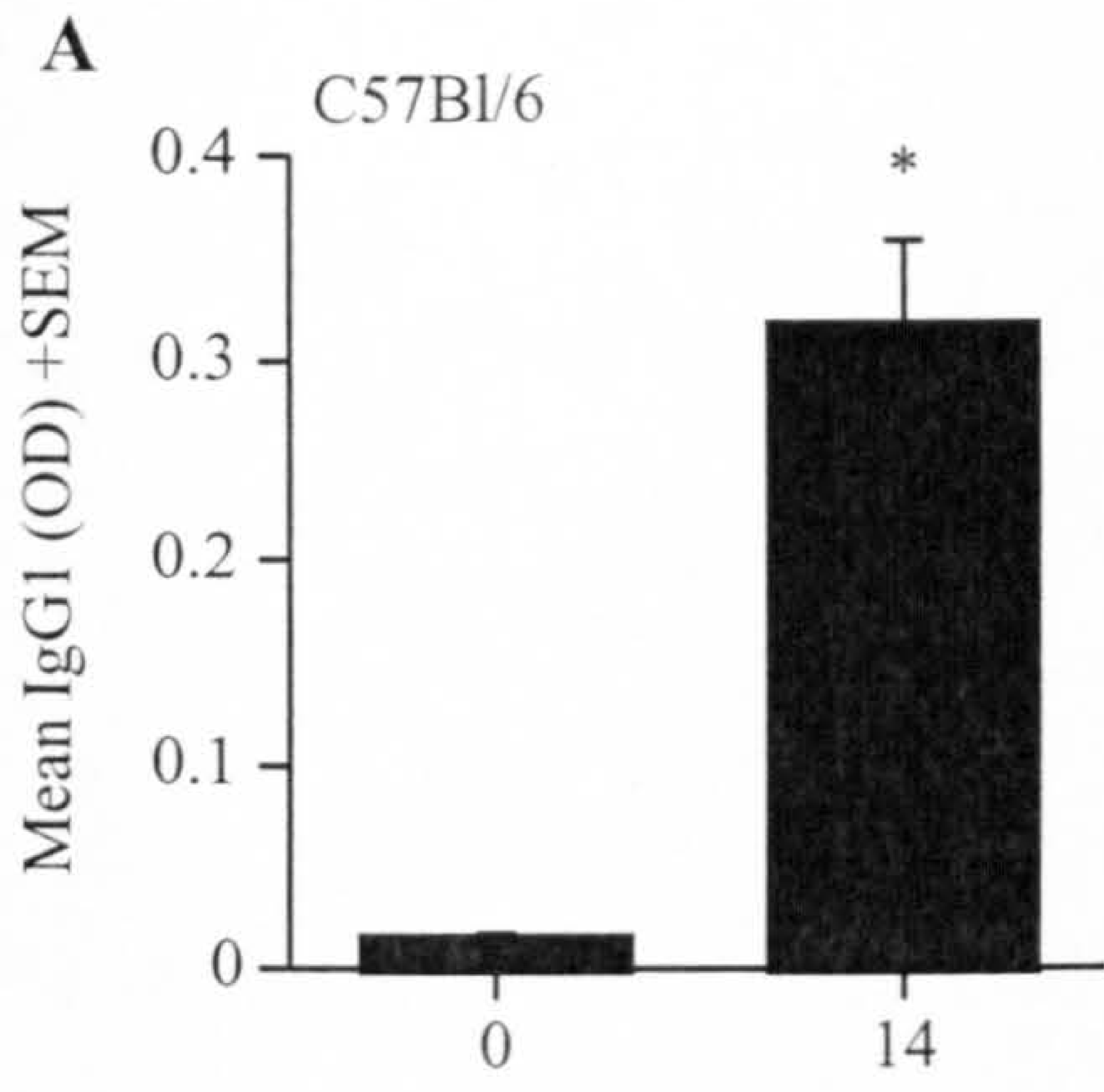
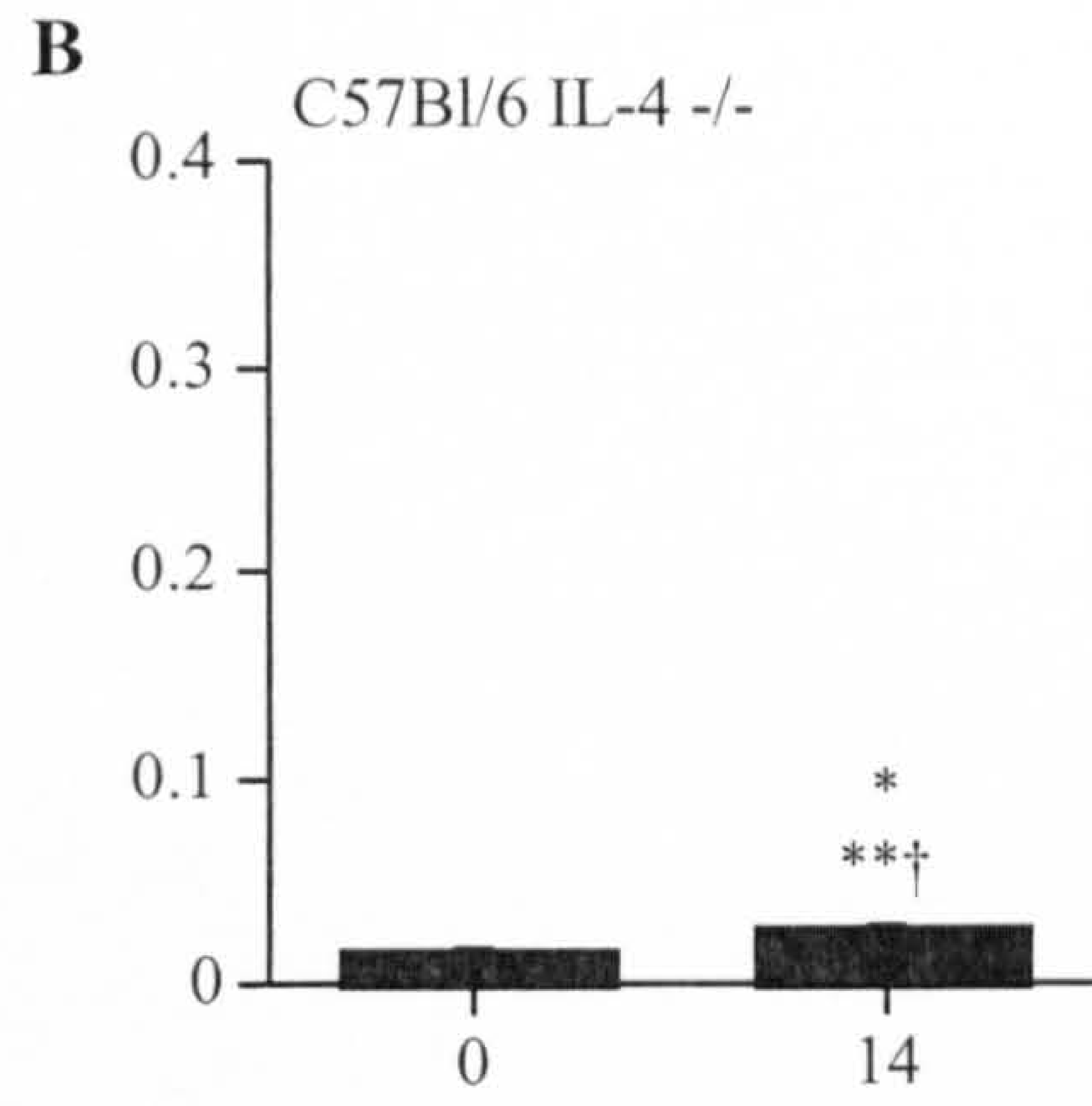
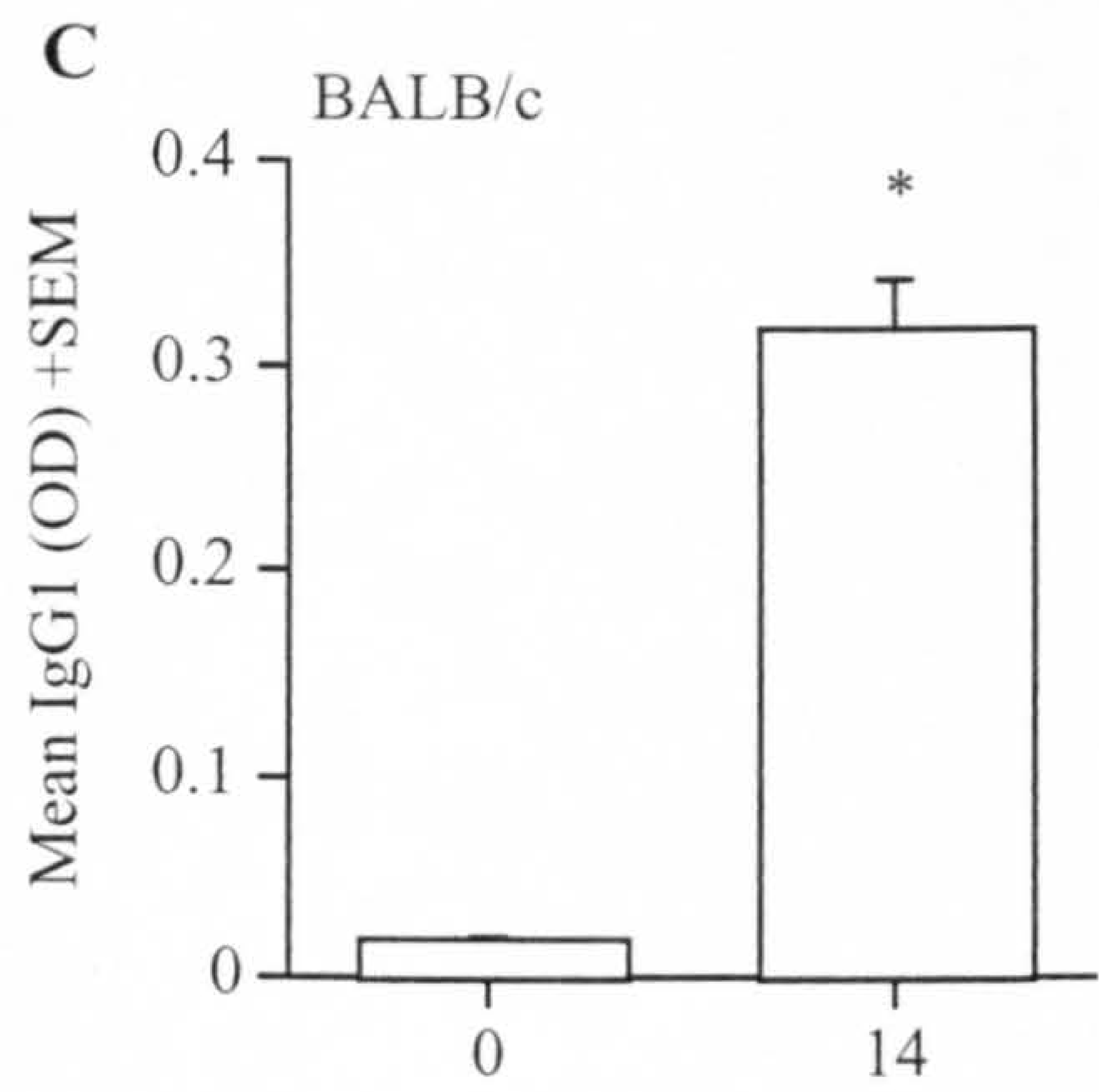
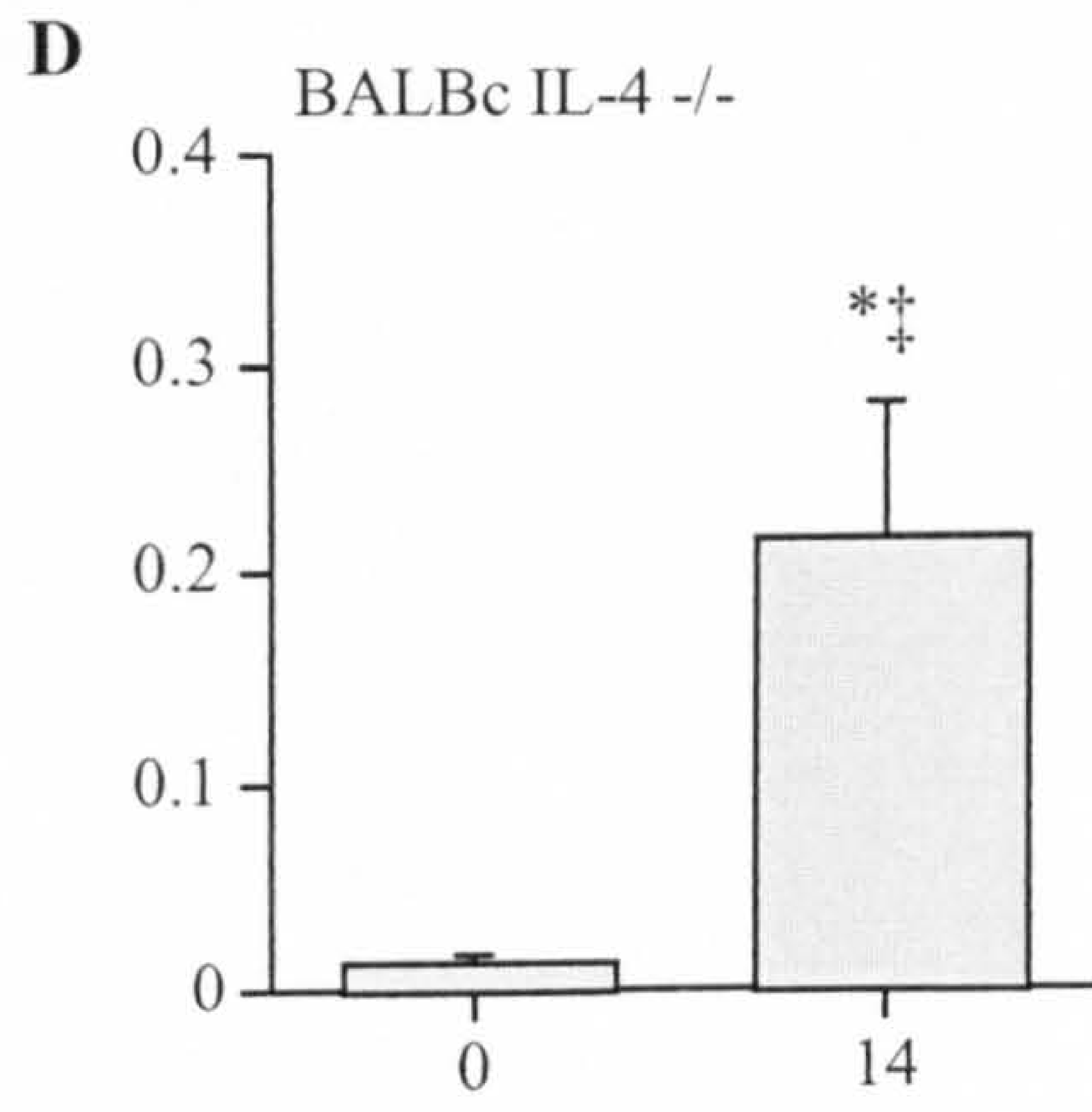

F
BALB/c IL-4Rα -/-
300
200
100
0
§
0
14
Days Post Infection

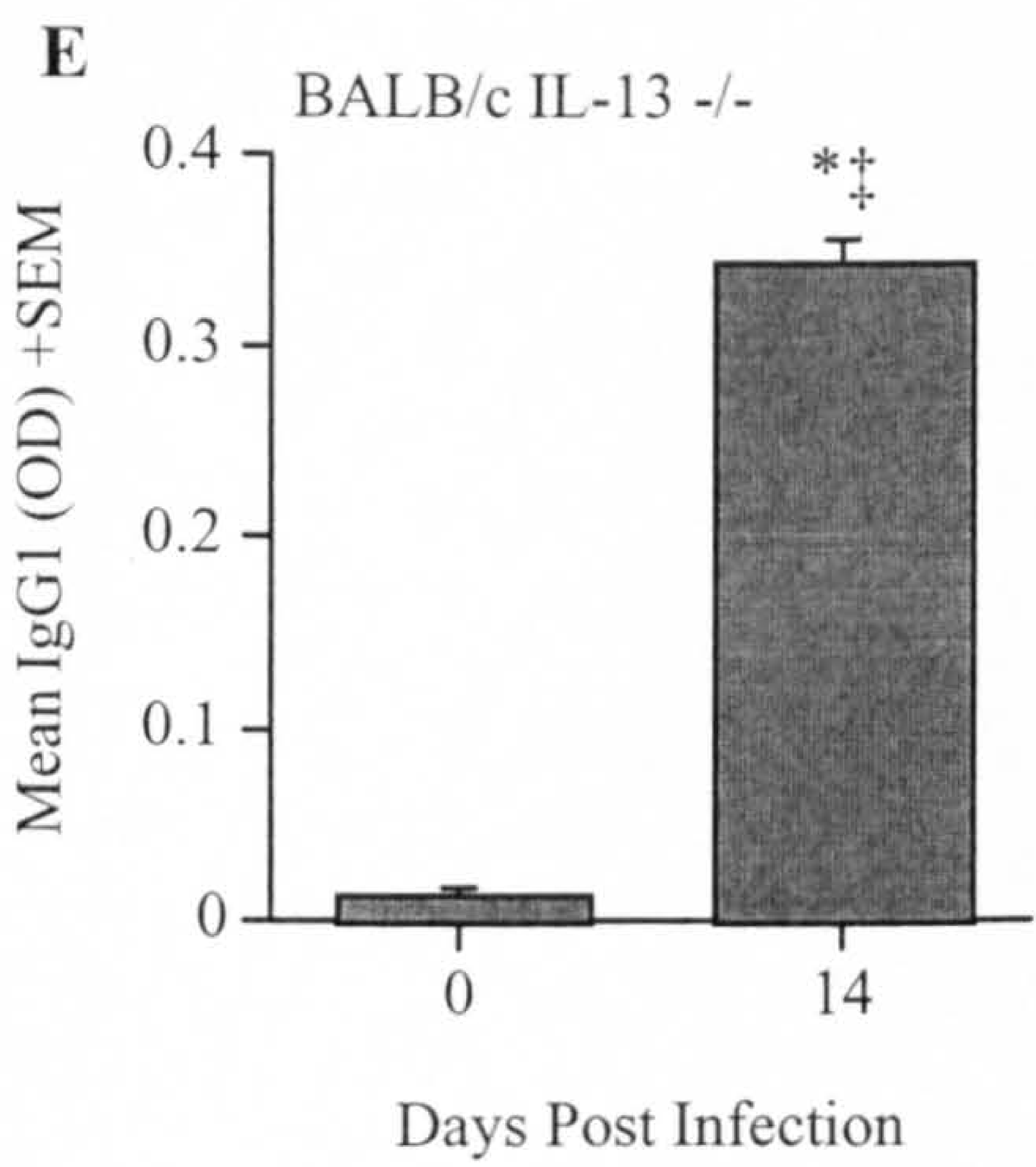
FIGURE 3.12: The role of host strain, IL-4, IL-13 and IL-4Rα in Antigen specific IgG1 responses following infection with *T. spiralis*. Antigen specific IgG1 titres were measured in sera taken uninfected and infected (day 14 p.i.) wild type C57BL/6 (A) C57BL/6 IL-4 -/- (B), wild type BALB/c (C), BALB/c IL-4 -/- (D), BALB/c IL-13 -/- (E), and BALB/c IL-4Rα -/- (F). Antigen specific IgG1 titres were assessed by ELISA using 96 well microtitre plates coated with *TAg* at 2μg/ml, sera was serially diluted and optical density readings at 1:640 dilution are shown. Data was expressed as mean optical density (OD) +SEM; five mice were used per group. *, represents significantly different to uninfected; **, represents significantly different to wild type C57BL/6 mice; §, represents significantly different to wild type BALB/c mice; †, represents significantly different to BALB/c IL-4 -/- mice; ‡, represents significantly different to BALB/c IL-4Rα -/- mice ($p<0.05$).


A
C57Bl/6
*
0.4
0.3
0.2
0.1
0
Mean IgG1 (OD) +SEM
0
14


B
C57Bl/6 IL-4 -/-
0.4
0.3
0.2
0.1
0
*
**†
0
14


C
BALB/c
*
0.4
0.3
0.2
0.1
0
Mean IgG1 (OD) +SEM
0
14


D
BALBc IL-4 -/-
*‡
0.4
0.3
0.2
0.1
0
0
14


E
BALB/c IL-13 -/-
*‡
0.4
0.3
0.2
0.1
0
Mean IgG1 (OD) +SEM
0
14
Days Post Infection

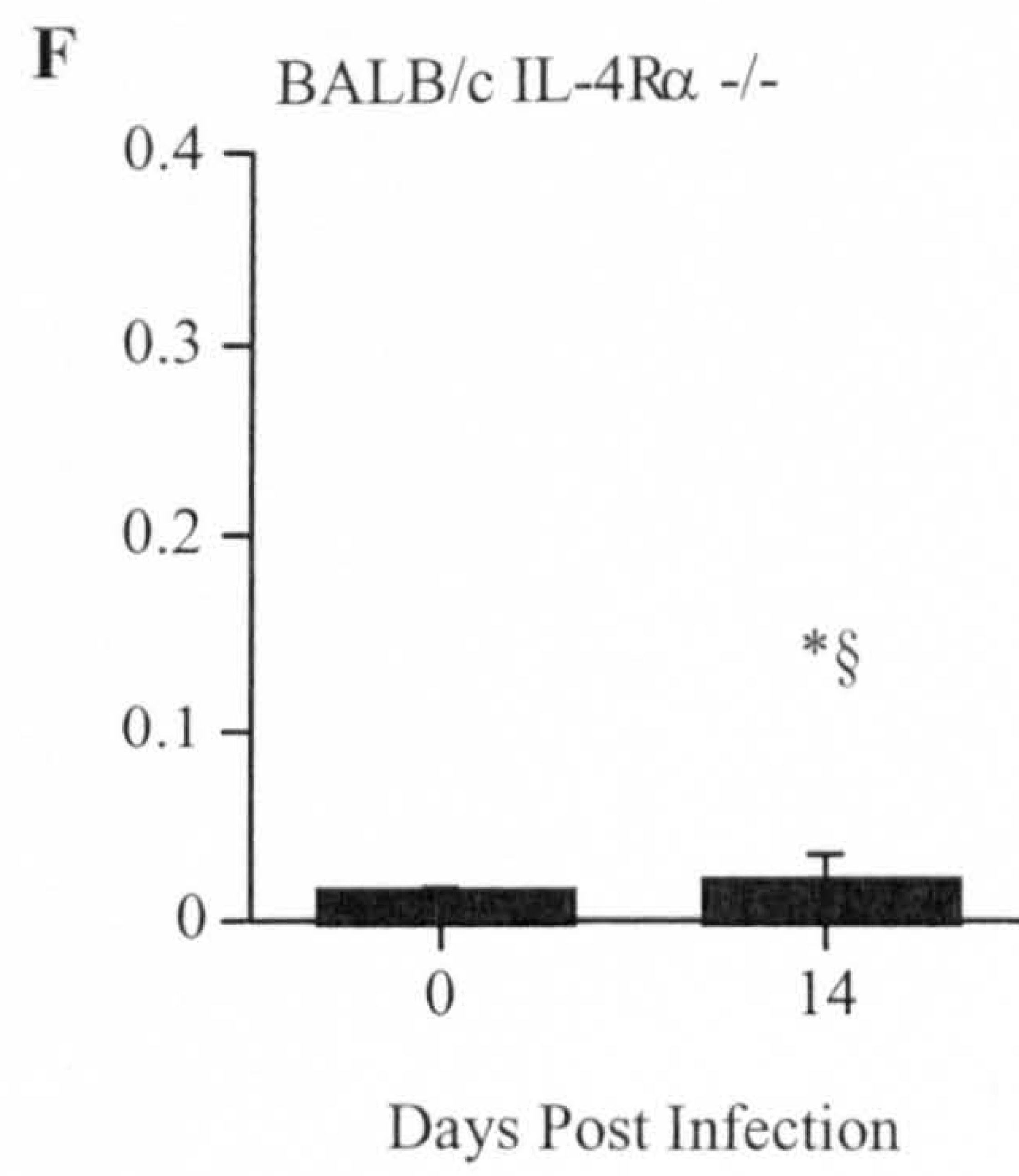
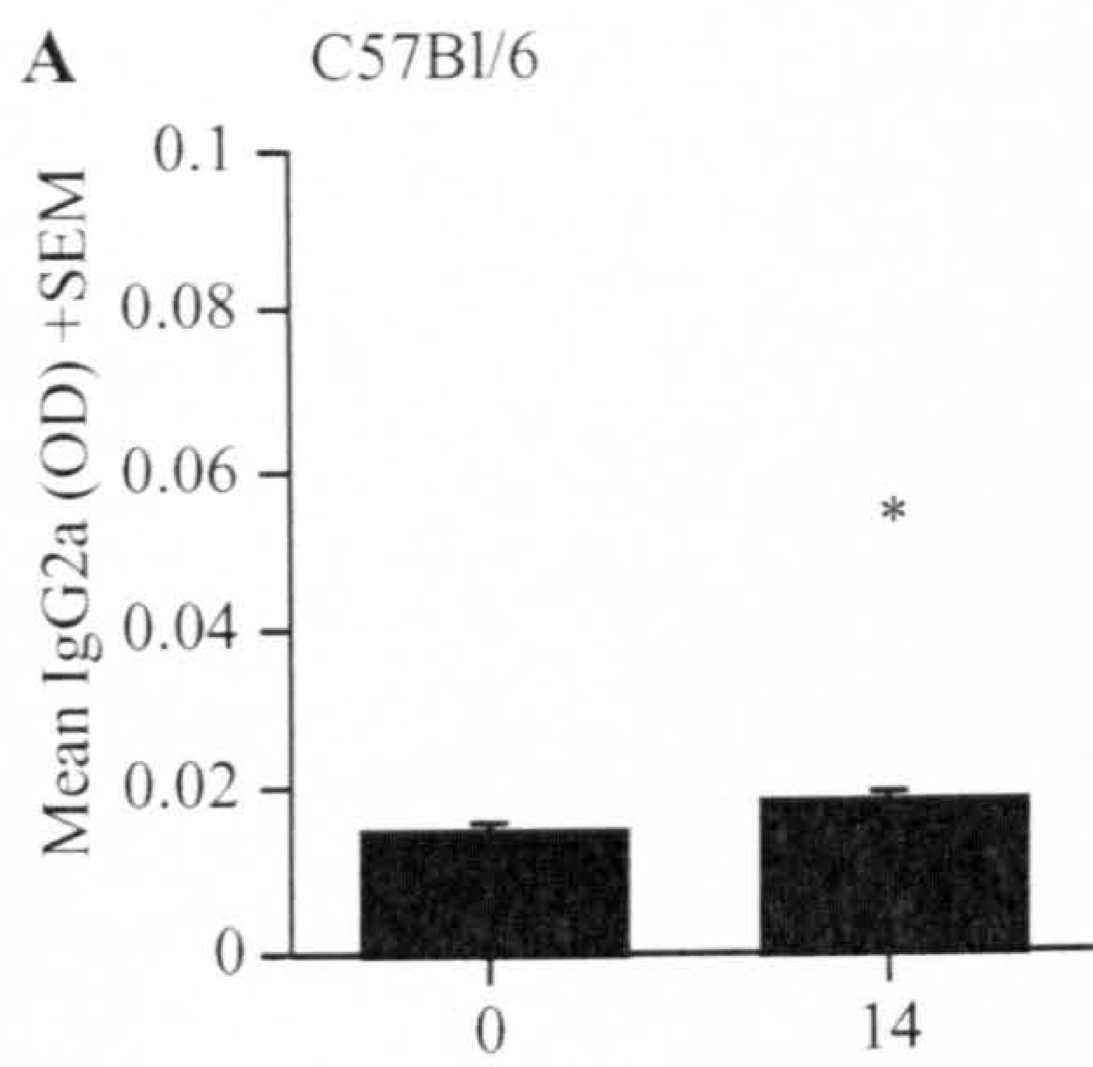
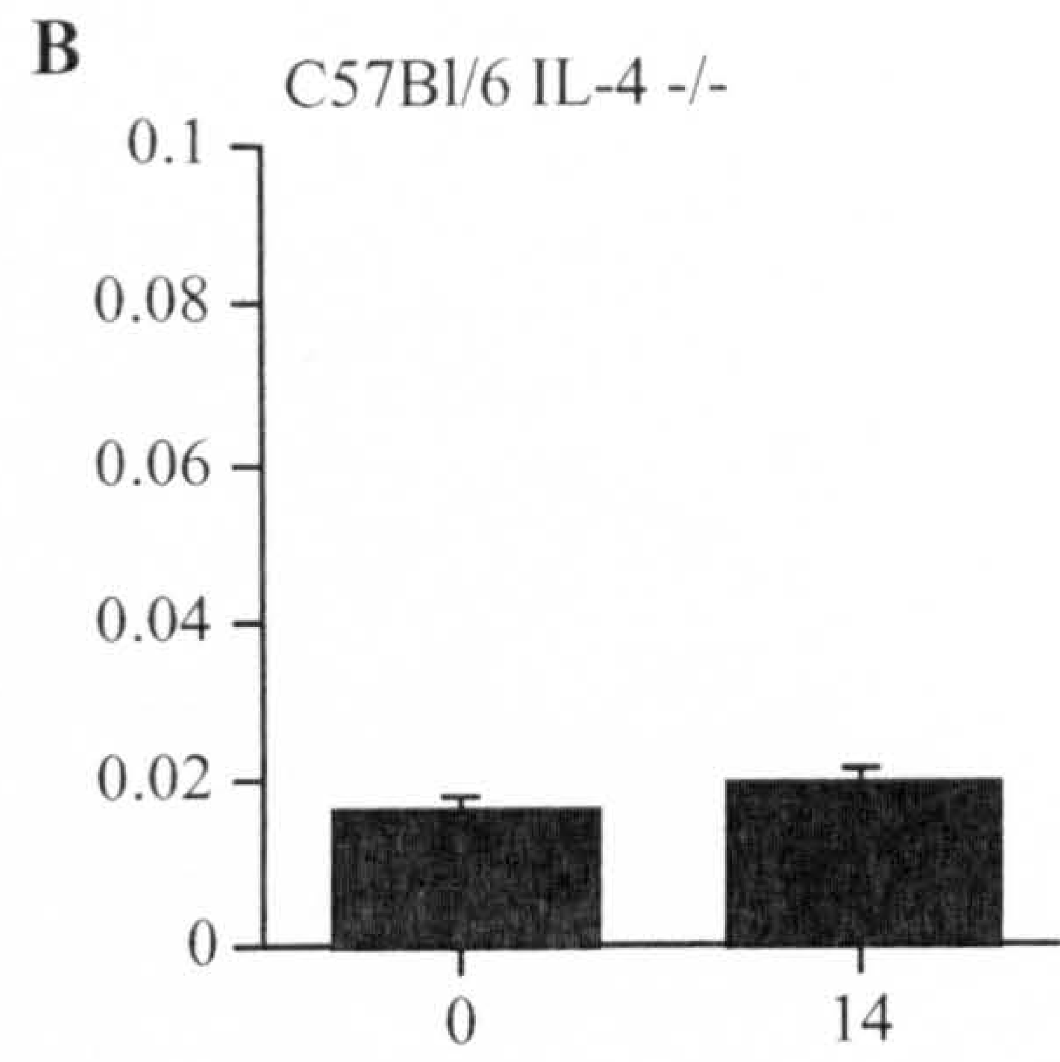
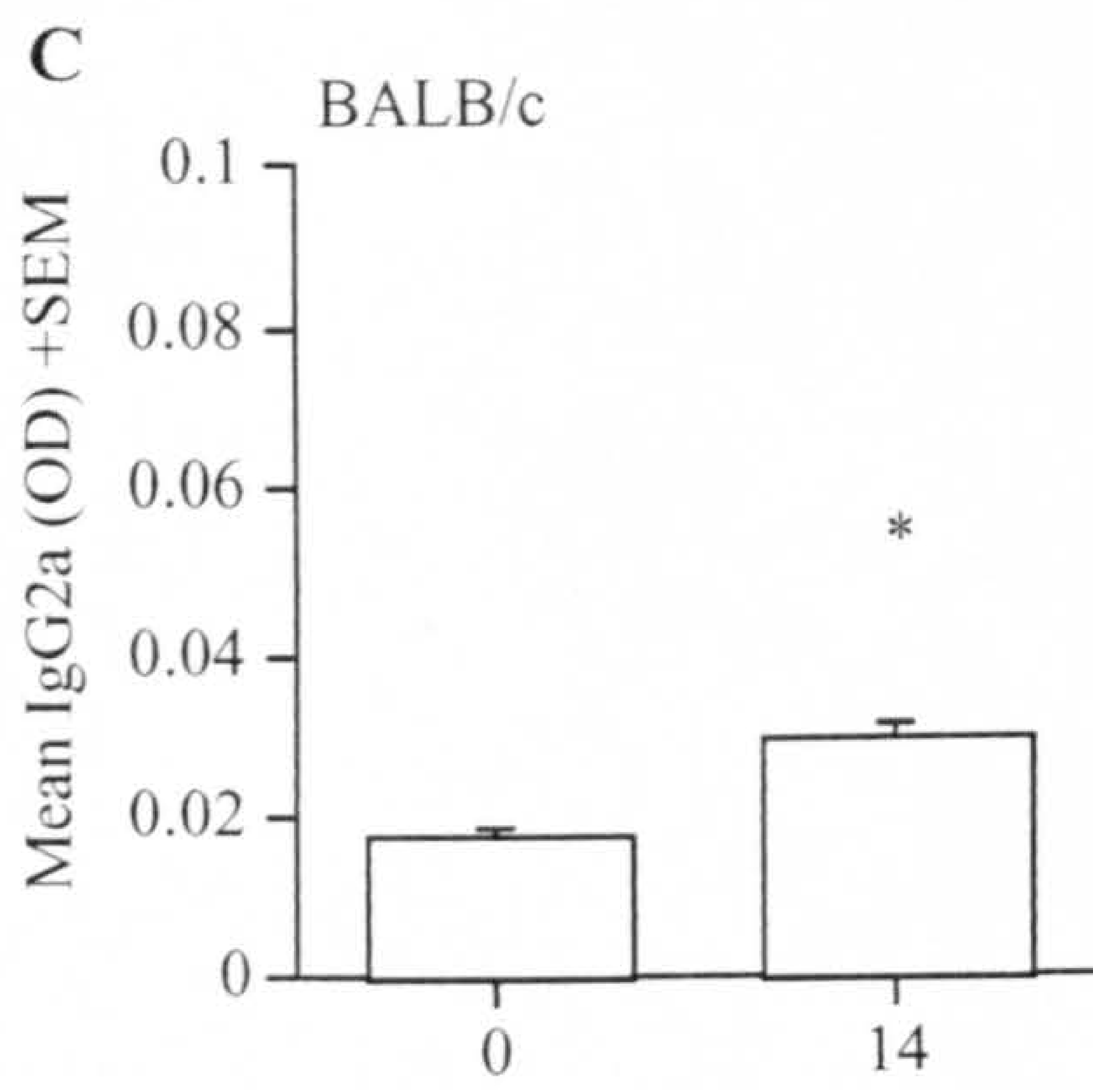
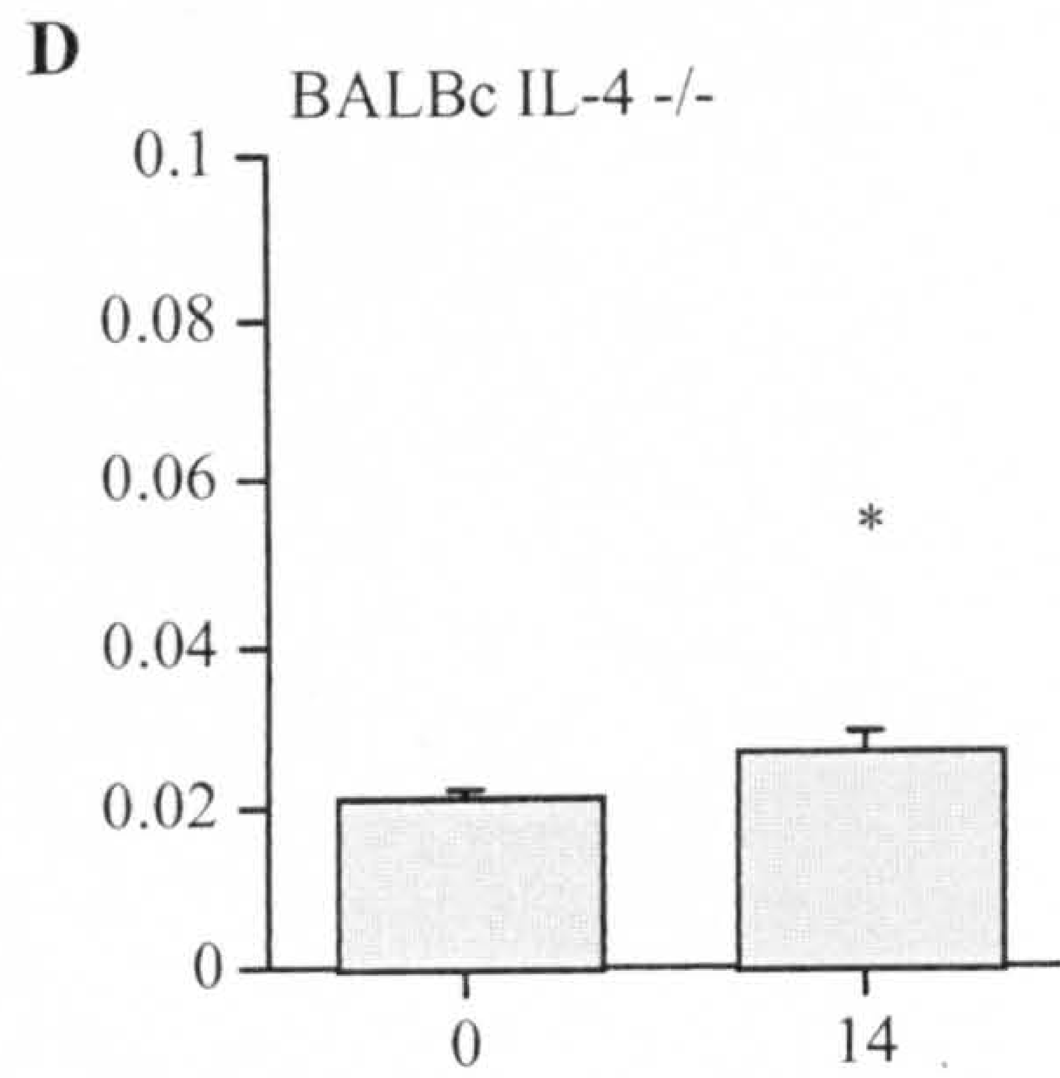

F
BALB/c IL-4Rα -/-
*§
0.4
0.3
0.2
0.1
0
0
14
Days Post Infection

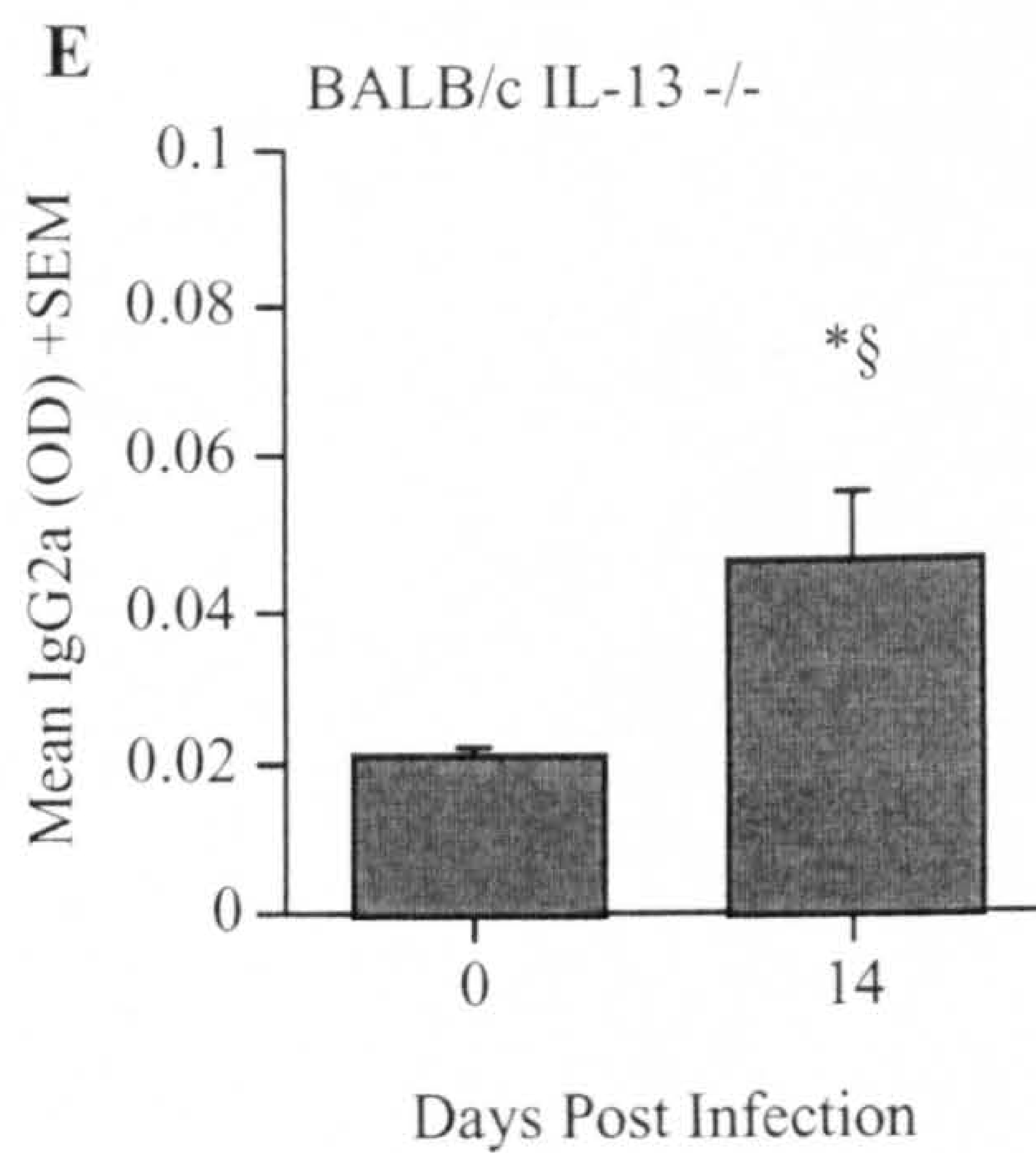
FIGURE 3.13: The role of host strain, IL-4, IL-13 and IL-4Rα in Antigen specific IgG2a responses following infection with *T. spiralis*. Antigen specific IgG2a titres were measured in sera taken uninfected and infected (day 14 p.i.) wild type C57BL/6 (A) C57BL/6 IL-4 -/- (B), wild type BALB/c (C), BALB/c IL-4 -/- (D), BALB/c IL-13 -/- (E), and BALB/c IL-4Rα -/- (F). Antigen specific IgG2a titres were assessed by ELISA using 96 well microtitre plates coated with *TAg* at 2μg/ml, sera was serially diluted and optical density readings at 1:640 dilution were used. Data was expressed as mean optical density (OD) +SEM; five mice were used per group. *, represents significantly different to uninfected; §, represents significantly different to wild type BALB/c mice; ^, represents significantly different to wild type C57BL/6 mice; +, represents significantly different to BALB/c IL-4 -/- mice; ~, represents significantly different to BALB/c IL-4Rα -/- mice ($p<0.05$).

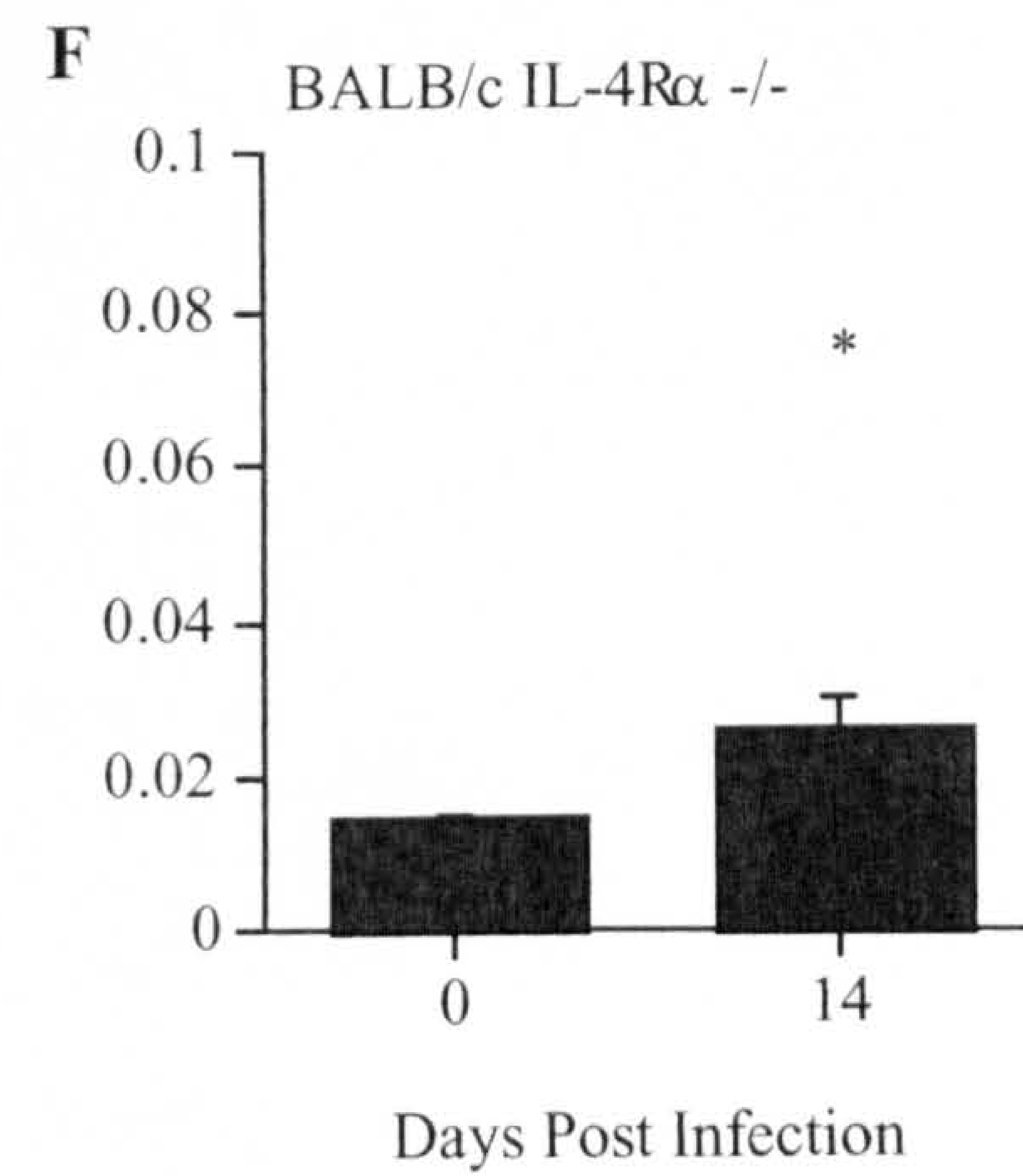

A
C57Bl/6
Mean IgG2a (OD) +SEM
0.1
0.08
0.06
0.04
0.02
0
*
0
14


B
C57Bl/6 IL-4 -/-
0.1
0.08
0.06
0.04
0.02
0
0
14


C
BALB/c
Mean IgG2a (OD) +SEM
0.1
0.08
0.06
0.04
0.02
0
*
0
14


D
BALBc IL-4 -/-
0.1
0.08
0.06
0.04
0.02
0
*
0
14


E
BALB/c IL-13 -/-
Mean IgG2a (OD) +SEM
0.1
0.08
0.06
0.04
0.02
0
*§
0
14
Days Post Infection


F
BALB/c IL-4Rα -/-
0.1
0.08
0.06
0.04
0.02
0
*
0
14
Days Post Infection

### *3.2.6 Expulsion of T. spiralis is not significantly effected by the absence of the IL-4Rα from cells of the macrophage/ neutrophil lineage.*

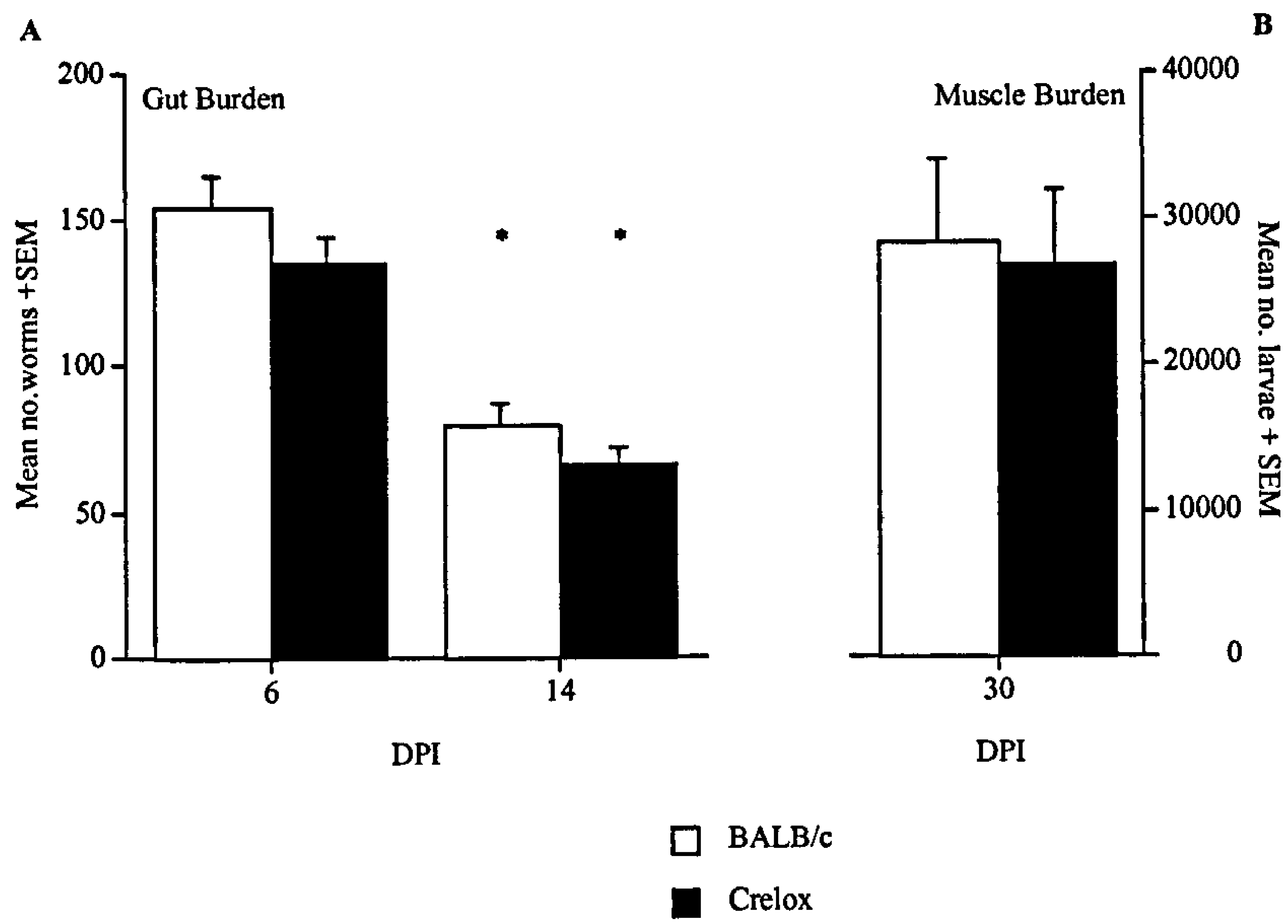
As shown above IL-4, IL-13 and their receptor IL-4Rα play a role in the development of both protective and pathological responses to *T. spiralis*. To evaluate role of signalling to macrophages and neutrophils via IL-4Rα, wild type and macrophage/neutrophil specific IL-4Rα -/- BALB/c mice were infected with 400 freshly isolated *T. spiralis* larvae. Briefly, macrophage/neutrophil lineage specific IL-4Rα -/- mice where generated using the crelox system which utilises *cre*, a bacteriophage recombinase, which mediates recombination of DNA between *loxP* sites. In order to generate tissue specific -/- mice, cre was placed under the control of the promoter for lysozyme (*lysM*) which is expressed in cells of the macrophage/neutrophil lineage and the *il-4rα* gene was flanked by two *loxP* sites (floxed). In cells of the macrophage/neutrophil lineage Cre is expressed and cleaves out the *il-4rα* gene, resulting in neutrophil/macrophage specific IL-4Rα -/- (LysM(Cre)IL-4Rα(-/flox)) mice (Herbert *et al* 2004). For simplicity neutrophil/macrophage specific IL-4Rα-/- mice will be referred to as Crelox mice in this chapter.

To determine the establishment of the worms in the small intestine wild type and Crelox mice were culled day 6 p.i. and the total number of adult worms present was counted, subsequently at day 14 p.i. further wild type and Crelox mice were culled and the total number of worms present in the small intestine counted to evaluate the expulsion of the

worms. The accumulation of larvae in the muscle of infected host provides a measure of the fecundity of the female *T. spiralis* in the intestine. The larvae harboured by wild type and Crelox mice were extracted at day 30 p.i., by the digestion of muscle tissue followed by the filtration and concentration of the supernatant; the number of larvae present were counted.

Worm establishment at day 6 p.i. was not significantly different between wild type and Crelox mice. Both wild type ($p=0.009$) and Crelox ($p=0.009$) mice had significantly lower numbers of adult worms at day 14 p.i. compared to day 6 p.i., however no significant difference was observed in the number of adult worms present in the intestine at day 14 p.i. between wild type and Crelox mice (Figure 3.14A). No significant difference was observed in the number of muscle larvae harboured by wild type and Crelox mice (Figure 3.14B).

FIGURE 3.14: The role of IL-4Rα signalling to macrophages and neutrophils in the expulsion of *T. spiralis* from the small intestine and in the accumulation of muscle larvae. The establishment and expulsion of *T. spiralis* was measured in wild type BALB/c, and Crelox mice at days 6, 14 and 30 p.i. The small intestine was excised and the total number of worms present was counted. The accumulation of muscle larvae was measured in wild type BALB/c and Crelox mice at day 30 p.i. Data is expressed as mean number of worms/mouse + SEM, five mice were used per group *, represents significantly different to mice at day 6 p.i. §, represents significantly different to wild type BALB/c mice. ($p<0.05$).


A
B
Gut Burden
Muscle Burden
200
150
100
50
0
40000
30000
20000
10000
0
Mean no.worms +SEM
Mean no. larvae + SEM
6
14
30
DPI
DPI
BALB/c
Crelox

### 3.2.7 *Villus atrophy following* T. spiralis *is increased in the absence of signalling through the IL-4Rα in cells of the macrophage/ neutrophil lineage.*

In order to evaluated the role of signalling through the IL-4Rα to cells of the macrophage/neutrophil lineage in the development of enteropathy following infection with *T. spiralis* the small intestine was removed from uninfected (day 0 p.i.) and infected (days 6 and 14 p.i.) wild type and Crelox mice. The development of villus atrophy and crypt hyperplasia is assessed in a sample of jejunum and cell division in the small intestine was also measured by counting the number of mitotic figures/VCU. The development of oedema and the accumulation of fluid in the small intestine were assessed at days 0, 6, and 14 p.i. by weighing the whole small intestine.

Significant villus atrophy and crypt hyperplasia was observed at days 6 and 14 p.i. in wild type (p=0.009 and 0.009) and Crelox (p=0.009 and 0.0143) mice compared to uninfected mice. Mean villus length in Crelox (p=0.0472) mice was significantly shorter compared to wild type, however, no significant differences were observed between mean crypt depth in the wild type and Crelox mice. Villus length and crypt depths were not significantly different between wild type and Crelox mice at days 0 or 14 p.i. (Figure 3.15A). The number of dividing cells or mitotic figures in the base of crypts was significantly increased at days 6 and 14 p.i. in both wild type (p=0.0088 and 0.0088) and Crelox (p=0.009 and 0.0143) mice compared to uninfected mice. No significant

differences were observed between wild type and Crelox mice at any time point analysed (Figure 3.15B).

The development of oedema and the accumulation of fluid in the intestinal lumen was assessed by weighing the small intestine. Intestinal weight was significantly increased at days 6 and 14 p.i. compared to uninfected in both wild type ($p=0.0143$ and 0.0143) and Crelox ($p=0.009$ and 0.009) mice. No significant differences were observed between wild type and Crelox mice at days 0 or 6 p.i.; however, at day 14 p.i. Crelox ($p=0.05$) mice had significantly higher median intestinal weight compared to wild type (Figure 3.16).

FIGURE 3.15: The role of IL-4Rα signalling to macrophages and neutrophils in the development of enteropathy following infection with *T. spiralis*. Villus and crypt lengths (μm) (A) were measured and the number of mitotic figures per crypt (B) was determined in wild type and Crelox mice at day 0 (uninfected) and at day 6 and 13 p.i. Data expressed as mean + SEM for five mice. *, represents significantly different to uninfected; §, represents significantly different to wild type ($p<0.05$).

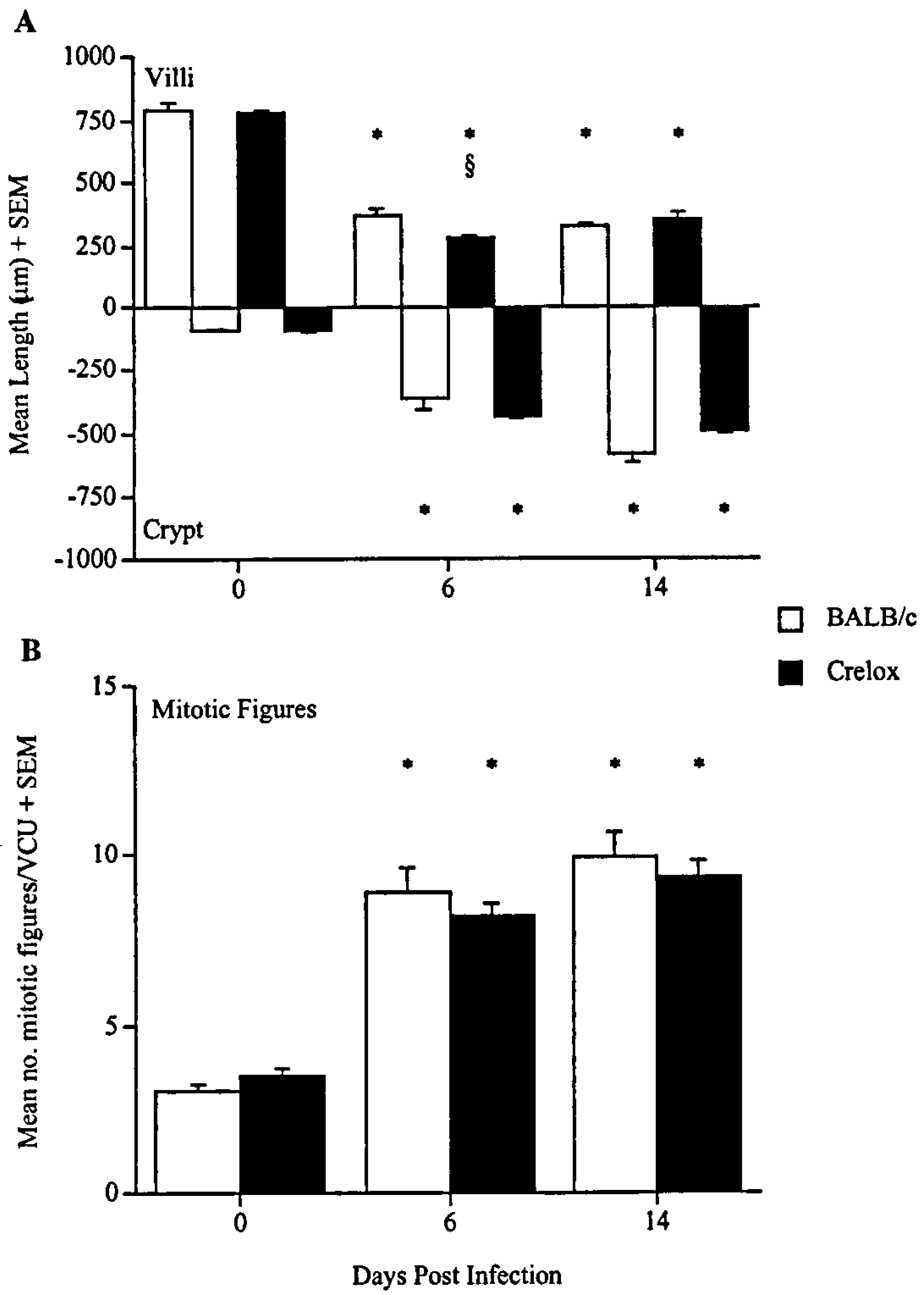
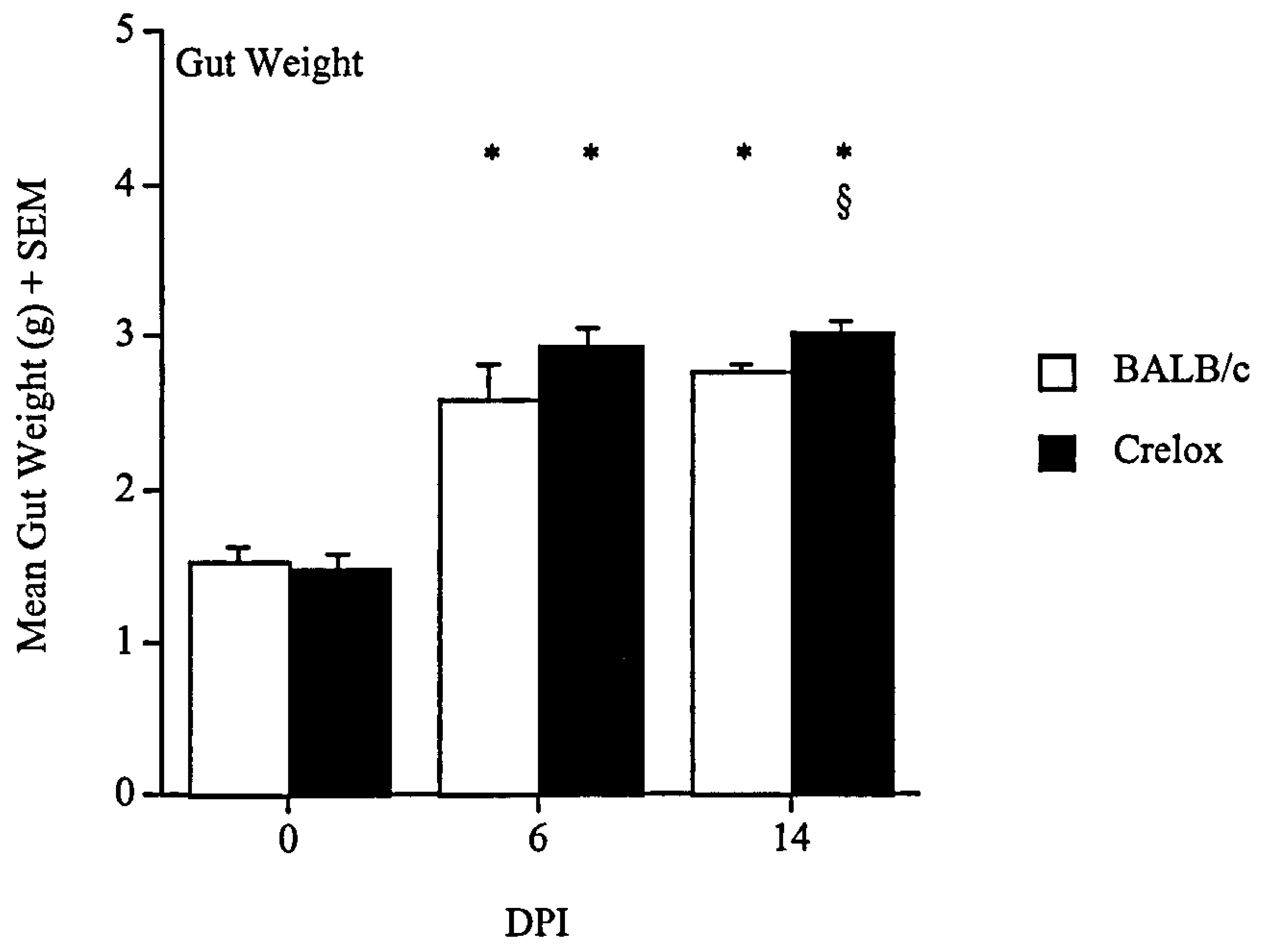

A
Villi
Crypt
Mean Length (μm) + SEM
1000
750
500
250
0
-250
-500
-750
-1000
0
6
14
§
B
Mitotic Figures
Mean no. mitotic figures/VCU + SEM
15
10
5
0
0
6
14
Days Post Infection
BALB/c
Crelox

FIGURE 3.16: The role of IL-4R$\alpha$ signalling to cells of the neutrophil/macrophage lineage in the development of intestinal oedema following infection with *T. spiralis*. The development of oedema was assessed in uninfected and infected (day 6 and 13 p.i.) in wild type BALB/c and Crelox mice by weighing the entire small intestine in grams. Data expressed as mean weight (g) + SEM for five mice. *, represents significantly different to uninfected; §, represents significantly different to wild type ($p<0.05$).

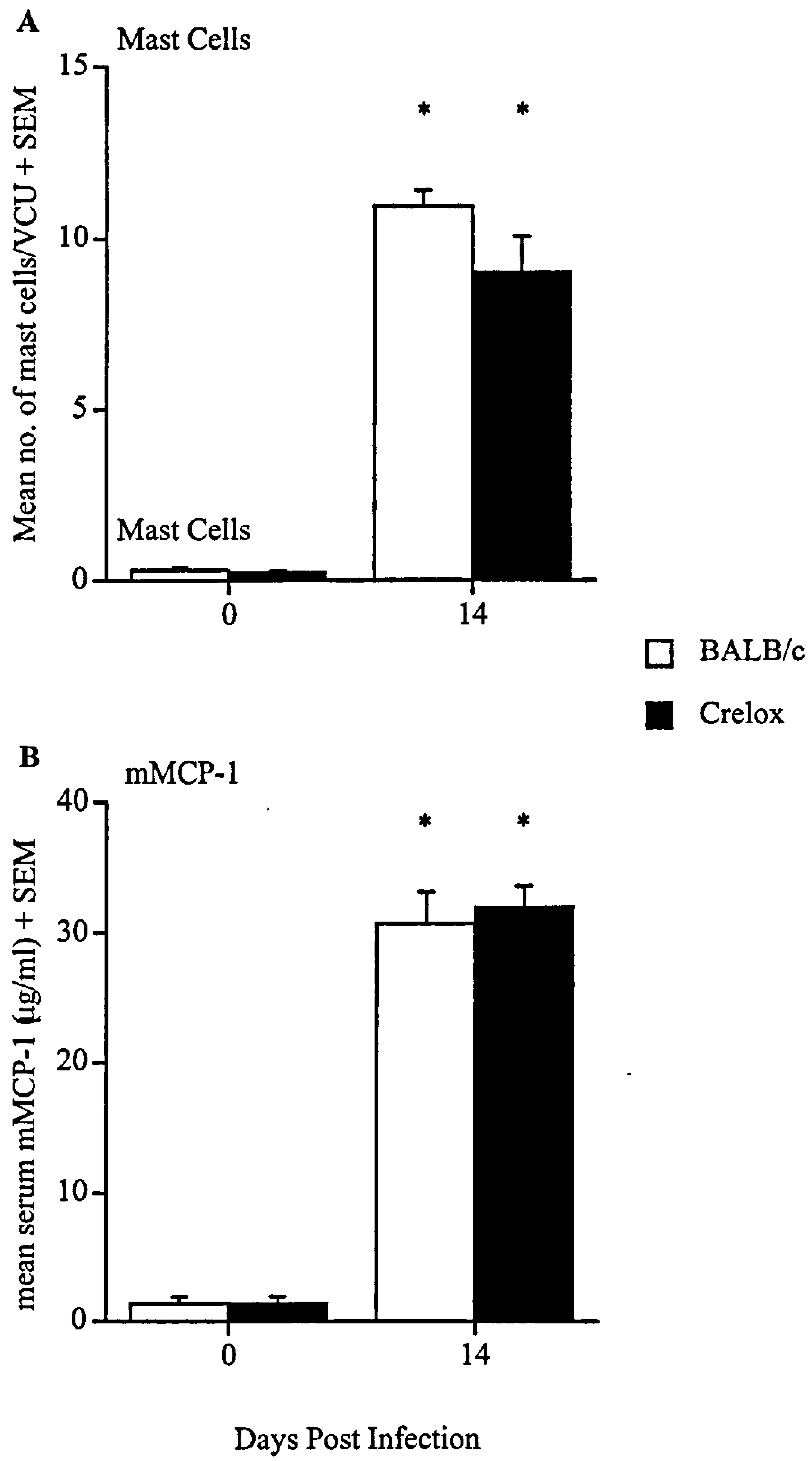

Gut Weight
Mean Gut Weight (g) + SEM
5
4
3
2
1
0
*
*
*
*
§
0
6
14
DPI
BALB/c
Crelox

### *3.2.8 The development of mastocytosis is not effected by the deficiency in IL-4Rα in cells of the macrophage/neutrophil lineage during infection with* T. spiralis.

The role of signalling through IL-4Rα to cells of the macrophage/ neutrophil lineage in the development of mastocytosis following infection with *T. spiralis* was evaluated histologically. Samples of jejunum from uninfected (day 0 p.i.) and infected (day 6 and 14 p.i.) wild type and Crelox mice were fixed in Carnoy's fluid, and subsequently processed into wax blocks. 5 m sections were cut and stained in toluidine blue and counter stained with nuclear fast red. The number of mast cells present in 10 villus crypt units (VCU) per sample was counted and the mean result recorded. The degranulation of mast cells was assessed by measuring concentrations of the granule protease mMCP-1 in the sera of uninfected and infected (day 14 p.i.) wild type BALB/c and Crelox mice.

The number of mast cells/VCU present in the intestines of both wild type (p=0.009) and Crelox (p=0.009) increased significantly following infection with *T. spiralis* (day 14 p.i.) compared to uninfected mice. No significant differences were observed between wild type and Crelox mice at any time point observed (Figure 3.17A). Mast cell degranulation as demonstrated by an increase in serum mMCP-1 levels increased significantly in both wild type BALB/c (p=0.009) and Crelox mice (p=0.009) at day 14 pi compared to uninfected mice. Consistent with mast cell numbers no differences were observed between serum mMCP-1 in wild type BALB/c and Crelox mice at any time point analysed (Figure 3.17B).

FIGURE 3.17: The role of IL-4Rα signalling to macrophages and neutrophils in the development of mastocytosis and mast cell degranulation following infection with *T. spiralis*. A) Carnoy's fixed jejunum from wild type BALB/c and Crelox mice at day 0 and 13 p.i. were processed and stained with 0.5% toluidine blue, revealing mast cells. The number of mucosal mast cells was counted in 20 randomly selected villus crypt units (VCU). Data expressed as the mean number of mast cells /VCU + SEM. B) The degranulation of mucosal mast cells in wild type BALB/c and Crelox mice at day 0 and 13 p.i. was assessed by measuring serum titres of mMCP-1 by ELISA against a recombinant protein standard. Data expressed as mean mMCP-1 concentration in pg/ml+ SEM. *, represents significantly different to uninfected, **, represents significantly different to day 6 p.i. §, represents significantly different to wild type. Five mice were used per group.


A
Mast Cells
Mean no. of mast cells/VCU + SEM
15
10
5
0
*
*
Mast Cells
0
14
BALB/c
Crelox
B
mMCP-1
mean serum mMCP-1 (µg/ml) + SEM
40
30
20
10
0
*
*
0
14
Days Post Infection

### 3.2.9 *Cytokine production by MLN cells stimulated ex-vivo with TAg was not altered by deficiency in IL-4Rα from cells of the macrophage /neutrophil lineage.*

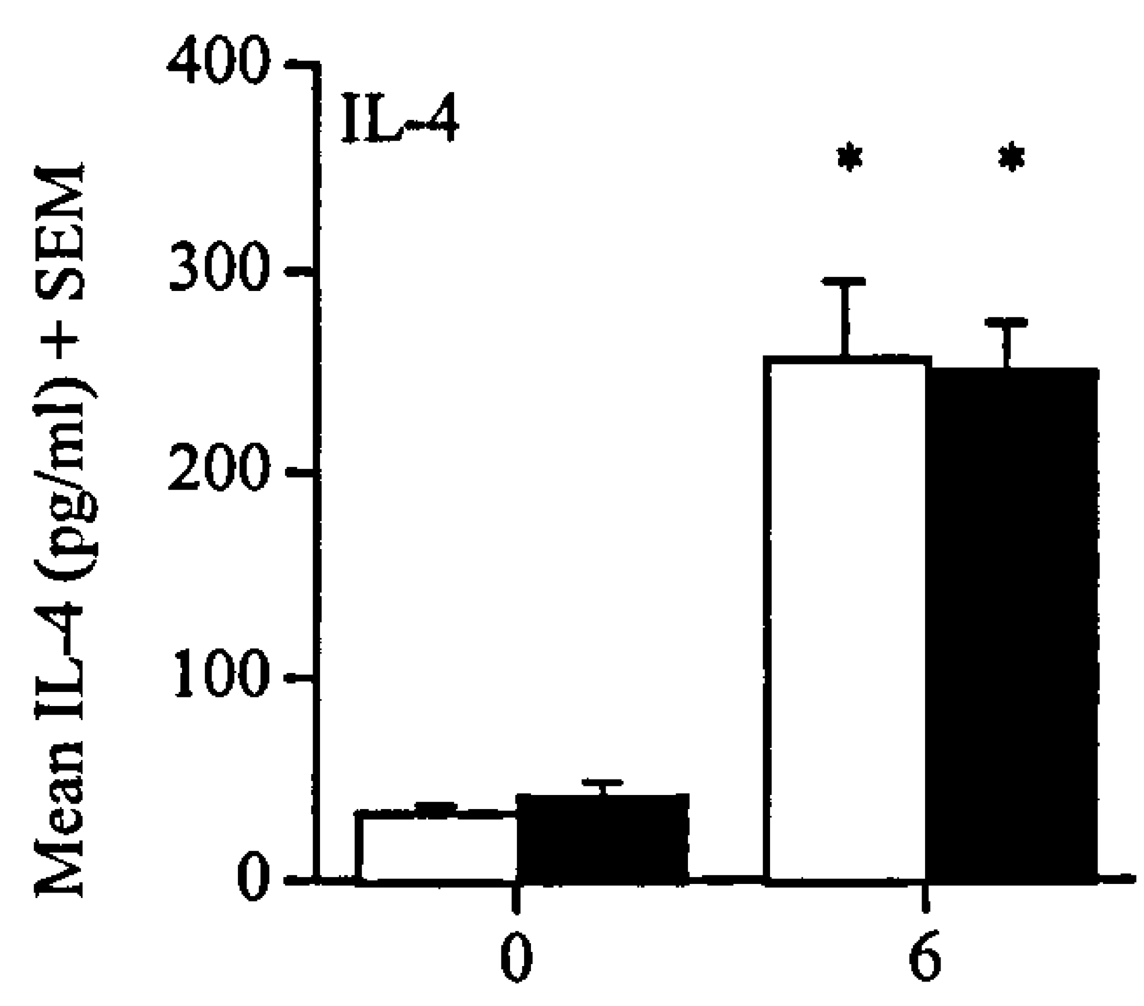
Infection with *T. spiralis* has previously been shown to illicit a strong Th2 response; IL-4 a major regulator of Th2 responses has been shown to be involved in the development of enteropathy and in the expulsion of the worms from the intestine (Lawrence *et al.*, 1998). In order to evaluate the effect of IL-4Rα deficiency in cells of the macrophage/neutrophil lineage on the Th1:Th2 balance following infection with *T. spiralis* the mesenteric lymph nodes (MLN) were removed from uninfected (0 dpi) and infected (6 dpi) wild type and Crelox mice. Single cell ($1\times10^6$ cells/ml) preparations were made from the MLN and the cells were cultured with and with out *Trichinella* antigen (TAg) at 50μg/ml for 24 hours. The cultures were centrifuged and the supernatants were removed and tested for the Th2 cytokines IL-4 and IL-13, and the Th1 cytokine IFN-γ by ELISA against recombinant protein standards.

Background levels of IL-4, IL-13 and IFN-γ were produced by MLN cells cultured without stimulation (data not shown). IL-4 secretion from MLN cells stimulated with TAg increased significantly from both wild type (p=0.009) and Crelox (p=0.0143) mice at day 6 p.i. compared to uninfected. No significant differences in IL-4 secretion were observed between strains at either time point analysed (Figure 3.18A). IL-13 secretion increased significantly from both wild type (p=0.009) and Crelox (p=0.0143) mice at day 6 p.i. compared to uninfected. No significant differences in IL-13 secretion were

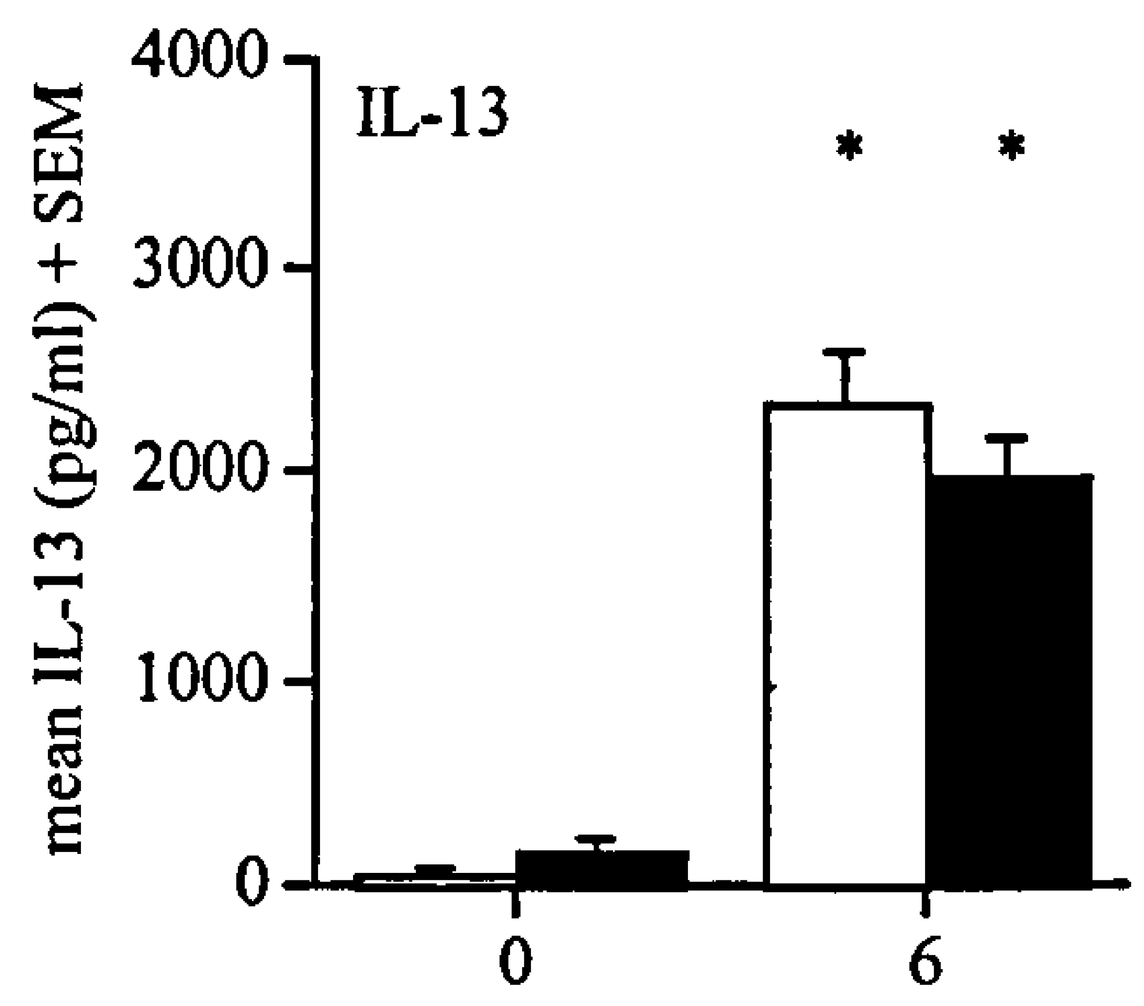
observed between strains at either time point analysed (Figure 3.18B). IFNγ production did not alter significantly following infection in either wild type or Crelox mice and no significant differences were observed between strains at either time point (Figure 3.18C).

FIGURE 3.18: The role of IL-4Rα signalling to macrophages and neutrophils in the development of mesenteric lymph node cytokine responses. MLN were removed from uninfected and infected (day 6 p.i.) wild type BALB/c and Crelox mice. Single cells suspensions produced and cells cultured at $1 \times 10^6$ with 50μg/ml TAg. The concentration of the cytokines IL-4 (A), IL-13 (B) and IFN-γ (C) were measured in the culture supernatants by ELISA. Data expressed as mean cytokine concentration in pg/ml+ SEM. *, represents significantly different to uninfected. Five mice were used per group, except uninfected Crelox mice where 4 mice were used.

A

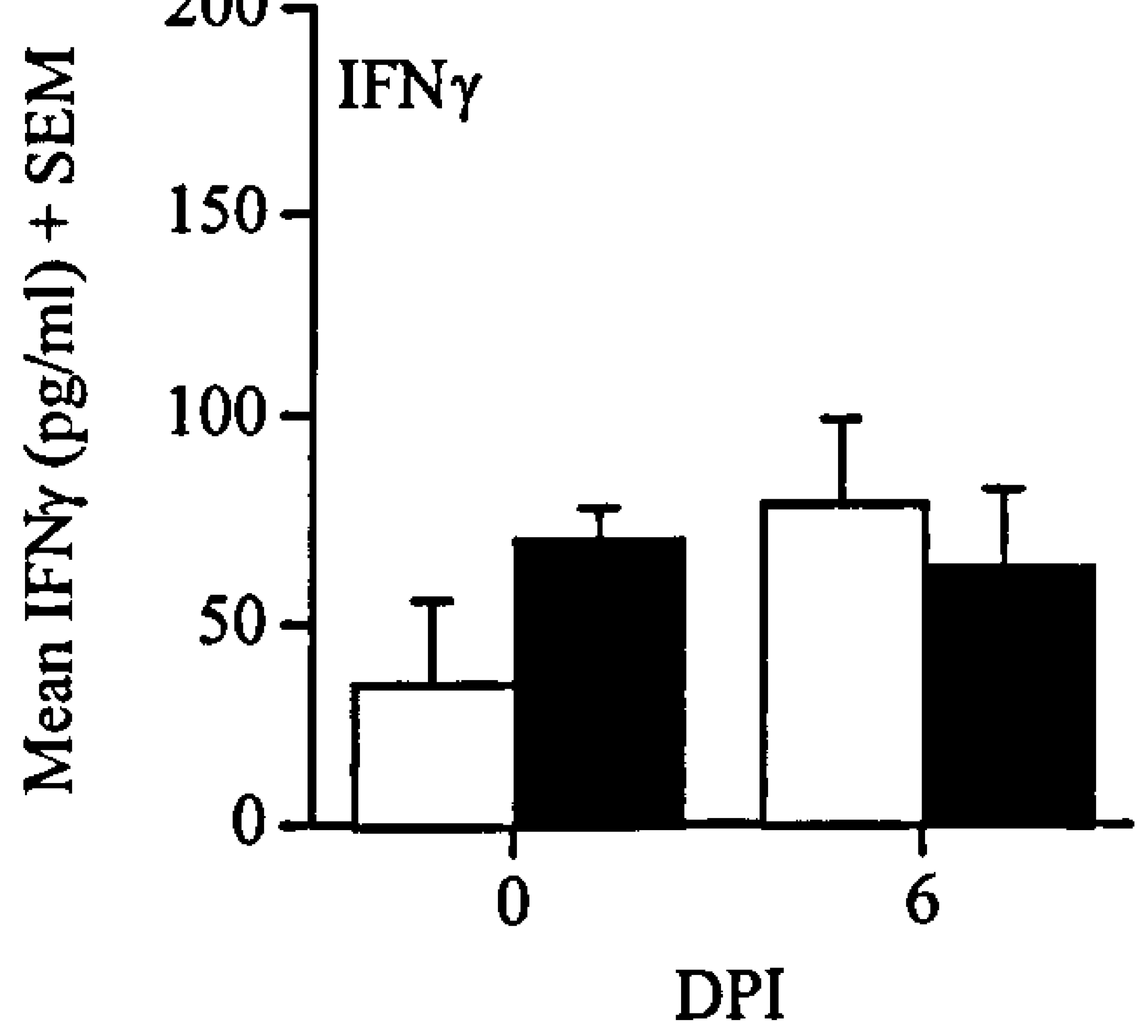



B




BALB/c
Crelox

C



### *3.2.10 Serum antibody titres were not effected by deficiency in IL-4Rα from cells of the macrophage/neutrophil lineage.*

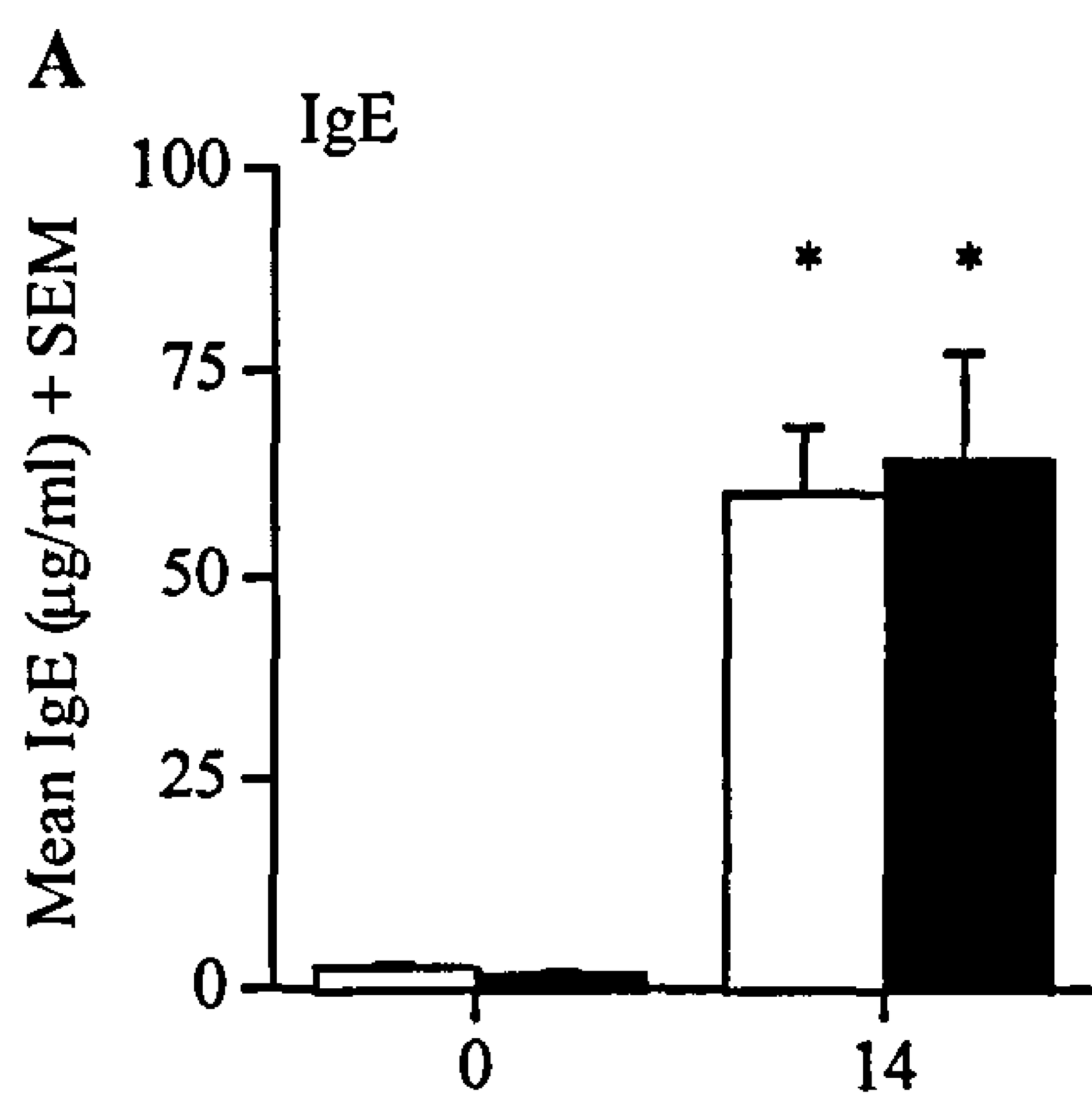
It has been previously shown that following infection with *T. spiralis* significant IgE and antigen specific IgG1 responses develop, while no antigen specific IgG2a develops (Lawrence *et al.*, 1998). In order to determine the effect of deficiency in IL-4Rα from cells of the macrophage/neutrophil lineage on the development of antibody responses to *T. spiralis* infection, total IgE and TAg specific IgG1 and IgG2a levels in sera were measured in uninfected and infected (day 14 p.i.) wild type and Crelox mice.

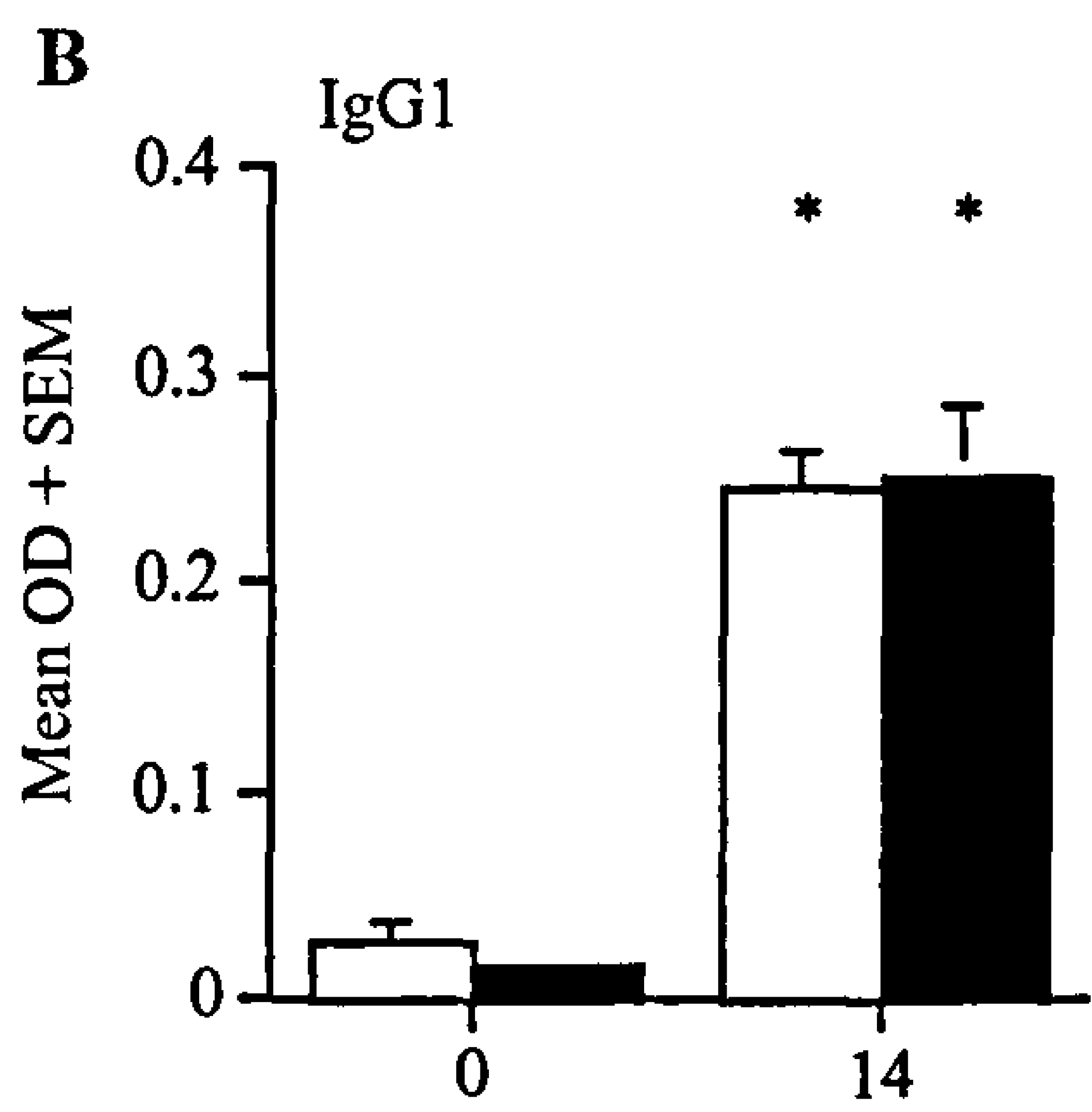
Blood samples were taken from uninfected and infected (day 14 p.i.) wild type and Crelox mice, the blood was stored over night at 4°C to allow clotting, the sera was then removed and stored at –20°C. Total IgE titres were measured in serum from uninfected and infected (day 14 p.i.) mice by ELISA against a standard and relative TAg specific IgG1 and IgG2a titres were in sera from uninfected and infected (day 14 p.i.) mice measured by ELISA, sera was serially diluted and the optical density was recorded, data for the 1 in 160 dilution is shown.

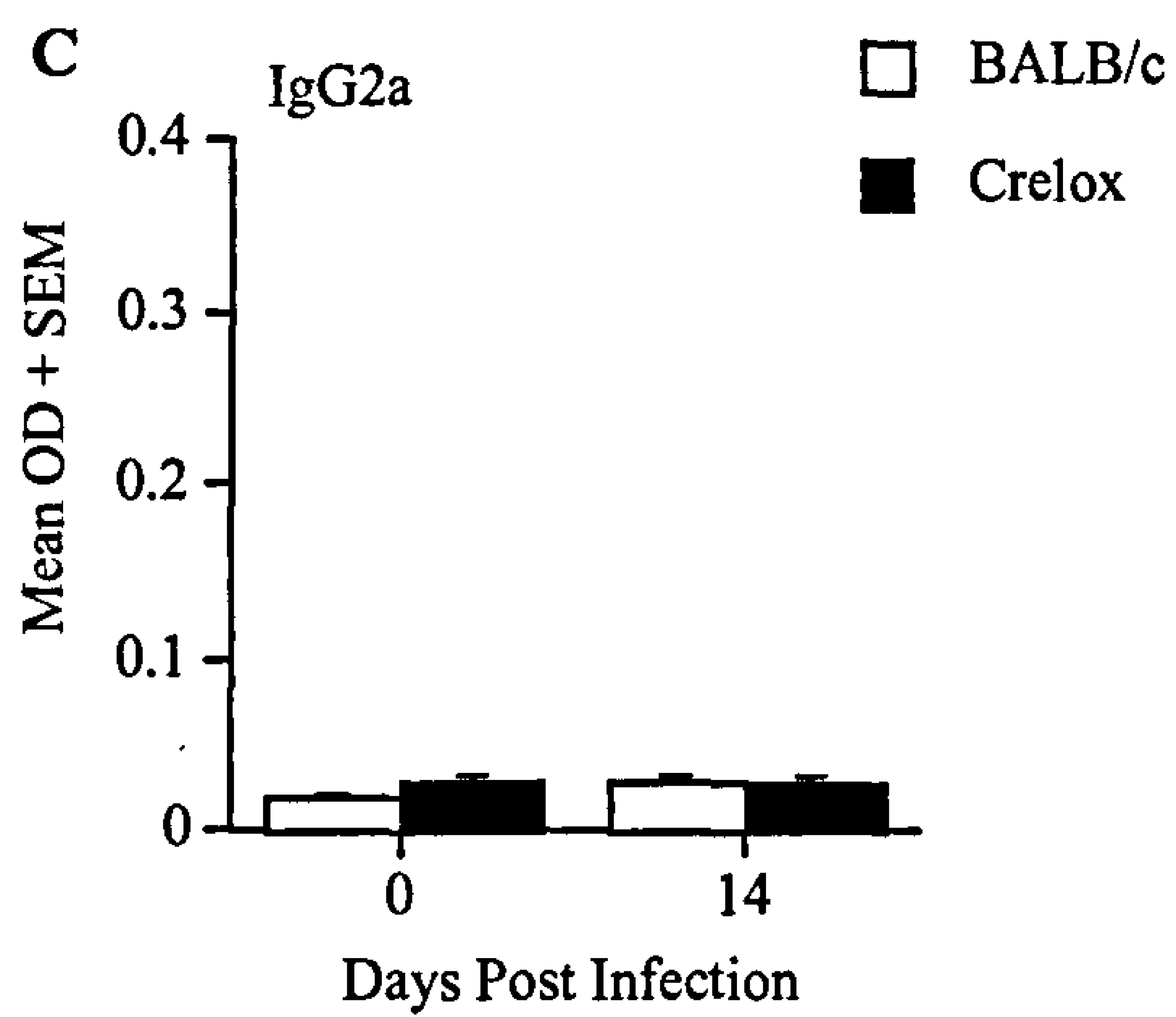
Total IgE titres were significantly increased in both wild type (p=0.009) and Crelox (p=0.0143) mice (day 14 p.i.) compared to uninfected. No significant differences were observed between wild type and Crelox mice at either time point analysed (Figure 3.19A).

Relative titres of antigen specific IgG1 were significantly increase day 14 p.i. in both wild type ($p=0.0088$) and Crelox ($p=0.0088$) mice compared to uninfected. No significant differences in IgG1 titres were observed between either infected or uninfected wild type and Crelox mice (Figure 3.19B). No significant differences in IgG2a titres were observed between uninfected and infected wild type mice or. No significant differences were observed between strains (Figure 3.19C).

FIGURE 3.19: The role of IL-4Rα signalling to macrophages and neutrophils in the development of IgE, IgG1 and IgG2a responses to *T. spiralis* infection. To further establish the in vivo Th1:Th2 balance serum samples were taken from uninfected (day 0) and infected (days 14 and 16 p.i.) wild type and Crelox mice. Total IgE (**A**) titres were measured in μg/ml by ELISA against a purified IgE standard. Data is expressed as mean IgE concentration (mg/ml) + SEM. Antigen specific IgG1 (**B**) and IgG2a (**C**) titres were measured by ELISA, using 96 well microtitre plates coated with TAg at 2μg/ml. Sera was serially diluted down the plate and relative optical density (OD) was recorded. Data for the 1 in 160 dilution is shown. Data is expressed as mean OD + SEM. *, represents significantly different to uninfected; §, represents significantly different to wild type.


A
IgE
Mean IgE (μg/ml) + SEM
100
75
50
25
0
*
*
0
14


B
IgG1
Mean OD + SEM
0.4
0.3
0.2
0.1
0
*
*
0
14


C
IgG2a
BALB/c
Crelox
Mean OD + SEM
0.4
0.3
0.2
0.1
0
0
14
Days Post Infection

## 3.3 Discussion.

This study has shown that IL-4, IL-13 and IL-4Rα are necessary for the generation of Th2 responses to *T. spiralis*. However, the role these responses play in the development of protection and enteropathy is less clear. Although Th2 responses are reduced in BALB/c IL-4 -/- mice neither expulsion nor enteropathy are different to wild type BALB/c, while lack of IL-13 inhibited expulsion and the development of enteropathy. In contrast, C57BL/6 IL-4 -/- exhibit delayed expulsion and reduced pathology suggesting that host genetics are important in the function of individual cytokines. Furthermore, expulsion was delayed in C57BL/6 mice compared to BALB/c mice without significant changes in the development of enteropathy, Th2 responses or mastocytosis. IL-4Rα deficiency in BALB/c mice lead to a further delay in parasite expulsion, development of enteropathy and Th2 responses. Specific deficiency of IL-4Rα in neutrophils and macrophages however, did not affect parasite expulsion or the development of Th2 responses but resulted in an increase in enteropathy.

Differences between host strain backgrounds in their responses to infection has previously been demonstrated in a variety of infection models including, the nematodes *Trichuris muris, Heligosomoides polygyrus* and the protozoan *Leishmania mexicana*, where host background leads to variation in susceptibility to these chronic infections. This is thought to be due to differences in the cytokine milieu stimulated by these parasites in different inbred strains. BALB/K and BALB/c mice, which are on the same

background but have different MHC H2 haplotypes (*k* and *d* respectively) infected with *T. muris*, develop an acute self-resolving infection, and have been shown to produce a predominantly Th2 cytokine profile (high IL-5 and IL-9; low IFN-γ). While AKR mice, which have the same H2 haplotye as BALB/K mice, develop a Th1 cytokine profile with elevated IFN-γ in response to *T. muris* infection and develop chronic trichuriasis (Cliffe *et al.*, 2005; Else *et al.*, 1992). Furthermore IgG2a titres and eosinophilia have been shown to be reliable indicators of Th1/Th2 polarisation and predictors of susceptibility phenotypes in *T. muris* infected mice (Else *et al.*, 1993). These data show the importance of the Th2 response in protection from *T. muris*, but that factors other than MHC H2 haplotype are involved important in the generation of Th2 responses. However, in *T. spiralis* infection, although different expulsion phenotypes have been observed in different inbred mouse strains, this was not associated with different cytokine profiles (Dehlawi and Goyal, 2003; Grencis *et al.*, 1991). These findings are consistent with the present study where C57BL/6 mice have been shown to expel there worms slower than BALB/c but showed no significant differences in Th responses were observed, suggesting that in *T. spiralis* other factors are associated with strain differences in the rate of expulsion.

It has been suggested that the increased loss of epithelial cells from the mucosa provide a mechanism for the expulsion of nematode parasites from their niche in the intestine. An association with parasite expulsion and increased epithelial turnover has been demonstrated during *T. muris* infection where a rapid increase in cell turnover is seen in

resistant BALB/c mice, and occurs slower in the susceptible AKR mouse. This increased epithelial turnover in the BALB/c mouse is dependent on IL-13 but is delayed in the AKR mouse by IFN-γ (Cliffe *et al.*, 2005). In *T. spiralis* infection, however, it has been shown that expulsion can occur in the absence of enteropathy (Lawrence *et al.*, 1998; Lawrence *et al.*, 2000). This may relate to differences in the niche occupied by these two parasites, *T. muris* is a ceacal dwelling parasite while *T. spiralis* resides in the small intestine. Furthermore in *T. muris* infection the AKR mice fail to expel their parasites despite developing an increased epithelial turnover at day 21 p.i. comparable to that observed in BALB/c mice at day 14 p.i., a time at which BALB/c mice are expelling their parasites. Between day 14 and day 21 p.i. *T. muris* undergoes a further moult and now resides higher in the crypt epithelium where it appears that increased epithelial turnover is no longer an effective means of parasite expulsion (Cliffe *et al.*, 2005).

This study has shown a slight delay in the expulsion of *T. spiralis* in C57BL/6 mice compared to BALB/c mice furthermore, the role of IL-4 in C57BL/6 compared to BALB/c mice appears to be different. It has previously been shown that in 129/C57 mice IL-4 is necessary for the development of enteropathy (Lawrence *et al.*, 1998). In contrast this study has shown that in the BALB/c mouse, IL-13, rather than IL-4, is the key player in the development of both protective responses and in the development of enteropathy, however in C57BL/6 IL-4 -/- mice expulsion is delayed and enteropathy is reduced, suggesting a more prominent role for IL-4 in both the development of enteropathy and in expulsion from the small intestine, in the C57BL/6 mouse compared

to the BALB/c mouse. Polymorphisms have been demonstrated in the IL-4Rα of BALB/c and C57BL/6 mice, in ovalbumin model of airway inflammation IL-4 was shown to be more potent when interacting with the BALB/c IL-4Rα, in the absence of IL-13 (Webb *et al.*, 2004). In this model, IL-13, in BALB/c mice in the absence of IL-4, appears to be able to compensate, but not in the C57BL/6 mice, suggesting that the polymorphism in the IL-4Rα gene may also influence IL-13-IL-4Rα interactions.

Mast cells and their granule products in particular mMCP-1 have been shown to play important role in both protective responses to *T. spiralis* and in the development of enteropathy (Donaldson *et al.*, 1996; Knight *et al.*, 2000; Lawrence *et al.*, 2004). A role for IL-4 in the development of mastocytosis has been shown in both BALB/c and C57BL/6 mice, with mast cell numbers significantly reduced in the absence of IL-4. This is consistent with pervious studies using 129/C57 IL-4 -/- mice were mastocytosis was inhibited (Lawrence *et al.*, 1998). It has also been shown that human intestinal mast cells *in vitro* proliferate when cultured with IL-4 and stem cell factor (SCF) but fail to significantly proliferate with SCF alone (Bischoff *et al.*, 1999). Taken together these data suggest that *in vivo* IL-4 is vital for the development of normal mast cell numbers following infection with *T. spiralis*. However, in this study the slight delay in expulsion observed in C57BL/6 compared to BALB/c mice did not coincide with a reduction in mucosal mast cell numbers or in serum mMCP-1 titres in the C57BL/6 mice, suggesting that other factors may also be important in strain specific resistance to infection with *T. spiralis*. Furthermore in BALB/c IL-4 -/- mice despite reduced mast cell numbers and

serum mMCP-1 both enteropathy and expulsion do not significantly differ from that seen in wild type mice. BALB/c IL-4Rα -/- mice, in which neither IL-4 or IL-13 can signal normally, fail to expel their parasites, develop mastocytosis or enteropathy; suggesting a role for IL-13 signalling via IL-4Rα.

In BALB/c IL-13 -/- mice, although the development of enteropathy is reduced and the expulsion of *T. spiralis* is delayed, mastocyosis and mast cell degranulation are increased. Studies in human intestinal derived mast cells showed that IL-13 alone did not affect the proliferation or survival *in vitro* (Lorentz *et al.*, 2005). However this study suggests that *in vivo* IL-13 may regulate the development of mastocytosis following infection with *T. spiralis*, this may be due to a direct effect of IL-13 on mast cells although a regulatory role for IL-13 in mast cell development has not been demonstrated or it may be due to an enhancement of IL-4 in the absence of IL-13, as both IL-4 and IL-13 signal via IL-4Rα the absence of IL-13 may enhance IL-4 signalling simply by reducing competition for the receptor (Zheng *et al.*, 2003). Furthermore it has been shown that the decoy IL-13Rα2, which is up regulated by IL-13 its self (Zheng *et al.*, 2003), can down regulate IL-4 signalling in transformed keratinocytes (Rahaman *et al.*, 2002). However, the effect of IL-13 on IL-4 signalling has not been fully described.

This study suggests that cells other than mast cells are important in the development of both protection and enteropathy in the absence of IL-4, as BALB/c IL-4 -/- mice expel *T. spiralis* and develop enteropathy while IL-13 -/- mice fail to expel their parasites or

develop enteropathy. This is consistent with the findings of a recent study which has also shown that SCID and athymic mice although unable to expel *T. spiralis* develop enteropathy, this appears to be due to a population of IL-13 secreting NK cells present in the jejunal mucosa (McDermott *et al.*, 2005). Although it has been shown that IL-4 in C57/129 mice appears to influence the development of enteropathy via the p55TNF-R (Lawrence *et al.*, 1998), no TNFα was detected in the mucosa of the SCID mice, suggesting that IL-13 may mediate enteropathy in a TNFα independent fashion (McDermott *et al.*, 2005).

Following infection with *T. spiralis*, BALB/c and C57BL/6 IL-4 -/- mice failed to generate IgE responses of a comparable magnitude to wild type mice, however a significant increase in serum IgE was observed. This contrasts with studies using ovalbumin/ALUM immunisation where in the absence of IL-4, IgE responses were ablated (Brewer *et al.*, 1999). However, IgE responses are initiated following prolonged allergen exposure in IL-4 -/- mice (Grunewald *et al.*, 2001) and in IL-4 -/- mice which over express IL-13 (Emson *et al.*, 1998). Similar results were obtained in this study where IgE responses following infection were significantly reduced in the absence of either IL-4 or IL-13. In BALB/c IL-4Rα -/- mice IgE responses were entirely ablated suggesting that IL-4 and IL-13 work together to generate Th2 responses, following *T. spiralis* infection.

Signalling to cells of neutrophil/macrophage lineage via IL-4Rα, although not altering host protection or the generation of Th2 responses, appears to play a role in the regulation of enteropathy. It has previously been shown that mice deficient in inducible nitric oxide synthase (iNOS) fail to develop pathology following infection with *T. spiralis* suggesting that enteropathy is mediated by Nitric oxide (NO) (Lawrence *et al.*, 2000). Activated macrophages produce NO (Lawrence *et al.*, 2000), except when exposed to IL-4, when they develop into an regulatory IL-10 producing phenotype (Nathan *et al.*, 1983; Padilla *et al.*, 2005), in the Crelox model macrophages are unable to respond to IL-4 and therefore may become activated to synthesise reactive oxygen intermediates including NO and therefore contribute further to enteropathy. Interestingly in a murine model of Schistosomiasis, IL-4Rα signalling to macrophages has been shown to be essential to limit the development of pathology associated with the egg granuloma (Herbert *et al.*, 2004).

Differences in the effect of signalling via IL-4Rα depending on the cell type expressing the receptor has been shown previously in a nematode model where the expression of IL-4Rα on non-bone marrow derived cells was necessary for expulsion (Urban *et al.*, 2001) and in a murine model of asthma where non-bone marrow derived cell expression of IL-4Rα was shown to be necessary for the development of goblet cell hyperplasia, while bone marrow derived cell expression of IL-4Rα was necessary for the development of eosinophilia (Kelly-Welch *et al.*, 2004). Although both neutrophils and macrophages have been shown to affect the development of Th responses it is perhaps

not surprising that the absence of signalling to the cells through the IL-4Rα does not influence the overall Th2 response to infection with *T. spiralis*. *T. spiralis* is a potent activator of Th2 responses stimulating significant infiltration into the small intestine of cells such as eosinophils and mast cells. Mast cells are important producers of Th2 cytokines such as IL-4, IL-13, IL-5 and IL-9 (Bradding *et al.*, 1993; Huels *et al.*, 1995; Lorentz *et al.*, 2000) and mast cells have been shown to present antigen to CD4+ T cells leading to a Th2 biased phenotype (Fox *et al.*, 1994; Frandji *et al.*, 1993; Frandji *et al.*, 1996) thus normal Th responses may be maintained in the absence of signalling via the IL-4Rα in neutrophils and macrophages. Furthermore it has been shown that infection with *T. spiralis* can down regulate a Th1 response to a heterologous antigen in an IL-4 independent manor. This is thought to be due to IL-10 (Boitelle *et al.*, 2005), which has also been shown to modulate macrophage and neutrophil activation (Bober *et al.*, 2000; Fiorentino *et al.*, 1991).

These data shown that the roles of the Th2 cytokines, IL-4 and IL-13 in the development of enteropathy following infection with *T. spiralis* are complex. Despite previous studies implicating mast cells in the development of enteropathy, enteropathy still developed in BALB/c IL-4 -/- mice where mast cell responses were diminished and enteropathy was ablated in BALB/c IL-13 -/- mice where mast cell responses were enhanced. This suggests that IL-13 plays a mast cell independent role in the development of enteropathy and protection and that mast cell mediated pathology and protection may rely on the presence of IL-13. While enhanced pathology in Crelox mice who are IL-4Rα deficient

in cells of the macrophage/neutrophil lineage suggest that either IL-13 or IL-4 acting through these cells plays a regulatory role in enteropathy but do not influence expulsion. A recent study has shown that NK cells are a potential source of IL-13 during *T. spiralis* infection, although T cells appear to be required for the development of IL-13 dependent worm expulsion but not for the development of IL-13 mediated enteropathy (McDermott *et al.*, 2005). IL-4Rα and IL-13R1α are expressed on a variety of cell types in the intestine but which are mediating pathological and protective effects of IL-13 remains uncertain.

In conclusion, this study confirms an essential role for Th2 responses in both protective and pathological responses to *T. spiralis* infection, it is also apparent that the role of IL-4 in host protection and in the development of pathology appears to be dependent on host background, thus in evaluating the function of any single molecule in an immune response it is necessary to be aware of the genetic background of the host organism. Differences in the function of a single molecule depending on the background genotype may relate to the complexity observed in out bred populations, such as humans, in the development and genetics of conditions such as asthma.

<u>Chapter Four</u>

# The role of co-stimulatory signals in protective and pathological immune responses induced by infection with *Trichinella spiralis.*

## The role of co-stimulatory signals in protective and pathological immune responses induced by infection with *Trichinella spiralis.*

### *4.1 Introduction*

The generation of appropriate T cell responses to antigens or infection depends on recognition by a specific T cell receptor (TCR), and on co-stimulatory signals from molecules present on the antigen-presenting cell and on the responding T cell. The most widely studied co-stimulatory molecules being CD28, CTLA-4, CD40 and their ligands B7-1 (CD80), B7-2 (CD86) and CD40L (CD154). However, the precise role of these molecules in Thl/Th2 polarisation remains controversial. More recently it has become apparent that a third signal may also be required to elicit full T cell responses. This third signal may be mediated by a number of recently discovered co-stimulatory molecules, for example, the CD28 homologue inducible co-stimulator (ICOS)(Coyle *et al.*, 2000; Hutloff *et al.*, 1999) or a member of the TNF receptor super-family, OX40 (Calderhead *et al.*, 1993; De Smedt *et al.*, 2002). There has been interest in the manipulation of these novel co-stimulatory molecules to either suppress unwanted immune responses that cause pathology or to promote beneficial responses, such as those against tumour cells or pathogens.

ICOS, although a homologue of CD28, does not bind either B7-1 or B7-2 but instead interacts with B7 related protein 1 (B7RP-1). ICOS is not present on naive T cells but is induced on all activated T cells while B7RP-1 is expressed constitutively on resting B

cells and DC and may be up regulated on monocytes, keratinocytes and a population of $CD3^+$ cells (Beier *et al.*, 2000; Coyle *et al.*, 2000; Mages *et al.*, 2000; Yoshinaga S.K. *et al.*, 1999) (Hutloff *et al.*, 1999). The interaction between ICOS and its ligand are important in T-B cell interactions and antibody class switching, and ICOS signalling also appears to be involved the generation and maintenance of both Th1 and Th2 responses.

ICOS may be of heightened importance in Th2 responses as expression is sustained on Th2 cells after chronic stimulation (Coyle *et al.*, 2000; Gonzalo *et al.*, 2001a; Kopf *et al.*, 2000; McAdam *et al.*, 2001; Riley *et al.*, 2001; Rottman *et al.*, 2001; Tesciuba *et al.*, 2001). However, Greenwald *et. al.* (2002) showed that in ICOS-/- mice infected in the footpad with *Leishmania mexicana* lesion growth and both Th1 and Th2 cytokine responses were significantly impaired in comparison to wild type mice (Greenwald *et al.*, 2002a). A role for ICOS co-stimulation has also been shown in the development of protective Th1 responses to the intracellular bacterium *Listeria monocytogenes* in wild type mice; bacterial burdens in the liver were significantly increased by ICOS blockade while IFN-γ levels were significantly reduced (Mittrucker *et al.*, 2002). ICOS has also been shown to play a role in the resistance to and the development of IFN-γ responses to another intracellular parasite *Toxoplasma gondii* in CD28-/- mice (Villegas *et al.*, 2002).

ICOS may also be important in controlling immunopathological responses as it has been shown to play a role in the development of T regulatory cells. ICOS blockade in the

BDC2.5/NOD mouse enhances the development of diabetes by inhibiting the development of $CD4^+$ $CD25^+$ T regulatory cells (Herman *et al.*, 2004). It has also been shown that the development of tolerance to nasally administered OVA is inhibited by the blockade of ICOS-ICOSL interactions or by neutralising IL-10 (Akbari *et al.*, 2002). Furthermore, it has been shown that $CD4^+CD25^{hi}$ cells which secrete IL-10, also express high levels of ICOS while $CD4^+CD25^{lo}$ cells which secrete IFN-γ express lower levels of ICOS (Lohning *et al.*, 2003).

OX40 (CD134) is a member of the TNFR superfamily, and is expressed on activated T cells (Calderhead *et al.*, 1993). While OX40L, a TNFα homologue is expressed at low levels on resting antigen-presenting cells (dendritic cells (DC) and B cells) and is upregulated following CD40-CD40L interactions. OX40 ligation on T cells enhances proliferation in response to the mitogen Con A and to antigen specific stimulation (Higgins *et al.*, 1999; Murata *et al.*, 2000). In OX40 deficient mice the development of memory T cell responses is impaired but humoral responses are normal (Gramaglia *et al.*, 2000; Pippig *et al.*, 1999). OX40, along with CD28, has also been shown to be necessary for the development of both Th1 and Th2 effector functions (Rogers and Croft, 2000). Inhibition of OX40/OX40L interactions *in vivo* has been shown to inhibit the development of Th1 mediated experimental allergic encephalomyelitis (EAE) (Weinberg *et al.*, 1999). In *L. major* infected BALB/c mice, treatment with anti-OX40 antibodies has been shown to reduce disease progression and result in reduced IgE and IgG1 titres in these mice (Akiba *et al.*, 2000). In murine asthma models OX40 -/- mice

have been shown to develop significantly reduced Th2 responses as demonstrated by IL-4, IL-5 and serum IgE levels and significantly attenuated pathology as characterised by airway hyper-reactivity and mucous secretion (Jember *et al.*, 2001; Salek-Ardakani *et al.*, 2002; Salek-Ardakani *et al.*, 2003). Where OX40/OX40L interactions are blocked at the time of sensitisation to airway antigens recall responses to the antigen are significantly diminished (Salek-Ardakani *et al.*, 2003). These data indicate a role for OX40/OX40L interactions in the development of Th2 responses and in the generation of memory responses mediated by Th2 *in vivo*. OX40 co-stimulation of T cells has also been demonstrated to be important in the development of Th1 responses such as those involved in the murine model for multiple sclerosis, experimental autoimmune encephalitis (EAE) (Chitnis *et al.*, 2001; Ndhlovu *et al.*, 2001; Nohara *et al.*, 2001). The importance of OX40/OX40L interactions has also been demonstrated in the development of IFN-γ and IgG2a responses to collagen induced arthritis in DBA mice (Yoshioka *et al.*, 2000).

Increased OX40 expression has been demonstrated in tissue biopsies from patients with the Th1 mediated inflammatory bowel conditions: ulcerative colitis, celiac and Crohn's disease (Stuber *et al.*, 2000), and is expressed on activated CD8+ murine intraepithelial lymphocytes (IEL) (Wang and Klein, 2001). Murine models have also further confirmed a role for OX40/OX40L interactions in intestinal inflammation. Treatment with the OX40-IgG fusion protein (OX40-Ig) which blocks OX40 signalling, but not the OX40L-IgG fusion protein (OX40L-Ig) which activates OX40 on T cells, of either IL-2 -/- mice

or mice with hapten-induced colitis reduced intestinal inflammation and lymphocyte infiltration (Higgins *et al.*, 1999). Further more transgenic C57/BL6 mice, expressing high levels of OX40L on T cells, resulting in constitutive OX40 co-stimulation, develop spontaneous intestinal inflammation (Murata *et al.*, 2002).

As regulators of Th responses, ICOS and OX40 may therefore play important roles in the development of both protective and pathological responses to *T. spiralis* infection in the intestine. In separate experiments we employed a blocking antibody against ICOS or OX40 and OX40L fusion proteins (OX40-Ig and OX40L-Ig) to investigate the contribution of these co-stimulatory molecules in the development of the immune responses induced during infection with *T. spiralis*. The use of a blocking antibody against ICOS prevents ICOS binding B7-RP thus preventing co-stimulation via this pathway. OX40 and OX40L fusion proteins, rather than simply blocking the interaction of receptor-ligand pairs, results in the activation of one half of the pair but prevents the activation of the other. Thus OX40-Ig binds to OX40L on the APC preventing co-stimulation of T cells, while OX40L-Ig binds to T cell OX40 providing co-stimulatory signals to T cells (Chapoval *et al.*, 2002).

To assess the role of ICOS co-stimulation in the development of both the pathological and expulsive responses to infection with *T. spiralis* wild type BALB/c mice are infected with 400 *T. spiralis* larvae and treated with 100μg either anti-ICOS or isotype control

antibodies at day –1 and 3 post infection (p.i). Mice were killed at day 0, 6, 13 and 30 p.i.

In a separate experiment the role of OX40/OX40L interactions in the development of pathological and expulsive responses to *T. spiralis* were assessed by infecting wild type BALB/c mice at day 0 p.i. and treating with 100μg OX40-Ig or OX40L-Ig fusion proteins on day 2 and 5 p.i. or left untreated. Untreated and treated mice were culled at day 0, 6, 14 and 30 p.i. and the establishment of and expulsion from the small intestine of adult worms, the accumulation of larvae in muscle tissue, the development of enteropathy, the development of mesenteric lymph node cytokine responses and the development of antibody responses were measured.

## 4.2 *Results*

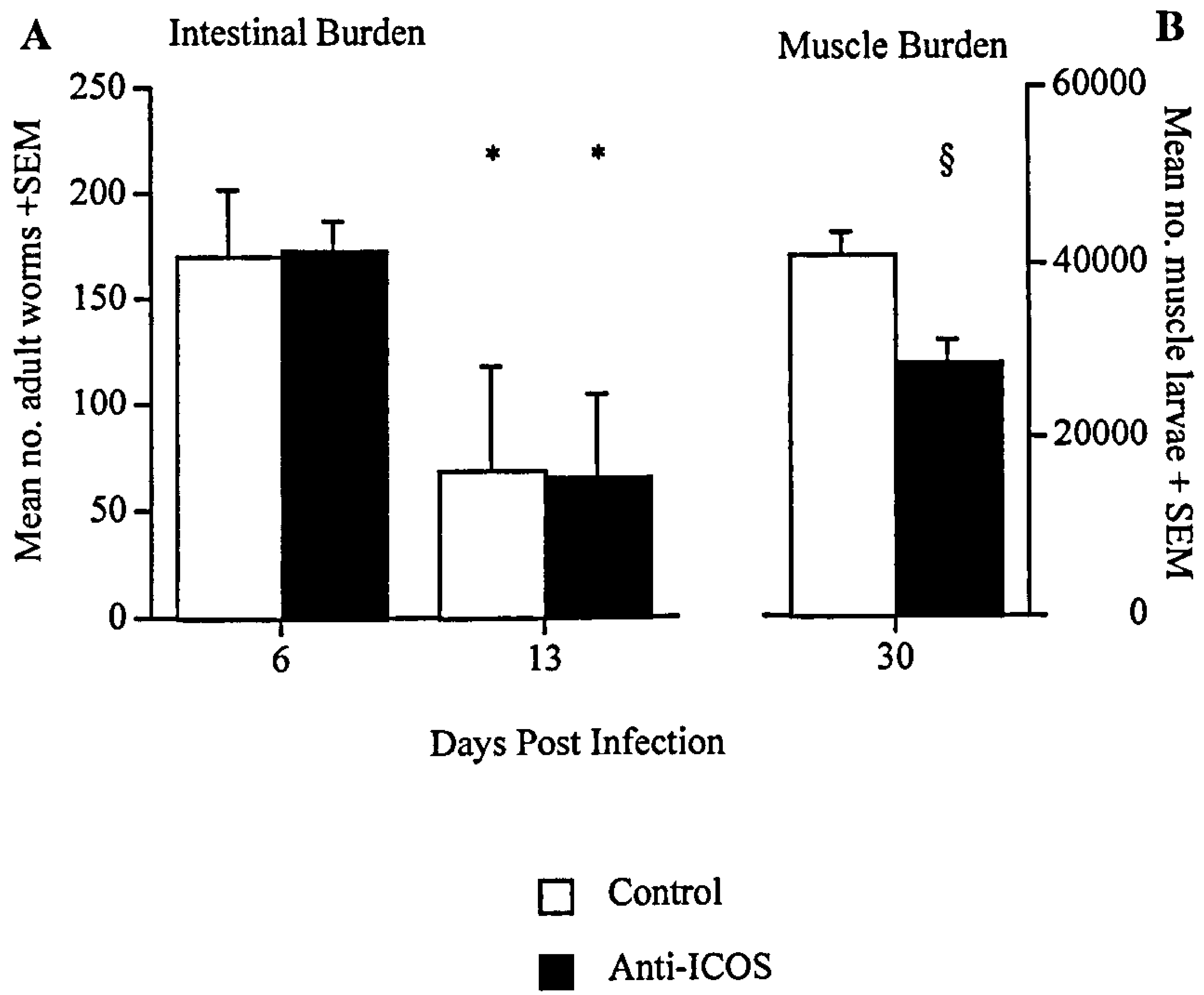
### 4.2.1 *Blocking ICOS has no effect on intestinal worm burdens but reduces muscle larvae loads.*

Anti-ICOS treated and untreated mice were infected with 400 *T. spiralis* larvae. By day 6 p.i. the larvae had matured and established as adults in the small intestine, expulsion of adult worms was evaluated by comparing the number of adult worms still present in the small intestine at day 13 p.i. compared to the number present at day 6 p.i. Establishment of adult worms at day 6 p.i. was equivalent in untreated and anti-ICOS treated mice. Although both untreated and anti-ICOS mice had significantly reduced their worm burdens by day 13 p.i. (p=0.0163 and 0.009), no significant differences were observed in the number of worms present in the small intestines at day 6 or 13 p.i. between anti-ICOS treated or untreated mice (Figure 4.1A).

While no significant differences were seen in gut worm burdens between anti-ICOS treated and untreated mice at any of the time points investigated, the numbers of muscle larvae retrieved at day 30 p.i. were significantly reduced (p=0.00163) in the anti-ICOS treated group compared with untreated group (Figure 4.1B). Following their release from adult female worms in the small intestine *T. spiralis* larvae migrate through the lymphatics and into circulation after which they colonise striated muscle tissue. The

degree of muscle tissue colonisation will vary, depending on the rate and timing of expulsion of adult parasites, parasite fecundity and the ability of larvae to penetrate the gut. This suggests that in anti-ICOS treated mice either worm fecundity or the numbers of resulting larvae invading and/or surviving in muscle cells was reduced.

FIGURE 4.1: The effect of anti-ICOS treatment on the expulsion of *T. spiralis* from the intestine and the accumulation of muscle larvae. A) The establishment and expulsion of *T. spiralis* was measured in untreated and anti-ICOS treated mice at day 6 and 13 p.i. The small intestine was excised and the total number of worms present was counted. B) The accumulation of muscle larvae was measured in untreated and anti-ICOS treated mice at day 30 p.i. *, represents significantly different to mice at day 6 p.i. §, represents significantly different to untreated mice ($p<0.05$). Data is expressed as mean number of worms/mouse + SEM, for 5 mice per group.

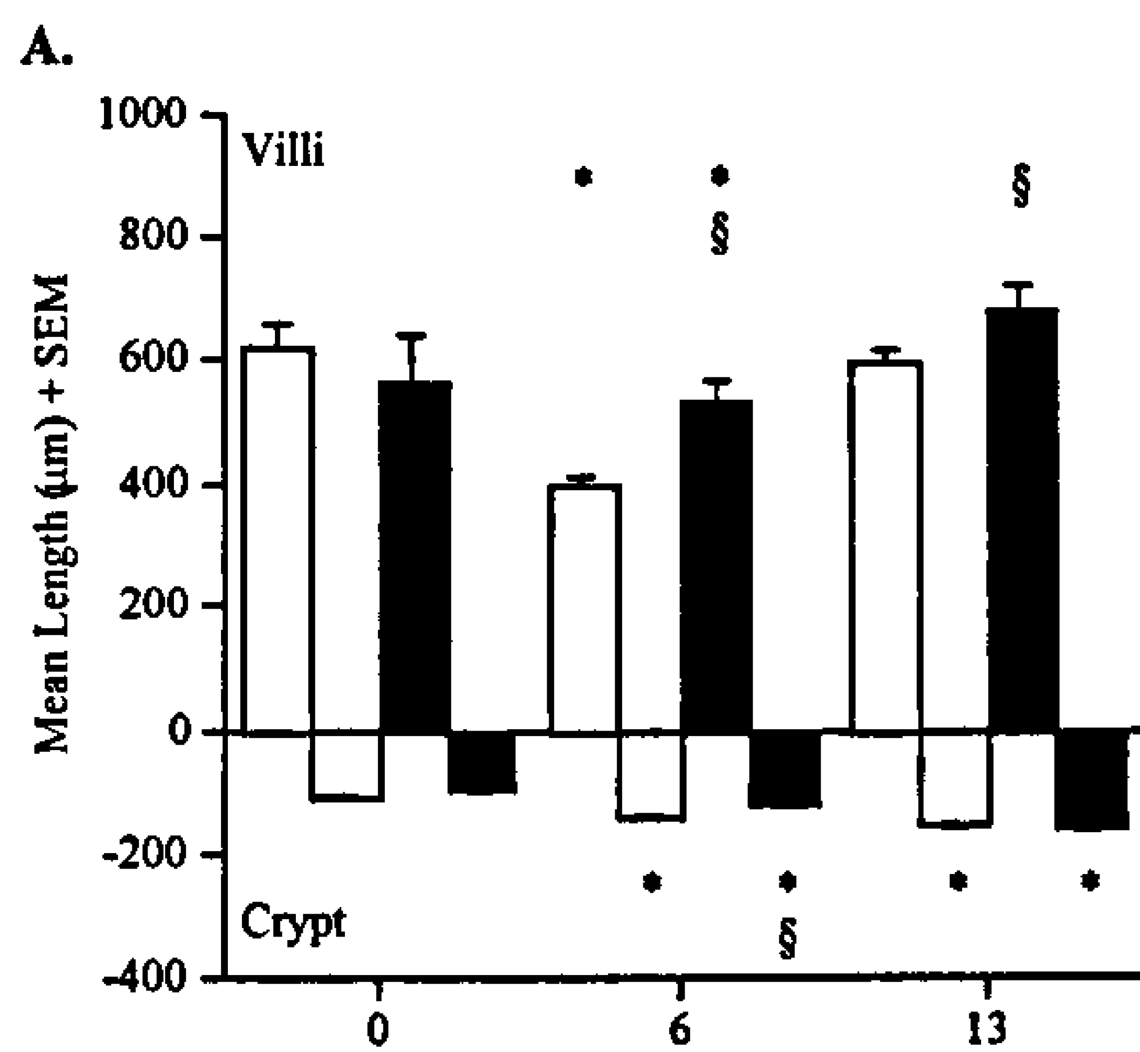

A
Intestinal Burden
Muscle Burden
B
250
200
150
100
50
0
Mean no. adult worms +SEM
*
*
§
60000
40000
20000
0
Mean no. muscle larvae + SEM
6
13
30
Days Post Infection
Control
Anti-ICOS

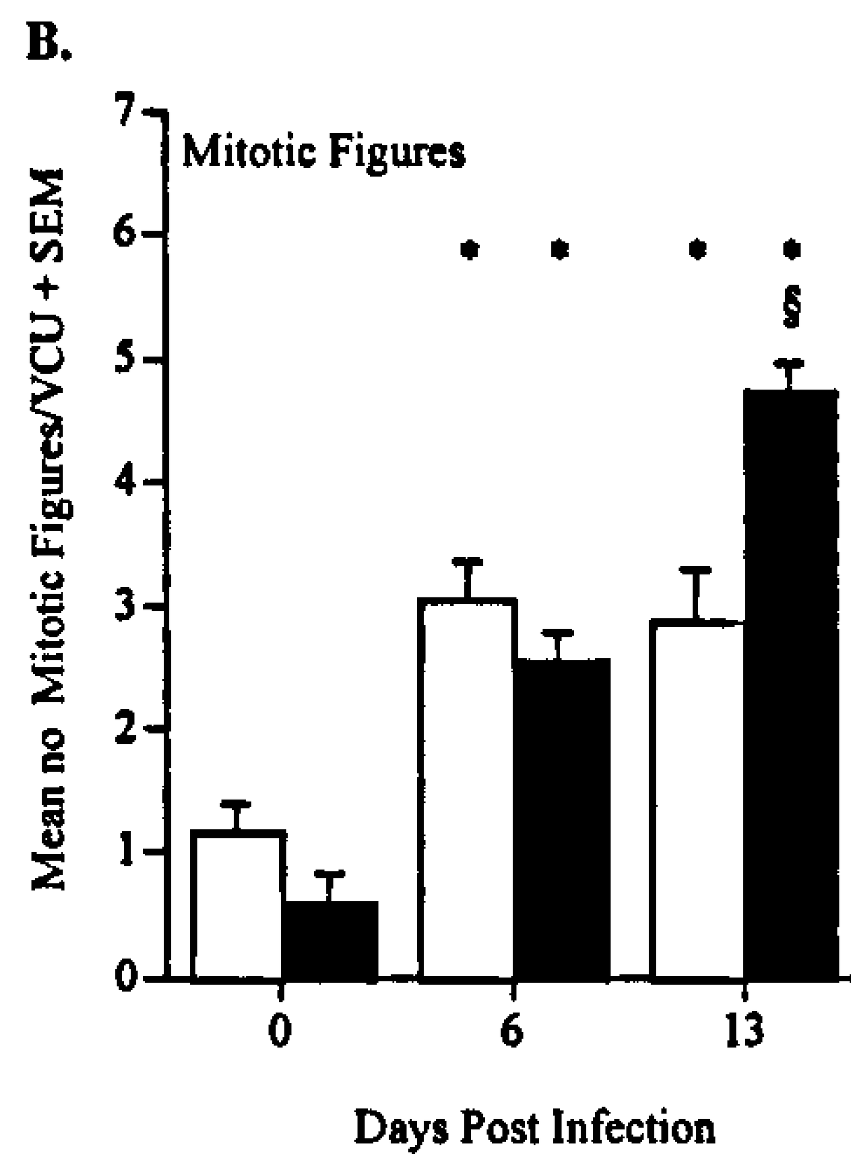
### *4.2.2 Blocking ICOS protects against intestinal pathology.*

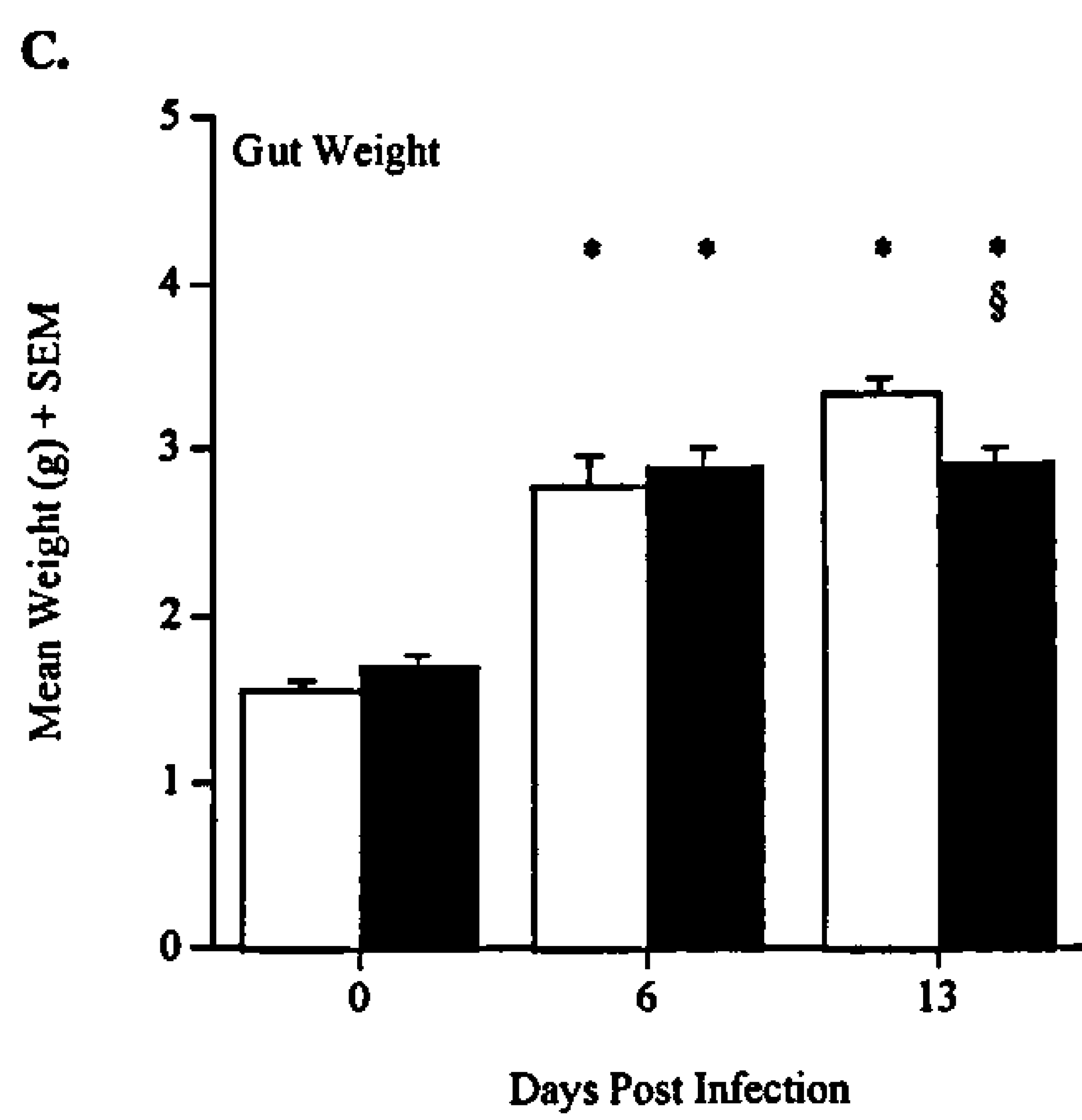
As described previously (Lawrence *et al.*, 1998) loss of adult parasites is associated with the development of enteropathy. Villus length, crypt depth and the proliferative capacity of the crypts in treated and untreated mice were determined. The development of oedema was assessed by weighing the entire small intestine. Enteropathy developed as previously seen in untreated mice, which exhibited significant villus atrophy and crypt hyperplasia, by day 6 p.i. (Figure 4.2A). Treatment of mice with anti-ICOS antibody resulted in significant amelioration of the enteropathy observed at day 6 p.i. reflected by a decrease in both villus atrophy (p=0.009) and crypt hyperplasia (p=0.0283) compared with the untreated group. By day 13 p.i., crypt depths were not significantly different between treated and untreated mice, however, villus lengths were significantly longer in the treated group than the untreated group (p=0.0443). This may be a reflection of the proliferative rates of the crypts, by day 6 p.i. the numbers of mitotic figures had increased significantly in both treated and untreated groups compared to uninfected (p=0.009 and 0.009). However, by day 13 p.i. the numbers of mitotic figures were significantly greater in the anti-ICOS treated group in comparison to the treated group (p=0.009) (Figure 4.2B). Taken together these data imply a delay in the development of enteropathy in anti-ICOS treated mice, which may partly be mediated by the increased proliferative rates of the crypts. The development of oedema following infection with *T. spiralis* infection is associated with an increase in the weight of the small intestine. The weight of the intestine of both treated and untreated mice increased significantly day 6

(p=0.009 and p=0.009) and 13 p.i. (p=0.0122 and p=0.009) compared to day 0 p.i., however at day 13 p.i. anti-ICOS treated mice had significantly lower gut weights than untreated mice (p=0.0088) indicating reduced oedema at day 13 p.i. in the anti-ICOS treated mice (Figure 4.2C).

FIGURE 4.2: The effect of anti-ICOS treatment on the development of enteropathy following infection with *T. spiralis*. Villus and crypt lengths (A) were measured and the number of mitotic figures per crypt (B) was determined untreated and anti-ICOS treated mice at day 0 (uninfected) and at day 6 and 13 p.i. The small intestine was removed from untreated and anti-ICOS treated mice at day 0, 6 and 13 p.i. and weighed in grams (C). Data expressed as mean + SEM (C) for five mice. *, represents significantly different to uninfected mice; §, represents significantly different to untreated mice ($p<0.05$).


A.
Villi
Crypt
Mean Length (µm) + SEM
1000
800
600
400
200
0
-200
-400
0
6
13


B.
Mitotic Figures
Mean no Mitotic Figures/VCU + SEM
7
6
5
4
3
2
1
0
0
6
13
Days Post Infection

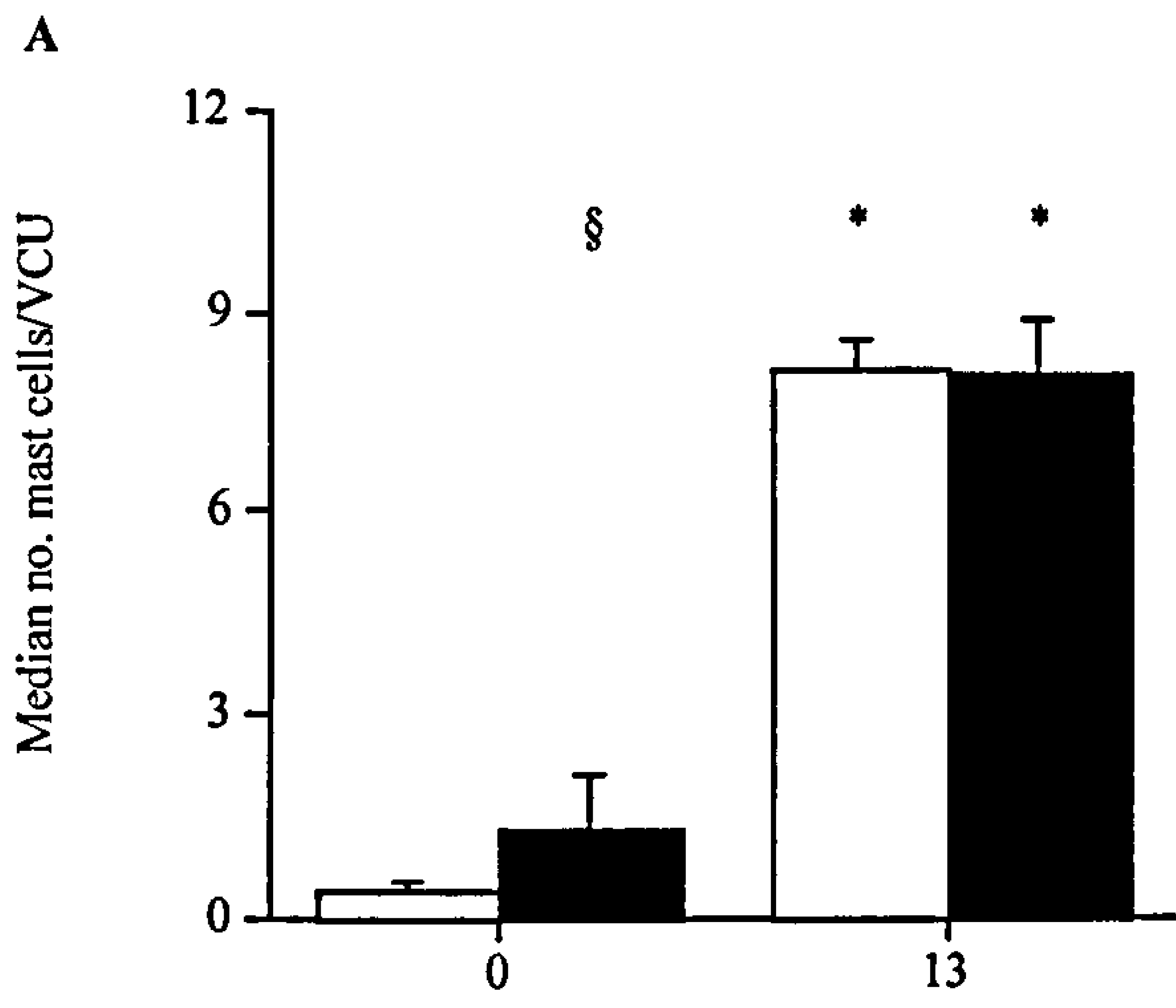

C.
Gut Weight
Mean Weight (g) + SEM
5
4
3
2
1
0
0
6
13
Days Post Infection
Untreated
Anti-ICOS

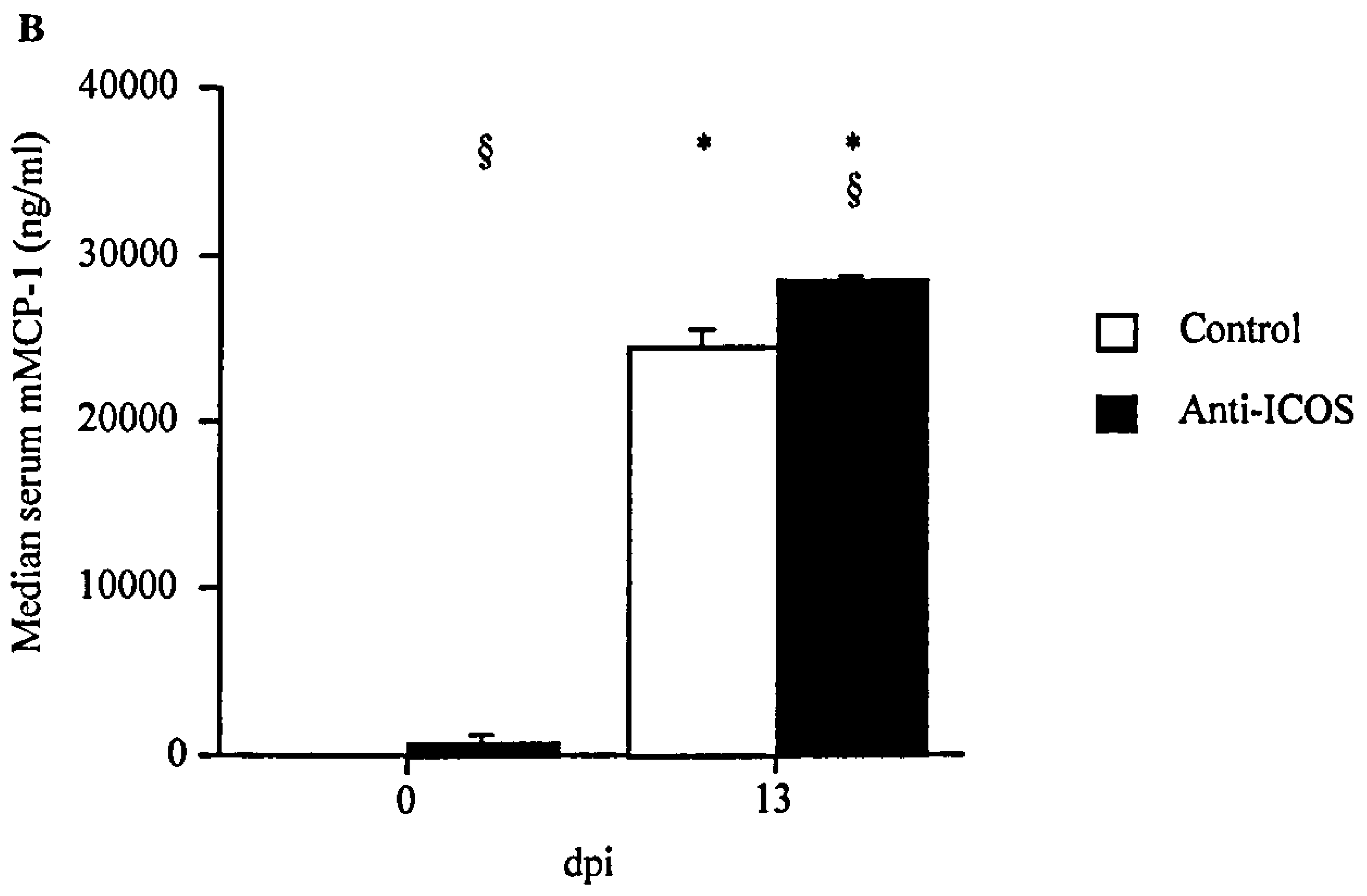
### *4.2.3 Serum mMCP-1 but not mastocytosis was increased by anti-ICOS treatment.*

The infiltration of mast cells into the mucosa has previously been shown to be associated with the development of enteropathy and with the expulsion of *T. spiralis* from the small intestine (Lawrence *et al.*, 2004). The role of ICOS in the development of mastocytosis was evaluated by counting total number of mast cells per VCU at day 0 and 13 p.i. for both treated and untreated mice (Fig. 5.3A). Pronounced mastocytosis was observed at day 13 p.i. in both anti-ICOS treated ($p=0.0143$) and untreated mice ($p=0.0209$) compared to uninfected mice although there was no difference between the groups. Interestingly, treatment of uninfected mice with anti-ICOS resulted in a significant increase in the number of mast cells in the small intestine in comparison to untreated control mice ($p=0.0143$).

Mast cell protease (mMCP)-1 is a marker of mast cell activation and has been shown to be important in the development of both enteropathy (Lawrence *et al.*, 2004) and worm expulsion (Knight *et al.*, 2000). The level of mMCP-1 was measured in serum samples from uninfected mice and mice at day 13 p.i. As observed for mast cell numbers, the level of mMCP-1 in sera from uninfected, anti-ICOS treated mice were significantly higher than in untreated, uninfected mice ($p=0.009$) (Figure 5.3B). Following infection with *T. spiralis* mMCP-1 levels significantly increased in both untreated ($p=0.009$) and anti-ICOS treated ($p=0.009$) groups, however levels of serum mMCP-1 in anti-ICOS treated mice were significantly higher than in untreated mice ($p=0.009$).

FIGURE 4.3: The effect of anti-ICOS treatment on the development of mucosal mastocytosis and mast cell degranulation following *T. spiralis*. A) Carnoy's fixed jejunum from untreated and anti-ICOS treated mice at day 0 and 13 p.i. were processed and stained with 0.5% toluidine blue, revealing mast cells. The number of mucosal mast cells were counted in 20 randomly selected villus crypt units (VCU). Data expressed as the mean number of mast cells /VCU + SEM. B) The degranulation of mucosal mast cells in untreated and anti-ICOS treated day 0 and 13 p.i. were assessed by measuring serum titres of mMCP-1 by ELISA against a recombinant protein standard. Data expressed as mean mMCP-1 concentration in pg/ml+ SEM. *, represents significantly different to uninfected. §, represents significantly different to untreated. Five mice were used per group.

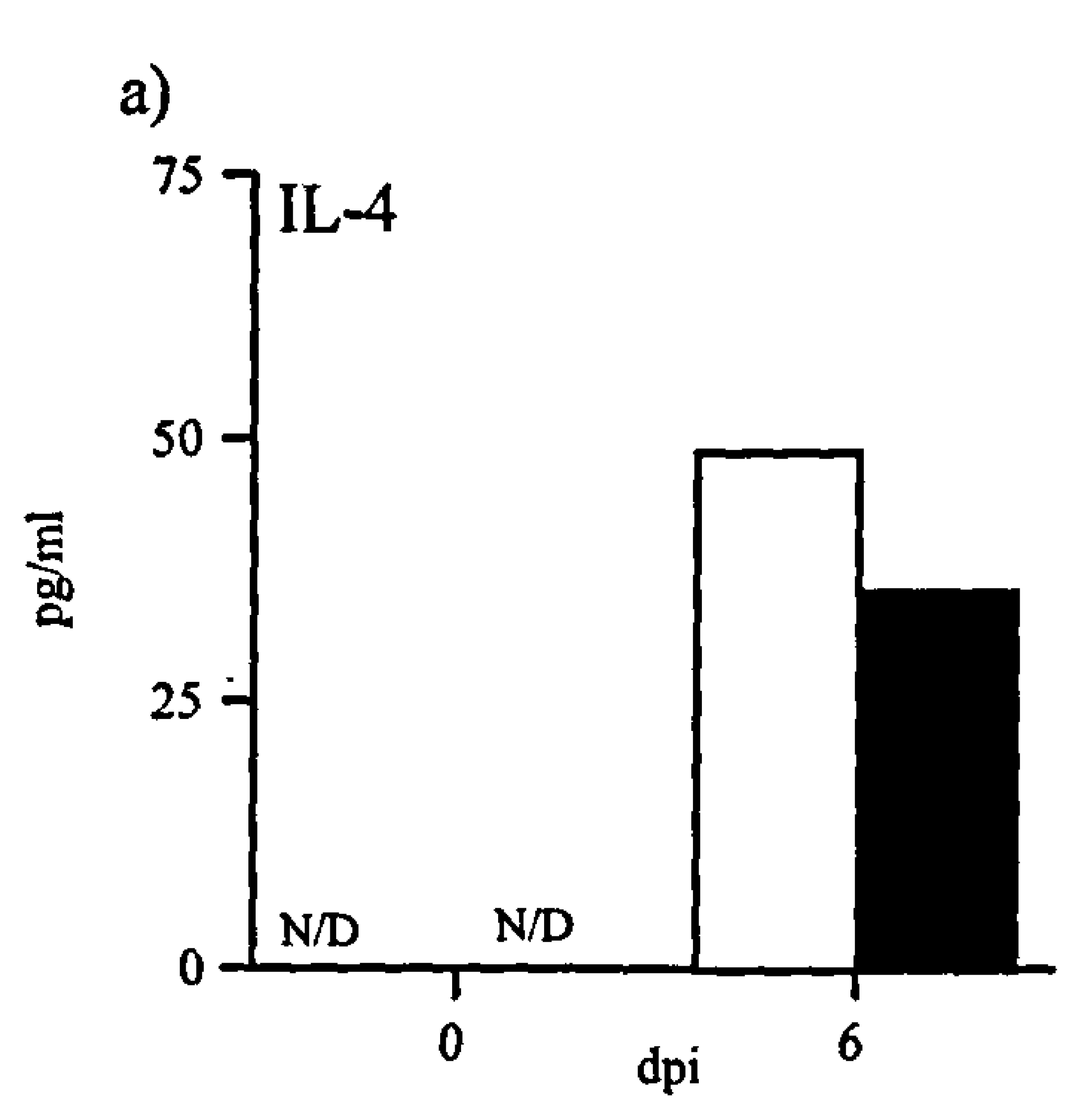

A
Median no. mast cells/VCU
12
9
6
3
0
§
*
*
0
13

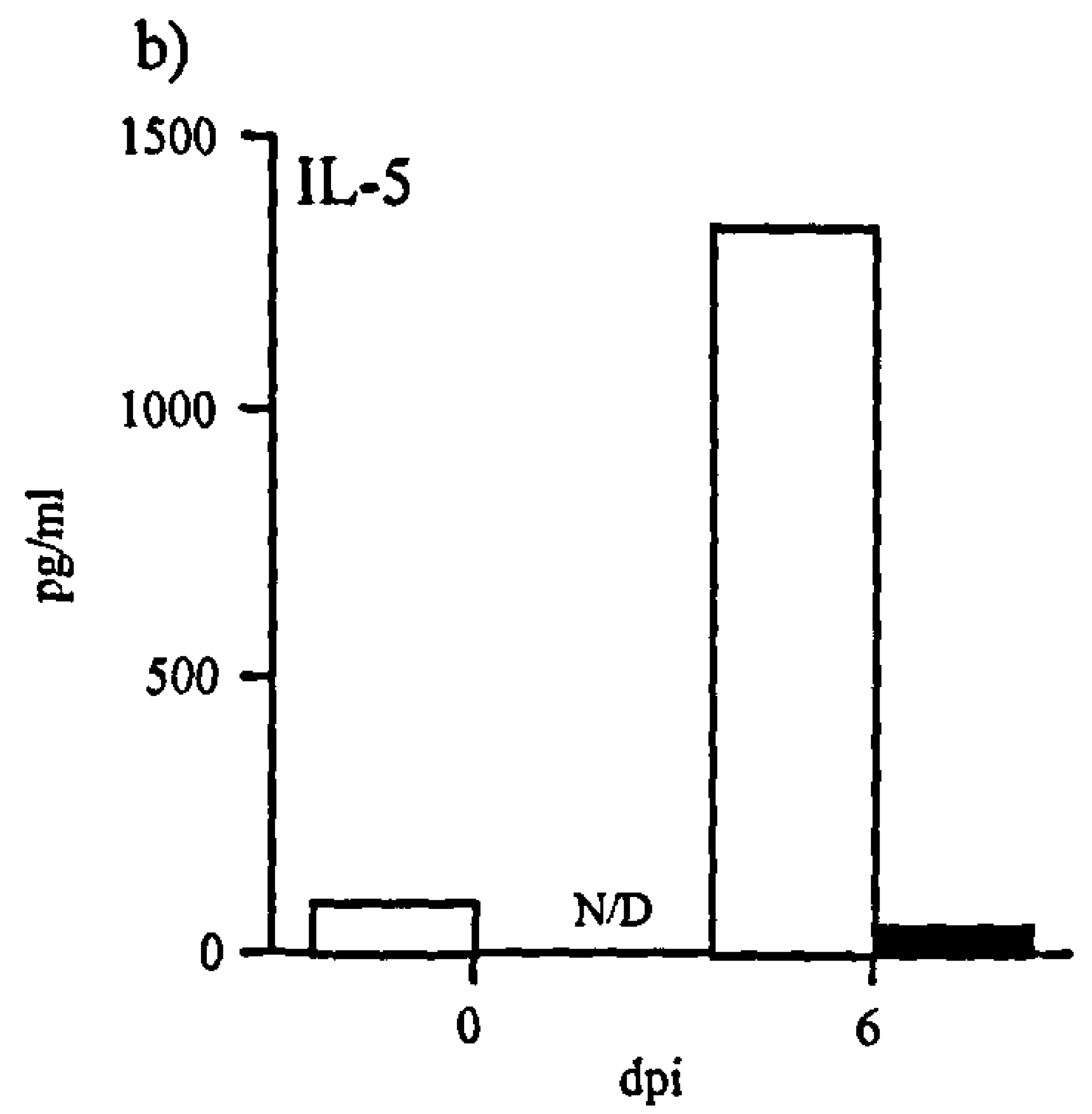

B
Median serum mMCP-1 (ng/ml)
40000
30000
20000
10000
0
§
*
*
§
0
13
dpi
Control
Anti-ICOS

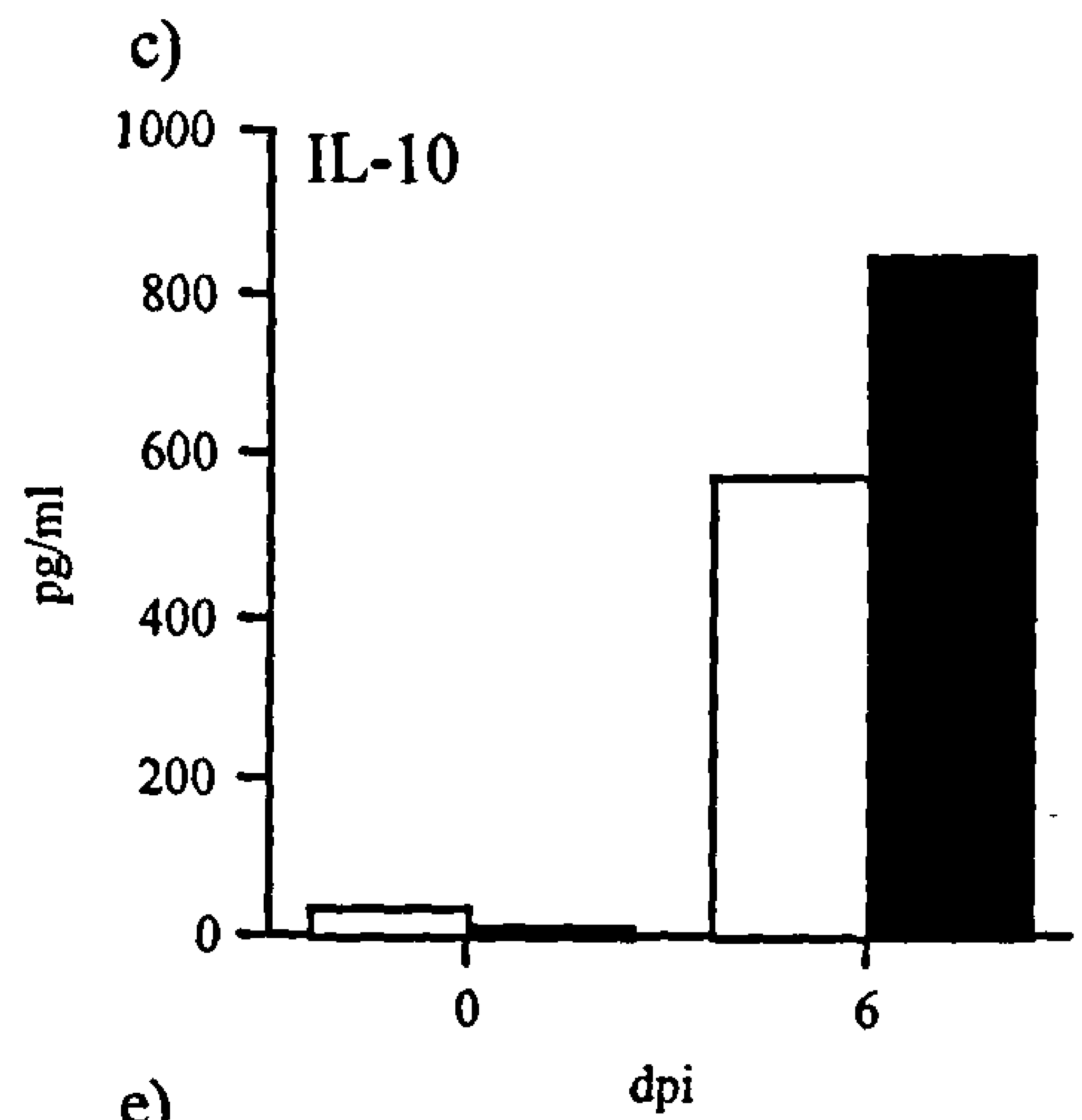
### *4.2.4 Anti-ICOS antibody treatment reduces IL-4 and IL-5 but increases IFN-γ, IL-10 and IL-13 secretion by MLN cells following infection with T. spiralis.*

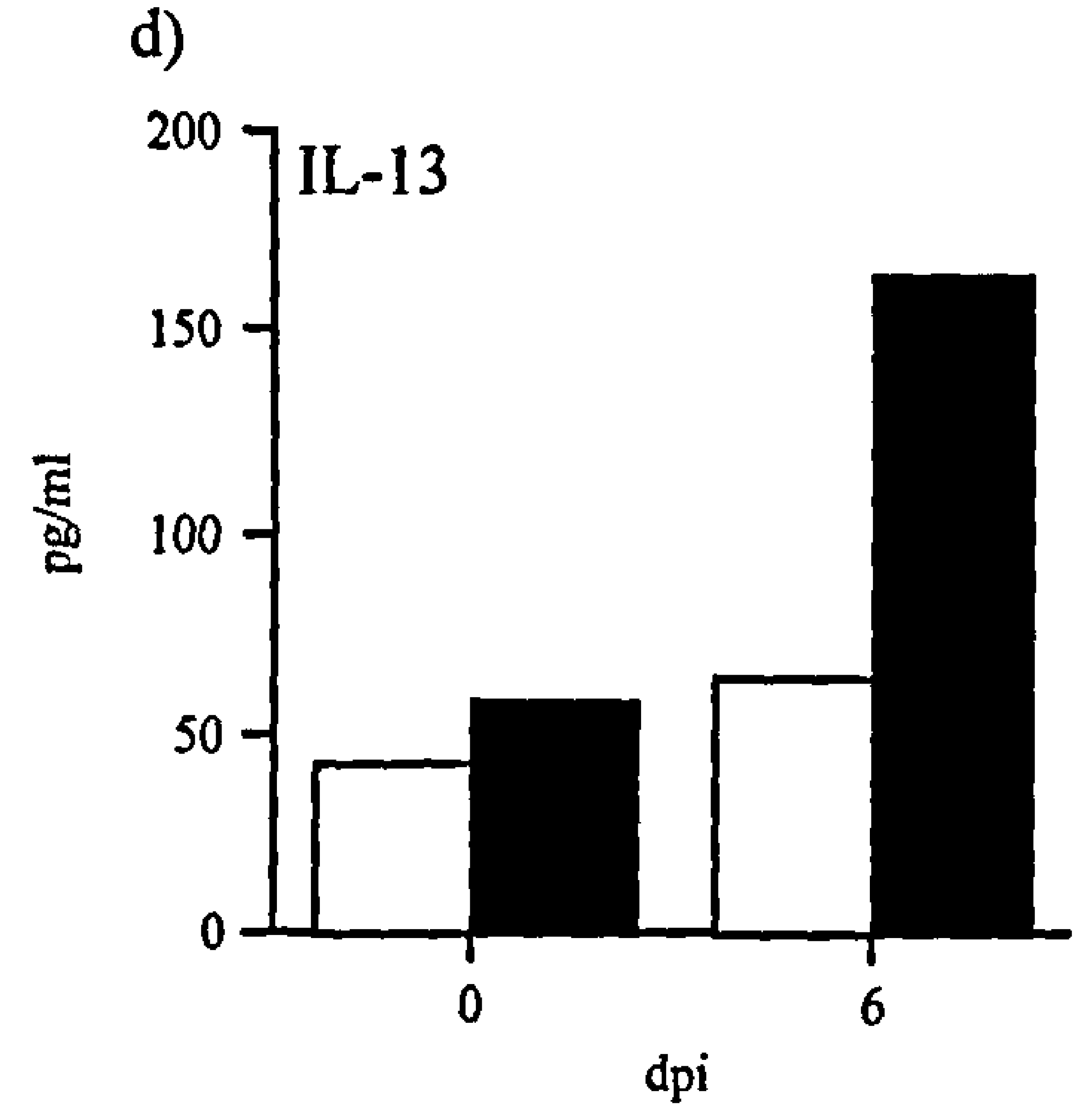
Expulsion of adult *T. spiralis* from the intestine is associated with increased production of Th2 cytokines and decreased production of Th1 cytokines. Cytokine production by mesenteric lymph node cells following re-stimulation with *Trichinella* antigen was measured at day 0 and 6 p.i. The production of IL-4, IL-5, IL-10, IL-13 and IFN-γ by MLN cells were increased in untreated mice following infection. Treatment of mice with anti-ICOS resulted in a slight decrease in IL-4 production (Figure 4.4A) compared to untreated mice at day 6 p.i. while IL-5 production in anti-ICOS treated mice is virtually ablated (Figure 4.4B). An increase in the secretion of the Th1 cytokine IFN-γ by anti-ICOS treated mice compared to untreated mice (Figure 4.4C) was observed at day 6 p.i. Surprisingly, production of IL-13 and IL-10 from MLN cells from anti-ICOS treated mice infected with *T. spiralis* (Figure 4.4D and E) was greatly increased in comparison to untreated mice.

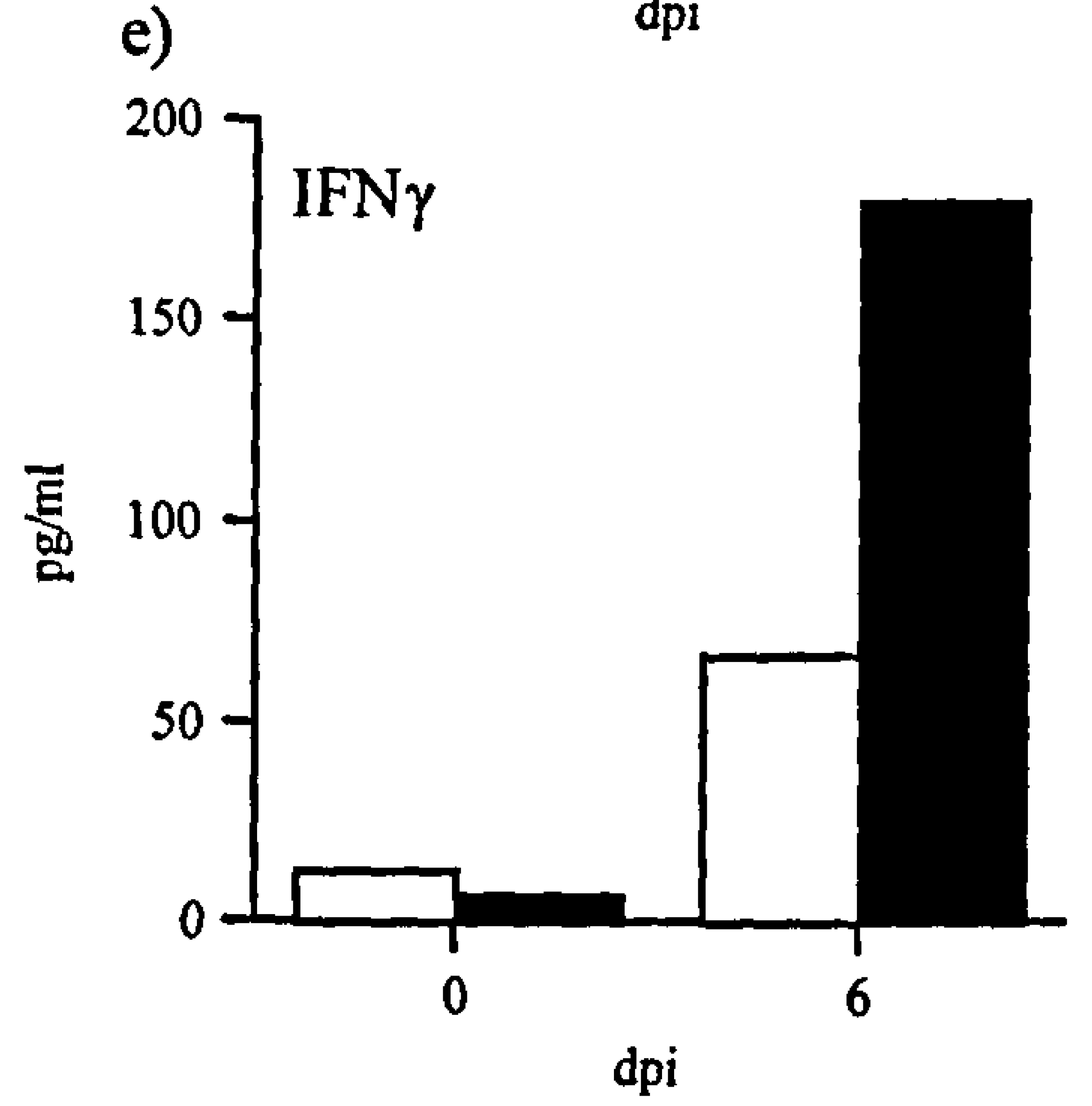
FIGURE 4.4: The effect of anti-ICOS treatment on MLN cytokine secretion following infection with *T. spiralis*. MLN were removed from untreated and anti-ICOS treated mice at day 0 and 6 p.i. Single cell suspensions were made, samples pooled and cultured at $1 \times 10^7$ cells in 1.5ml with 50μg/ml TAg. The secretion of the cytokines IL-4 (A), IL-5 (B), IL-10 (C), IL-13 (D), and IFN-γ (E) were measured in the culture supernatants by ELISA against recombinant standards. Data expressed cytokine concentration in pg/ml.



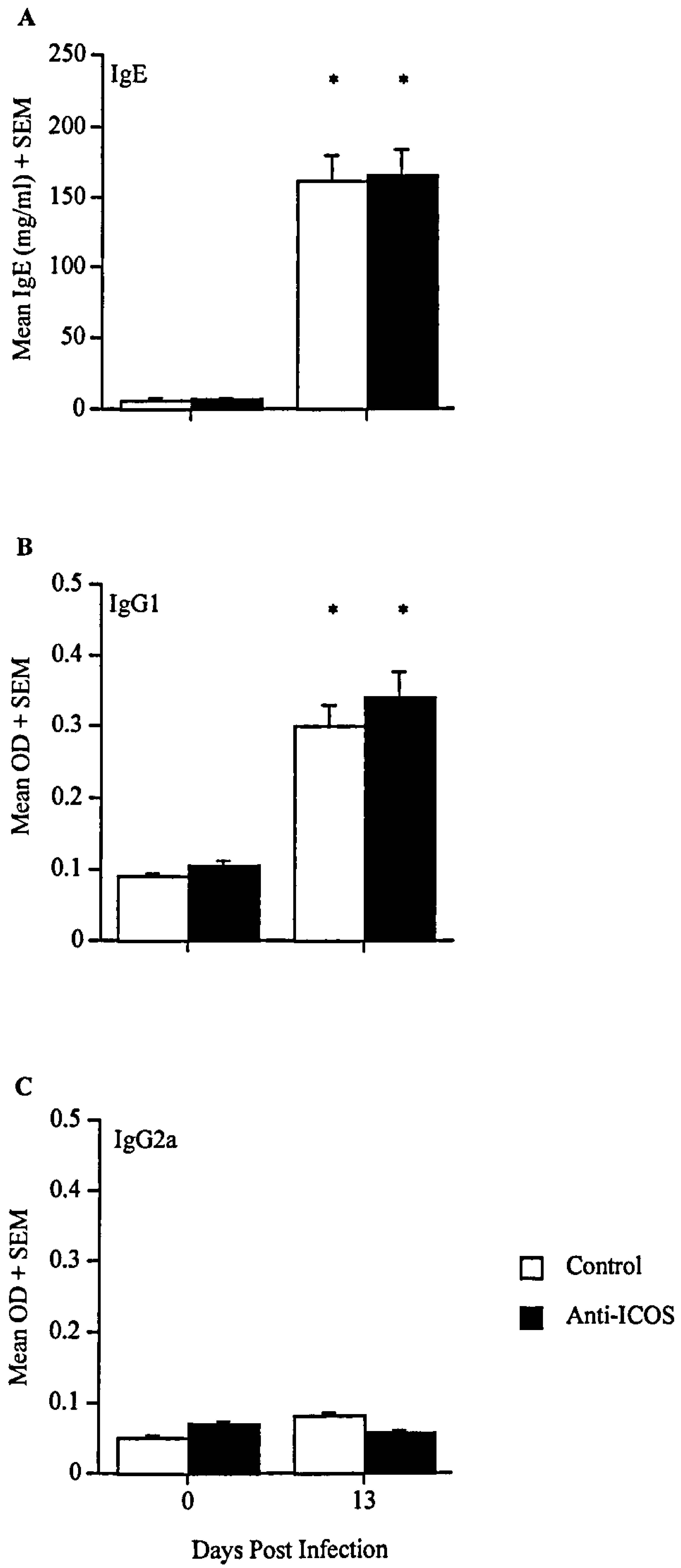

untreated

anti-ICOS treated

*4.2.5 Serum antibody levels are not significantly affected by anti-ICOS treatment.*

Antibodies, in particular IgG1 and IgE, have been implicated in protection against many helminths. Total serum IgE and antigen specific IgG1 and IgG2a were measured at day 0 and 13 p.i. Levels of total IgE increased significantly in both treated and untreated mice following infection (p=0.009 and 0.009) but treatment with anti-ICOS did not significantly alter the levels of total IgE at day 13 p.i. (Figure 4.5A). Treatment with anti-ICOS did not significantly affect the production of parasite specific IgG2a or IgG1 at day 13 p.i. with a significant increase in IgG1 being observed following infection in both treated and untreated mice (p=0.0143 and 0.009) while no significant alteration in IgG2a was observed (Figure 4.5B and C).

FIGURE 4.5: The effect of anti-ICOS treatment on antibody responses following infection with *T. spiralis*. Total IgE (A) and TAg specific IgG1 (B) and IgG2a (C) titres were measured in sera taken at day 0 and 13 p.i. from Untreated and anti-ICOS treated mice. IgE titres were measured by sandwich ELISA against a purified IgE standard and were expressed as mean µg/ml + MAD. While antigen specific IgG1 and IgG2a titres were assessed by ELISA using 96 well microtitre plates coated with *TAg* at 2µg/ml, sera was serially diluted and optical density readings at 1:2560 dilution were used. Data was expressed as mean optical density (OD) +SEM. *, represents significantly different to uninfected ($p \leq 0.05$). Five mice were used per group.

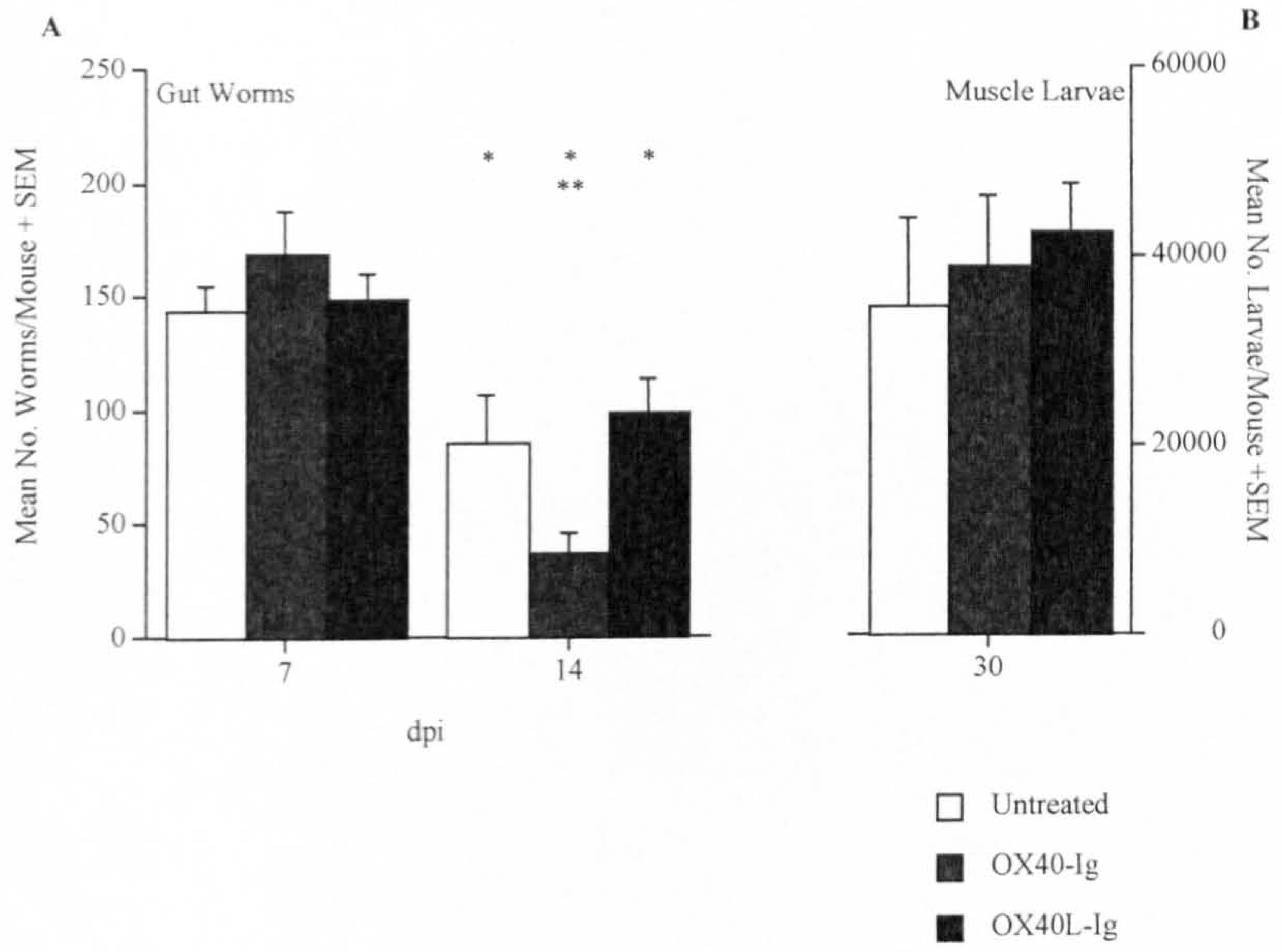

A
IgE
Mean IgE (mg/ml) + SEM
250
200
150
100
50
0
B
IgG1
Mean OD + SEM
0.5
0.4
0.3
0.2
0.1
0
C
IgG2a
Mean OD + SEM
0.5
0.4
0.3
0.2
0.1
0
0
13
Days Post Infection
Control
Anti-ICOS

### *4.2.6 OX40-Ig and OX40L-Ig treatment does not effect the establishment in, or expulsion from the small intestine of adult worms or the accumulation of muscle larvae.*

OX40 has, like ICOS, been shown to be important in the development of T helper cell responses, with the role of OX40 in the development of the Th1/Th2 balance remaining controversial. Here the role of OX40/OX40L interactions in the development of the immune response, worm expulsion and enteropathy during infection with *T. spiralis* was investigated. Wild type BALB/c mice were infected with 400 freshly isolated *T. spiralis* larvae and treated at day 2, 5 and 8 p.i. with 100μg of either OX40-Ig, which blocks OX40 signalling to T cells, or OX40L-Ig which promotes OX40 signalling to T cells or left untreated. Worm establishment in the small intestine was measured day 7 p.i., no significant differences in the number of worms retrieved were observed between untreated, OX40-Ig and OX40L-Ig treated mice. Worm expulsion is evaluated by measuring the difference in the numbers of worms present at day 14 p.i. compared to day 7 p.i. Untreated, OX40-Ig and OX40L-Ig treated mice all had significantly fewer worms at day 14 p.i. compared to day 7 p.i. ($p=0.0472$, 0.009 and 0.0163). However no significant differences were seen in the expulsion of worms by OX40-Ig or OX40L-Ig treated mice compared to the untreated controls, however OX40-Ig treated mice had significantly fewer worms than OX40L-Ig treated mice ($p=0.009$) (Figure 4.6A).

Larval worm burdens provide a measure of the longevity of adult worms, the ability of larvae to penetrate the mucosa and to survive until they are able to enter a muscle cell. Larval worm establishment in the muscle was measured at day 30 p.i., no significant differences were observed in OX40-Ig or OX40L-Ig treated mice compared to untreated controls (Figure 4.6B).

FIGURE 4.6: The effect of OX40-Ig and OX40L-Ig treatment on the expulsion of *T. spiralis* from the intestine and the accumulation of muscle larvae. A) The establishment and expulsion of *T. spiralis* was measured in untreated, OX40-Ig and OX40L-Ig treated mice at day 6 and 14 p.i. The small intestine was excised and the total number of worms present were counted. B) The accumulation of muscle larvae was measured in untreated, OX40-Ig and OX40L-Ig treated mice at day 30 p.i. *, represents significantly different to mice at day 7 p.i. §, represents significantly different to untreated mice ($p<0.05$). Data is expressed as mean number of worms/mouse + SEM, for 5 mice per group. *, represents significantly different to day 7 p.i.


A
B
Gut Worms
Muscle Larvae
Mean No. Worms/Mouse + SEM
Mean No. Larvae/Mouse +SEM
250
200
150
100
50
0
60000
40000
20000
0
* * *
**
7
14
30
dpi
Untreated
OX40-Ig
OX40L-Ig

### *4.2.7 OX40-Ig or OX40L-Ig treatment does not significantly alter the development of villus atrophy or crypt hyperplasia but OX40-Ig treatment delays the reduction oedema at day 14 p.i.*

The enteropathy associated with infection with *T. spiralis* is characterised by the development of villus atrophy, crypt hyperplasia and intestinal oedema. The effect of OX40-Ig and OX40L-Ig treatment on the development of enteropathy was assessed by measuring the villi length, crypt depth, proliferative capacity, and the gut weight in mice at day 0, 7 and 14 p.i.

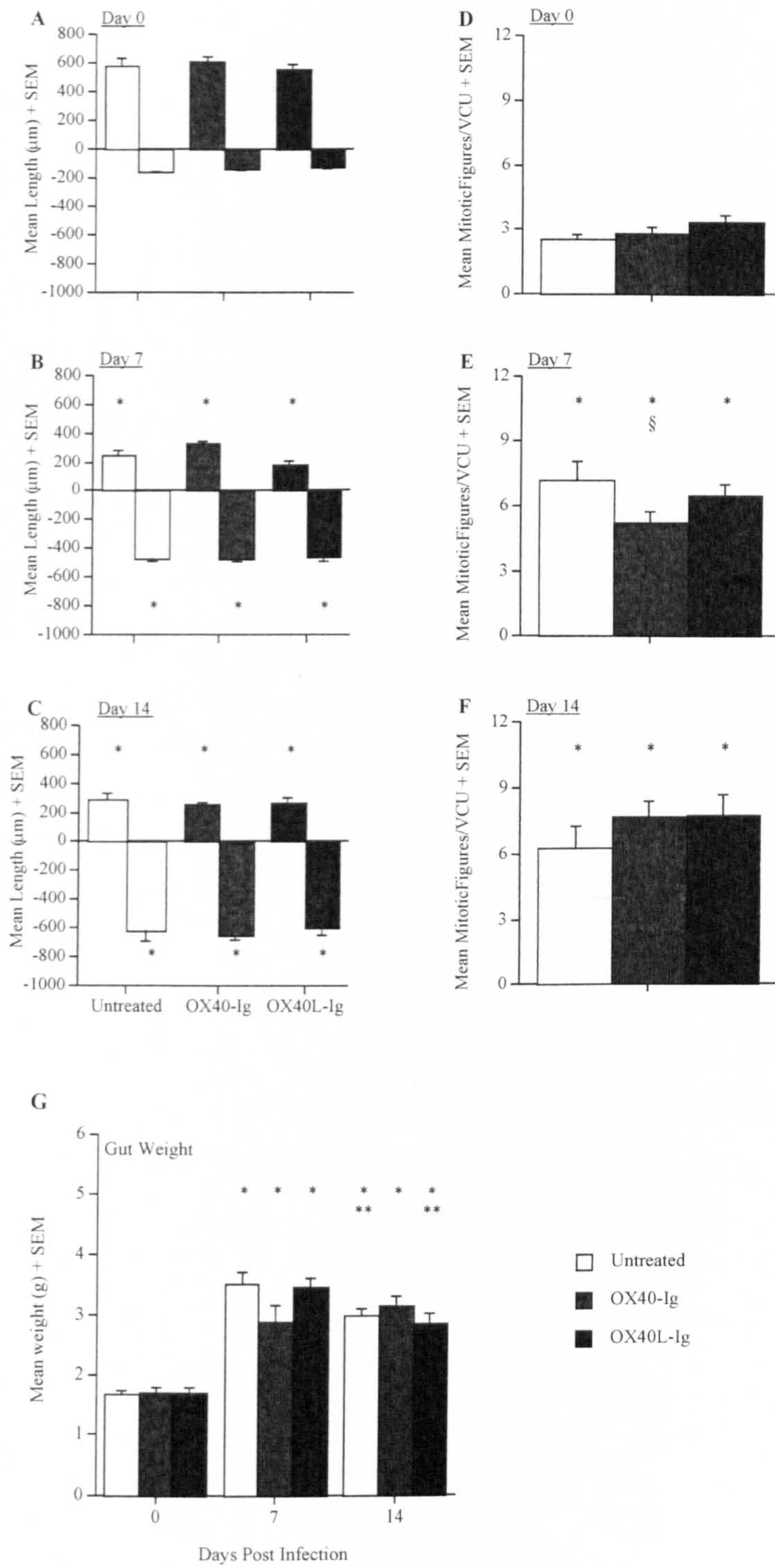
Significant villus atrophy and crypt hyperplasia was present in untreated control, OX40-Ig and OX40L-Ig treated mice at day 7 (villus atrophy p=0.009, 0.009 and 0.009; crypt hyperplasia p=0.009, 0.009 and 0.009) (Figure 4.7B) and 14 p.i. (villus atrophy p=0.0143, 0.009 and 0.009; crypt hyperplasia p=0.0143, 0.009 and 0.009) (Figure 4.7C) compared to uninfected mice (Figure 4.7A). No significant differences where observed between OX40-Ig and OX40L-Ig treated mice and untreated control mice at day 0, 6 or 14 p.i. (Figure 4.7A, B and C).

The number of mitotic figures / VCU had increased significantly in untreated, OX40-Ig, and OX40L-Ig treated at day 6 (p=0.009, 0.009 and 0.009) (Figure 4.7D) and 14 p.i. (p=0.0143, 0.009 and 0.009) (Figure 4.7E) compared to day 0 (Figure 4.7F) indicating increased proliferative capacity relative to uninfected mice. However, despite there

being no significant differences in the development of crypt hyperplasia there were significantly fewer mitotic figures/ VCU in the OX40-Ig treated mice compared to untreated mice at day 7 p.i. ($p=0.0472$), by day 14 p.i. however there were no longer any significant differences between OX40-Ig treated and untreated mice.

Consistent with the development of oedema, the weight of the small intestine from untreated ($p=0.0143$ and 0.0143), OX40-Ig ($p=0.0163$ and 0.009) and OX40L-Ig ($p=0.009$ and 0.009) treated mice significantly increased following infection (day 7 and 14 p.i.) with *T. spiralis*. Although no significant differences where observed between the gut weights from treated and untreated mice at day 7 p.i., gut weight appears to be slightly lower in OX40-Ig treated mice. By day 14 p.i. gut weight from untreated and OX40L-Ig treated mice was significantly lower than at day 7 p.i. while gut weights from OX40-Ig treated mice were not significantly altered (Figure 4.8G).

FIGURE 4.7: The effect of OX40-Ig and OX40L-Ig treatment on the development of enteropathy following infection with *T. spiralis*. Villus and crypt lengths (A) were measured and the number of mitotic figures per crypt (B) were determined untreated OX40-Ig and OX40L-Ig treated mice at day 0 (uninfected) and at day 7 and 14 p.i. The small intestine was removed from untreated OX40-Ig and OX40L-Ig treated mice at day 0, 7 and 14 p.i. and weighed in grams (C). Data expressed as mean + SEM for 5 mice per group. *, represents significantly different to uninfected; §, represents significantly different to untreated ($p<0.05$).



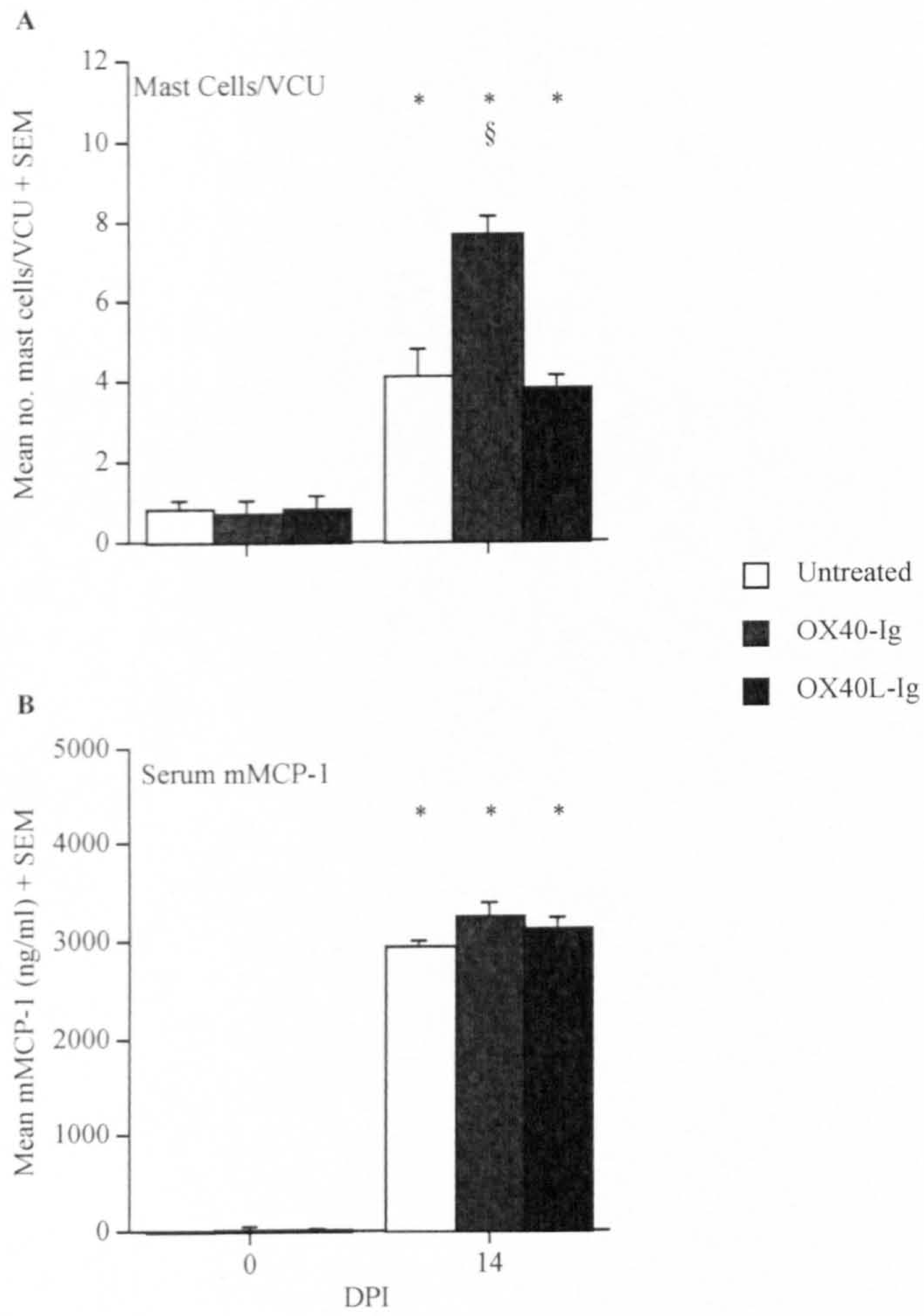
A
Day 0
Mean Length (µm) + SEM
B
Day 7
C
Day 14
Untreated
OX40-Ig
OX40L-Ig
D
E
F
Mean MitoticFigures/VCU + SEM
G
Gut Weight
Mean weight (g) + SEM
Days Post Infection
Untreated
OX40-Ig
OX40L-Ig

### *4.2.8 OX40-Ig treatment significantly enhances* T. spiralis *induced mucosal mastocytosis and but does not effect serum mMCP-1 levels.*

Mast cells and the mast cell protease mMCP-1 have previously been shown to be important in the development of enteropathy and in the expulsion of adult *T. spiralis* worms from the intestine. The development of mastocytosis following infection was measured in intestinal samples from mice at day 0 and 14 p.i., sectioned and stained with toluidine blue (pH0.3). Untreated control, OX40-Ig and OX40L-Ig treated mice had significantly elevated numbers of mast cells per villus-crypt unit (VCU) at day 14 p.i. compared to day 0 p.i. (p=0.0143, 0.0062, and 0.0202). OX40-Ig treated mice also had significantly more mast cells per VCU at day 14 p.i. compared to untreated control and OX40L-Ig treated mice (p=0.0143 and 0.0143) (Figure 4.9A).

Serum mucosal mast cell protease (mMCP)-1 concentration reflects the activation and degranulation of mast cells in the mucosa; increased numbers of mast cells would be expected to increase the serum concentration of mMCP-1. Untreated control, OX40-Ig and OX40L-Ig treated mice all developed significantly increased serum mMCP-1 levels day 14 p.i. compared to uninfected mice (p=0.009, 0.0143 and 0.009). Interestingly despite the significantly greater number of mast cells/VCU found in the OX40-Ig treated mice at day 14 p.i. compared to untreated and OX40L-Ig treated, serum mMCP-1 levels are not significantly altered in OX40-Ig or OX40L-Ig treated mice compared to untreated controls at day 14 p.i.

FIGURE 4.8: The effect of OX40-Ig and OX40L-Ig treatment on the development of mucosal mastocytosis and mast cell degranulation following *T. spiralis.* A) Carnoy's fixed jejunum from untreated, OX40-Ig and OX40L-Ig treated mice at day 0 and 13 p.i. were processed and stained with 0.5% toluidine blue, revealing mast cells. The number of mucosal mast cells were counted in 20 randomly selected villus crypt units (VCU). Data expressed as the mean number of mast cells /VCU + SEM. B) The activation and degranulation of mucosal mast cells in untreated and anti-ICOS treated mice 0 and day 13 p.i. was assessed by measuring serum titres of mMCP-1 by ELISA against a recombinant protein standard. Data expressed as mean mMCP-1 concentration in pg/ml+ SEM. *, represents significantly different to uninfected. §, represents significantly different to untreated. Five mice were used per group.


A
Mast Cells/VCU
Mean no. mast cells/VCU + SEM
12
10
8
6
4
2
0
* * *
§
Untreated
OX40-Ig
OX40L-Ig
B
Serum mMCP-1
Mean mMCP-1 (ng/ml) + SEM
5000
4000
3000
2000
1000
0
* * *
0
14
DPI

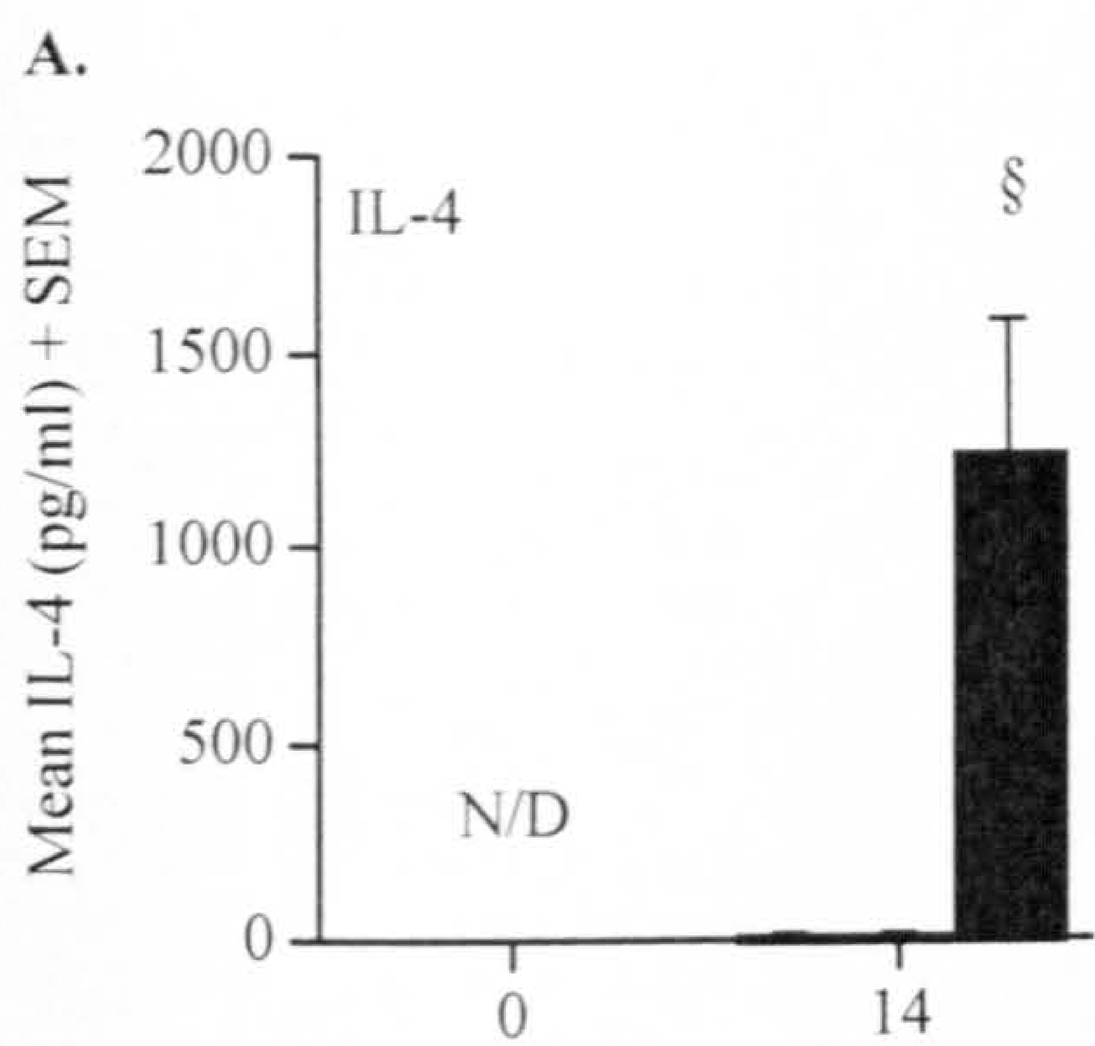
### *4.2.9 OX40L-Ig treatment enhances IL-4, IL-13 and IL-18 but not IFN-γ secretion by mesenteric lymph node cells stimulated* ex-vivo *with TAg.*

The MLN from uninfected, untreated mice and from untreated, OX40-Ig and OX40L-Ig from mice day 14 p.i. were extracted and cultured with TAg. The effect of OX40-Ig and OX40L-Ig treatment on the development of Th1 and Th2 cytokine responses following infection with *T. spiralis* was assessed by measuring the secretion of the cytokines IL-4, IL-13, IL-18 and IFN-γ by MLN cells stimulated ex-vivo with TAg.

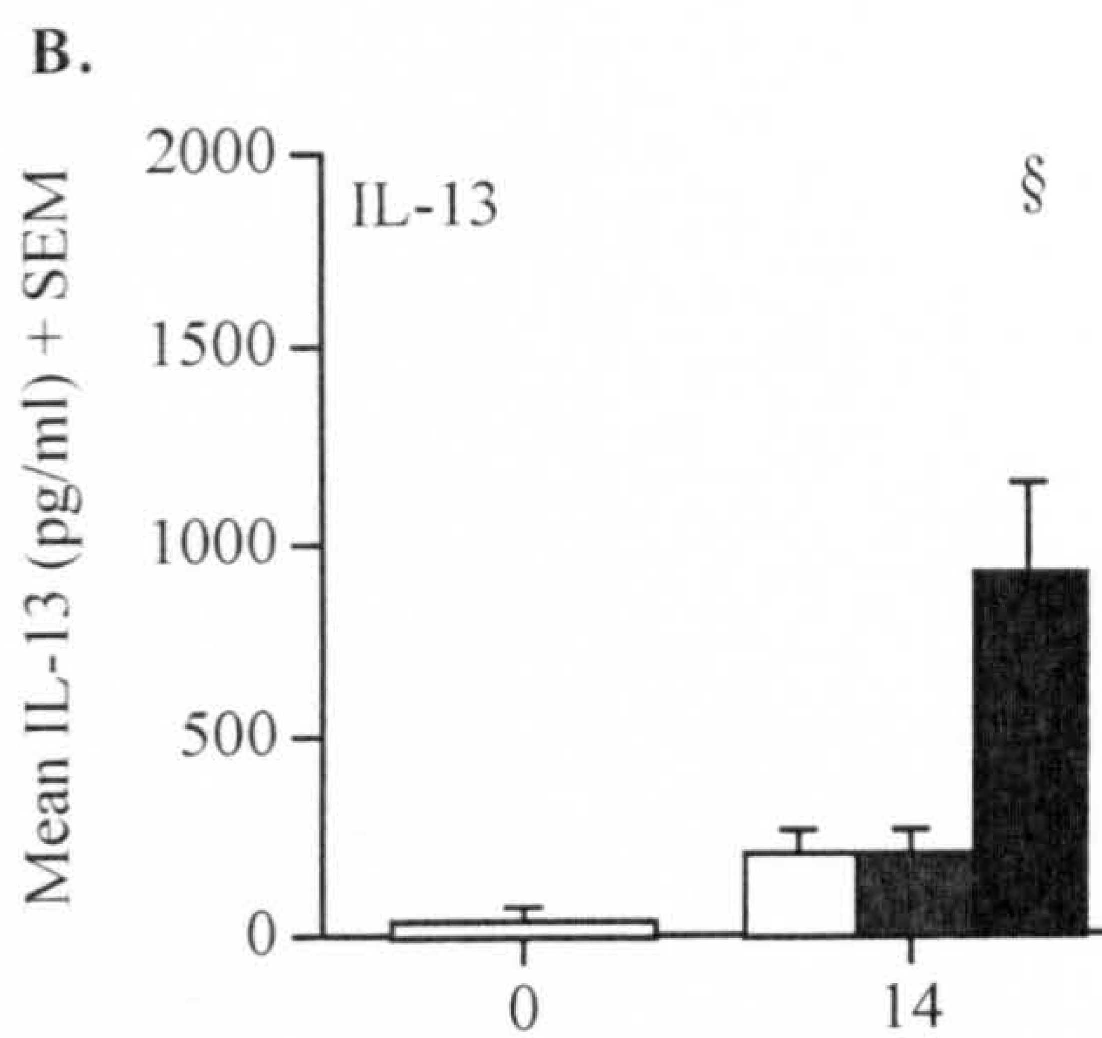
IL-4 and IL-13 are the major regulators of Th2 responses and are significantly elevated in mice treated with OX40L-Ig at day 14 p.i. compared to untreated or OX40L-Ig treated mice (Figure 4.9A and B). The secretion of IFN-γ, a regulator of Th1 responses, is not effected by either OX40-Ig or OX40L-Ig treatment compared to untreated at day 14 p.i. (Figure 4.9C), surprisingly IL-18 secretion a cytokine originally identified as IFN-γ inducing factor is enhanced in mice treated with OX40L-Ig (Figure 4.9D).

FIGURE 4.9: The effect of OX40-Ig and OX40L-Ig treatment on MLN cytokine secretion following infection with *T. spiralis*. MLN were removed from untreated, uninfected mice (n=2) and from untreated (n=5), OX40-Ig (n=4) and OX40L-Ig (n=4) treated mice at day 14 p.i. and $1 \times 10^6$ cells in 0.2ml were cultured with 50µg/ml TAg. The secretion of the cytokines IL-4 (**A**), IL-13 (**B**), IL-18 (**C**), and IFN-γ (**D**) were measured in pg/ml by ELISA against recombinant protein standards. Data is expressed as mean cytokine concentration (pg/ml) + SEM. §, represents significantly different to untreated at day 6 p.i. ($p<0.05$). N/D, cytokine not detected as concentrations below the assays limit of sensitivity.

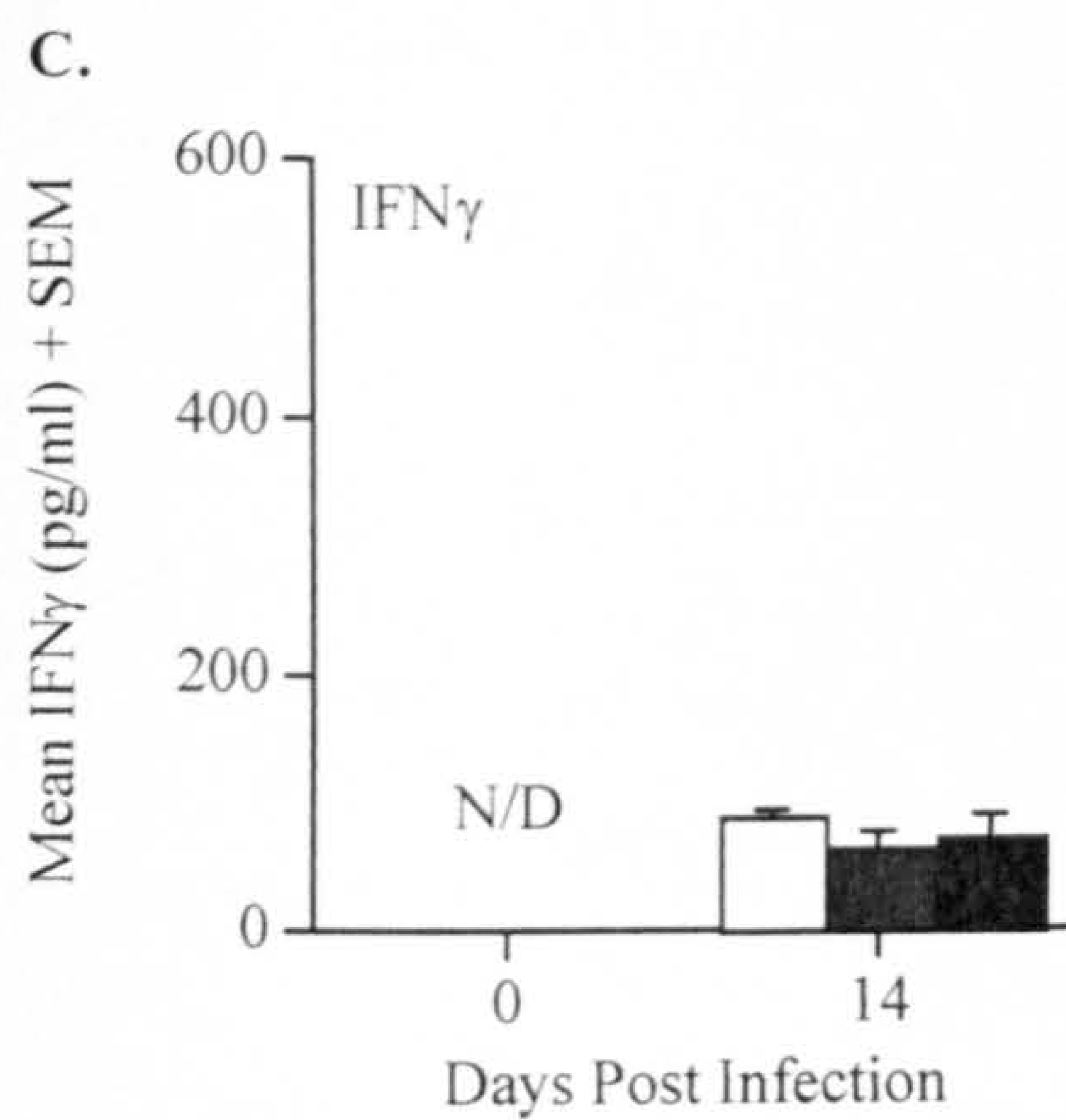
A.



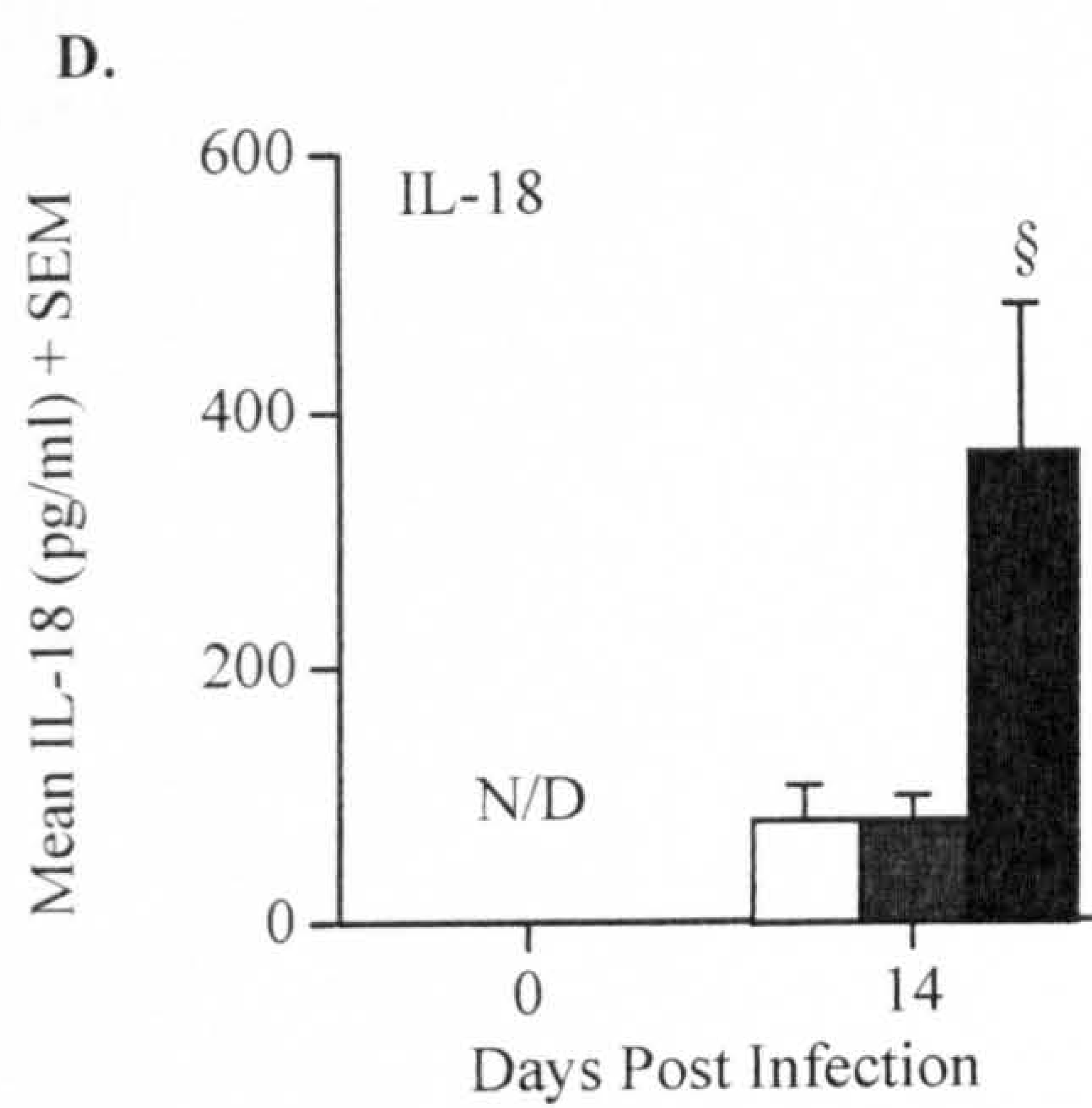

B.




C.




D.




□ Untreated

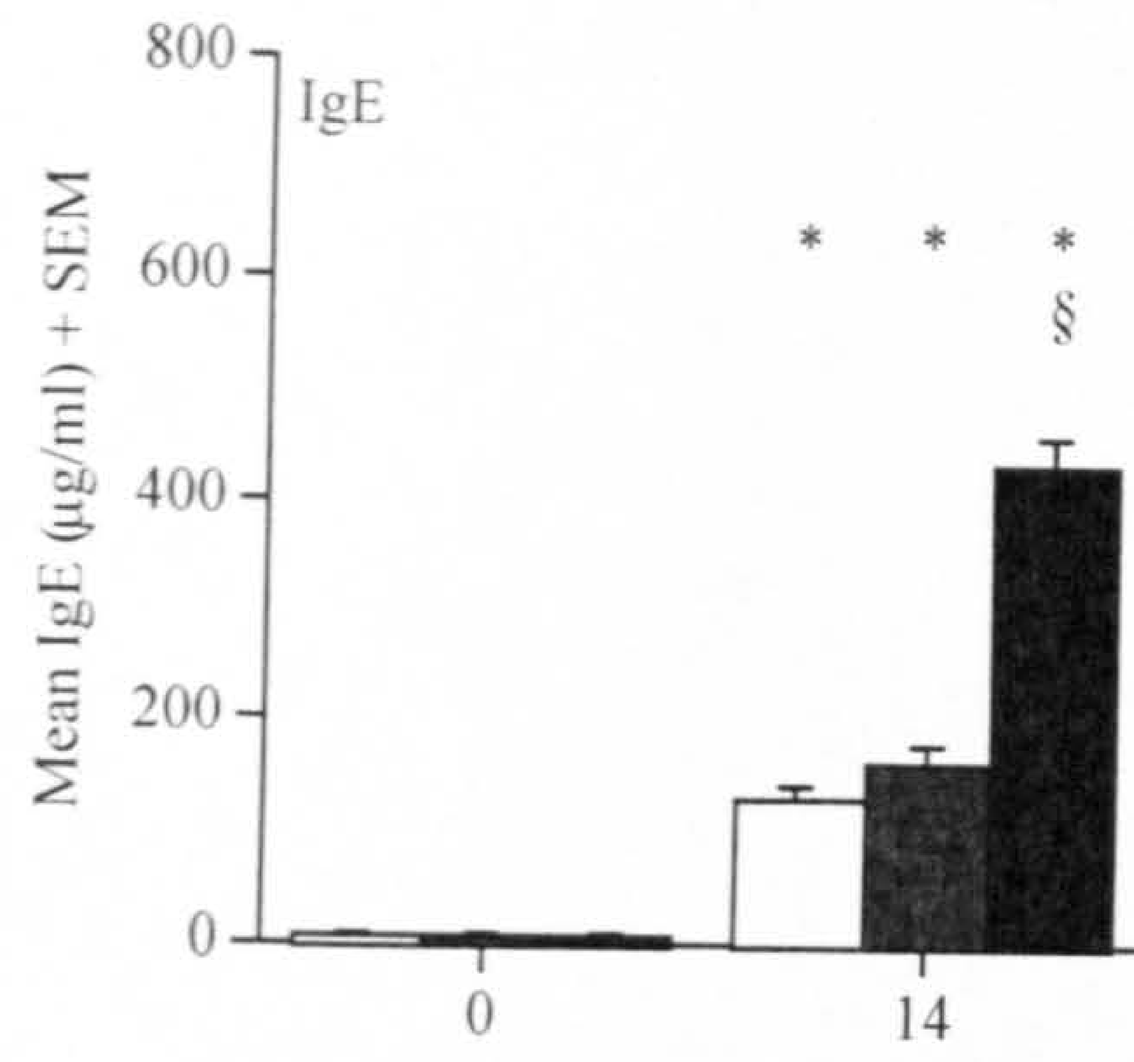
■ OX40-Ig

■ OX40L-Ig

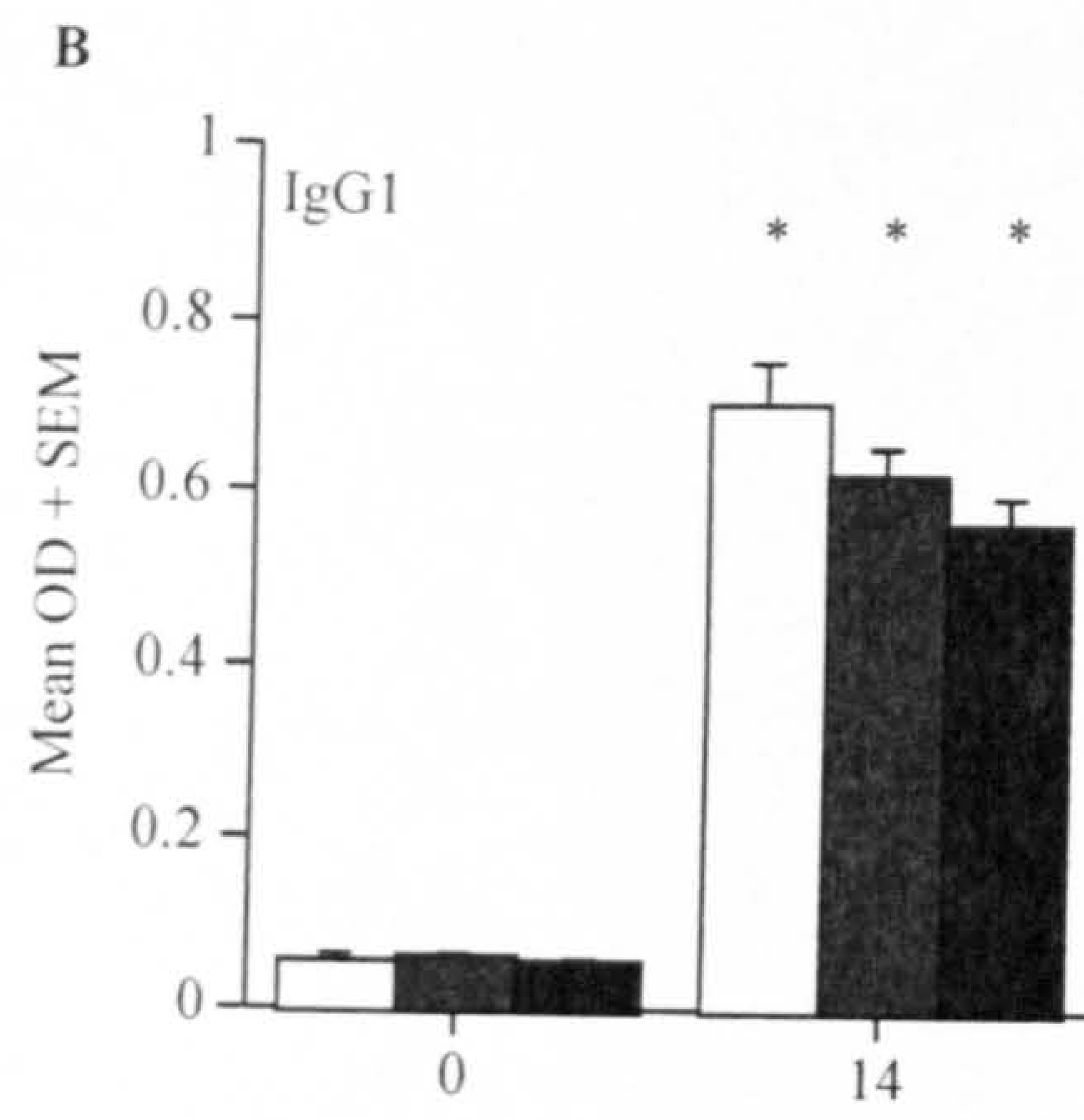
### *4.2.10 OX40L-Ig treatment increases total IgE but does not effect TAg specific IgG1 or IgG2a titres.*

Total IgE and antigen specific IgG1 and IgG2a titres were measured in serum samples collected from untreated mice and mice treated with either OX40-Ig or OX40L-Ig at day 0 and 14 p.i. Total IgE titres were elevated in untreated and treated mice day 14 p.i. compared to day 0 p.i. (p=0.009, 0.009, and 0.0088), Interestingly OX40L-Ig treated mice showed significantly higher IgE titres compared to untreated (p=0.009) (Figure 4.10A). Antigen specific IgG1 titres were, like IgE, elevated following infection with *T. spiralis* (p=0.009, 0.009 and 0.009), while IgG2a titres were unaffected compared to day 0 p.i. in untreated, OX40-Ig and OX40L-Ig treated mice. No significant differences were observed in IgG1 or IgG2a titres between untreated and treated mice (Figure 4.10B and C).

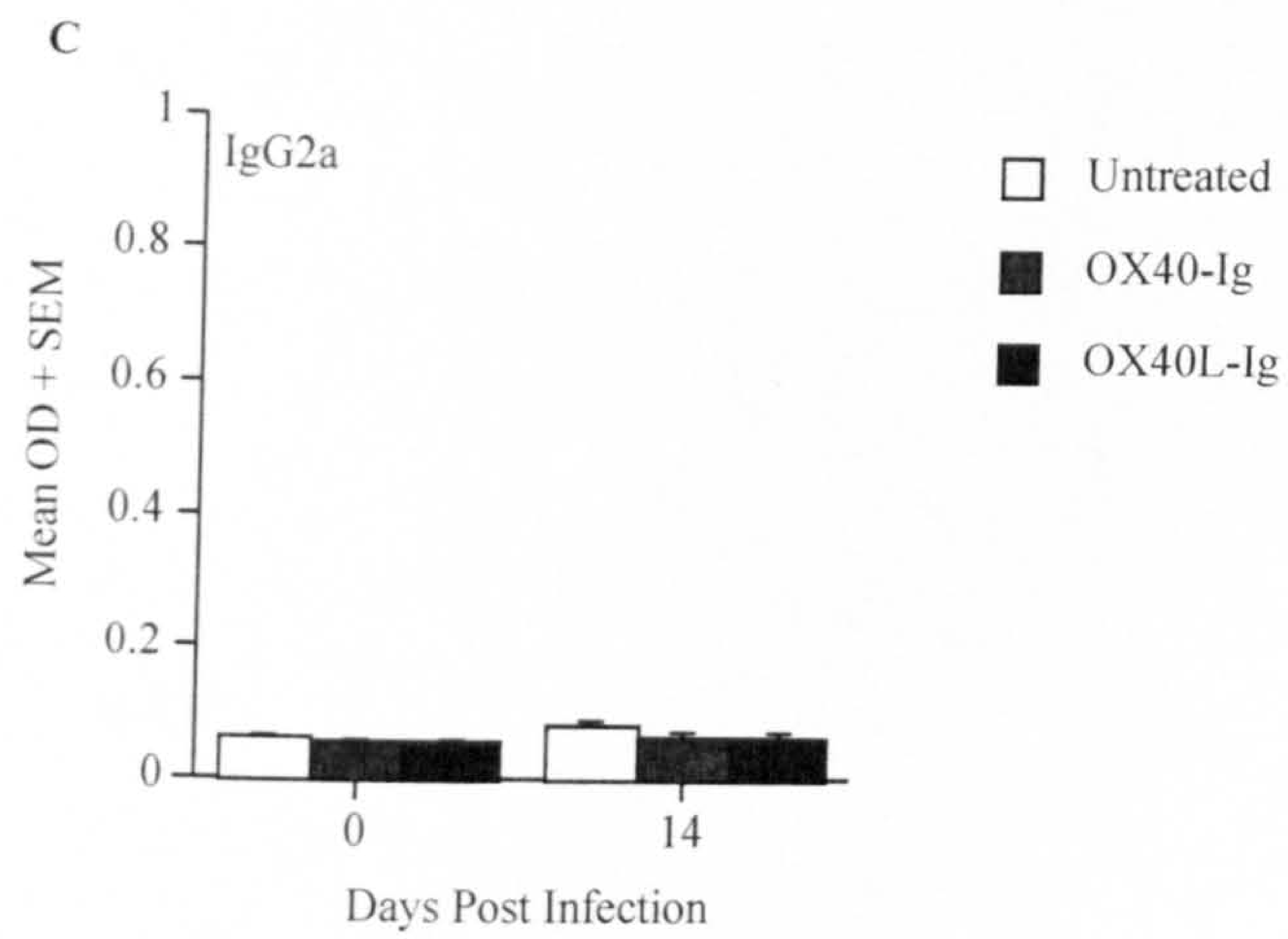
FIGURE 4.10: The effect of OX40-Ig and OX40L-Ig treatment on antibody responses following infection with *T. spiralis*. Total IgE (A) and TAg specific IgG1 (B) and IgG2a (C) titres were measured in sera taken at day 0 and 14 p.i. from untreated, OX40-Ig and OX40L-Ig treated mice. IgE titres were measured by sandwich ELISA against a purified IgE standard and were expressed as mean μg/ml + MAD. While antigen specific IgG1 and IgG2a titres were assessed by ELISA using 96 well microtitre plates coated with *TAg* at 2μg/ml, sera was serially diluted and optical density readings at 1:640 dilution were used. Data was expressed as mean optical density (OD) +SEM. *, represents significantly different to uninfected ($p \leq 0.05$). Five mice were used per group.




B




C



## *4.3 Discussion*

These studies show that modulating co-stimulatory signals to T cells during infection with *T. spiralis* can alter immune responses to the parasite and the development of enteropathy following infection.

ICOS

While blocking ICOS reduced the pathological consequences of the intestinal phase of *T. spiralis* infection it had no effect on the expulsion of adult worms from the gut. In sharp contrast ICOS blockade decreased the accumulation of muscle larvae. These changes were associated with a decrease in the Th2 responses as assessed by reduced levels of IgE and IL-4 and IL-5 production and a corresponding increase in IFN-γ production by MLN cells. Conversely, however, levels of IL-13 and IL-10 were elevated following treatment with anti-ICOS.

ICOS is thought to be involved in the maintenance of Th2 responses rather than in the induction of T helper response (Coyle *et al.*, 2000; McAdam *et al.*, 2000) and our model helps to confirm this. IL-4 and IL-5 levels were reduced while IFN-γ production was increased following treatment with anti-ICOS. Contrary to previous studies where blocking ICOS or the use of ICOS knock out mice resulted in decreased production of IL-4, IL-13 and IL-10, the production of IL-10 and IL-13 were increased by blocking ICOS in *T. spiralis* infection. While Th2 responses are an absolute requirement for the

expulsion of gastrointestinal helminths (Else *et al.*, 1993; Else *et al.*, 1994; Helmby *et al.*, 2001; Lawrence *et al.*, 1998), expulsion kinetics were unaffected by the decrease in the Th2 cytokines IL-4 and IL-5 following blocking ICOS. However, the increase in levels of IL-13 may have provided sufficient stimulus for the expulsion of the parasite. Indeed, IL-13 plays an important role in the expulsion of both *T. spiralis* and *Nippostrongylus brasiliensis* (Urban *et al.*, 1998). This enhanced response may also account for the reduction in muscle larvae burden following blocking ICOS. This apparent contradictory response in which IL-4 levels are reduced while IL-13 is increased has also been observed in studies where the CD28-B7 interaction was blocked during infection with *T. muris*; however, in this case this resulted in impaired expulsion of the parasite (Urban *et al.*, 2000). Blockade of ICOS during infection with *N. brasiliensis* also resulted in diminished IL-4 and IL-5 production; however, levels of IL-10 and IFN-γ were also reduced (Kopf *et al.*, 2000). The differences observed in the effects that this blockade has on the expulsion of the parasites might be the result of differences in the protective mechanism required for the expulsion of these two parasites. For instance, expulsion of *N. brasiliensis* is mediated by IL-13 and goblet cells with IL-4 and mast cells playing little or no role (Barner *et al.*, 1998; Crowle and Reed, 1981; Ishikawa *et al.*, 1993; Khan *et al.*, 1995), while expulsion of *T. spiralis* is mediated by a combination of IL-4 and IL-13 and mast cells (Donaldson *et al.*, 1996; Faulkner *et al.*, 1997; Lawrence *et al.*, 1998)

Previous work has suggested a role for mast cells and mMCP-1 in the expulsion of *T. spiralis* (Grencis *et al.*, 1993; Knight *et al.*, 2000). Although anti-ICOS treatment elevated the number of mast cells in uninfected mice it did not significantly alter the development of intestinal mastocytosis. However, serum mMCP-1 levels were significantly higher following treatment of anti-ICOS suggesting increased mast cell degranulation. Although this had no effect on the expulsion of the adult parasite it significantly reduced the accumulation of muscle larvae, indeed we have shown that while expulsion of worms from mMCP-1 -/- mice was slightly delayed, the decrease in the muscle larvae burden was more substantial (Knight *et al.*, 2000)

Consistent with a role for ICOS is mediating Th2-type responses, blocking ICOS or the use of ICOS deficient mice results in an amelioration of pathology in various models including allergic airway disease and chronic graft-versus-host disease (GvHD) (Ogawa *et al.*, 2001) (Gonzalo *et al.*, 2001b) (Tesciuba *et al.*, 2001) while exacerbating Th1 mediated pathologies such as experimental autoimmune encephalomyelitis (EAE) and GvHD (Guo *et al.*, 2002; Rottman *et al.*, 2001). Intestinal pathology in our model is mediated by IL-4, and this enteropathy was ameliorated following blocking of ICOS. While this reduction in pathology by blocking ICOS may mainly be mediated by the decreased production of IL-4 the increased production in IL-10 may also be important. This is in contrast to studies where the use of ICOS -/- mice or blockade of ICOS resulted in decreased levels of IL-10. IL-10 plays a crucial role in preventing immunopathology and it is thought that this is through the induction of T regulatory cells (Asseman *et al.*, 1999). IL-10 also plays a role in the pathology induced during

infection with GI parasites with IL-10 -/- mice suffering increased mortality compared to wild type mice when infected with *T. muris* (Schopf *et al.*, 2002). It is well known that nematode parasites exert immunomodulatory effects on their hosts and these appear to be mediated by IL-10 independent of Th2 responses (Paterson *et al.*, 2002) (Boitelle *et al.*, 2003). We therefore suggest that suppression of IL-10 by anti-ICOS in other models may be the result of a reduction in production of IL-10 by Th2 cells whereas blockade of ICOS during infection with *T. spiralis* may result in an elevation in IL-10 production possibly as a result of amplification of the regulatory T cell response.

Although expulsion of adult parasites was unaffected following treatment with anti-ICOS the muscle larvae burden was significantly lower suggesting some protection is afforded by the blockade of ICOS signalling. The killing of newborn larvae, is mediated by Th2 stimulated IgE and eosinophilia, (Bass and Szejda, 1979; Buys *et al.*, 1984) therefore it would be anticipated that blocking ICOS would enhance the accumulation of muscle larvae. The changes in permeability of the epithelium seen in the inflamed intestine (McDermott *et al.*, 2003) may facilitate the invasion of host tissues by newborn larvae. Consequently, reduced muscle larvae burdens obtained following anti-ICOS treated may be a reflection of the reduction in enteropathy during the intestinal phase of the infection.

In conclusion, we show that blocking ICOS during infection with a GI helminth decreased the production of the Th2 associated cytokines IL-4 and IL-5 but this had no significant effect on the expulsion of the adult parasite. Conversely, levels of IL-13 and

IL-10 were enhanced and this was associated with an enhanced response against the larval stage of the parasite and, importantly, an amelioration of the associated enteropathy. Moreover, this data confirms that enteropathy is not an essential component of protective response but may be beneficial to the parasite enhancing the penetration of newborn larvae through the mucosa and the resulting increase in muscle larvae. The mechanism by which IL-4 and IL-5 are suppressed by blocking ICOS while IL-13 and IL-10 are elevated requires further investigation.

OX40/OX40L

In contrast to anti-ICOS treatment OX40L and OX40L-Ig treatment does not alter the development of villus atrophy and crypt hyperplasia following infection with *T. spiralis*, the expulsion of adult worms from the small intestine or the accumulation of muscle larvae. OX40-Ig treatment however enhances the development of mucosal mastocytosis; despite the increase in mast cell numbers observed in the small intestine no increase were seen in serum mMCP-1 titres. The parasite associated Th2 response, as demonstrated by increased total IgE and MLN cell secretion of IL-4 and IL-13 was increased by treatment with OX40L-Ig but not OX40-Ig. Interestingly IL-18 secretion was also increased while IFN-γ secretion is not altered by OX40L-Ig.

In previous studies it has been shown that the development of enteropathy is dependent on the generation of IL-4 (Lawrence *et al.*, 1998) and Mast cell (Lawrence *et al.*, 2004) responses to *T. spiralis* infection. However despite the increase in IL-4 secretion seen in

MLN stimulated *ex vivo* with TAg from mice treated with OX40L-Ig no significant effects were observed in the development of enteropathy. In OX40-Ig treated mice a reduction in the proliferative capacity of crypt cells, as demonstrated by reduced mitotic figures/VCU was observed in spite of the lack of effect of OX40-Ig treatment on the development of crypt hyperplasia or villus atrophy, suggesting that treatment may be affecting the differentiation of stem cells in the crypt into enterocytes but also reducing the loss of cell from the tips of the villi. Even with a reduced rate of cell division, cells may therefore fail to differentiate into mature enterocytes and thus may build up in the crypt resulting in crypt hyperplasia.

This data suggests a complex relationship between the development of immune responses to *T. spiralis* and the development of enteropathy. The development of protective responses to *T. spiralis* infection has, like enteropathy, been shown to be dependent on IL-4 and on IL-13 (Lawrence *et al.*, 1998), and an important role for mast cells and the mast cell granule protease mMCP-1 has also been demonstrated (Donaldson *et al.*, 1996; Knight *et al.*, 2000). However despite increased IL-4, IL-13 secretion in OX40L-Ig treated and an increase in mast cell numbers in OX40-Ig treated mice, no significant effects were seen on either the establishment or the expulsion of *T. spiralis* from the small intestine.

In most cases an increase in mast cell numbers in the small intestine is been associated with an increase in serum mMCP-1 titres however in these experiments despite the enhanced mastocytosis observed in OX40-Ig treated mice, serum mMCP-1 titres were

not significantly altered. This suggests that while mast cell infiltration in to the small intestine is increased, possibly resulting from increased expression of integrins or chemokines, signalling to mast cells once in the intestine that results in degranulation are not significantly altered. Activated T cells have been shown to be able to stimulate the degranulation of bone marrow derived mast cells following direct cell-cell interaction *in vitro* (Bhattacharyya *et al.*, 1998), while human mast cells have been shown express OX40L and induce T cell proliferation via OX40 (Kashiwakura *et al.*, 2004). Thus treatment with OX40-Ig; the prevention of OX40L mediated T cell co-stimulation may result in T cells that are unable to fully activate mast cells. This may explain why despite enhancing mastocytosis OX40-Ig treatment failed to alter the development of enteropathy or the expulsion of adult worms from the small intestine. The longevity and fecundity of adult worms and the survival and establishment of larval worms are not significantly altered by OX40-Ig or OX40L-Ig treatment.

OX40-Ig and OX40L-Ig treatment during infection with *T. spiralis* fails to affect either the establishment or expulsion of adult worms, the accumulation of muscle larvae or the development of villus atrophy and crypt hyperplasia. OX40-Ig but not OX40L-Ig treatment also results in significantly increased accumulation of mast cells in the small intestine. However mast cell activation as measured by serum mMCP-1 levels are not elevated compared to un-infected or OX40L-Ig treatment. The mechanisms by which OX40-Ig treatment enhances mastocytosis requires further study, this treatment may affect the expression of molecules on the surface of intestinal vascular endothelial cells or on the surface of the mast cells themselves, that are involved in the migration of mast

cells into the mucosa. For example it has been shown that the integrins alpha-4 and beta-7 have been shown to be necessary for the entry of mast cells into the mucosa of *Nippostrongylus brasiliensis* infected mice (Issekutz *et al.*, 2001), thus if OX40 signalling has an inhibitory effect on the expression of molecules such as alpha-7 and beta-7 OX40-Ig treatment would result in increased numbers of mast cells in the small intestine. Another possibility is alteration in either the rate at which mast cell progenitors develop in the bone marrow or the rate at which they exit the bone marrow. As mMCP-1 titres are not altered in OX40-Ig treated mice compared to untreated the maturation of mast cells once in the small intestine may be impaired.

OX40L-Ig treatment, the activation of T cells via OX40, significantly enhances IL-4, IL-13 and IL-18 secretion by MLN cells stimulated *ex-vivo* with TAg and total serum IgE titres however MLN IFN-γ levels were unaffected as were antigen specific IgG1 and IgG2a. Both IL-13 and IL-4 have been implicated in the development of protective responses to *T. spiralis*. The expulsion of *T. spiralis* by IL-4 -/- mice is significantly delayed and the development of enteropathy ameliorated (Lawrence *et al.*, 1998), while IL-4Rα -/- mice show similar levels of enteropathy to IL-4 -/- (Lawrence *et al.*, 1998), expulsion of the adult worms is further delayed (Lawrence unpublished data). As both IL-13 and IL-4 signal via the IL-4Rα, the increased susceptibility of IL-4Rα -/- mice compared to IL-4 -/- mice is likely to be due to the loss of IL-13 function. IL-13 has been shown to be necessary for expulsion of other gastrointestinal nematodes such as *T. muris* (Bancroft *et al.*, 1998). It is therefore surprising that despite the increase in these

important cytokines in the OX40L treated mice that no effects were observed in either the development of enteropathy or in the expulsion of adult worms from the intestine.

IL-18 was originally identified and described as IFN-γ inducing factor, however further work has shown that IL-18 while being able to induce IFN-γ secretion, IL-18 also functions independently of IFN-γ in both Th1 and Th2 models. For example IL-18 -/- C57/BL6 mice have been shown to expel *T. spiralis* more rapidly and develop less pathology than wild type mice (Helmby and Grencis, 2002). However OX40L-Ig treatment resulted in elevated MLN IL-18 secretion *ex-vivo* but was not associated with increased resistance to *T. spiralis* or to the development of enteropathy. We have previously shown that IL-18 deficiency on a BALB/c background fails to affect worm expulsion or the development of enteropathy (Lawrence *et al* unpublished data). The role played by IL-18 during infection with *T. spiralis* may be dependent on host strain.

This data confirms a role for OX40/OX40L in the co-stimulation of Th2 cytokine responses however the inability of OX40L-Ig to alter Th2 dependent worm expulsion and enteropathy suggests that the downstream effects of these cytokines are not enhanced either because other important signals were not up regulated in this model by enhancing OX40 signalling to T cells. Local cytokine responses in the small intestine may also not have been as greatly affected as that seen in the MLN when stimulated ex-vivo due to the presence of other regulatory mechanisms.

Conclusions

Both ICOS and OX40 modulate the development of Th2 cytokine responses and mastocytosis, however while ICOS modulates the development of enteropathy and the accumulation of muscle larvae, OX40 was shown to play minimal role in enteropathy, worm expulsion and the accumulation of muscle larvae.

Both ICOS and OX40 have been shown to play important roles in the development of both Th1 and Th2 responses and in a variety of model systems and diseases such as arthritis (Iwai *et al.*, 2002; Yoshioka *et al.*, 2000), *Leishmania* infection (Akiba *et al.*, 2000; Greenwald *et al.*, 2002b) colitis, (Higgins *et al.*, 1999; Malmstrom *et al.*, 2001) and airway inflammation (Gonzalo *et al.*, 2001b; Jember *et al.*, 2001). We have shown that ICOS blockade results in a reduction in serum IgE and MLN IL-4 and IL-5 secretion while blocking OX40 did not affect Th2 responses. In contrast enhancing OX40 signalling to T cells, using OX40L-Ig resulted in enhanced MLN cytokine production and increased serum IgE titres. This suggests that in terms of the development of Th2 responses to *T. spiralis* ICOS plays a more important role than OX40 despite OX40's apparent ability to stimulate Th2 responses. Co-stimulatory signals have in a number of models been shown to play roles of differing importance CD28 B7-1/2 signalling in most cases has been shown to be the most important signal with the roles played by other co-stimulatory signals more clear with the use of anti-CD28, CD28 -/- mice or CTLA-4 fusion proteins (Chitnis *et al.*, 2001; Ozkaynak *et al.*, 2001; Tamura *et al.*, 2001; Villegas *et al.*, 2002). Thus it may be possible to dissect the precise functioning of ICOS and OX40 during *T. spiralis* infection by blocking CD28.

The blockade of both ICOS and OX40 affects mucosal mast cells. ICOS blockade resulted not in increased mast cell numbers but in increased mast cell degranulation as measured by serum mMCP-1 concentrations. Conversely, OX40 blockade increased the number of mast cells in the mucosa but not the degranulation of these mast cells. It has been shown that the activation of human mast cells can be stimulated by activated T cells (Bhattacharyya *et al.*, 1998), and human mast cells have been shown to express OX40L (Kashiwakura *et al.*, 2004). It has also been shown that mast cells contribute to the development of Th2 responses (Aoki *et al.*, 1999) and are able to act as antigen presenting cells (Fox *et al.*, 1994; Frandji *et al.*, 1993; Frandji *et al.*, 1996; Frandji *et al.*, 1995). These data taken together suggest a two-way relationship between T cell activation and the development of mast cells and mast cell activation.

In conclusion, ICOS blockade inhibits the development of Th2 mediated enteropathy as well as the Th1 enteropathies. Unlike ICOS, OX40 did not appear to play a significant role in *T. spiralis* induced enteropathy, despite the role for OX40 in Th1 mediated enteropathies (Malmstrom *et al.*, 2001; Obermeier *et al.*, 2002; Souza *et al.*, 1999; Totsuka *et al.*, 2002).

Importantly, blocking the interaction of ICOS could prevent the induction of pathological responses without compromising the development of protective responses. These results suggest that ICOS may be a useful target for Th2 mediated inflammatory conditions whilst not affecting Th2-mediated protective responses.

Inhibition OX40-Ig interactions in our model failed to significantly alter the Th2 response to *T. spiralis,* while reducing the development of intestinal oedema, this in conjunction with evidence that the blockade of OX40 ameliorates Th1 induced enteropathy in mouse models (Higgins *et al.*, 1999) suggests that OX40 blockade could provide a potential therapeutic target for Th1 but not Th2 mediated pathologies. Furthermore in murine Influenza, weight loss and pulmonary inflammation are significantly ameliorated by the blockade of OX40 signalling by OX40-Ig (Humphreys *et al.*, 2003b). However it has been shown that OX40 signalling is essential for the development of airway inflammation in murine asthma (Jember *et al.*, 2001) while enhancing OX40 signalling using OX40L-Ig enhances protective responses to *Cryptococcus neoformans*, reducing pulmonary eosinophilia in an IFN-γ, IL-12 dependent fashion (Humphreys *et al.*, 2003a). The enhancement of intestinal mastocytosis by OX40-Ig, however could prove problematic in humans if there are pre-existing mast cell mediated conditions such as atopy. Enhancing OX40 co-stimulation in this model enhanced Th2 responses but did not alter the development of pathology or protection. However it has been suggested that enhancing OX40 co-stimulation may have potential use as adjuvants in anti-cancer vaccine therapies (Kjaergaard *et al.*, 2000; Weinberg *et al.*, 2000), these data suggest that this may enhance unwanted Th2 responses which could potentially be hazardous in asthmatic or atopic patients.

These data suggest that the manipulation of OX40 or ICOS co-stimulatory signals may have potential use in therapies. However, further study is required into the effects that this may have on unrelated responses.

# Chapter Five

## The role of transmembrane TNFα, soluble TNFα and LTα in the development of enteropathy and protective responses.

# 5 The role of transmembrane TNFα, soluble TNFα and LTα in the Development of enteropathy and protective responses following infection with *Trichinella spiralis*.

## *5.1 Introduction*

TNFα is synthesised as a 26kDa precursor, which may be proteolytically cleaved by the membrane bound matrix metalloprotease, TNFα converting enzyme (TACE) (Moss *et al.*, 1997) into a 17kDa-secreted monomer that forms trimers (sTNFα) (McGeehan *et al.*, 1994) (Gearing *et al.*, 1994). However, if TNFα is not cleaved, it will then be present as a type II transmembrane protein (Kriegler *et al.*, 1988), tmTNFα. Lymphotoxin (LT)α is a member of the TNF superfamily that can form homotrimers ($LT\alpha_3$) or heterotrimers with the transmembrane protein LTβ ($LT\alpha_1LT\beta_2$ or $LT\alpha_2LT\beta_1$) (Browning *et al.*, 1993), $LT\alpha_3$ signals via the p55 and p75 TNF-Rs while $LT\alpha_1LT\beta_2$ signals via the lymphotoxin β receptor (LTβR).

LTα, LTβ and TNFα have been shown to play important roles in the development of the structure of the immune system, i.e. lymph node organogenesis and germinal centre and marginal zone development in nodes and spleen. Signalling via LTβR has been shown to be essential for the development of peripheral lymph nodes (although a small percentage of LTβR -/- mice may have mesenteric or cervical nodes); for the proper formation of germinal centres (Futterer *et al.*, 1998), and for the development of Peyer's patches,

where signalling via LTβR has been shown to up regulate the expression of the chemokine CCL20 in the intestinal epithelium (Rumbo *et al.*, 2004), which is important in the recruitment of immature CD11d+ myeloid dendritic cells into Peyer's patches (Dieu-Nosjean *et al.*, 2000; Iwasaki and Kelsall, 2000). LTα -/- mice also do not possess peripheral lymph nodes, and lack Peyer's patches (Alimzhanov *et al.*, 1997), while TNFα -/- mice possess normal lymph nodes but fail to form proper germinal centres (Pasparakis *et al.*, 1997).

TNFα is a pro-inflammatory cytokine generally considered to play important roles in Th1 mediated protective and pathological responses. TNFα has been shown to be of particular importance in the control of *Mycobacterium tuberculosis* infection, in both mice and humans. In mice, latent *M. tuberculosis* infection can be established using chemotherapy following i.v. infection, in TNFα -/- mice. This latent infection is rapidly reactivated and fatal following cessation of chemotherapy (Botha and Ryffel, 2003). Similarly in humans, where latent *M. tuberculosis* infection occurs naturally, the reactivation of latent *M. tuberculosis* is an important side effect of anti-TNFα therapy for conditions such as rheumatoid arthritis and Crohn's disease (Ehlers, 2003). TNFα has also been shown to play an important role in the development of neutrophil infiltration and protective responses at the site of bacterial infection (Malaviya *et al.*, 1996). The *in vitro* lysis of L929 cells secreting a soluble p55 TNF-R, which prevents TNFα binding to membrane p55 TNF-R, by L929 reactive cytotoxic lymphocytes (CTL)

is reduced suggesting that TNFα is important in CTL mediated immunity (Selinsky *et al.*, 1998).

In spite of the importance of TNFα in protective responses to pathogens and tumour cells, TNFα has also been implicated in a variety of immunopathological conditions, for example, astrocytes from rat strains susceptible to experimental autoimmune encephalitis (EAE) express TNFα at higher levels in response to the Th1 cytokine IFN-γ when compared to more resistant strains (Chung *et al.*, 1991). TNFα has also been shown to be essential for the development of autoimmune arthritis in IL-1 receptor antagonist deficient mice, while anti-TNFα treatment has been shown to inhibit the exacerbation of established collagen induced arthritis in mice (Williams *et al.*, 1994). In murine graft-vs-host disease anti-TNFα treatment has been shown to inhibit the development of intestinal and skin lesions (Piguet *et al.*, 1987). Further more, anti-TNFα therapy (inifliximab) has been successfully used to treat patients following intestinal transplantation suffering from acute graft rejection (Pascher *et al.*, 2005).

A number of studies also implicate TNFα in Th2 mediated immune responses. For example *in vitro* treatment of purified human blood eosinophils with TNFα has been shown to increase viability and to induce cytokine secretion (Uings *et al.*, 2005). Expulsion of *T. muris* has been shown to be dependent on Th2 mediated cytokine responses in particular IL-13, anti-TNFα treatment of IL-4 -/- mice delays parasite expulsion, suggesting that TNFα plays an important role in the IL-13 mediated

expulsion of *T. muris* (Artis *et al.*, 1999). Mast cells have also been shown to be an important source of TNFα in humans (Bischoff *et al.*, 1999) and in mice (Gordon and Galli, 1990). Furthermore TNFα has been shown to be necessary for the development of mast cell mediated gastric allergic inflammation (Furuta *et al.*, 1997).

TmTNFα has been shown to be the major activating ligand of p75TNF-R (Grell *et al.*, 1995), which has been shown to suppress the pro-inflammatory activity of TNFα signalling via p55TNF-R (Peschon *et al.*, 1998) thus as an activator of p75TNF-R, tmTNFα may play a role in the inhibition of TNFα mediated pathology. However, evidence for important pro-inflammatory functions for tmTNFα in both protection against pathogens and in immunopathologies have been demonstrated. Mueller *et al* (1999) have generated a non-cleavable murine TNFα and cloned it into TNFα/LTα -/- mice (Mueller *et al.*, 1999). TmTNFα and soluble TNFα have been shown to exert different effects both in vitro and in vivo. The expression of tmTNFα on CD4+ T cells, has been shown to provide a co-stimulatory signal to human B cells (Aversa *et al.*, 1993). TmTNFα-tg mice have also been shown to be resistant to *M. bovis* bacillus Calmette-Guérin while TNFα/LTα -/-, TNFα -/- and LTα -/- mice succumb to infection. TmTNFα-tg mice were also more resistant to the more virulent *M. tuberculosis* infection than TNFα/LTα -/- mice but, show a higher bacterial load than wild type mice (Olleros *et al.*, 2002). Mice over expressing tmTNFα are prone to developing arthritis (Alexopoulou *et al.*, 1997) and mice expressing only tmTNFα are susceptible to ConA induced liver disease (Kusters *et al.*, 1997). While tmTNFα-tg mice, treated with LPS

and D-galactosamine, suffer reduced mortality compared to wild type mice. These data suggest that tmTNFα and sTNFα play independent roles during immune responses.

LTα, LTβ and the LTβR have also been shown to play important roles in the development of inflammation and in protection against pathogens. For example, wild type mice reconstituted with LTα -/- bone marrow have been shown to be more susceptible to hepatic *Leishmania donavani* than wild type mice (Engwerda *et al.*, 2004). The blockade of LTβR signalling using a soluble LTβR immunoglobulin fusion protein prevented the development of colitis in CD45RB$^{hi}$CD4+ reconstituted SCID mice (Mackay *et al.*, 1998). While in *Citrobacter rodentum* induced colitis the blockade of LTβR signalling resulted in increased pathology and bacterial loads (Spahn *et al.*, 2004). LTβ -/- mice have been shown develop impaired cytotoxic T cell responses to lymphocytic choriomeningitis virus, this is thought to relate to impaired splenic structure (Berger *et al.*, 1999).

TNFα is thought to play an important role in the development of inflammatory bowel diseases (IBD), such as ulcerative colitis and Crohn's disease, with increased TNFα secretion from mononuclear cells isolated from the lamina propria of IBD patients (Noguchi *et al.*, 1998). Furthermore, it has been shown that the treatment of wild type mice with exogenous TNFα leads to the rapid development of severe intestinal lesions characterised by villus atrophy, with apoptotic epithelial cells and oedema (Guy-Grand *et al.*, 1998). LTα has also been shown to play a role in the development of colitis in

murine models, for example the blockade of signalling via LTβR inhibits the development of colitis in CD45Rb$^{hi}$CD4+ reconstituted SCID mice (Mackay *et al.*, 1998).

Previous studies using p55 TNFR -/- mice suggest that TNFα plays a role in the development of enteropathy but not in the expulsion of *T. spiralis* from the small intestine (Lawrence *et al.*, 1998) while p75 TNFR -/- mice developed increased pathology suggesting that signalling via this receptor played a role in the regulation of pathology (Lawrence, unpublished data). Earlier studies implicate TNFα signalling through the TNFR1 (p55) in the development of Th2 mediated enteropathy during infection with *T. spiralis;* furthermore these studies showed that this pathological response is also dependent on IL-4 (Lawrence *et al.*, 2000). TNFR1 signalling does not however appear to play a role in the development of protective responses to *T. spiralis* whilst IL-4 did (Lawrence *et al.*, 1998 and Lawrence *et al.*, 2000). Conversely during *Trichuris muris* infection, TNFα in conjunction with IL-13 has been shown to be necessary for the development of protection (Artis *et al.*, 1999). TNFR2 (p75) -/- mice infected with *T. spiralis* have been shown to suffer increased enteropathy in comparison with wild type mice suggesting that TNFα signalling through TNFR2 may inhibit the development of pathology (Lawrence *et al.* unpublished data). These findings are consistent with studies suggesting that TNFR2 acts as an antagonist of TNFR1 signalling (Peschon *et al.*, 1998).

Mast cells have been implicated in the development of both enteropathy and protective responses during infection with *T. spiralis*. The expulsion of the gastrointestinal parasite *T. spiralis* has been shown to coincide with the development of mucosal mastocytosis in both rats (Woodbury *et al.*, 1984) and mice (Lawrence *et al.*, 1998), and be delayed in mice lacking mucosal mast cell protease (mMCP)-1 (Knight *et al.*, 2000). Enteropathy also appears to be dependent on mast cells, and mMCP-1 (Lawrence *et al.*, 2004). Interestingly it has been shown that mast cells contain pre-formed TNFα (Bischoff *et al.*, 1999) and are an important source of TNFα in the intestine (Bischoff *et al.*, 1999).

Thus, it is of interest to further evaluate the roles played by TNFα and LTα in the development of immune responses to *T. spiralis* infection. In this study the roles of tmTNFα, sTNFα and LTα in the development of protective and pathological responses to *T. spiralis* was investigated using TNFα/LTα -/- and tmTNFα mice. The TNFα/LTα -/- mice were on a C57BL/6 background while the tmTNFα-tg mice were on a C57BL/6 × 129 background deficient in TNFα and LTα.

### 5.2 *Results.*

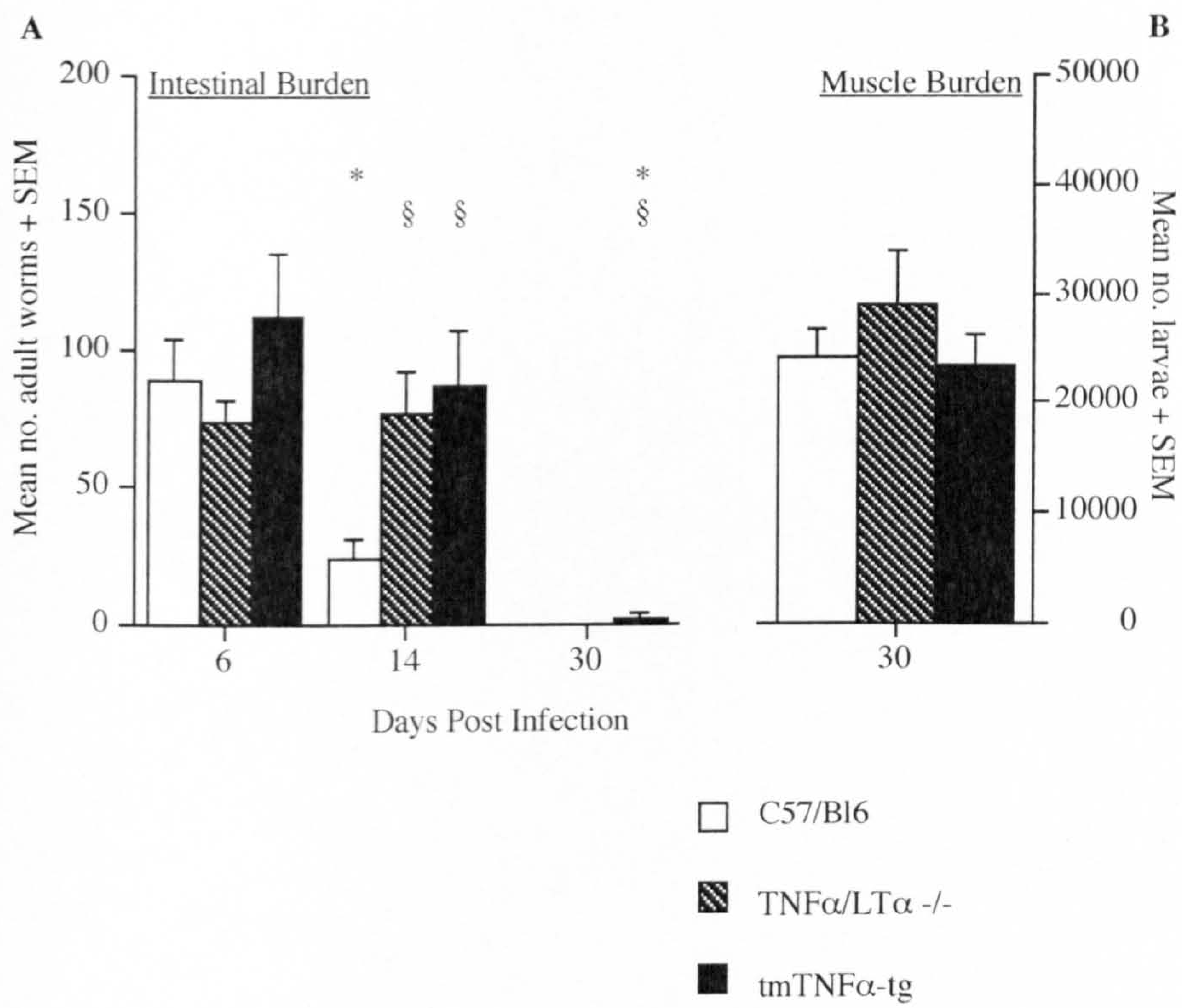
#### 5.2.1 *Expulsion of adult* T. spiralis *is delayed in the absence of soluble TNFα and LTα.*

To assess the effect of the absence of TNFα and LTα and the role played by tmTNFα in the development of immune expulsion of *T. spiralis* wild type, TNFα/LTα -/- and tmTNFα-tg mice were infected with 300 freshly isolated *T. spiralis* larvae. Gut burdens were measured at day 6 p.i. to assess the establishment of the parasite within the small intestine and again at days 14 and 30 p.i. to measure the expulsion of the worms. The number of muscle larvae present may act as an indicator of the length of time adult worms were present in the intestine and as a measure of the viability of the adult worms. The total number of larvae harboured by wild type, TNFα/LTα -/- and tmTNFα-tg mice was measured at day 30 p.i.

Adult *T. spiralis* establishment at day 6 p.i. in the intestine is not significantly different between the strains. However wild type mice have significantly reduced intestinal worm burdens at day 14 p.i. (p=0.009) compared to day 6 p.i. Both TNFα/LTα-/- and tmTNFα-tg mice however, had failed to expel the adult worms having significantly higher intestinal worm burdens compared to wild type mice at day 14 p.i. (p=0.016 and 0.0283). No significant differences were observed between TNFα/LTα -/- and tmTNFα-tg mice at day 14 p.i. By day 30 p.i. both, wild type and TNFα/LTα-/- mice had

completely expelled their worms while a few worms were still found in the intestines of tmTNFα-tg mice ($p=0.0367$ compared to wild type mice) suggesting that worm expulsion was more severely delayed in the tmTNFα-tg mice compared to TNFα/LTα -/- mice (Figure 5.1A). Muscle burdens obtained 30dpi from tmTNFα-tg and TNFα/LTα -/- mice were not significantly different from those obtained from wild type mice (Figure 5.1B).

FIGURE 5.1: The role of LTα, sTNFα and tmTNFα in the expulsion of *T. spiralis* from the intestine and the accumulation of muscle larvae. A) The establishment and expulsion of *T. spiralis* was measured in wild type, TNFα/LTα -/- and tmTNFα-tg mice at day 6 and 14 p.i. The small intestine was excised and the total number of worms present in the were counted. B) The accumulation of muscle larvae was measured in wild type, TNFα/LTα -/- and tmTNFα-tg mice at day 30 p.i. Data is expressed as mean number of worms/mouse + SEM, five mice were used per group except for uninfected, tmTNFα-tg where four mice were used. *, represents significantly different to mice at day 6 p.i. §, represents significantly different to wild type mice ($p<0.05$).


A
B
Intestinal Burden
Muscle Burden
200
150
100
50
0
Mean no. adult worms + SEM
*
§ §
*
§
6
14
30
Days Post Infection
50000
40000
30000
20000
10000
0
Mean no. larvae + SEM
30
C57/Bl6
TNFα/LTα -/-
tmTNFα-tg

### *5.2.2 Enteropathy is enhanced in the absence of soluble TNFα and LTα.*

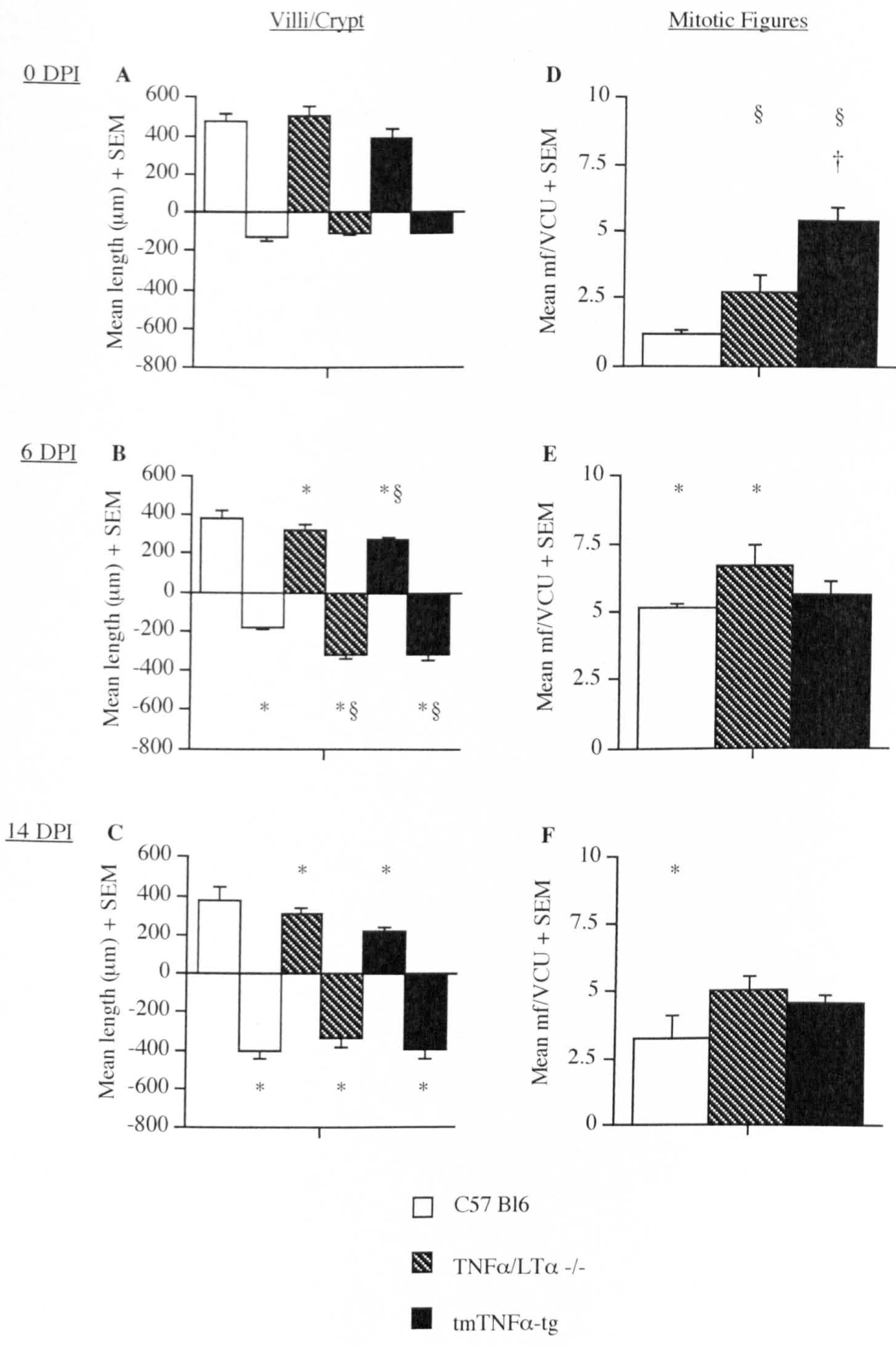
In order to assess the effect of the absence of TNFα and LTα and the role played by tmTNFα in enteropathy, jejunum samples were taken from uninfected and infected, wild type, TNFα/LTα -/- and tmTNFα-tg mice at day 6 and 14 p.i. These samples fixed in Clarke's fluid and were stained with Schiff's reagent and micro-dissected. Villus and crypt lengths were measured using an eyepiece graticule and the numbers of dividing cells (mitotic figures) in crypts were counted.

No significant evidence of gross intestinal abnormality was seen in uninfected mice of any strain used in this experiment (Figure 5.2A). As expected crypt hyperplasia had developed by day 6 and 14 dpi ($p=0.0283$ and 0.0143) in wild type mice. (Figure 5.2A, B and C). Villi lengths are significantly longer in TNFα/LTα -/- and tmTNFα-tg mice at days 6 ($p=0.0275$ and 0.05) and 14 p.i. ($p=0.05$ and 0.0143) compared to uninfected TNFα/LTα -/- and tmTNFα-tg mice (Figure 5.2A and B). Villus lengths at day 6 p.i. were significantly shorter in tmTNFα-tg ($p=0.0282$) compared to those of wild type mice. Crypt lengths were also significantly longer in TNFα/LTα -/- ($p=0.0143$) and tmTNFα-tg ($p=0.009$) mice compared to wild type mice at day 6 p.i. No significant differences were observed between tmTNFα and TNFα/LTα -/- mice at day 6 p.i. (Figure 5.2B). At day 14 p.i. the villi lengths of tmTNFα-tg mice were reduced compared to wild type mice ($p=0.0282$). No significant differences were observed in

villi or crypt lengths between TNFα/LTα -/- and tmTNFα-tg mice at any time point assessed (Figure 5.2C).

Although crypt and villus lengths in uninfected TNFα/LTα -/- and tmTNFα-tg mice were not significantly different to wild type mice, the number of mitotic figures per VCU were significantly higher in uninfected TNFα/LTα -/- ($p= 0.0189$) and tmTNFα-tg (0.0139) mice compared to uninfected wild type mice, furthermore uninfected tmTNFα-tg mice had significantly more mitotic figures/VCU than TNFα/LTα -/- mice ($p=0.0209$) (Figure 5.2A). Following infection with *T. spiralis* the number of mitotic figures/VCU were increased in infected wild type ($p=0.0086$ and 0.0143) mice at days 6 and 14 p.i. compared to uninfected wild type mice (Figure 5.2C, D and E). The number of mitotic figures in infected TNFα/LTα -/- mice was significantly higher than uninfected TNFα/LTα -/- mice at day 6 p.i. ($p=0.0209$) however by day 14 p.i. the number of mitotic figures was not different from that observed in uninfected mice. The number of mitotic figures is not significantly increased in tmTNFα-tg -/- at days 6 and 14 p.i. compared to uninfected tmTNFα-tg mice. No significant differences were observed in the number of mitotic figures /VCU between the strains at days 6 and 14 p.i. (Figure 5.2C, D and E).

FIGURE 5.2: The role of LTα, sTNFα and tmTNFα in the development of enteropathy following infection with *T. spiralis*. Villus and crypt lengths were measured in uninfected mice (A) and in infected mice at days 6 (B) and 14 p.i. (C). The number of mitotic figures per crypt was determined in uninfected mice (D) and in infected mice at days 6 (D) and 14 p.i. (E). Data is expressed as mean + SEM, five mice were used per group except for uninfected, tmTNFα-tg where four mice were used. *, represents significantly different to uninfected mice; §, represents significantly different to wild type mice; †, represents significantly different to TNFα/LTα -/- mice ($p<0.05$).

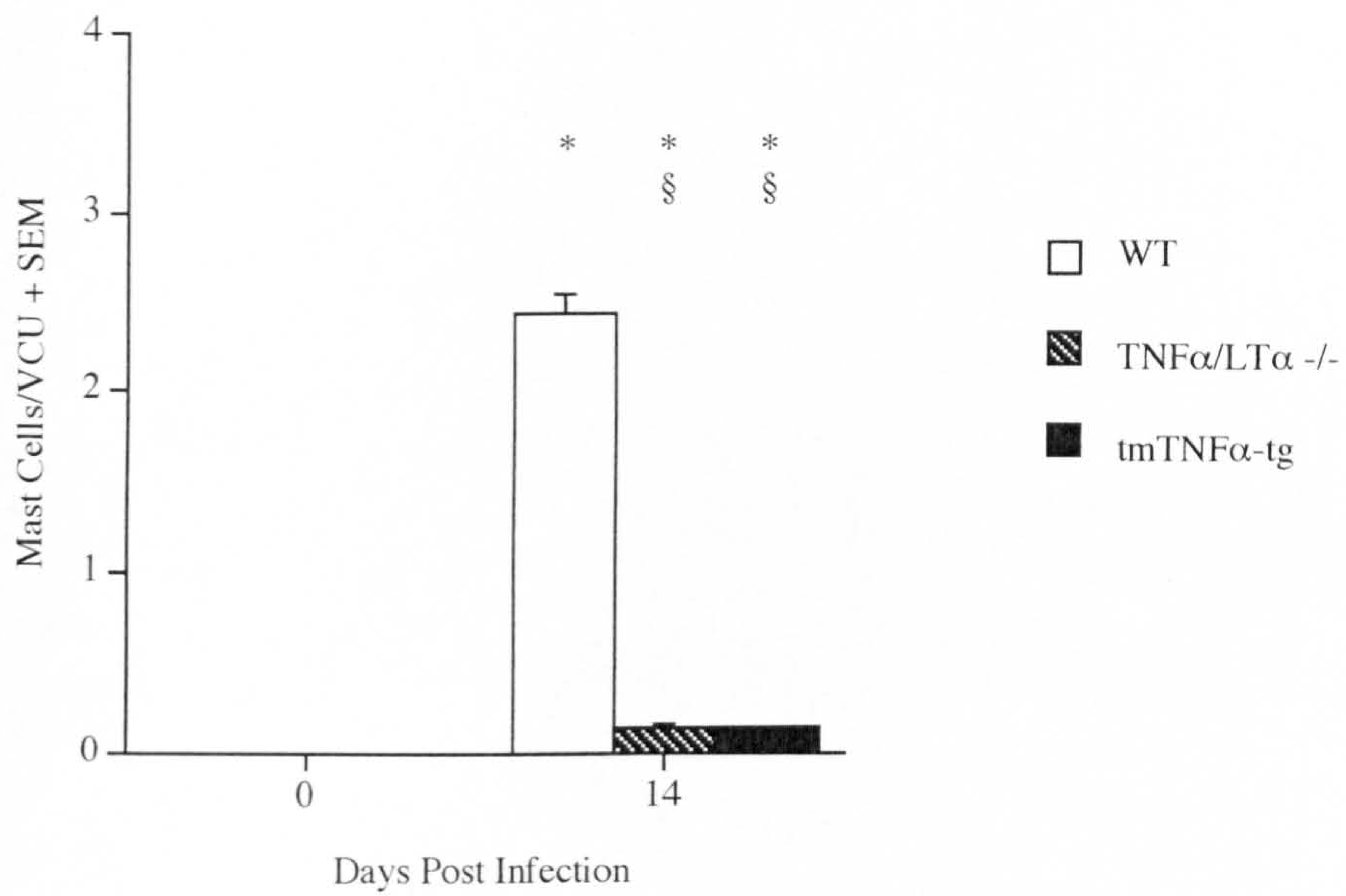

Villi/Crypt
Mitotic Figures
0 DPI
A
D
6 DPI
B
E
14 DPI
C
F
Mean length (μm) + SEM
Mean mf/VCU + SEM
600
400
200
0
-200
-400
-600
-800
10
7.5
5
2.5
0
C57 Bl6
TNFα/LTα -/-
tmTNFα-tg

### 5.2.3 *Mucosal mastocytosis is significantly reduced in the absence of soluble TNFα and LTα.*

Mastocytosis is an important feature of infection with *T. spiralis* and mast cells have been implicated in the development of both enteropathy and expulsion following infection (Knight *et al.*, 2000; Lawrence *et al.*, 2004). To assess the role of LTα, sTNFα and tmTNFα in the development of mastocytosis following infection with *T. spiralis* the number of mast cells/ VCU was counted in toluidine blue stained sections of jejunum from uninfected (day 0) and infected (day 14 p.i.), wild type, TNFα/LTα -/- and tmTNFα-tg mice.

No significant differences were observed between uninfected wild type, TNFα/LTα -/- and tmTNFα-tg mice. Significant mastocytosis had developed in wild type ($p=0.009$), TNFα/LTα -/- ($p=0.0285$) and tmTNFα-tg ($p=0.0339$) mice by 14 dpi. The number of mast cells observed in the TNFα/LTα -/- ($p=0.0082$) and tmTNFα-tg ($p=0.0215$) mice was significantly lower than the number observed in wild type mice (Figure 5.3).

FIGURE 5.3: The role of LTα, sTNFα and tmTNFα in the development of mucosal mastocytosis following infection with *T. spiralis*. Carnoy's fixed jejunum from uninfected and infected (day 14 p.i) wild type, TNFα/LTα -/- and tmTNFα-tg mice were processed and stained with 0.5% toluidine blue, revealing mast cells. The numbers of mucosal mast cells were counted in 20 randomly selected villus crypt units (VCU). Data expressed as mean + SEM, Five mice were used per group, except for uninfected tmTNFα-tg mice where four mice were used. *, represents significantly different to uninfected; §, represents significantly different to wild type ($p<0.05$).



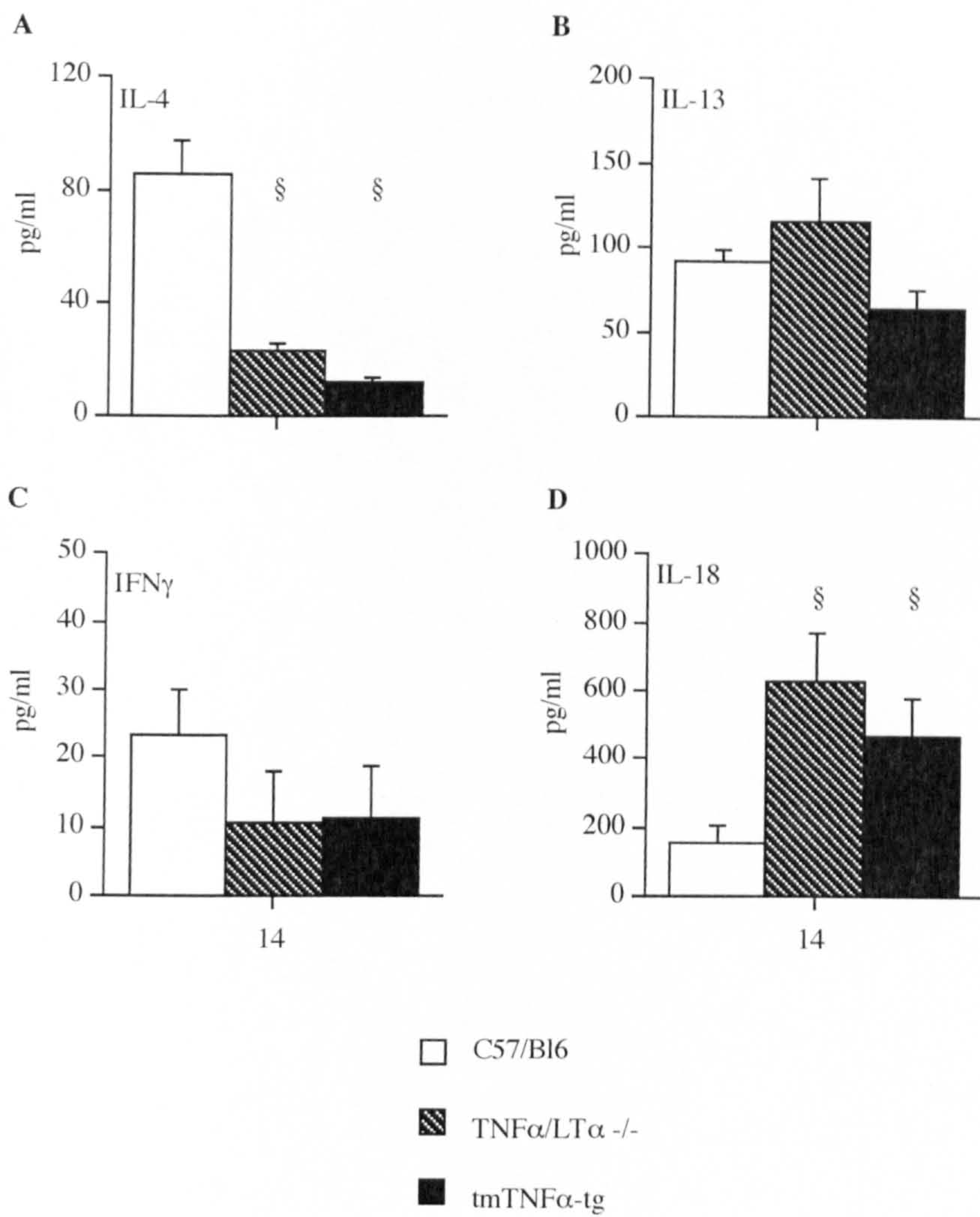
Mast Cells/VCU + SEM
4
3
2
1
0
*
*
§
*
§
0
14
Days Post Infection
WT
TNFα/LTα -/-
tmTNFα-tg

### 5.2.4 *In the absence of soluble TNFα and LTα the production of IL-4 is reduced but secretion of IL-13 and IFNγ are unaffected.*

In order to assess the effect of the knock out and the transgenic on the Th1:Th2 balance during *T. spiralis* infection spleen cells from wild type, TNFα/LTα and tmTNFα-tg mice 14 dpi were cultured in the presence or absence of *Trichinella* antigen (TAg). Spleen cells were used in this experiment because TNFα/LTα -/- and tmTNFα-tg mice lack mesenteric lymph nodes. The concentration of the Th2 cytokines IL-4 and IL-13 and the Th1 cytokine IFNγ secreted into the growth media was measured by ELISA against purified recombinant protein standards.

IL-4 production TAg stimulated spleen cells *ex vivo* at day 14 p.i. was significantly reduced in TNFα/LTα -/- ($p = 0.009$) and tmTNFα-tg ($p = 0.009$) mice compared to wild type mice. Production of IL-4 was not significantly different between tmTNFα-tg and TNFα/LTα -/- mice (Figure 5.4A). IL-13 production was not significantly altered in either strain of mouse compared to wild type mice (Figure 5.4B). IFNγ production was not significantly altered in either strain of mice compared to wild type (Figure 5.4C). IL-18 production by TAg stimulated spleen cells cultured ex vivo was significantly increased in TNFα/LTα -/- ($p = 0.0163$) and tmTNFα-tg ($p = 0.0163$) mice compared to wild type at day 14 p.i.

FIGURE 5.4: Role of LTα, sTNFα and tmTNFα on spleen cell cytokine secretion following infection with *T. spiralis*. Spleens were removed from wild type, TNFα/LTα -/- and tmTNFα-tg mice at day 14 p.i. Single cell suspensions were made, samples pooled and cultured at $1\times10^6$ cells with 50μg/ml TAg. The secretion of the cytokines IL-4 (A), IL-13 (B), IFN-γ (C) and IL-18 (D) were measured in the culture supernatants by ELISA against recombinant standards. Data expressed mean cytokine concentration in pg/ml + SEM, five mice were used per group; §, represents significantly different to wild type mice ($p<0.05$).


A
120
IL-4
80
§
§
pg/ml
40
0
B
200
IL-13
150
pg/ml
100
50
0
C
50
IFNγ
40
pg/ml
30
20
10
0
14
D
1000
IL-18
§
§
800
pg/ml
600
400
200
0
14
C57/Bl6
TNFα/LTα -/-
tmTNFα-tg

### *5.2.5 Total serum IgE is significantly reduced in the absence of soluble TNFα and LTα.*

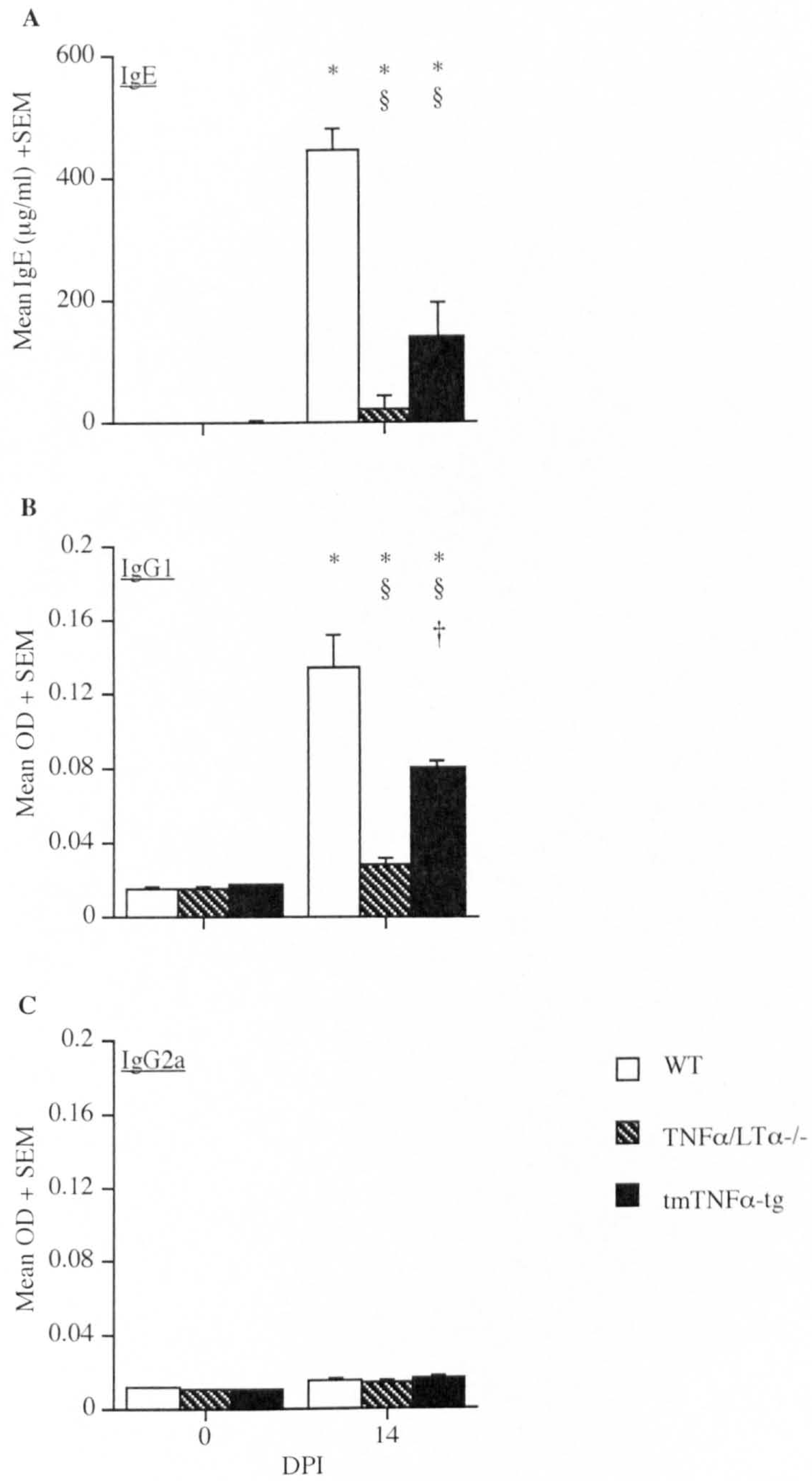
In order to further evaluate the in vivo Th1:Th2 balance serum samples were taken from uninfected (0 dpi) and *T. spiralis* infected (days 14 and 30 p.i.) wild type, TNFα/LTα -/- and tmTNFα-tg mice. Total IgE titres were measured in serum from uninfected and infected (14 and 30 dpi) mice by ELISA against a standard and relative TAg specific IgG1 and IgG2a titres were in sera from uninfected and infected (day 14 p.i.) mice measured by ELISA, sera was serially diluted and the optical density was recorded, data for the 1 in 640 dilution is shown.

Serum total IgE is measured at days 0, 14 and 30 p.i., IgE titres were increased at day 14 p.i. compared to uninfected (day 0 p.i.) in wild type, TNFα/LTα -/-, tmTNFα-tg mice. However IgE levels were reduced in TNFα/LTα -/- and tmTNFα-tg mice compared to wild type at day 14 p.i. ($p=0.0082$ and $p=0.0088$) and at day 30 p.i. ($p=0.0088$ and $p=0.0088$) (Figure 5.5A).

TAg specific IgG1 titres were elevated following infection in wild type, TNFα/LTα -/- and tmTNFα-tg mice compared to uninfected ($p=0.0$). However, TAg specific IgG1 levels were significantly lower in TNFα/LTα -/- ($p=0.009$) and tmTNFα-tg($p=0.009$) mice compared to wild type mice at 14 dpi. tmTNFα-tg also had significantly higher IgG1 titres than TNFα/LTα -/- mice ($p=0.009$) at day 14 p.i. (Figure 5.5B). TAg specific IgG2a titres were not significantly elevated following infection and no significant

differences were observed between wild type, TNF$\alpha$/LT$\alpha$ -/- or tmTNF$\alpha$-tg mice (Figure 5.5C).

FIGURE 5.5: Role of LTα, sTNFα, tmTNFα in antibody responses following infection with *T. spiralis*. Total IgE (A) and TAg specific IgG1 (B) and IgG2a (C) titres were measured in sera taken uninfected and infected (day 14 p.i.) wild type, TNFα/LTα and tmTNFα-tg mice. IgE titres were measured by sandwich ELISA against a purified IgE standard and were expressed as mean μg/ml + SEM. While antigen specific IgG1 and IgG2a titres were assessed by ELISA using 96 well microtitre plates coated with *TAg* at 2μg/ml, sera were serially diluted and optical density readings at 1:640 dilution were used. Data was expressed as mean optical density (OD) + SEM; five mice were used per group, except for uninfected tmTNFα-tg mice where four mice were used. *, represents significantly different to uninfected; §, represents significantly different to wild type mice; †, represents significantly different to TNFα/LTα -/- mice ($p<0.05$).



A
600
400
200
0
IgE
Mean IgE (μg/ml) +SEM
*
*
§
*
§
B
0.2
0.16
0.12
0.08
0.04
0
IgG1
Mean OD + SEM
*
*
§
*
§
†
C
0.2
0.16
0.12
0.08
0.04
0
IgG2a
Mean OD + SEM
0
14
DPI
WT
TNFα/LTα-/-
tmTNFα-tg

*5.3 Discussion.*

In this study we have demonstrated that in the absence of LTα and sTNFα mice show significantly delayed expulsion of *T. spiralis* from the intestine and enhanced enteropathy. Expulsion in tmTNFα-tg mice was further delayed and villus atrophy was significantly enhanced compared to TNFα/LTα-/- mice. The IL-4 response elicited by *ex vivo* re-stimulation of spleen cells was significantly reduced in TNFα/LTα-/- and tmTNFα-tg mice whilst IL-13 production was unaffected. IL-18 secretion was significantly increased; however, despite IL-18 being first identified as IFNγ inducing factor, IFNγ production was not significantly different in TNFα/LTα -/- and tmTNFα-tg mice compared to wild type mice. In agreement with the reduced IL-4 secretion, IgE production was significantly reduced in TNFα/LTα -/- and tmTNFα-tg mice.

The expulsion of *T. spiralis* has previously been shown to be mediated by T cells (Korenaga *et al.*, 2001; Manson-Smith *et al.*, 1979), in particular Th2 cells (Grencis *et al.*, 1991), expulsion being delayed in IL-4 -/- mice (Lawrence *et al.*, 1998) and IL-13 -/- mice (McDermott *et al.*, 2005). It has also been shown that p55TNF-R -/- (Lawrence *et al.*, 1998) and p75 TNF-R -/- (Lawrence *et. al.* unpublished data) mice expel *T. spiralis* with the same kinetics as wild type mice. In contrast, this study shows that *T. spiralis* expulsion is significantly delayed in TNFα/LTα -/- and tmTNFα-tg mice. LTα can form homotrimers ($LT\alpha_3$) which signals through p55 and p75 TNF-Rs, and heterotrimers with

LTβ ($LT\alpha_1LT\beta_3$) that signals through the LTβR, thus, it is possible that LTα interacting with LTβ and LTβR plays a protective role during infection with *T. spiralis*.

Muscle larvae accumulation is believed to depend on the duration of the intestinal phase of *T. spiralis* infection however, despite the prolonged survival of adult *T. spiralis* in the small intestine in TNFα/LTα -/- and tmTNFα-tg mice, the number of muscle larvae obtained from these mice at day 30 p.i. was not significantly different from wild type mice. This may be due to a number of factors, the adult worms although surviving may be less able to reproduce, either throughout their lifespan or late in infection, or the survival of the larvae themselves may be impaired. Assuming that adult fecundity is unaltered, protection responses to larvae has been suggested to be mediated by Th1 responses, although protective immunity to *T. spiralis* larvae is not well studied nor understood. In this model the splenic secretion of the Th2 cytokine IL-4 was reduced while IL-18, but not IFN-γ was increased. IL-18 is a pleotropic cytokine capable of potentiating both Th1 and Th2 responses (Nakanishi *et al.*, 2001) and thus may be assisting in the control of muscle larvae burdens.

TNFα is an important mediator in intestinal pathology, anti-TNFα treatment of patients with inflammatory bowel disease (IBD) has been shown to be efficacious in ameliorating pathology, furthermore in RAG2 -/- mice the induction of colitis following the adoptive transfer of CD4+CD45RBhi T cells has been shown to be dependant on non-cell derived TNFα (Corazza *et al.*, 1999). Increased TACE activity has been

demonstrated in the colonic mucosa of ulcerative colitis patients suggesting that the conversion of TNFα from the transmembrane to the soluble form may be important (Brynskov *et al.*, 2002). Interestingly the transfer of $CD4^{+}CD45RB^{hi}$ expressing only tmTNFα has been shown induce colitis in the RAG2 -/- mouse (Corazza *et al.*, 2004), suggesting that increased TACE activity in ulcerative colitis patients may be an anti-inflammatory mechanism. In this study, however, enteropathy was exacerbated in the absence of soluble TNFα and LTα while no significant effect of tmTNFα on the development of enteropathy following infection was observed. Although, antigen specific IgG1 responses were reduced in both TNFα/LTα -/- and tmTNFα-tg mice compared to wild type mice this was to a lesser degree in the tmTNFα-tg mouse suggesting a role for tmTNFα in the development of Th2 responses to *T. spiralis.*

The development of enteropathy during infection with *T. spiralis,* has been shown to be significantly reduced in p55TNFR -/- mice suggesting that TNFα may play a role in the development of enteropathy (Lawrence *et al.*, 1998) while p75TNFR -/- mice developed enhanced enteropathy (Lawrence *et. al.* unpublished data). TmTNFα has been shown to be an important activating ligand for p75TNFR. However, in this study enteropathy was significantly enhanced in TNFα/LTα -/- and tmTNFα-tg mice suggesting that LTα may play a role in limiting enteropathy following infection with *T. spiralis.* This may relate to a role for LTα in protecting against bacteria, *Citrobacter rodentium* infected LTβR -/- mice have been shown to suffer increased pathology and an increase in intestinal bacterial abscesses compared to wild type mice (Spahn *et al.*, 2004). It has been shown

that tissue penetrating nematodes can carry bacteria into host tissues where the bacteria may cause pathology, for example piglets co-infected with *Ascaris suum* and *Escherichia coli* developed lung abscesses, from *E. coli* carried from the intestine by migrating larval *A. suum* (Adedeji *et al.*, 1989). It has also been shown that colitis was reduced in pigs infected with *Trichuris suis* and treated with antibiotics suggesting that enteropathy following nematode infection may in part be due to the intestinal flora (Mansfield and Urban, 1996). Thus it would be of interest in this model to evaluate whether a failure to control bacterial infiltration into the mucosa may be responsible for the exacerbation of enteropathy in the absence of LTα.

Mucosal mastocytosis is a significant histological feature of *T. spiralis* infected intestine, peak mastocytosis coincides with the expulsion of the adult worms from the intestine (Lawrence *et al.*, 1998). Deficiency in mucosal mast cell protease (mMCP)-1 in mice has also been shown to delay the expulsion of *T. spiralis* from the intestine (Knight *et al.*, 2000) and reduces enteropathy compared to wild type mice (Lawrence *et al.*, 2004). Human mast cells co-cultured with T cells have been shown to produce TNFα (Bhattacharyya *et al.*, 1998) while mast cells from human intestinal tissue have been shown to contain preformed TNFα (Bischoff *et al.*, 1999; Furuta *et al.*, 1997; Gordon and Galli, 1990). Interestingly this study showed that in the absence of soluble TNFα and LTα mucosal mast cell responses following infection with *T. spiralis* are significantly ablated. This may be due to diminished IL-4 responses in the TNFα/LTα -/- and tmTNFα-tg mice, as IL-4 is necessary for the development of mast cells or TNFα

and LTα may play a role directly in mast cell development however a role for TNFα or LTα in mast cell development has not been shown.

Both total IgE and antigen specific IgG1 titres were significantly reduced in TNFα/LTα -/- and tmTNFα-tg, compared to wild type mice following infection with *T. spiralis*. This is consistent with the reduced IL-4 secretion from spleen cells from infected TNFα/LTα -/- and tmTNFα-tg mice following ex-vivo re-stimulation with TAg. The failure to initiate normal Th2 responses may in part explain the delayed expulsion of adult *T. spiralis* from the intestines of TNFα/LTα -/- and tmTNFα-tg mice compared to wild type mice. Reduced IL-4 secretion by spleen cells was accompanied by elevated IL-18 secretion. IL-18 was first named IFN-γ inducing factor, as it was shown to induce the production of IFN-γ by T cells (Okamura *et al.*, 1995), and is structurally related to IL-1β(Bazan *et al.*, 1996). IL-18 has been implicated in the development of Th1 mediated enteropathies such as Crohn's disease where the expression of IL-18 mRNA has been shown to be increased (Monteleone *et al.*, 1999; Pizarro *et al.*, 1999) and in TNBS induced colitis in mice where both anti-IL-18 treatment and IL-18 deficiency attenuates the development of pathology (Kanai *et al.*, 2001). However, it has also been shown that exogenous IL-18 induces elevated serum IgE, IL-4 and IL-13 in mice (Yoshimoto *et al.*, 1997) and with IL-3 stimulates basophils to release IL-4 and IL-13 *in vitro* (Yoshimoto *et al.*, 1999). Thus, IL-18 appears to function as a mediator of both Th1 and Th2 responses depending on the cytokine environment, in this model with reduced IL-4 secretion increased IL-18 may indicate a shift to a more Th1 profile.

Exogenous IL-18 has also been shown to inhibit the development of mastocytosis following infection with *T. spiralis* and IL-18 -/- mice have been shown to be resistant to infection with *T. spiralis* expelling their parasites more rapidly than wild type mice (Helmby and Grencis, 2002). In this model the delay in parasite expulsion from the small intestine observed in TNFα/LTα -/- and tmTNFα-tg mice may therefore relate to elevated IL-18 secretion by spleen cells *ex vivo*.

The role of LTα and TNFα in the development of Th2 responses is unclear, however, it has been shown that LTα is essential for the development of Th2 responses following pulmonary challenge with Schistosome egg antigen (SEA). In LTα -/- mice, the resulting pulmonary inflammation and airway occlusion is compared to wild type mice. In wild type mice this inflammation is associated with elevated IgE and Th2 cytokine responses, in contrast LTα -/- mice fail to develop significant IgE responses to SEA, and develop a more Th1 cytokine profile. In LTα -/- mice the inflammation can be ameliorated by reconstitution with endogenous IgE, suggesting that LTα and IgE are important in the modulation of Th1-Th2 balance and pathology (Kang *et al.*, 2003). These data are consistent with the reduced IgE, IgG1 and IL-4 and the increased enteropathy seen in both the TNFα/LTα -/- and tmTNFα-tg mice compared to wild type mice following infection with *T. spiralis*, suggesting that LTα may play a role in balancing Th1 and Th2 mediated pathologies. While TNFα has been shown to have anti-inflammatory properties by inhibiting the production of IL-12 by macrophages stimulated with IFN-γ *in vitro*, and by protecting mice against fatal inflammation

mediated by IL-12 following infection with *Corynebacterium parvum* (Hodge-Dufour *et al.*, 1998). A further anti-inflammatory role for TNFα has been shown in both, human head injury patients, where cerebro-spinal fluid (CSF) IL-18 levels have been shown to correlate inversely with CSF TNFα levels and in a murine model of closed head injury were endogenous TNFα has been shown to depress intracranial IL-18 levels (Schmidt *et al.*, 2004). This suppression of IL-18 in the brain by TNFα correlates with the elevated IL-18 secretion seen in *T. spiralis* infected mice in the absence of TNFα and LTα in this study, suggesting that TNFα may play a role in regulating pro-inflammatory IL-18 responses.

The precise roles played by LTα and TNFα however, are difficult to assess as deficient mice show a variety of abnormalities in the development of the structure of their immune systems. Both LTα and TNFα play a role in the development of lymphoid organs and in gut associated lymphoid tissue (GALT). Mice deficient in both LTα and TNFα lack peripheral and mesenteric lymph nodes; show abnormal spleen architecture (Ngo *et al.*, 1999) and a variety of immune abnormalities. The lymph node draining the site of an infection, in the case of *T. spiralis*, the mesenteric lymph node, provide the site and support to T cells and antigen presenting cells allowing optimal T cell activation (Eugster *et al.*, 1996). Thus this defect in the structure of the immune system may affect the generation of effective normal immune responses against antigens and pathogens independently from the lack of the cytokine itself.

In order to further define the direct roles of these cytokines during *T. spiralis* infection it will be of interest to block the action of the cytokine using either antibodies or fusion proteins during the infection in wild type mice whose immune systems are structurally normal. It is also important to assess the role played by different secondary immune structures, in the development of effective gastrointestinal immune responses to this parasite. LTα and TNFα single -/- mice have been shown have different immune system developmental abnormalities for example LTα -/- mice lack Peyer's patches and lymph nodes, whilst TNFα -/- have defects in the GALT (Koni and Flavell, 1998). LTα has also been shown to be important in the maintenance of splenic architecture and humoural responsiveness in adult mice (Mackay *et al.*, 1997).

In conclusion, this study has shown a role for soluble TNFα and LTα in the expulsion of *T. spiralis* and in limiting the development of enteropathy, however, this data is not consistent with previous studies using TNFR1 -/- mice where expulsion was normal and enteropathy was reduced (Lawrence *et al.*, 1998). This suggests that LTα may be important in both host protection from *T. spiralis* and in limiting enteropathy, however the picture is complicated by the structural abnormalities in the immune systems of TNFα/LTα -/- and tmTNFα-tg mice.

<u>Chapter Six</u>

# Protease activated receptor-2 plays a role in the development of enteropathy but not in the expulsion of *T. spiralis* from the small intestine.

# 6 Protease activated receptor-2 plays a role in the development of enteropathy but not in the expulsion of *T. spiralis* from the small intestine.

## *6.1 Introduction.*

Proteases, such as tryptases, collasgenases, elastases and matrix metalloproteases, have long been known to play an important function in a variety of physiological processes, including inflammation. Proteases break down the proteins of connective tissue and tight junctions between cells, allowing the tissue remodelling and the development of oedema that accompanies inflammation (Pender *et al.*, 1996; Pender *et al.*, 1997). However the discovery of a group of novel G-protein coupled seven transmembrane receptors comprising protease activated receptor (PAR)-1 to PAR-4, in particular PAR-2, suggests that proteases may play a role in the development of inflammation as inter-cellular signals (Mackie *et al.*, 2002).

The PARs are activated by a novel mechanism involving the cleavage of an extracellular N-terminal domain by a serine protease. The cleavage of the extracellular N-terminal domain reveals a new N-terminus, termed the tethered ligand, which is then free to bind to the receptor (Figure 6.1) (Dery *et al.*, 1998; Kawabata and Kuroda, 2000). PAR-1, 3 and 4 are activated by thrombin (Derian *et al.*, 2002; Dery *et al.*, 1998), while PAR-2 is activated by trypsin and tryptase (Al-Ani and Hollenberg, 2003; Miike *et al.*, 2001).

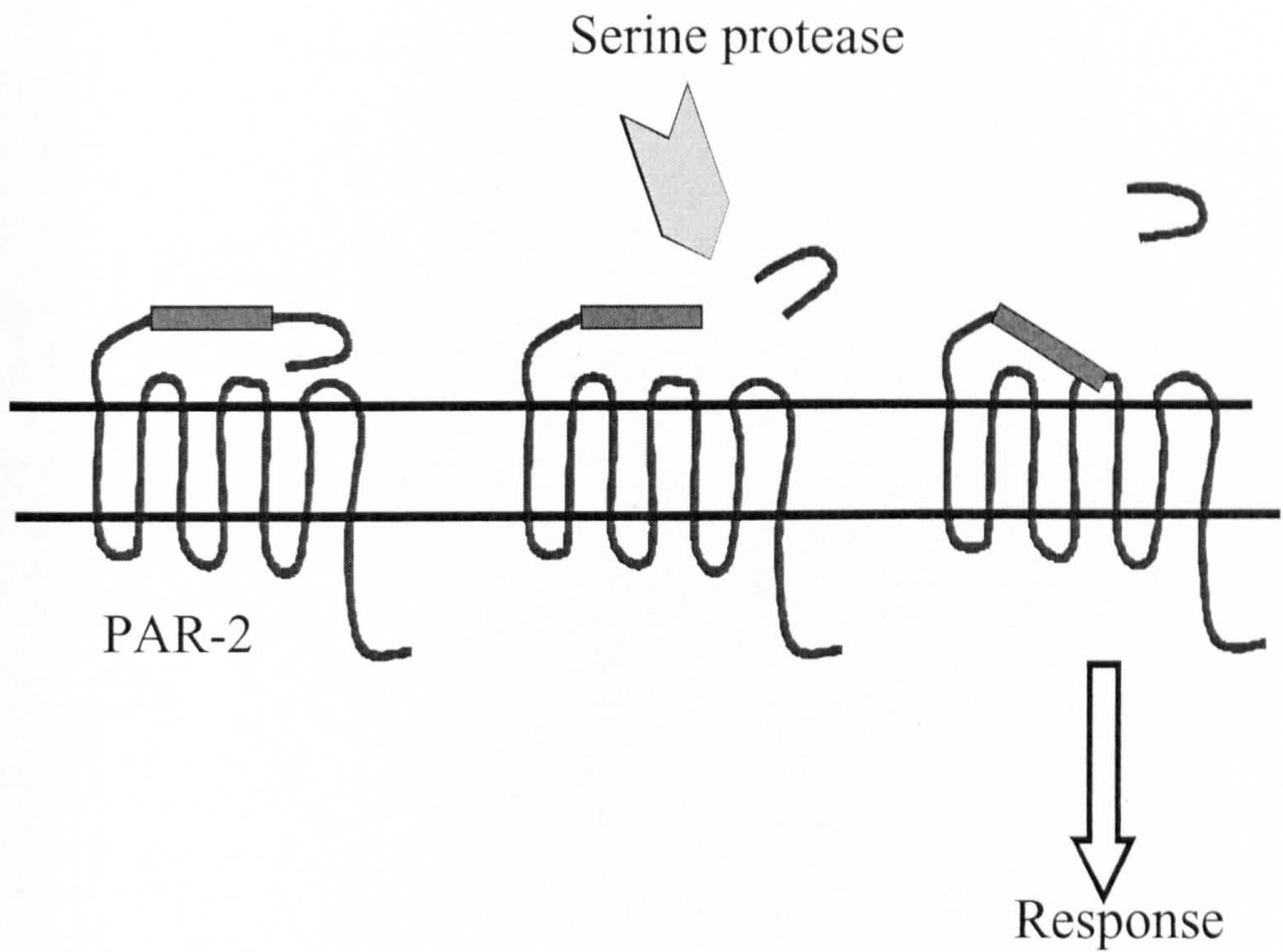



FIGURE 6.1: The cleavage of the extracellular N-terminal domain by serine proteases such as tryptase frees the tethered ligand to irreversibly activate the receptor. Figure adapted from Macfarlane *et al* (2001).

The PARs may also be activated in the absence of proteolytic cleavage using either the native peptide sequences or synthetic peptides corresponding to the tethered ligand (Hansen *et al.*, 2004; Hollenberg *et al.*, 1999). For example PAR-2 may be activated in the absence of trypsin or tryptase by peptide of the sequences Ser-Leu-Ile-Gly-Arg-Leu-amide (SLIGRL-$NH_2$) or by the synthetic peptide 2-furoyl- Leu-Ile-Gly-Lys-Val (2f-LIGKV-OH or ASKH-95) (Figure 6.2). The activation of PAR-2 has been shown to result in an increase in intracellular $Ca^{2+}$ (Berger *et al.*, 2001) and to result in mitogen activated protein kinase activation (Belham *et al.*, 1996).

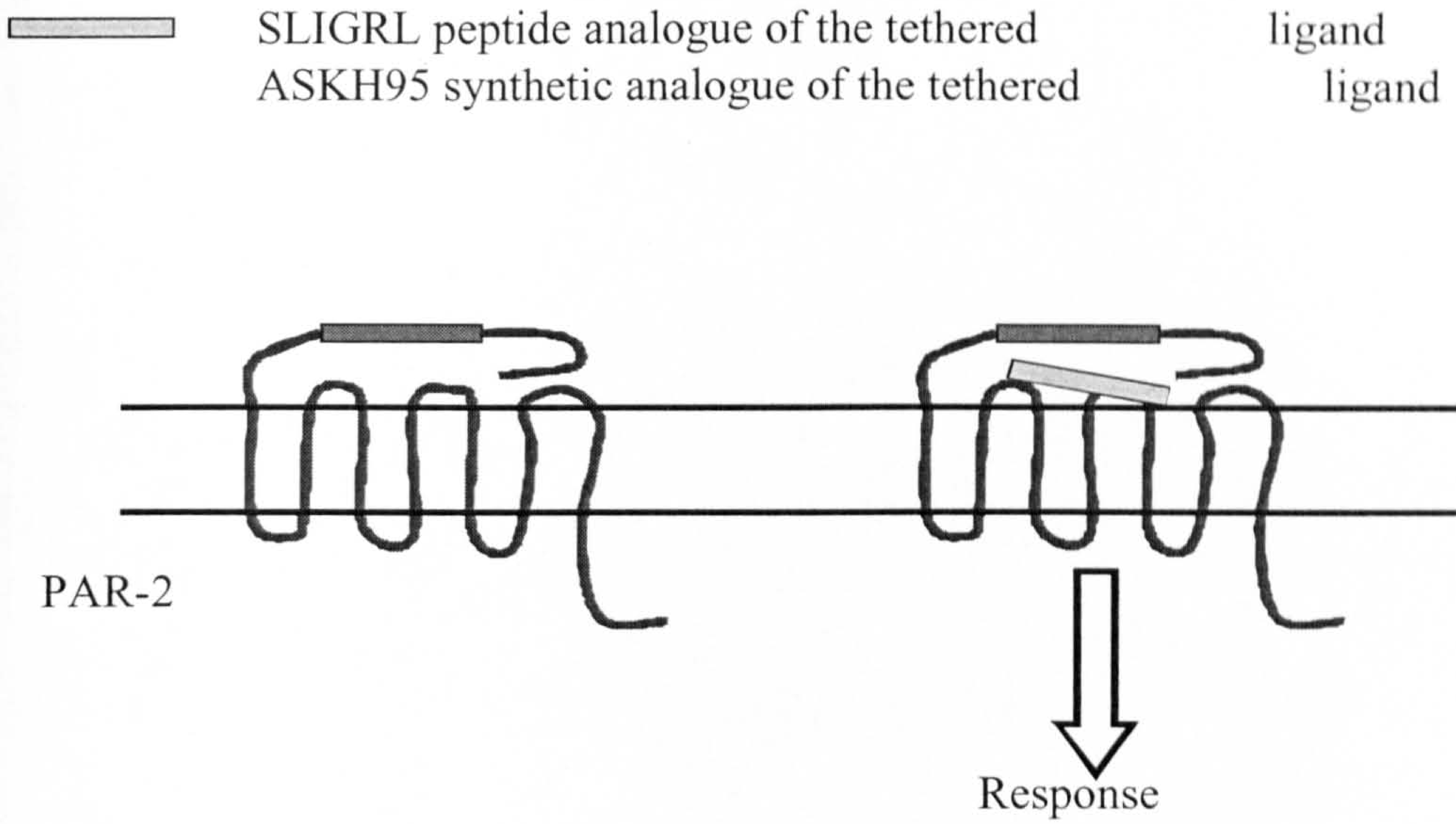



FIGURE 6.2: The activation of PAR-2 by agonist peptides in the absence of proteolytic cleavage. Native synthetic peptide sequences may activate PAR-2 by mimicking the action of the freed tethered ligand in the absence of serine proteases. Diagram adapted from Macfarlane *et al* (2001) (Macfarlane *et al.*, 2001).

Termination of signalling from these receptors occurs when the receptor is endocytosed and degraded within the cell. This leads to a period where cells stimulated *in vitro* with either serine proteases or agonist peptides are unable to respond to further stimulation. This refractory period lasts until the cell surface receptors have been replaced (Dery *et al.*, 1998; Kawabata and Kuroda, 2000).

PAR-2 is expressed in a variety of tissues and cell types including, smooth muscle (Berger *et al.*, 2001), respiratory epithelium (Vliagoftis *et al.*, 2000), vascular endothelium (Shpacovitch *et al.*, 2002), neuronal tissue (Gao *et al.*, 2002; Matej *et al.*, 2002; Vergnolle *et al.*, 2001), skin (Scott *et al.*, 2001; Steinhoff *et al.*, 1999; Steinhoff *et al.*, 2003), leukocytes (Dugina *et al.*, 2003) and throughout the intestinal tract (Cenac *et al.*, 2002; Kawabata *et al.*, 2001). Activation of PAR-2 therefore has a variety of effects depending on the tissue or on the circumstances. Activation of PAR-2 in the vascular endothelium leads to increased vascular permeability (Kawabata *et al.*, 1998), and studies using PAR-2 agonists have shown that PAR-2 activation leads to increased leukocyte adhesion, rolling, and extravasation (Vergnolle, 1999). Furthermore PAR-2 agonist treatment of A549 and BEA5-2B cell lines and of primary human bronchioepithelial cells have been shown to stimulate the release of the pro-inflammatory cytokines IL-6 and IL-8 (Asokananthan *et al.*, 2002).

PAR-2 activation thus appears to provide pro-inflammatory signals and this is further supported by experiments in murine models of inflammatory disease, including allergic and contact dermatitis have been shown to be significantly reduced in PAR-2 -/- mice

compared to wild type (Kawagoe *et al.*, 2002; Takizawa *et al.*, 2002) (Seeliger *et al.*, 2003). In addition adjuvant-induced arthritis is reduced in PAR-2-/- mice and is enhanced in mice treated with peptide agonists (Ferrell *et al.*, 2003). In the respiratory system, PAR-2 activation is believed to facilitate the development of asthma, playing a role in airway hyper-responsiveness by modulating airway smooth muscle contractility (Berger *et al.*, 2001; Lan *et al.*, 2000).

PAR-2 is widely expressed throughout the gastrointestinal tract (Kawao *et al.*, 2002), and as a pro-inflammatory signal the role of PAR-2 in intestinal function and inflammation is of interest. Studies of guinea pig myenteric neurons have shown that serine proteases such as mast cell tryptase regulate neuronal signalling through PAR-2 (Corvera *et al.*, 1999; Gao *et al.*, 2002). PAR-2 agonists have also been shown to induce relaxation and contraction in rat duodenal muscle (Kawabata *et al.*, 1999) and when delivered intraperitoneally facilitates intestinal transit in the mouse in a dose dependant manner (Kawabata *et al.*, 2001). *T. spiralis* infection is associated with neuromuscular dysfunction, PAR-2 may therefore play a role in *T. spiralis* induced neuromuscular dysfunction in the intestine (Blennerhassett *et al.*, 1992; Vermillion and Collins, 1988; Vermillion *et al.*, 1991). PAR-2 activation by agonist peptides has also been shown to increase gastric mucosal blood flow (Kawabata *et al.*, 2003). Thus increased blood flow and gastrointestinal motility in response to PAR-2 agonists suggest that PAR-2 is likely to provide pro-inflammatory signal in the intestine.

However, the role of PAR-2 during intestinal inflammation is not clear, in PAR-2-/- mice the development of colitis was inhibited and agonist treatment has been shown to

promote the development of colitis in mice (Cenac *et al.*, 2002), which could be prevented by co-treatment with nitric oxide synthase inhibitor (Cenac *et al.*, 2003). While, in contrast administering PAR-2 agonist peptides has been shown to be protective against TNBS-induced colitis in mice (Fiorucci *et al.*, 2001).

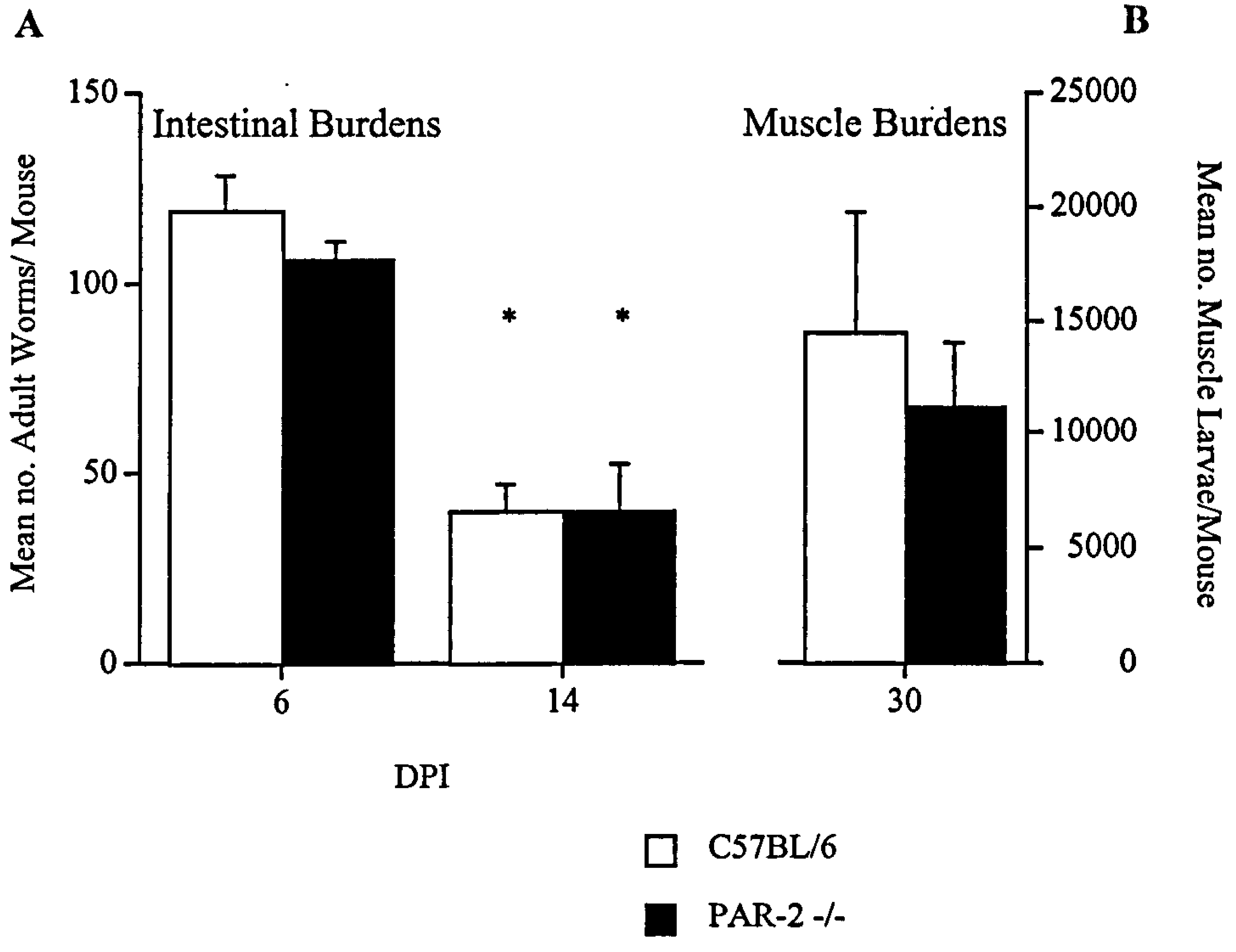
Here, the role of PAR-2 in the development of enteropathy and protective responses to *T. spiralis* infection was analysed using PAR-2 -/- mice and the PAR-2 agonists SLIGRL-$NH_2$ and ASKH-95. Wild type C57Bl6 and PAR-2 -/- C57Bl6 mice were infected with 400 freshly isolated larvae and were subsequently culled at day 0, 6, 14, 16 and 30 p.i. In a separate experiment the effect of enhanced PAR-2 activation on the development of pathological and expulsive responses to *T. spiralis* was assessed by infecting wild type C57Bl6 mice at day 0 and treating daily from day –1 to 16 p.i. with 30μg of SLIGRL-$NH_2$, ASKH-95 or with the scramble peptide control LRGILS-$NH_2$. Untreated and treated mice were culled at day 0, 14, 16 and 30 p.i. In both experiments, parasite establishment and expulsion, accumulation of larvae in muscle tissue, development of enteropathy, development of Th2 cytokine responses and development of antibody responses were measured.

## *6.2 Results*

### *6.2.1* T. spiralis *expulsion from the small intestine and the accumulation of muscle larvae is unaltered in PAR-2 -/- mice compared to wild type controls.*

Wild type C57/Bl6 and PAR-2 -/- mice were infected with 400 freshly isolated *T. spiralis* larvae. To assess the establishment and expulsion of *T. spiralis* the number of worms in the small intestine were counted at days 6, 14 and 16 p.i. No significant differences were observed in the establishment of worms in the small intestine at day 6 p.i. between PAR-2-/- and wild type controls. Both strains had significant worm loss at days 14 (p=0.009 and 0.009) and 16 p.i. (p=0.009 and 0.009) (data not shown) but no significant differences were observed between wild type and PAR-2 -/- mice at either time point (Figure 6.3A). The accumulation of muscle larvae may act as an indicator of the length of time adults spend in the small intestine or of the suitability of the intestinal environment for the adults. The total number of muscle larvae harboured by mice was measured at day 30 p.i., however no significant differences were observed between wild type and PAR-2 -/- (Figure 6.3B).

FIGURE 6.3: Effect of PAR-2 deficiency on the expulsion of *T. spiralis* from the intestine and the accumulation of muscle larvae. A) Establishment and expulsion of *T. spiralis* was measured in wild type and PAR-2 -/- mice at day 6 and 14 p.i. The small intestine was excised and the total number of worms present were counted. B) Accumulation of muscle larvae was measured in wild type and PAR-2 -/- mice at day 30 p.i. *, represents significantly different to mice at day 6 p.i. ($p<0.05$). Data are expressed as mean number of worms/mouse + SEM. Five mice were used for each experimental group, data is representative of two independent experiments.


A
B
Intestinal Burdens
Muscle Burdens
150
100
50
0
Mean no. Adult Worms/ Mouse
25000
20000
15000
10000
5000
0
Mean no. Muscle Larvae/Mouse
*
*
6
14
30
DPI
C57BL/6
PAR-2 -/-

### 6.2.2 T. spiralis *induced villus atrophy is reduced while crypt hyperplasia is in creased in PAR2-/- mice compared to wild type controls.*

The development of enteropathy was assessed in uninfected and infected mice at day 6, 14, and 16 p.i. by weighing the small intestine and by dissecting single rows of villi and crypts from a Clarke's fixed, Schiff's stained section of the jejunum, and measuring the lengths of the villi and crypts using an eye piece graticule. The level of cell division in the small intestine was also measured by counting the number of mitotic figures/VCU.

Gut weight had increased significantly in wild type mice from day 0 to 6 p.i. (p=0.0367), however in PAR-2 -/- mice the increase in gut weight was delayed until day 14 p.i. (p=0.0104). By day 14 p.i. gut weights were significantly higher in both wild type (p=0.009) and PAR-2 -/- mice (p=0.0104) compared to day 0 p.i., although no significant differences were observed in gut weights between wild type and PAR-2 -/- mice (Figure 6.4). No significant alteration was observed in the gut weights of wild type and PAR-2 -/- mice at day 16 p.i. (data not shown) compared to day 14 p.i.

Villi lengths from uninfected mice were significantly longer in PAR-2 -/- compared to wild type mice (p=0. 0143) but crypt lengths were not significantly different. Significant villus atrophy developed in wild type mice following infection at day 6 p.i. (p=0.009) compared to day 0 p.i., in PAR-2 -/- mice villi lengths were significantly longer than wild type (p=0.009) at day 6 p.i. In both wild type and PAR-2 -/- mice

significant crypt hyperplasia had developed day 6 p.i. (p=0.009 and 0.0061), however crypt hyperplasia was significantly greater in PAR-2 -/- compared to wild type (p=0.0472). In both wild type and PAR-2 -/- mice crypts were significantly longer at day 14 p.i. (p=0.009 and 0.061) compared to uninfected mice. No significant differences were observed in villus or crypt lengths at day 14 p.i. between PAR-2 -/- and wild type mice (Figure 6.5A). Villus atrophy and crypt hyperplasia were not significantly different at day 16 p.i. (data not shown) to day 14 p.i.

The number of dividing cells or mitotic figures increased in both wild type and PAR-2 -/- mice at day 6 p.i. (p=0.0088 and 0.0056) and at day 14 p.i. (p=0.0088 and 0.0062) compared to uninfected. By day 16 p.i. the number of mitotic figures/ VCU in both wild type and PAR-2 -/- mice was no longer significantly different to uninfected mice (data not shown). No significant differences were observed between wild type and PAR-2 -/- at any time point evaluated (Figure 6.5B).

FIGURE 6.4: The role of PAR-2 in the development of intestinal oedema following infection with *T. spiralis*. The small intestine was removed from uninfected and infected mice at day 6 and 14 p.i., the entire intestine was weighed. Five mice were used per group. Data expressed as mean weight (g) + SEM and is representative of two independent experiments. *, significantly different to uninfected mice, §, significantly different to wild type mice.

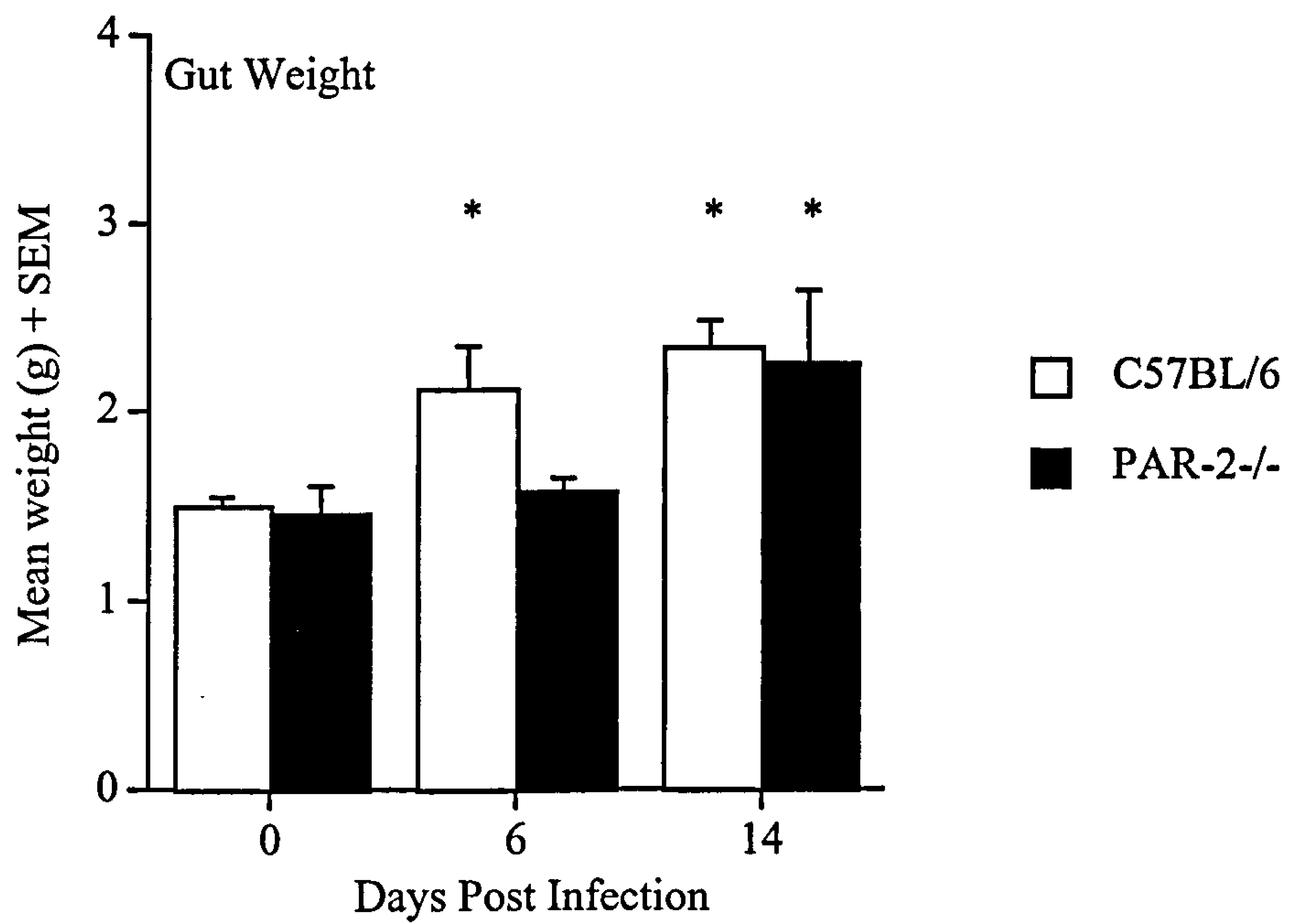
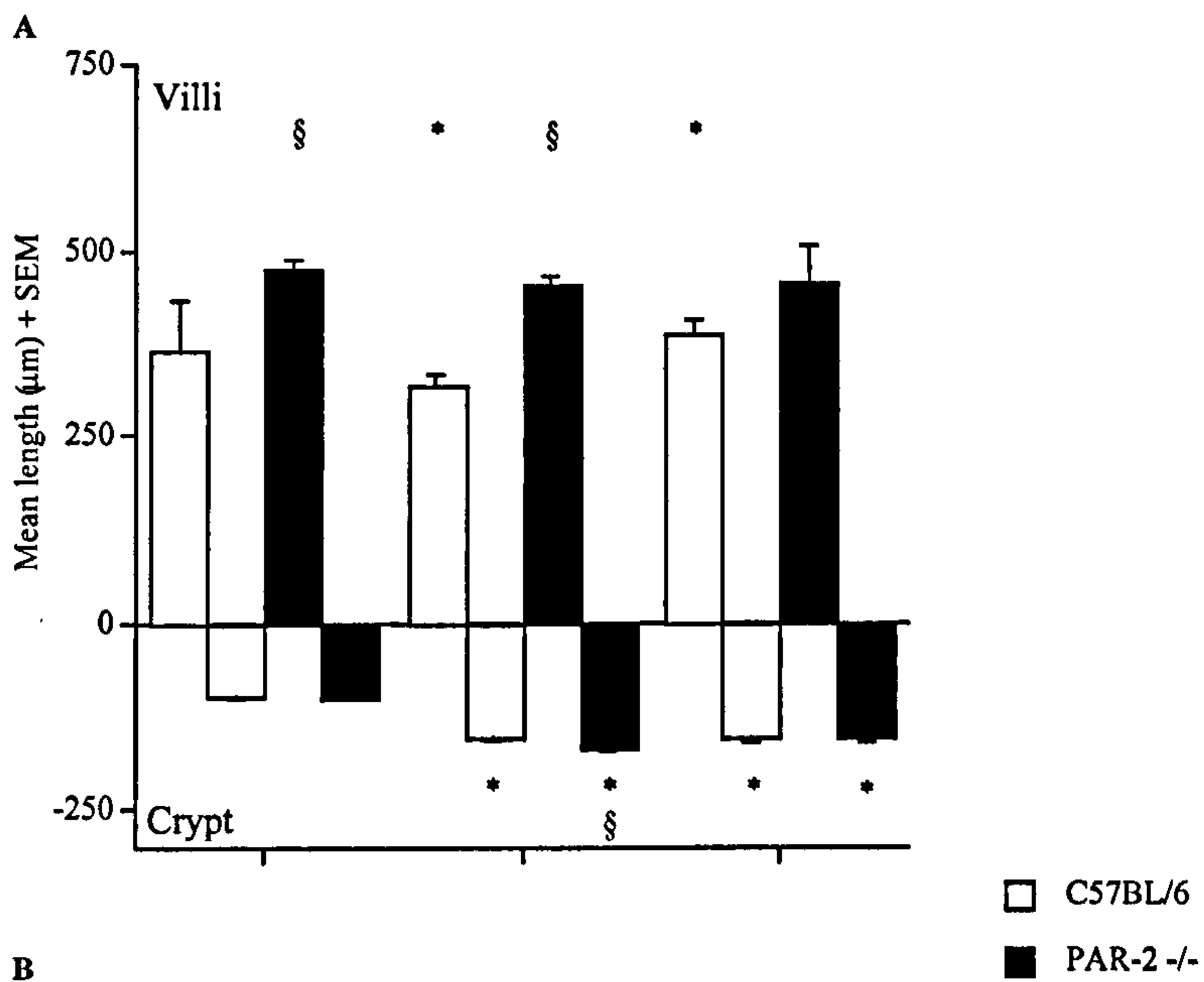
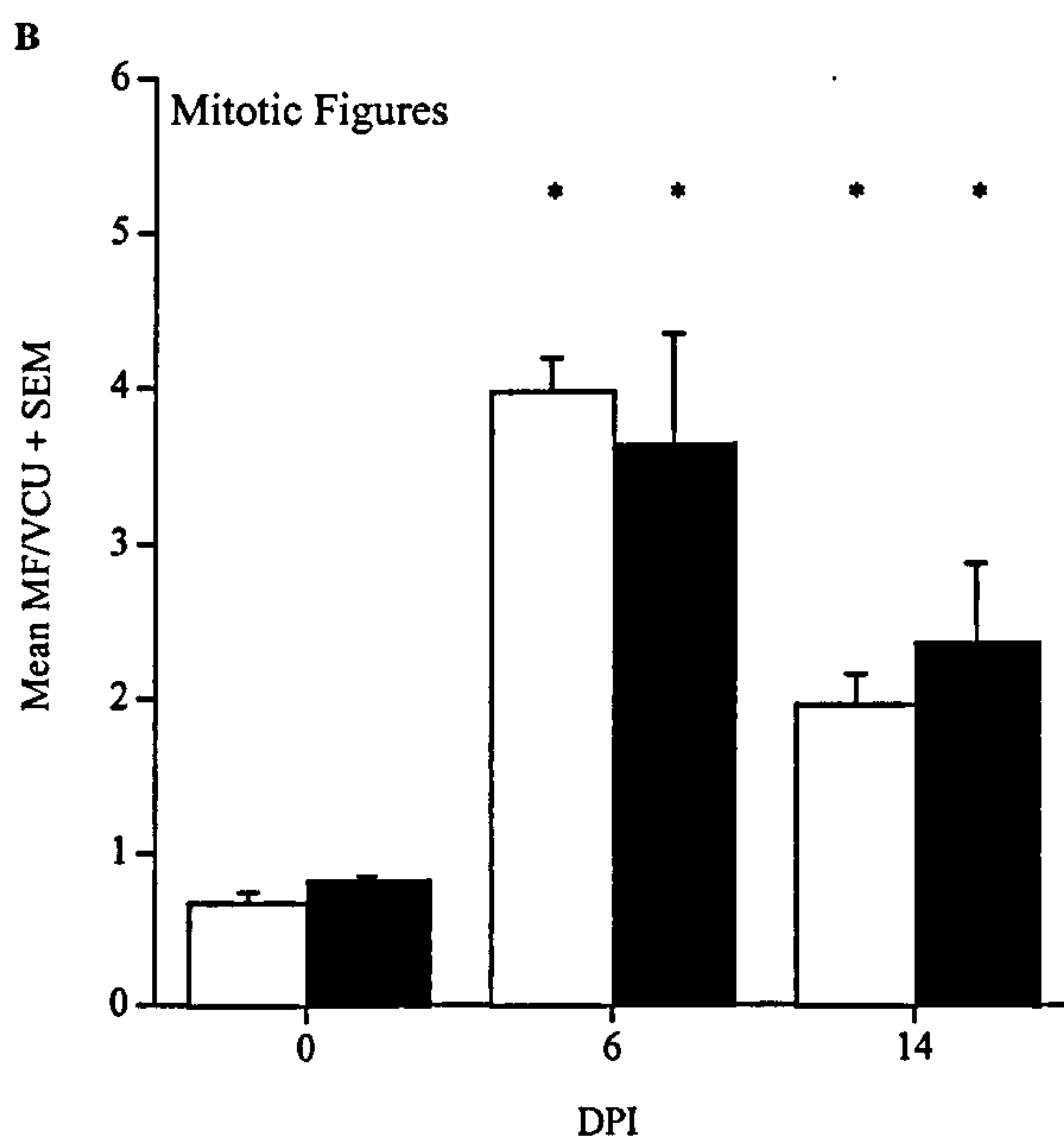

Gut Weight
Mean weight (g) + SEM
4
3
2
1
0
*
*
*
C57BL/6
PAR-2-/-
0
6
14
Days Post Infection

FIGURE 6.5: The role of PAR-2 in the development of enteropathy following infection with *T. spiralis*. Villus and crypt lengths (A) were measured and the number of mitotic figures per crypt (B) was determined in uninfected and infected at day 6 and 13 p.i. Five mice were used per group. Data expressed as mean + SEM and is representative of two independent experiments. *, represents significantly different to uninfected; §, represents significantly different to wild type ($p<0.05$).


A
750
500
250
0
-250
Villi
Crypt
Mean length (μm) + SEM
§
*
§
*
*
*
§
*
*
C57BL/6
PAR-2 -/-

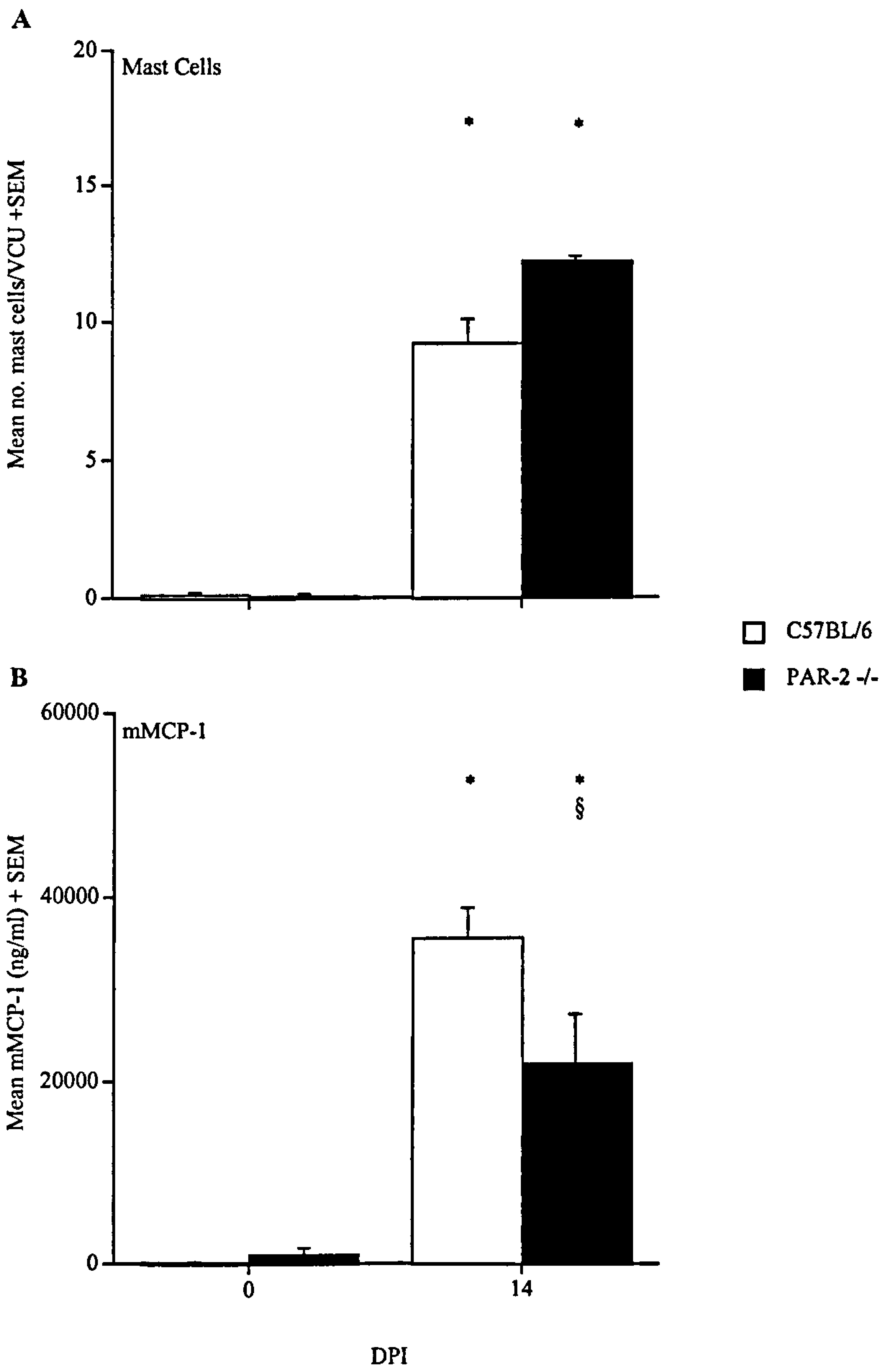

B
Mitotic Figures
Mean MF/VCU + SEM
6
5
4
3
2
1
0
*
*
*
*
0
6
14
DPI

### *6.2.3 Mastocytosis developed normally in PAR2-/- mice compared to wild type mice during infection however degranulation, as indicated by serum mMCP-1 levels, was decreased.*

The development of the mastocytosis in the mucosa was measured in uninfected mice and at day 14 and 16 p.i. by counting the number of mast cells / VCU in sections of Carnoy's fluid fixed ileum stained with Toluidine Blue. mMCP-1, which is released on mast cell degranulation, was measured by ELISA in serum samples taken from uninfected and infected at day 14 and 16 p.i. from both wild type and PAR-2 -/- mice.

The number of mucosal mast cells/ VCU had significantly increased by day 14 p.i. in both wild type and PAR-2 -/- mice (p=0.0088 and 0.0086) however no significant differences were observed between the two strains (Figure 6.6A). Serum mMCP-1 levels significantly increased in both strains of mice by day 14 p.i. compared to uninfected in both wild type and PAR-2 -/- mice (p=0.009 and 0.0106) but were significantly lower in PAR-2 -/- mice at day 14 p.i. compared to wild type mice at day 14 p.i. (p=0.0472) (Figure 6.6B). No significant differences in mast cell numbers or serum mMCP-1 titres were observed at day 16 p.i. between wild type and PAR-2 -/- mice (data not shown).

FIGURE 6.6: The role of PAR-2 in the development of mucosal mastocytosis and on serum mMCP-1 titres following infection with *T. spiralis*. A) Carnoy's fixed jejunum from uninfected and infected mice at day 14 p.i. were processed and stained with 0.5% toluidine blue, revealing mast cells. The number of mucosal mast cells were counted in 20 randomly selected villus crypt units (VCU). B) Serum mMCP-1 concentration was measured in uninfected and infected mice at day 14 p.i., by ELISA against a recombinant protein standard. Data expressed as mean mMCP-1 concentration in pg/ml+ SEM. Five mice were used per group. Data expressed as mean + SEM, and is representative of two independent experiments. *, represents significantly different to uninfected. §, represents significantly different to untreated.

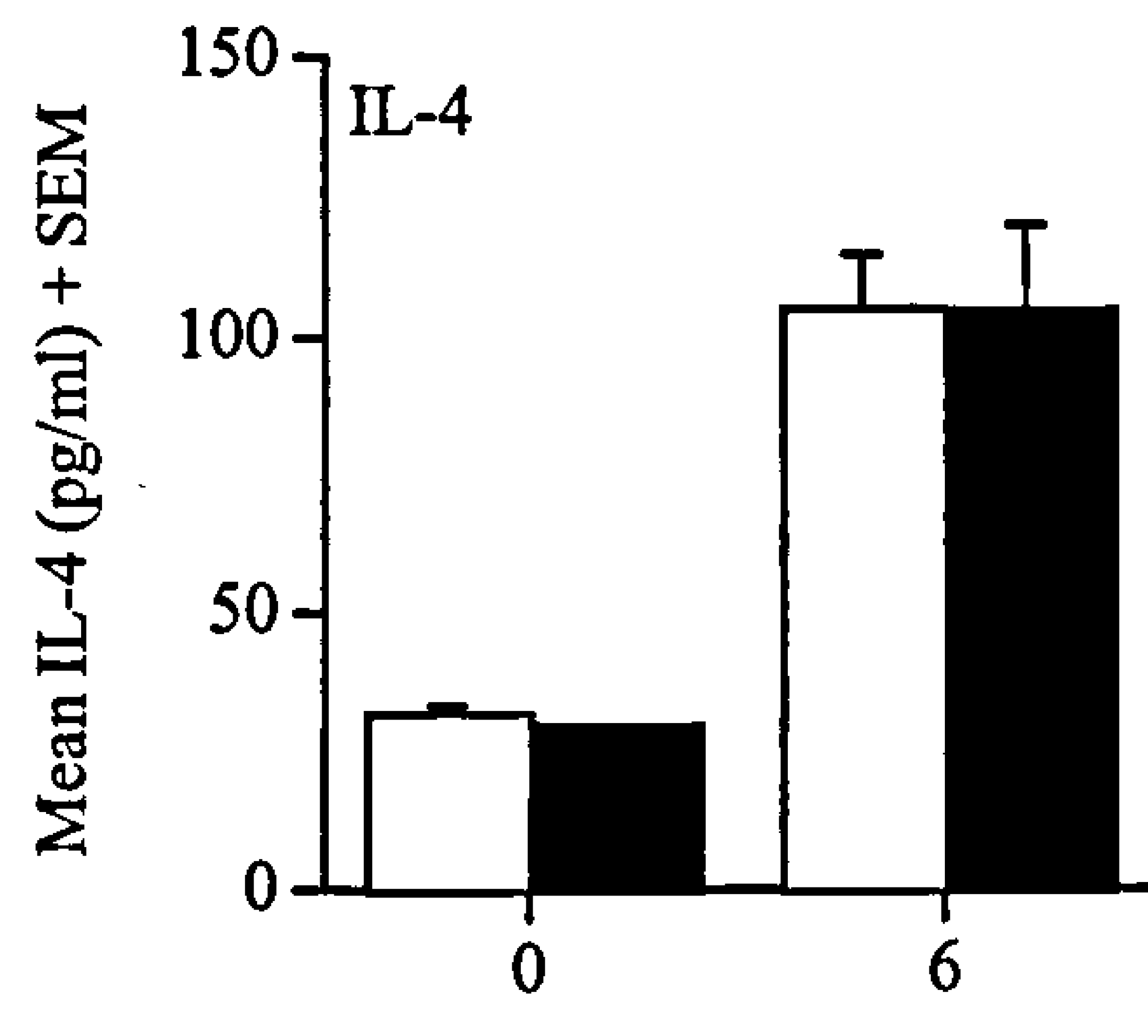

A
Mast Cells
Mean no. mast cells/VCU +SEM
20
15
10
5
0
*
*
C57BL/6
PAR-2 -/-
B
mMCP-1
Mean mMCP-1 (ng/ml) + SEM
60000
40000
20000
0
*
*
§
0
14
DPI

### *6.2.4 Cytokine production by MLN cells stimulated ex-vivo with TAg is not altered in PAR2-/- mice compared to wild type.*

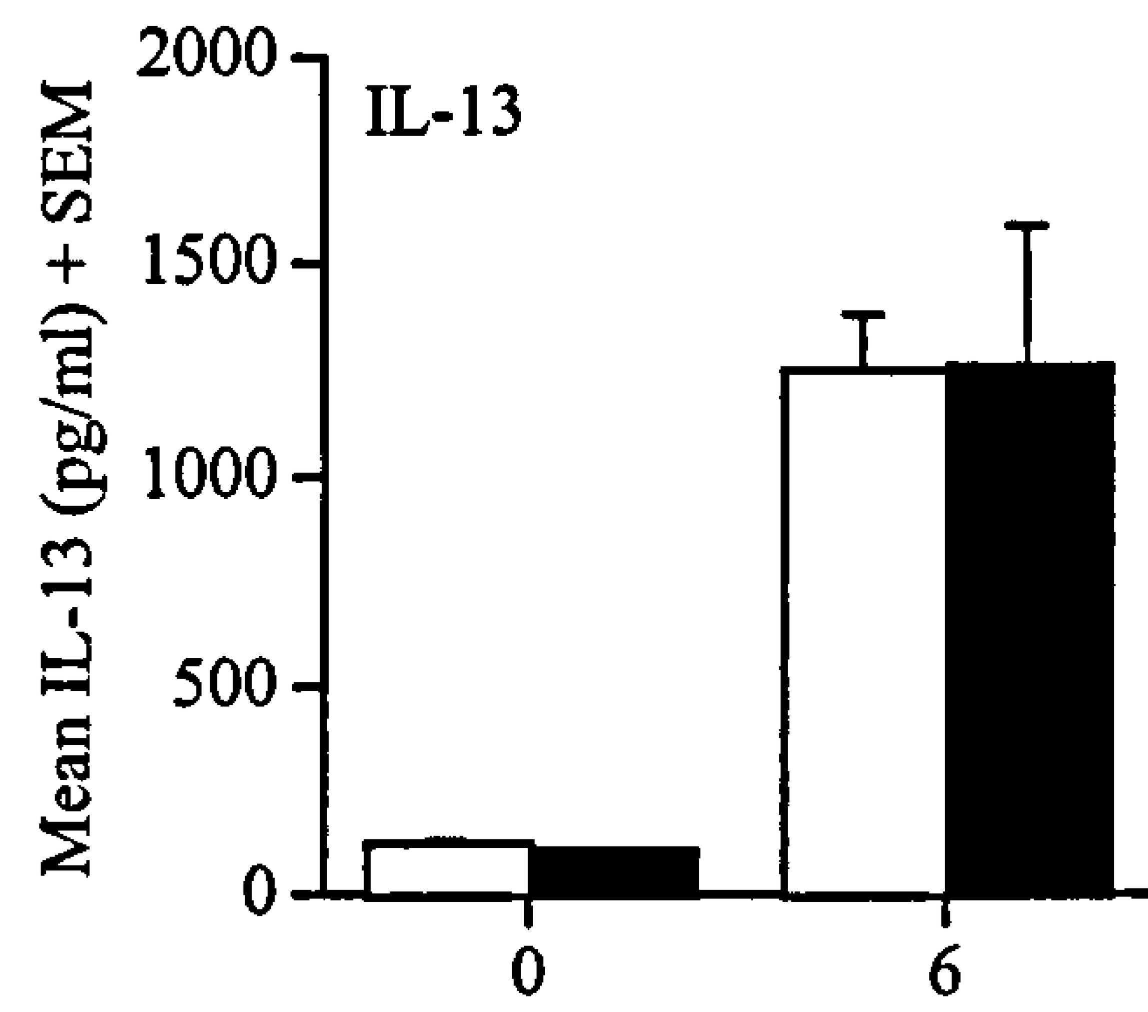
The mesenteric lymph nodes were removed from infected and uninfected, wild type and PAR-2 -/- mice at day 6 p.i., single cell suspensions were formed, were cultured with TAg. The secretion of the cytokines IL-4, IL-13 and IFNγ into the culture media was measured by ELISA. IL-4 and IL-13 secretion by MLN from both wild type and PAR-2 -/- mice was increased at day 6 p.i. compared to MLN from uninfected mice when cultured with TAg (Figure 6.7A and B respectively). IFNγ production was not altered at day 6 p.i. compared to uninfected mice of either strain (Figure 6.7C). Cell numbers obtained from uninfected mice were low so samples were pooled and therefore statistical analysis of any differences in cytokine secretion between uninfected and infected mice was not possible. At day 6 sufficient cells were obtained to culture MLN cell from individual animals separately however, no significant differences in cytokine production were observed between strains at day 6 p.i.

FIGURE 6.7: The role of PAR-2 in the secretion of the cytokines IL-4, IL-13, and IFNγ from MLN cells stimulated *ex vivo* with TAg. MLN were removed from uninfected and infected mice at day 6 p.i. and $1\times10^6$ cells were cultured with 50μg/ml TAg. Cell numbers from both uninfected mice were low and samples were pooled, for wild type mice two samples were obtained, while for PAR-2 -/- mice only one sample was obtained. Infected wild type and PAR-2 -/- mice yielded sufficient cells for 5 individual samples. The secretion of the cytokines IL-4 (A), IL-13 (B), and IFNγ (C) was assessed by measuring their concentration in the supernatants by ELISA against recombinant protein standards. Data is expressed as median cytokine concentration (pg/ml) + median absolute deviation.

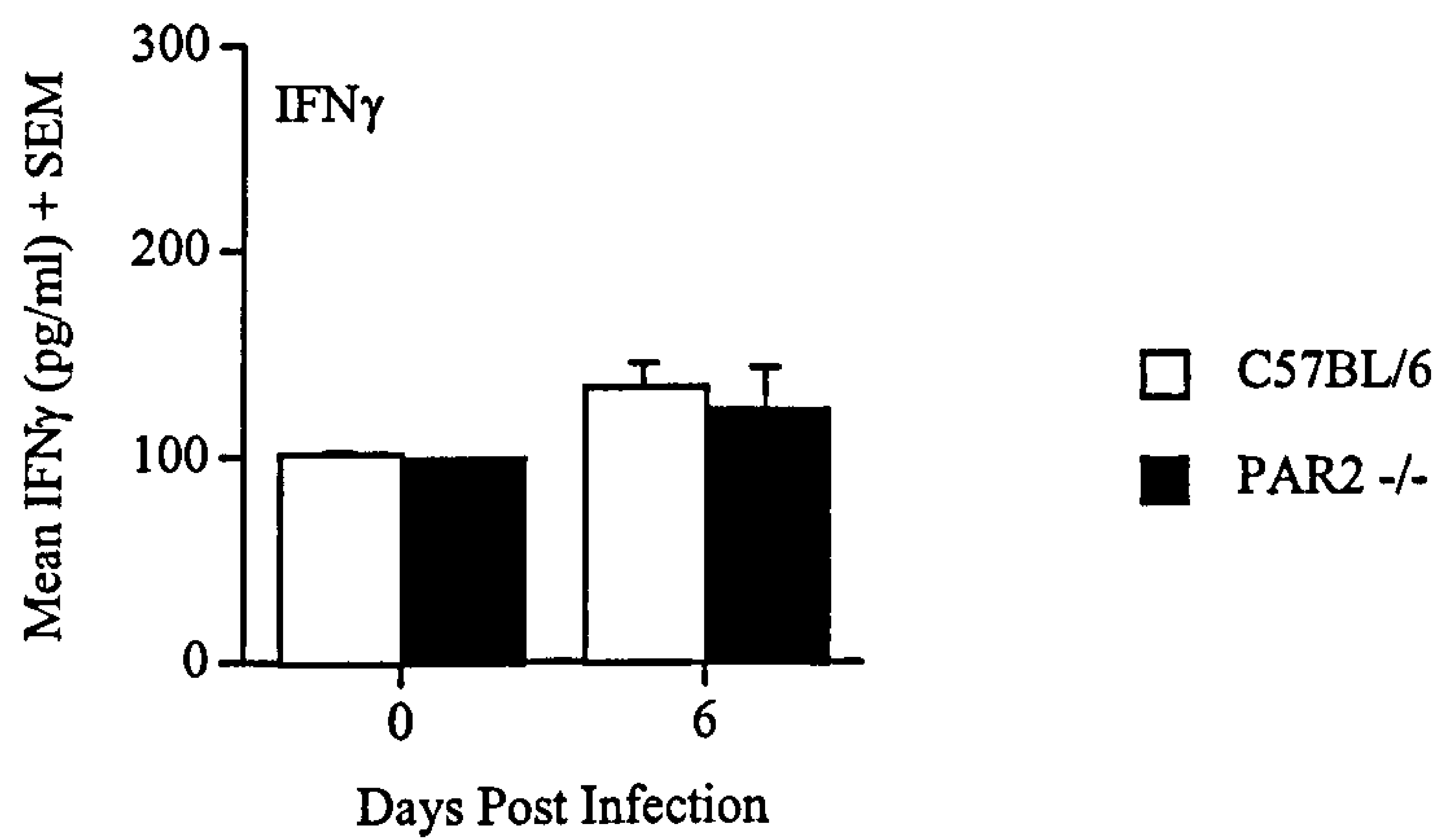
A




B



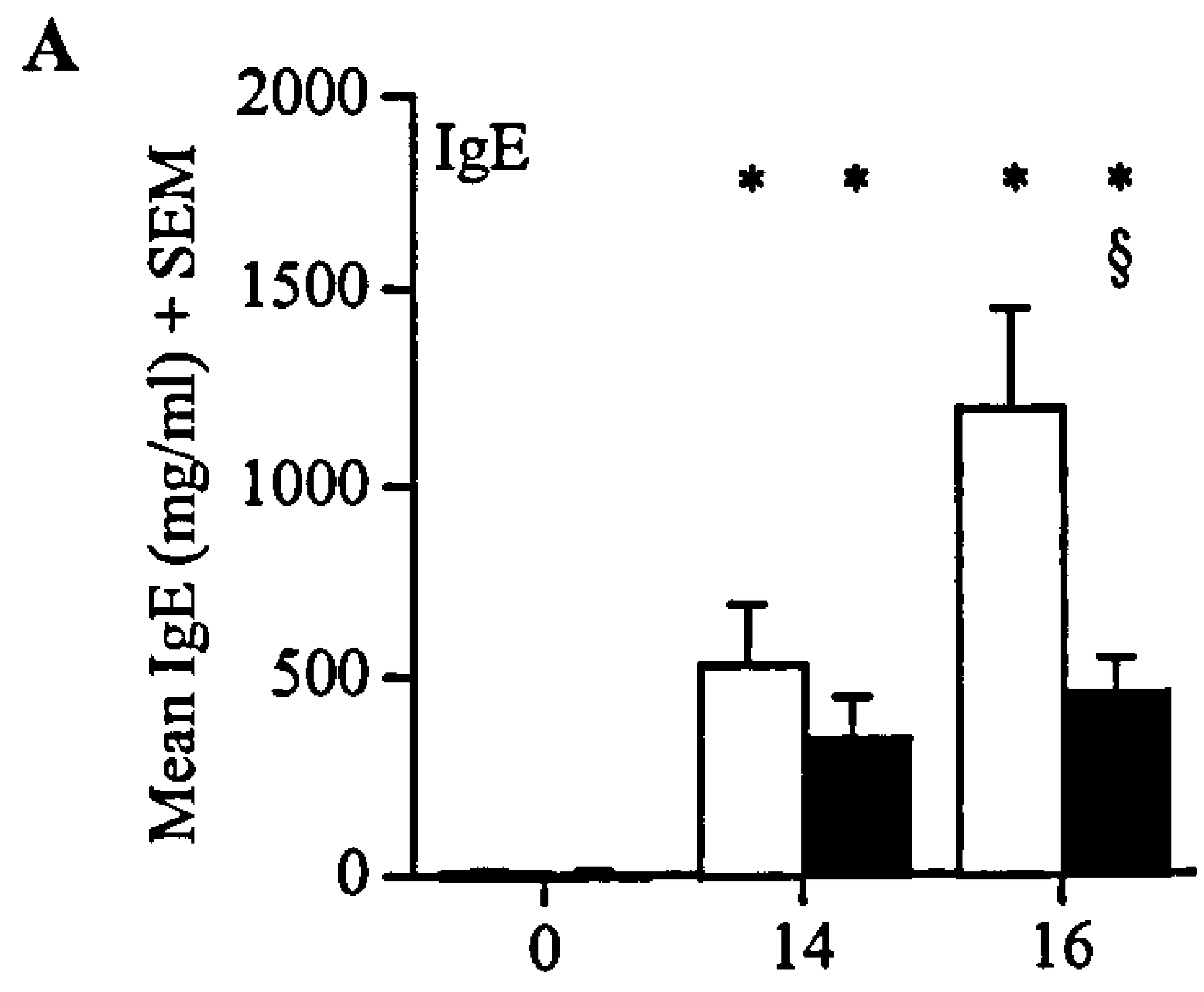

C

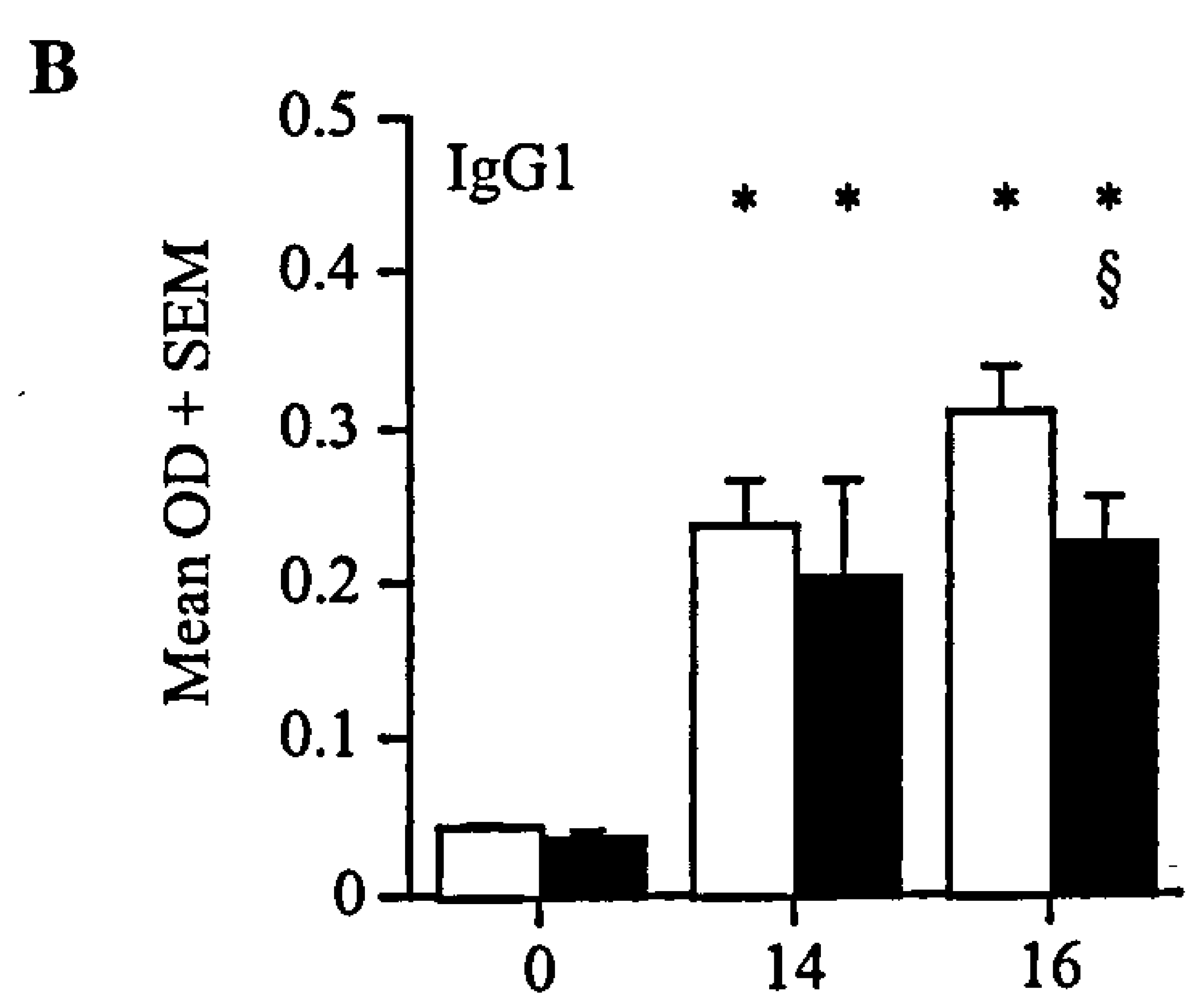


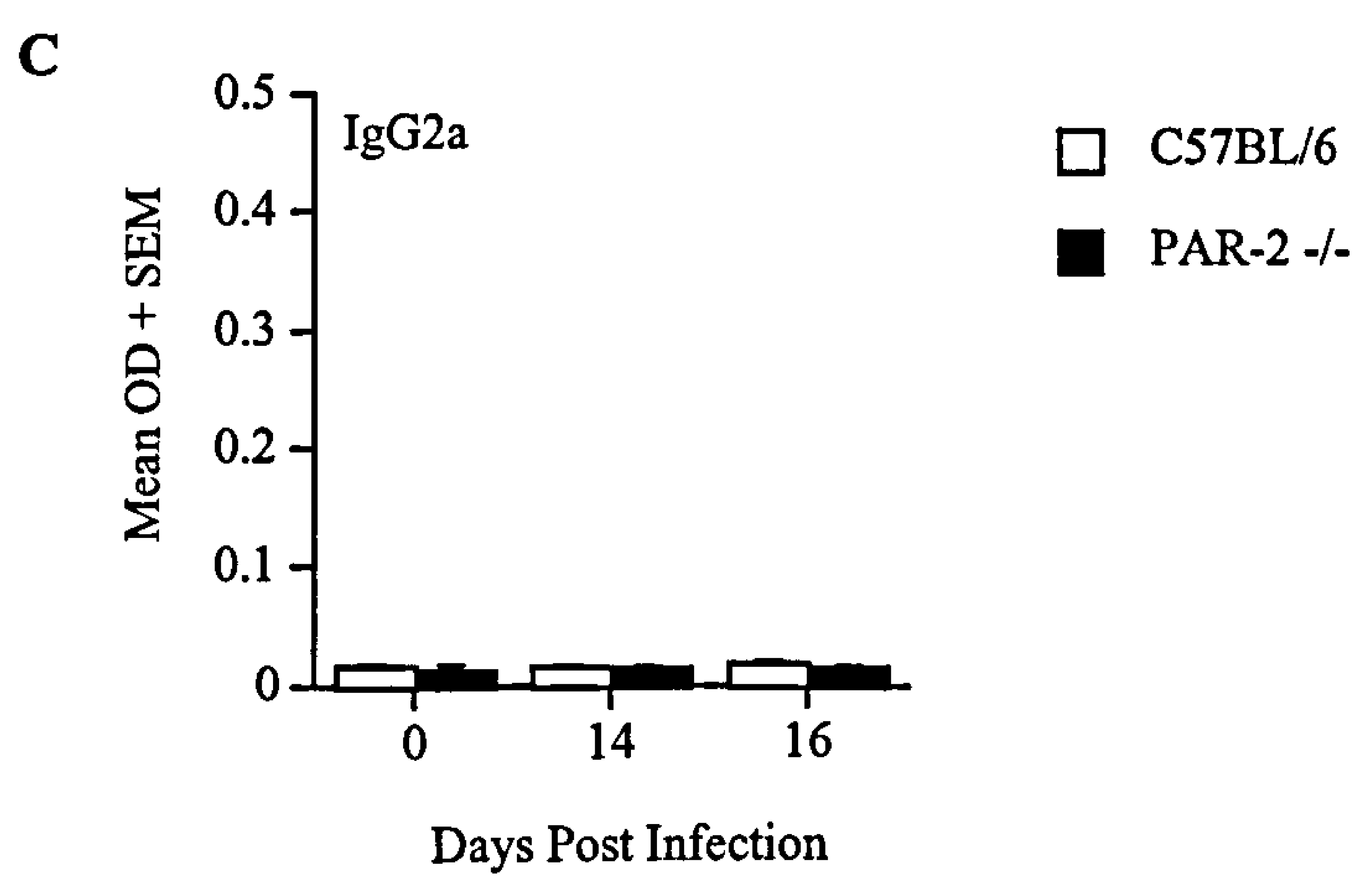
### *6.2.5 Total IgE and TAg specific IgG1 levels in the sera of PAR-2-/- mice are reduced compared to wild type mice at day 16 p.i.*

Serum samples were taken from uninfected and infected at day 14 and 16 p.i. wild type C57/Bl6 and PAR-2 -/- mice. Total serum IgE and TAg specific IgG1 and IgG2a were measured by ELISA. Both wild type and PAR-2-/- mice develop significantly elevated serum levels of total IgE at day 14 ($p=0.009$ and 0.0062) and 16 p.i. ($p=0.009$ and 0.0062) compared to uninfected mice. PAR-2 -/- mice however developed significantly lower levels of total IgE compared to wild type at 16 dpi ($p=0.0283$) (Figure 6.8A). TAg specific IgG1 levels are elevated in both wild type and PAR-2 -/- mice day 14 ($p=0.0088$ and 0.0103) and 16 p.i. ($p=0.0062$ and 0.009). PAR-2 -/- mice showed significantly lower IgG1 titres compared to wild type mice at day 16 p.i ($p=0.0472$) (Figure 6.8B). IgG2a titres were not altered in either wild type or PAR-2 -/- mice following infection with *T. spiralis* (Figure 6.8C).

FIGURE 6.8: The role of PAR-2 in the development of antibody responses following infection with *T. spiralis*. Total IgE (A) and TAg specific IgG1 (B) and IgG2a (C) titres were measured in sera taken at uninfected and infected mice at day 14 p.i. IgE titres were measured by sandwich ELISA against a purified IgE standard. While antigen specific IgG1 and IgG2a titres were assessed by ELISA using 96 well microtitre plates coated with *TAg* at 2μg/ml, sera were serially diluted and optical density readings at 1:2560 dilution used. Data expressed mean + SEM and is representative of two independent experiments. *, significantly different to uninfected mice. §, significantly different to wild type mice.


A
IgE
Mean IgE (mg/ml) + SEM
2000
1500
1000
500
0
0
14
16
§


B
IgG1
Mean OD + SEM
0.5
0.4
0.3
0.2
0.1
0
0
14
16
§

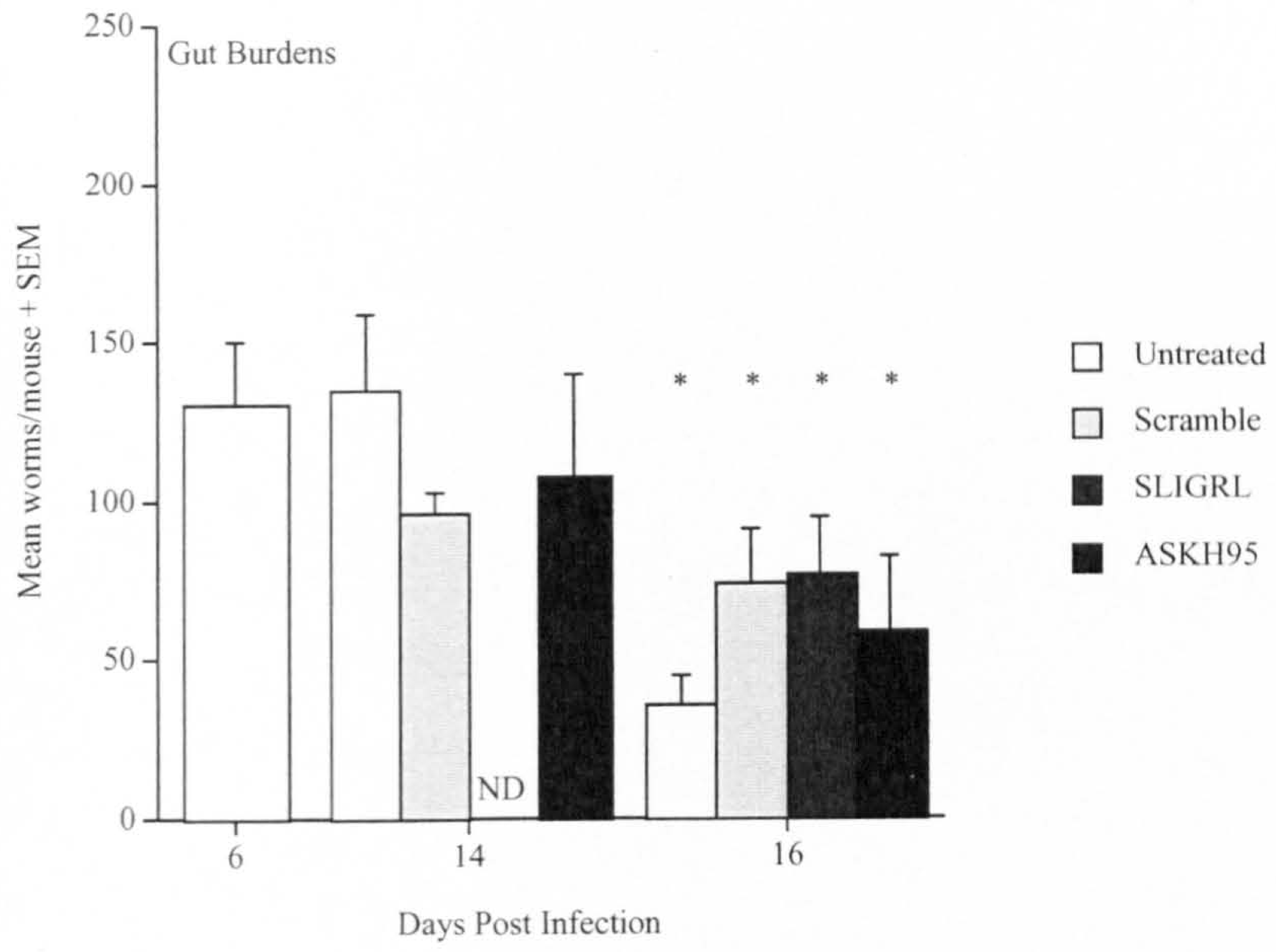

C
IgG2a
Mean OD + SEM
0.5
0.4
0.3
0.2
0.1
0
0
14
16
Days Post Infection
C57BL/6
PAR-2 -/-

### 6.2.6 *T.* spiralis *expulsion from the small intestine is not affected by PAR-2 agonist treatment.*

To further evaluate the role played by PAR-2 in the development of immune responses to *T. spiralis* we enhance signalling through PAR-2 was enhanced using the PAR-2 agonists SLIGRL-NH2 and ASKH-95. SLIGRL-NH2 is homologous to the native tethered ligand and ASKH-95, a synthetic furoylated derivative of the native peptide, both peptides bind and activate PAR-2. ASKH-95 has been shown to be a more potent activator of PAR-2 than SLIGRL-NH2 (Ferrell *et al.*, 2003; Kawabata *et al.*, 2004). LRGILS-NH2, the reversed ligand sequence fails to activate PAR-2 and is used as control treatment. Wild type C57/Bl6 mice were infected with 400 freshly isolated larvae and treated daily with 30μg per mouse of LRGILS-$NH_2$, SLIGRL-$NH_2$, or ASKH-95 by intraperitoneal injection from day –1 to 16 p.i.

Worm establishment was assessed in untreated mice at day 6 p.i. The expulsion of worms from the small intestine was measured in untreated, scramble peptide, SLIGRL-NH2 and ASKH-95 treated mice at day 14 and 16 p.i. by counting the number of worms present. Untreated, scramble peptide, SLIGRL-NH2, and ASKH-95 treated mice had significantly expelled their worms at day 16 p.i. compared to untreated mice at day 6 p.i. ($p=0.009$; 0.0472; 0.005 and 0.0209). No significant differences were observed between untreated; scramble peptide, SLIGRL-NH2, or ASKH-95 treated groups at day 14 or 16 p.i. (Figure 6.9).

FIGURE 6.9: The effect of treatment with PAR-2 agonists on the expulsion of *T. spiralis* from the small intestine. The establishment of adult *T. spiralis* in the small intestine was evaluated by counting the total number of worms harboured by untreated C57/Bl6 mice at day 6 p.i. To evaluate the effect of PAR-2 agonist treatment on the expulsion of *T. spiralis* from the small intestine the total number of worms harboured by untreated and LRGILS-NH2, SLIGRL-NH2 or ASKH-95 treated C57/Bl6 mice. Data is expressed as mean worm burden + SEM. *, Significantly difference between day 6 and 14 p.i. ND, not determined.


Gut Burdens
Mean worms/mouse + SEM
250
200
150
100
50
0
ND
* * * *
6
14
16
Days Post Infection
Untreated
Scramble
SLIGRL
ASKH95

### *6.2.7 PAR-2 Agonist treatment does not alter the development of enteropathy.*

The development of enteropathy was assessed in Clarke's fixed and Schiff's stained sections of jejunum taken from uninfected and infected (day 14 p.i.), untreated, scramble peptide, SLIGRL-NH2 and ASKH-95 treated mice. The sections were microdisected and the length of villi and depth of crypts measured in μm, while the number of dividing cells (mitotic figures) in crypts was counted.

No significant differences in villus length were observed between uninfected, untreated, and uninfected scramble peptide, SLIGRL-NH2 or ASKH-95 treated mice. No significant differences were observed in crypt length between uninfected untreated and uninfected scramble peptide or ASKH-95 treated mice, however uninfected SLIGRL-NH2 treated mice had shorter crypts than uninfected, untreated mice ($p=0.009$). Significant villus atrophy ($p=0.0283$, 0.0163, 0.009, 0.009) was present in untreated, scramble peptide, SLIGRL-NH2 and ASKH-95 treated mice at day 14 p.i. compared to uninfected mice. No significant differences are seen in villi and crypt lengths at day 16 p.i. compared to uninfected mice. No significant differences were observed between treatment groups at any time point (Figure 6.10A-D). The number of dividing cells per VCU was significantly higher at day 14 in untreated, LRGILS-NH2, SLIGRL-NH2, and ASKH-95 treated mice at day 14 p.i. ($p=0.009$, 0.0163, 0.009 and 0.009) and at day 16 p.i. in untreated, SLIGRL-NH2 and ASKH-95 treated mice ($p=0.0253$, 0.0367, and

0.0143) compared to uninfected mice in all treatment groups. No significant differences were observed between treatment groups at day 14 or 16 p.i. (Figure 6.10E).

To assess the degree of oedema developing in the gut, the entire small intestine was removed from uninfected and at day 14 and 16 p.i. untreated, scramble peptide, SLIGRL-NH2 or ASKH-95 treated mice and weighed. Intestinal weights were significantly higher in all treatment groups at day 14 (p=0.0143; 0.009; 0.009 and 0.009) and 16 p.i. (p=0.009; 0.009; 0.009 and 0.0143) (data not shown) compared to uninfected mice. No significant differences where observed between treatment groups at any time point measured (Figure 6.11).

FIGURE 6.10: Effect of PAR-2 agonist treatment on the development of enteropathy following infection with *T. spiralis*. Villus and crypt lengths were measured in uninfected and infected (day 14 p.i.), untreated (A), scramble peptide (B), SLIGRL-NH2 (C) and ASKH-95 (D) treated mice. The number of mitotic figures per crypt was determined in uninfected and infected (day 14 p.i.), untreated, scramble peptide, SLIGRL-NH2 and ASKH-95 treated mice (E). Five mice were used per group. Data is expressed as mean + SEM. *, represents significantly different to uninfected.

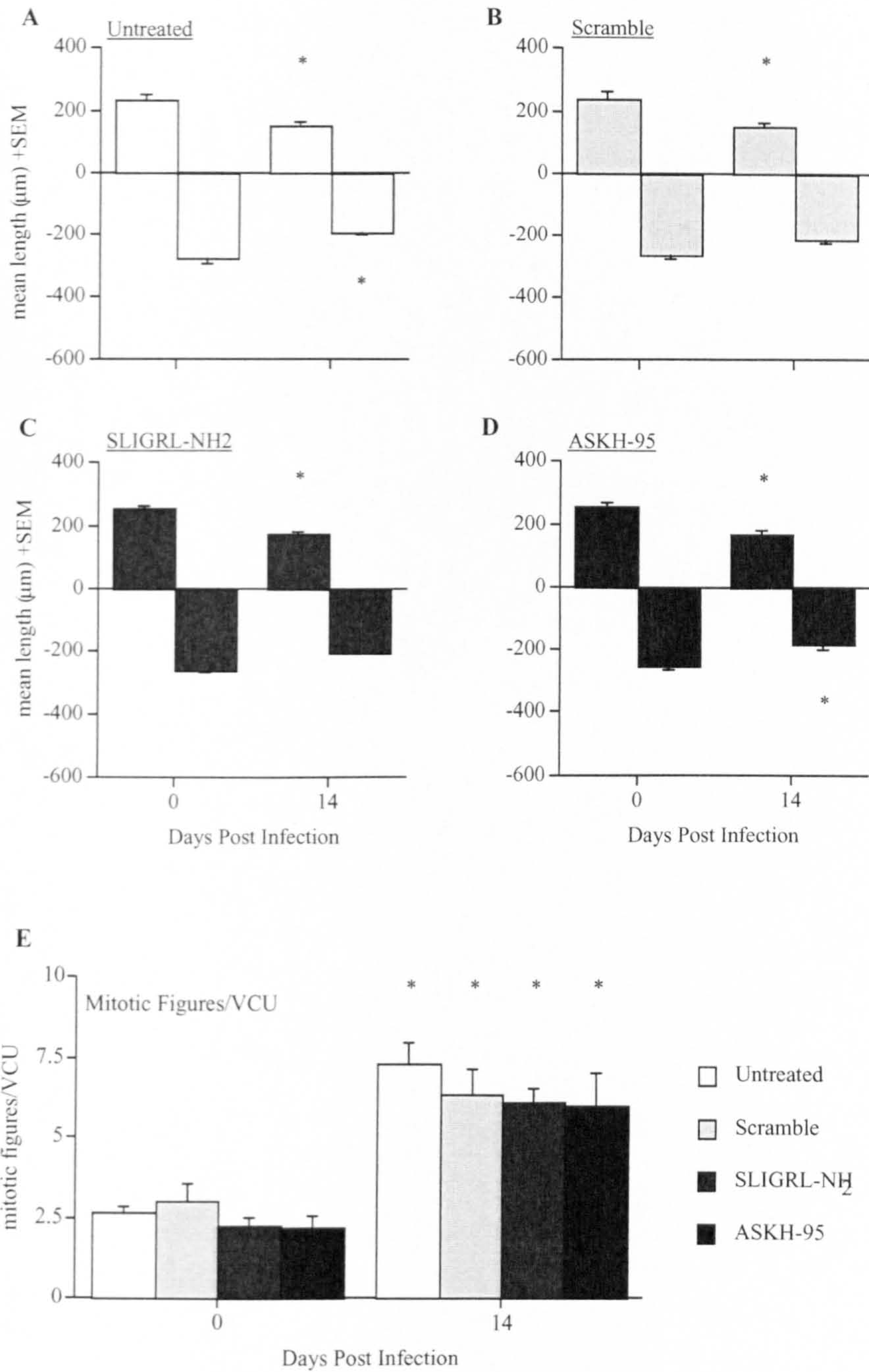
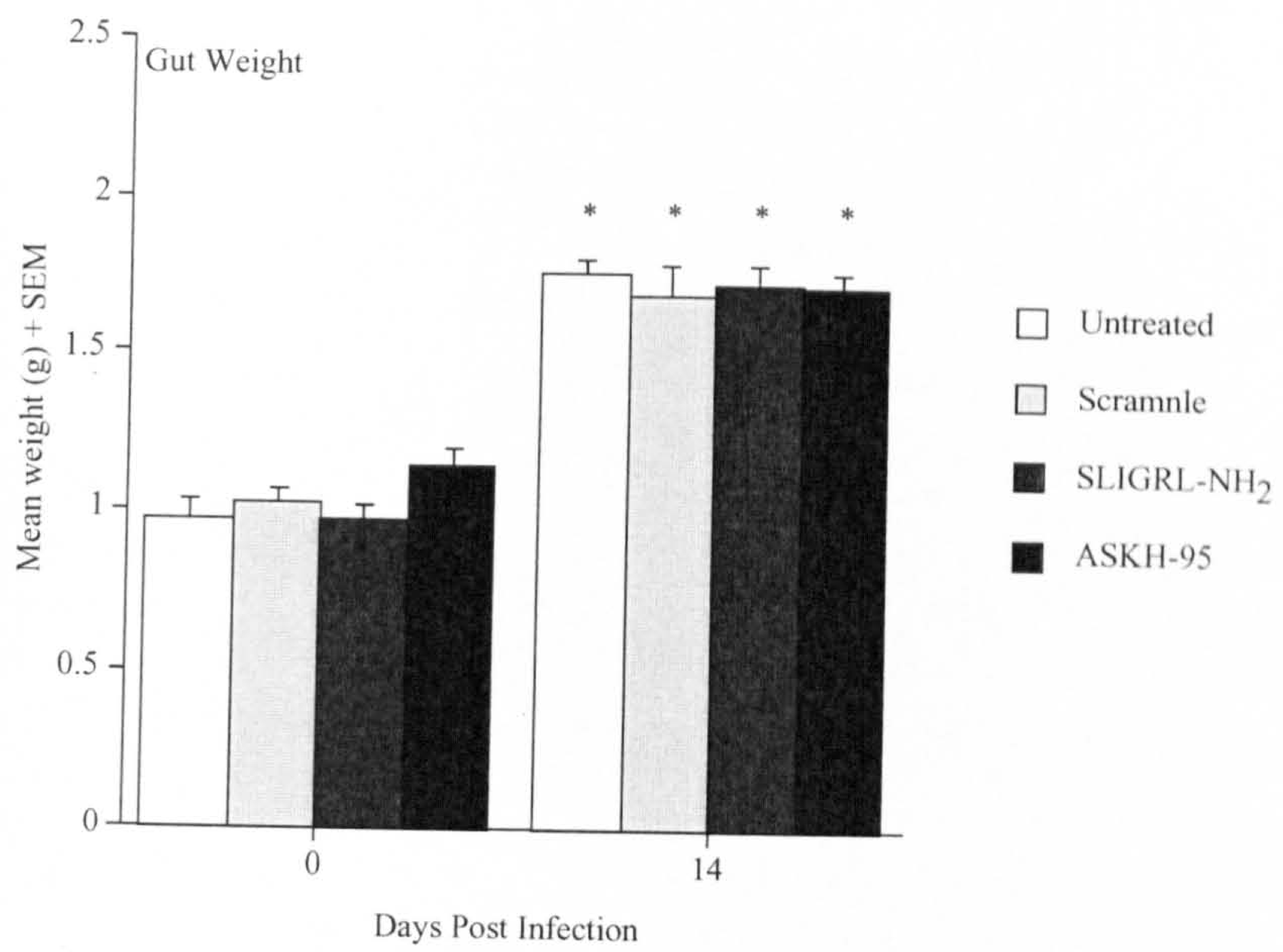

A
Untreated
B
Scramble
C
SLIGRL-NH2
D
ASKH-95
mean length (μm) +SEM
400
200
0
-200
-400
-600
0
14
Days Post Infection
E
Mitotic Figures/VCU
mitotic figures/VCU
10
7.5
5
2.5
Untreated
Scramble
SLIGRL-NH2
ASKH-95

FIGURE 6.11: The effect of PAR-2 agonist treatment on the development of oedema following infection with *T. spiralis*. To assess the effect of PAR-2 agonist treatment on the development of oedema following infection with *T. spiralis* the small intestine was removed from uninfected and infected (day 14 and 16 p.i.) untreated or LRGILS-NH2, SLIGRL-NH2 and ASKH-95 treated C57/Bl6 mice and weighed in grams. Data is expressed as mean weight (grams) + SEM, five mice were used for each group. *, represents significantly different to uninfected.


2.5
Gut Weight
2
1.5
1
0.5
0
Mean weight (g) + SEM
*
*
*
*
Untreated
Scramnle
SLIGRL-NH2
ASKH-95
0
14
Days Post Infection

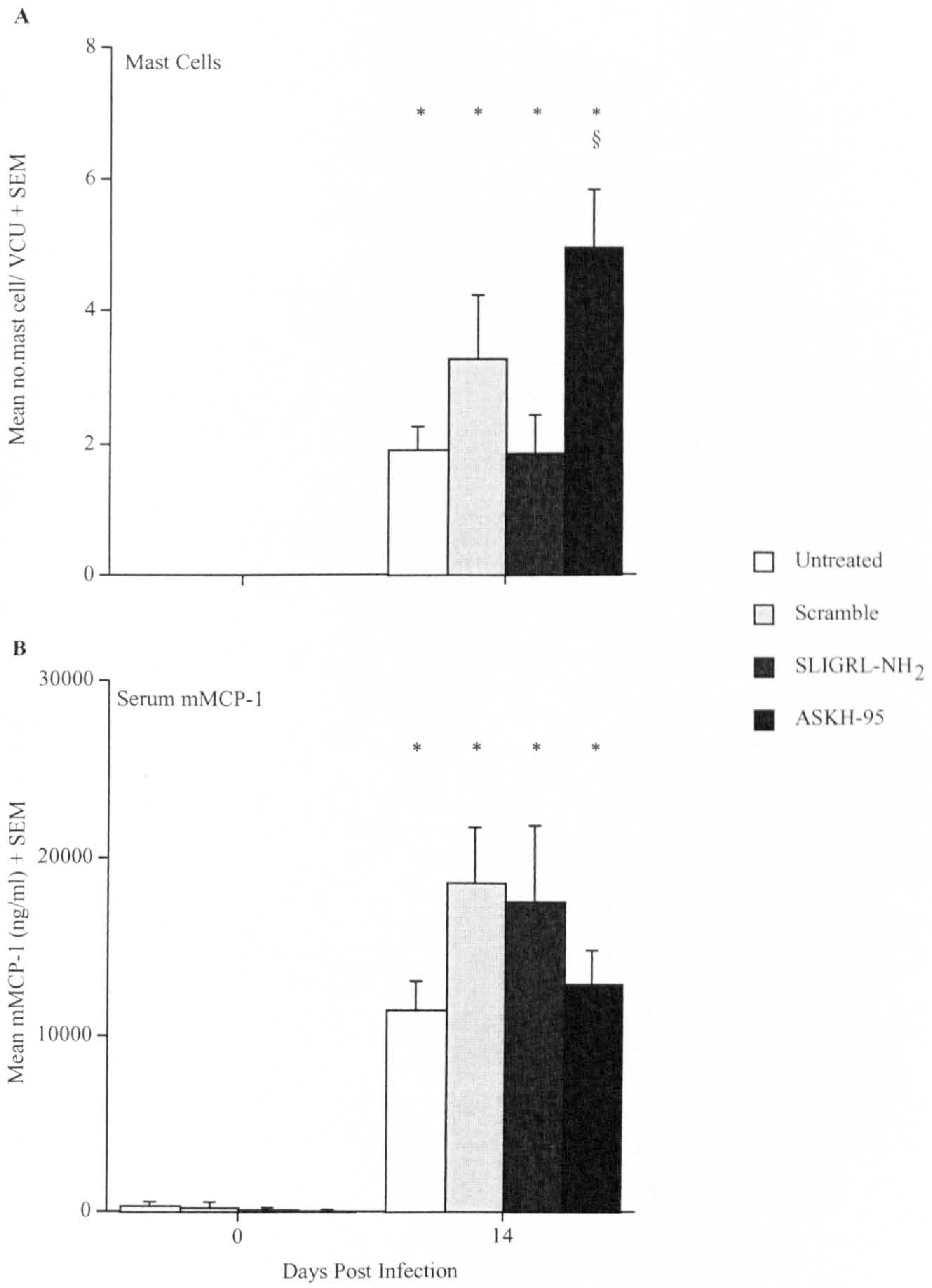
### *6.2.8 The development of peak mastocytosis is accelerated by ASKH-95 treatment but degranulation is not significantly altered by agonist treatment.*

The number of mast cells / VCU were counted in 5μm sections of jejunum taken from the small intestines of untreated controls and scramble peptide; SLIGRL-NH2 or ASKH-95 treated C57/Bl6 mice 0, 14 and 16 dpi fixed in Carnoy's fluid and stained with toluidine blue at pH 0.3. mMCP-1 is released from mast cells on degranulation and serum levels of mMCP-1 from untreated scramble peptide, SLIGRL-NH2 or ASKH-95 treated C57/Bl6 mice on days 0, 14 and 16 p.i. were analysed by ELISA.

The number of mast cells present in each VCU was significantly higher at days 14 (p=0.009, 0.0143, 0.009 and 0.009) and 16 p.i. (p=0.009, 0.0209, 0.009 and 0.0143) compared to uninfected mice in untreated and treated groups. No significant differences were observed between treatment groups and the untreated controls at days 0 and 16 p.i. However, at day 14 p.i. ASKH-95 treated mice had significantly more mast cells/ VCU than the untreated controls (p=0.0160), no significant differences were observed between scramble peptide or SLIGRL-NH2 and the untreated control mice at day 14 p.i. (Figure 6.12A). Serum mMCP-1 levels had significantly increased in the untreated controls and in the different treatment groups at days 14 (p=0.009, 0.009, 0.009 and 0.009) and 16 p.i. (p=0.009, 0.009, 0.009 and 0.0143) . Despite the increase in mast cell numbers observed in the ASKH-95 treated mice at 14 dpi no significant differences in

mMCP-1 levels was detected at 14 or 16dpi between the untreated controls and the treatment groups (Figure 6.12B).

FIGURE 6.12: The effect of PAR-2 agonist treatment on the development of mucosal mastocytosis and on serum mMCP-1 titres following infection with *T. spiralis*. A) The development of mastocytosis is assessed in sections of jejunum taken from untreated uninfected mice and from untreated, LRGILS-NH2, SLIGRL-NH2 and ASKH-95 treated mice at day 14 p.i., processed and stained with toluidine blue. The number of mucosal mast cells in 20 VCU were counted and the mean calculated for each individual. These values were then used to determine the mean number of mast cells/VCU for the group. Data is expressed as the mean number of mast cells /VCU + SEM. B) The activation and degranulation of mucosal mast cells in untreated uninfected mice and in untreated, LRGILS-NH2, SLIGRL-NH2 and ASKH-95 treated mice at day 14 p.i., was assessed by measuring serum titres of mMCP-1 by ELISA against a recombinant protein standard. Data is expressed as mean mMCP-1 concentration in pg/ml + SEM. *, represents significantly different to uninfected; §, represents significantly different to untreated.

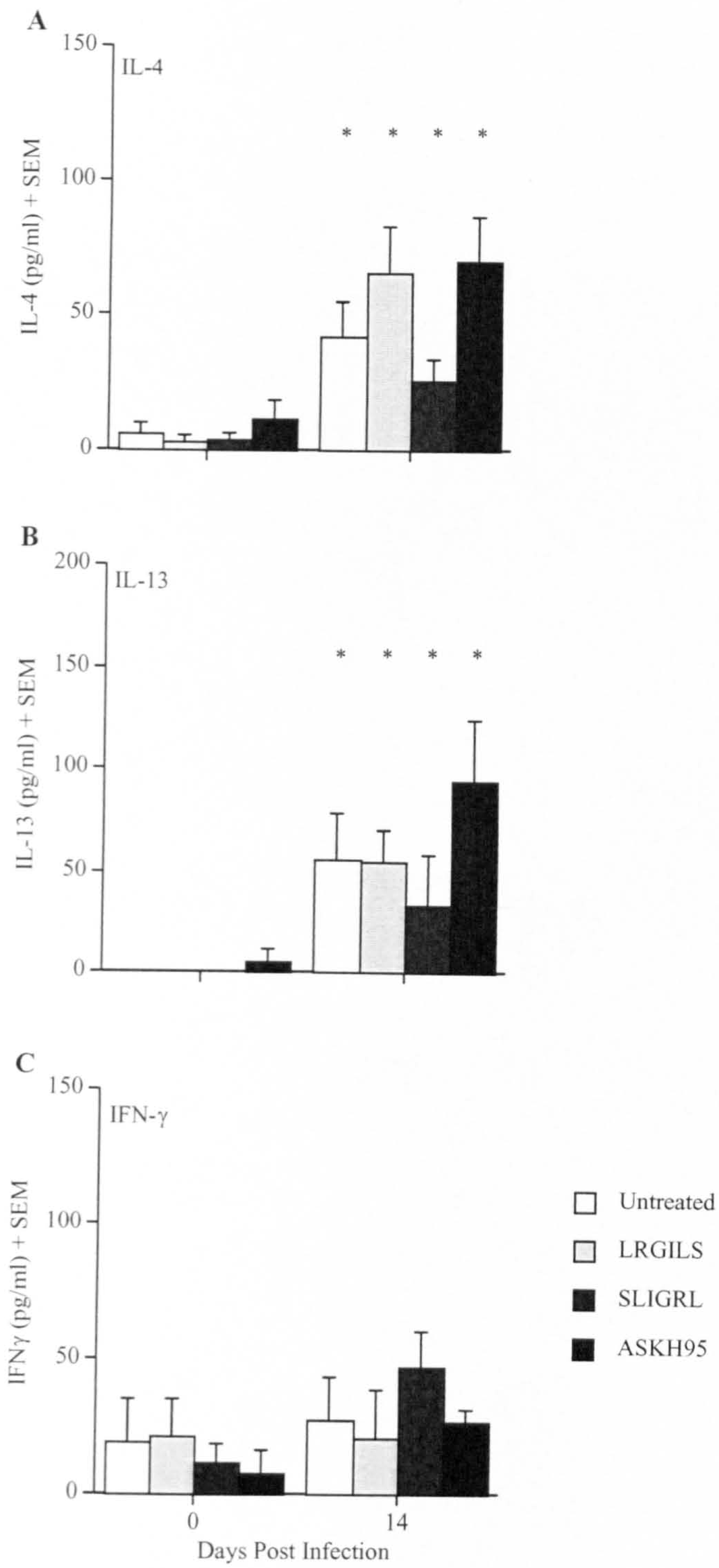

A
Mast Cells
Mean no.mast cell/ VCU + SEM
8
6
4
2
0
*
*
*
*
§
B
Serum mMCP-1
Mean mMCP-1 (ng/ml) + SEM
30000
20000
10000
0
*
*
*
*
0
14
Days Post Infection
Untreated
Scramble
SLIGRL-NH2
ASKH-95

### *6.2.9 Cytokine production at day 14 p.i. is not significantly affected by PAR-2 agonist treatment.*

To determine the effect of enhanced PAR-2 activation by agonist treatment on the development of Th responses lymphocytes were isolated from mesenteric lymph nodes removed from uninfected and infected (day 14 p.i.), untreated, scramble peptide, SLIGRL-NH2 and ASKH-95 treated C57/Bl6 mice and cultured with TAg. The secretion of IL-4, IL-13, and IFNγ was measured by ELISA in the culture supernatant.

Unstimulated cells produced only background levels of IL-4, IL-13 and IFNγ at 0 and 14 dpi (Figure 6.13A-C). MLN cells from untreated, scramble peptide, SLIGRL-NH2, and ASKH-95 treated mice at day 14 p.i. secreted significantly elevated levels of IL-4 (p=0.0264; 0.009; 0.0339 and 0.0275) and IL-13 (p=0.05; 0.0186; 0.2888 and 0.0384) when cultured with TAg compared to uninfected mice (Figure 6.13A, and B). IFNγ secretion did not significantly change between uninfected mice and mice at day 14 p.i. in any treatment group (Figure 6.13C). No significant differences in cytokine profiles were observed between treatment groups at any time point analysed.

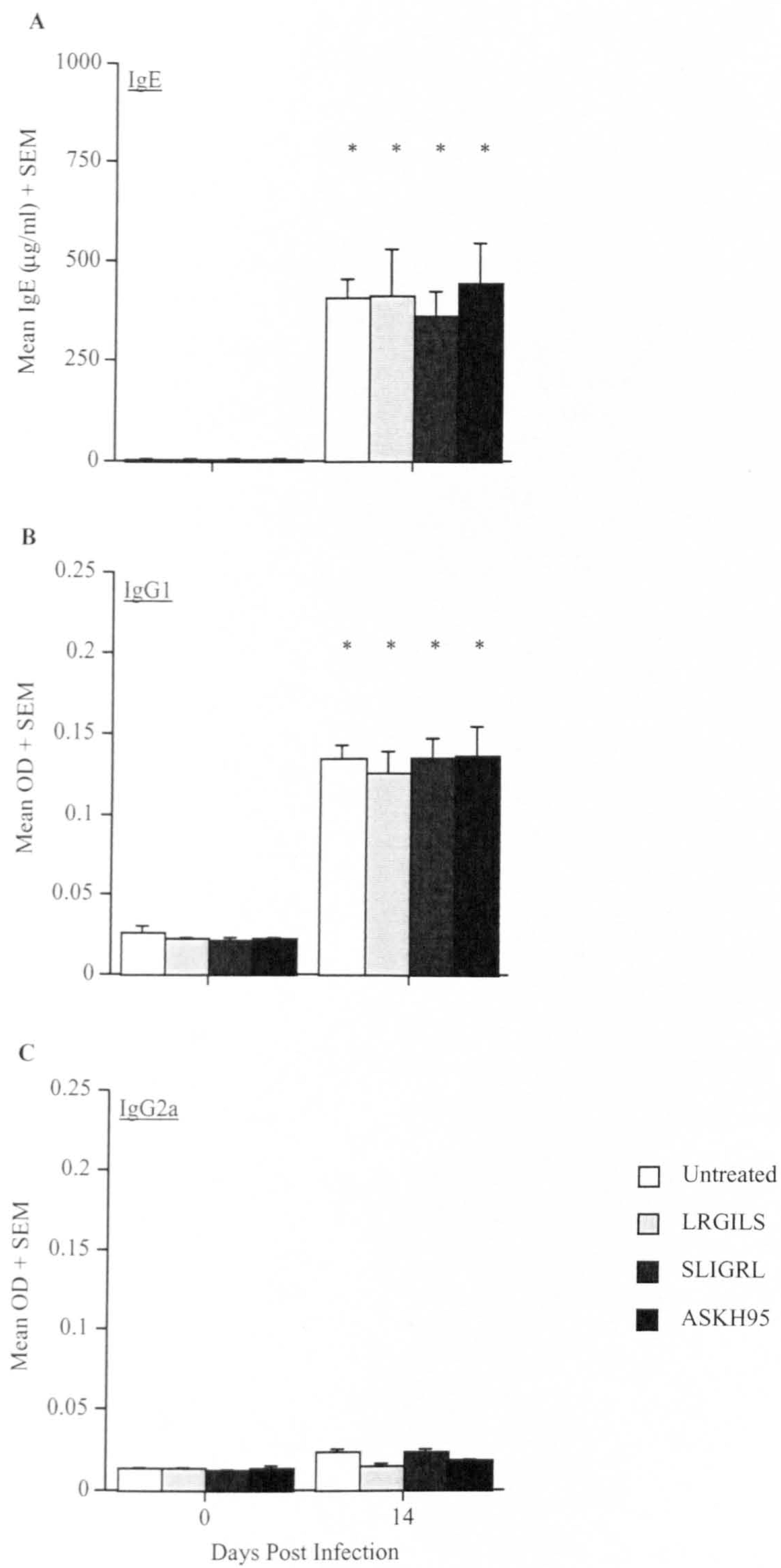
FIGURE 6.13: The effect of PAR-2 agonist treatment on the secretion of the cytokines IL-4, IL-13, and IFNγ from MLN cells stimulated *ex vivo* with TAg. MLN were removed from uninfected untreated and from infected untreated, LRGILS-NH2, SLIGRL-NH2, and ASKH-95 mice at day 14 p.i. and $1 \times 10^6$ cells were cultured with 50μg/ml TAg. The secretion of the cytokines IL-4 (**A**), IL-13 (**B**), and IFN-γ (**C**) were measured in pg/ml by ELISA against recombinant protein standards. Data is expressed as mean cytokine concentration (pg/ml) + SEM. *, represents significantly different to untreated, uninfected mice ($p<0.05$).


A
IL-4
IL-4 (pg/ml) + SEM
150
100
50
0
* * * *
B
IL-13
IL-13 (pg/ml) + SEM
200
150
100
50
0
* * * *
C
IFN-γ
IFNγ (pg/ml) + SEM
150
100
50
0
0
14
Days Post Infection
Untreated
LRGILS
SLIGRL
ASKH95

### *6.2.10 Total IgE and TAg specific IgG1 and IgG2a responses are not significantly different in PAR-2 agonist treated mice compared to scramble peptide treated or untreated controls.*

To further analyse the effect of PAR-2 agonist treatment on the Th response total IgE, and TAg specific IgG1 and IgG2a titres were measured in sera from untreated, scramble peptide, SLIGRL-NH2 or ASKH-95 treated wild type C57/Bl6 mice at 0, 14, and 16 dpi. Levels of total serum IgE increased significantly by day 14 ($p=0.009$; 0.009; 0.009 and 0.009) and 16 p.i. ($p=0.009$; 0.009; 0.0143 and 0.0143) (data not shown) compared to uninfected mice. No significant differences were observed between untreated and treated groups at any time point analysed (Figure 6.14A). TAg specific IgG1 is increased 14 ($p=0.009$; 0.009; 0.009 and 0.009) and 16 dpi ($p=0.009$; 0.009; 0.0143 and 0.0143) compared to uninfected mice in untreated and treated groups. However, no significant differences were observed between untreated and treated groups at any time point (Figure 6.14B). TAg specific IgG2a levels were not significantly elevated in any of the untreated or treated groups at day 14 and 16 p.i. compared to uninfected. No significant differences were observed between untreated and treated groups at any time point (Figure 6.14C).

FIGURE 6.14: The effect of PAR-2 agonist treatment on the development of antibody responses following infection with *T. spiralis*. Total IgE (A) and TAg specific IgG1 (B) and IgG2a (C) titres were measured in sera taken at days 0 and 14 p.i. from wild type and PAR-2 -/- mice. IgE titres were measured by sandwich ELISA against a purified IgE standard and were expressed as mean IgE (μg/ml) + SEM. While antigen specific IgG1 and IgG2a titres were assessed by ELISA using 96 well microtitre plates coated with TAg at 2μg/ml, sera was serially diluted and optical density readings at 1:2560 dilution were used. Data was expressed as mean optical density (OD) + SEM. *, represents significantly different to uninfected.


A
IgE
Mean IgE (µg/ml) + SEM
1000
750
500
250
0
B
IgG1
Mean OD + SEM
0.25
0.2
0.15
0.1
0.05
0
C
IgG2a
Mean OD + SEM
0.25
0.2
0.15
0.1
0.05
0
0
14
Days Post Infection
Untreated
LRGILS
SLIGRL
ASKH95

## *6.3 Discussion.*

PAR-2 has been shown to play a role in the development of inflammation and pathology in a variety of murine models, including colitis (Cenac *et al.*, 2002), allergic and contact dermatitis (Kawagoe *et al.*, 2002; Takizawa *et al.*, 2002), and arthritis (Ferrell *et al.*, 2003). *T. spiralis* infection induces a Th2 response and the development of enteropathy, mucosal mastocytosis and mast cell degranulation, these studies show that PAR-2 plays a role in the development of these responses.

The development of villus atrophy during *T. spiralis* infection and oedema leading to increased gut weight was delayed in PAR-2 -/- mice, although no significant differences were observed in the development of crypt hyperplasia in PAR-2 -/- compared to wild type mice. However, the activation of PAR-2 does not appear to be necessary for the development of protective responses, as the expulsion of *T. spiralis* from the small intestine was not significantly altered in PAR-2 -/- mice compared to wild type mice at day 14 or 16 p.i. PAR-2-/- mice showed no significant differences the development of mastocytosis during infection compared to wild type however the peak mast cell degranulation as shown by serum mMCP-1 levels at day 14 p.i. was reduced in the absence of PAR-2. Total serum IgE responses were elevated following infection in both strains, but were significantly lower in the PAR-2 -/- mice compared to wild type. This antibody data suggests that PAR-2 -/- produce a reduced Th2 response to *T. spiralis* however *ex vivo* re-stimulation of MLN lymphocytes at day 6 p.i. with TAg resulted in

no significant differences in production of the cytokines IL-4, IL-13, and IFNγ in PAR-2 -/- compared to wild type mice.

PAR-2 agonist treatment did not significantly affect expulsion, the development of enteropathy or the cytokine and antibody responses. The native peptide SLIGRL-NH2 failed to alter the development of mastocytosis, however treatment with the synthetic furoylinated peptide ASKH-95 increased in the number of mast cells in the intestine by day 14 p.i. Mast cell degranulation as indicated by serum mMCP-1 titres is not altered by SLIGRL-NH2 or ASKH-95 treatment.

Increased gastrointestinal transit has been suggested as a possible mechanism for the expulsion of gastrointestinal nematodes. It has been shown that peak intestinal muscle contractility coincides with the expulsion of *T. spiralis*, occurring earlier in the more resistant NIH Swiss mice compared to the more susceptible B10.BR mice (Vallance *et al.*, 1997). PAR-2 activation has been shown promote gastrointestinal transit by increasing smooth muscle contractility (Kawabata *et al.*, 2001). Increased gastrointestinal transit may also be due to increased water retention in the lumen, increased blood flow to the intestine and increased secretion of $Cl^-$ into the lumen. The injection of PAR-2 agonists has been shown to lead to increased vascular permeability and oedema in the hind paw of rats (Kawabata *et al.*, 1998) and to vasodilatation in humans (Robin *et al.*, 2003). Worm expulsion is unaltered in PAR-2 -/- mice compared to wild type thus PAR-2 regulation of intestinal transit does not appear to be necessary

for *T. spiralis* expulsion. However, the delay in the development of oedema in *T. spiralis* infected PAR-2 -/- mice may relate to the role played by PAR-2 in increasing gastrointestinal transit, blood flow to the intestine (Kawabata *et al.*, 2003) and vascular permeability (Kawabata *et al.*, 1998).

Th2 responses have been shown to be essential for the development of protective responses and enteropathy following infection with *T. spiralis* (Lawrence *et al.*, 1998). The Th2 antibody responses, IgE and IgG1, in PAR-2 -/- mice were reduced compared to wild type. Although the Th2 antibody titres were reduced, the Th1 antibody IgG2a titres were not increased in the PAR-2 -/- mice compared to wild type. Suggesting a reduction in the inflammatory response to *T spiralis* without a shift in Th1:Th2 bias. However, *ex vivo* re-stimulation of MLN cells from PAR-2 -/- mice, with TAg does not result in reduced IL-4 or IL-13 or increased IFNγ secretion compared to MLN cells from wild type mice.

In PAR-2 -/- mice villus atrophy was reduced while crypt hyperplasia was increased this suggests that activation of PAR-2 may be involved in the apoptosis of cells at the tip of the villi but not in the division of cells in the crypt. This may occur directly, as PAR-2 has been shown to be expressed on enterocytes where trypsin has been shown to result in the release of prostaglandin E2 (Kong *et al.*, 1997). Alternatively the PAR-2 agonist SLIGRL-$NH_2$ has been shown to induce colitis in mice and this colitis has been shown to be dependant on nitric oxide synthase (Cenac *et al.*, 2002; Cenac *et al.*, 2003), we

have also shown that enteropathy following *T. spiralis* infection is dependant on the production of NO via inducible nitric oxide synthase (iNOS) (Lawrence *et al.*, 2000). However, the role of PAR-2 in NO mediated enteropathies requires further investigation.

*T. spiralis* infection results in the mastocytosis and in the release of mMCP-1 which has been implicated in the expulsion of *T. spiralis* and in the development of enteropathy following infection. (Brown *et al.*, 1981; Knight *et al.*, 2000; Lawrence *et al.*, 2004). Mast cells and their proteases, in particular the mucosal mast cell protease (mMCP)-1 have been implicated in the expulsion of *T. spiralis* from the small intestine (Knight *et al.*, 2000). mMCP-1 is a serine protease and therefore may potentially activate the PAR-2 receptor. Expulsion is not significantly different between PAR-2-/- and wild type mice suggesting that mMCP-1 functions in expulsion by a PAR-2 independent mechanism. Although mastocytosis was not affected by PAR-2 deficiency, degranulation was reduced as shown by reduced serum mMCP-1 levels. Mast cell proteases have been shown to activate human mast cells in the skin (Steinhoff *et al.*, 1999) and signalling through PAR-2 has been shown to activate human colon mast cells (He *et al.*, 2004). Similar results have been obtained in the activation of PAR-2 in guinea pig myenteric neurons (Corvera *et al.*, 1999) and rat colonic myocytes (Corvera *et al.*, 1997) by mast cell tryptases. Thus the activation of PAR-2 may occur through mast cell proteases furthermore it has been shown that human tonsil mast cells are stimulated to secrete tryptase by PAR-2 agonist (SLIGKV-$NH_2$) treatment *in vitro* (He *et al.*, 2005). Thus PAR-2 appears to play a role in the activation and degranulation of mucosal mast cells

but not in the development of mastocytosis following infection with *T. spiralis*. mMCP-1 is a serine protease and therefore may potentially activate the PAR-2 receptor.

In the intestine mast cells are a major source of TNFα which is stored preformed in granules (Bischoff *et al.*, 1999), our previous work has shown that enteropathy is dependant on signalling through TNFR1 and subsequent activation of inducible nitric oxide synthase (Lawrence *et al.*, 1998; Lawrence *et al.*, 2000). Thus reduced mast cell degranulation as well reducing mMCP-1 release may also reduce the local release of TNFα.

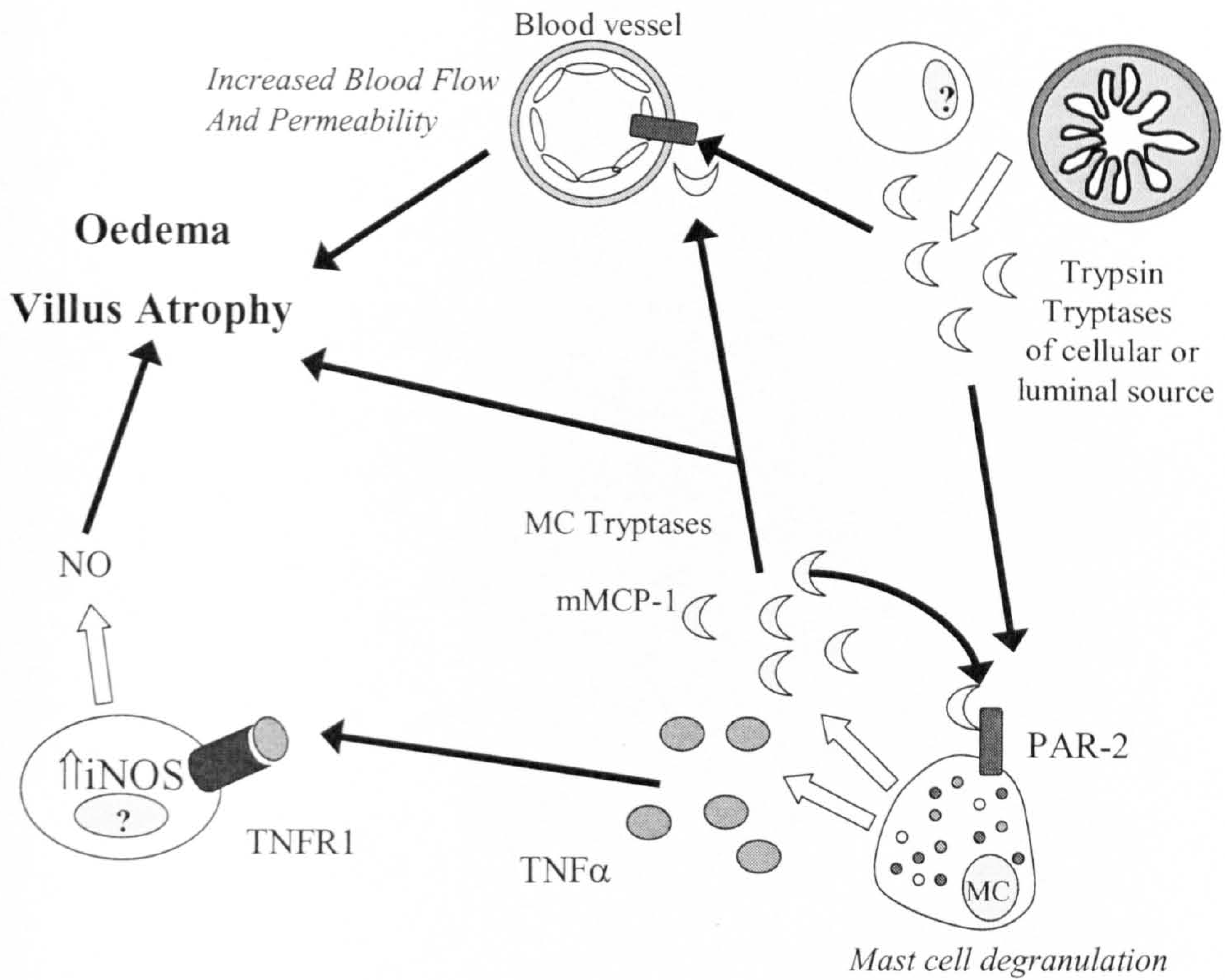



FIGURE 6.15: Possible role for PAR-2 in the development of enteropathy following infection with *T. spiralis*.

The treatment of mice with the agonists SLIGRL-$NH_2$ and ASKH-95 did not effect either the expulsion of *T. spiralis* or the development of enteropathy this may reflect the fact that *T. spiralis* infection is highly pro-inflammatory and once PAR-2 is activated further activation can only occur once the cell has synthesised new receptors and translocated them to the cell surface (Dery *et al.*, 1998). So the limiting factor in the activation of PAR-2 during infection with *T. spiralis* is the presence of sufficient inactive receptor rather than the presence of factors activating PAR-2 such as serine proteases or agonists. Interestingly mast cell numbers were increased in ASKH-95 treated mice at day 14 p.i. compared to untreated or SLIGRL-$NH_2$ treated mice. However no significant differences in mast cell numbers were observed at day 16 p.i. ASKH-95 is furoylinated peptide analogue of the tethered ligand has previously been shown to be a more potent agonist of PAR-2 than the native peptide SLIGRL-$NH_2$. Furoylination of agonist peptides has been shown to increase the resistance to degradation by aminopeptidases (Kawabata *et al.*, 2004), furthermore ASKH-95 has been shown to induce a more prolonged artheritic infammation in comparison to the native peptide (Ferrell *et al.*, 2003). Thus the increased potency of ASKH-95 may allow it to promote the development of mastocytosis following *T. spiralis* infection, by acting more rapidly than SLIGRL-$NH_2$ or endogenous serine proteases released following infection or due to ASKH-95 increased resistance to degradation (Kawabata *et al.*, 2004).

PAR-2 activation appears to provide a pro-inflammatory signal during infection with *T. spiralis*, stimulating the development of villus atrophy and the development of intestinal

oedema, although crypt hyperplasia is stimulated by mechanisms distinct from PAR-2. The infiltration of mast cells into the mucosa during infection with *T. spiralis* is not dependant on signalling through PAR-2, however PAR-2 activation appears to be important in the degranulation of mucosal mast cells. Despite the pro-inflammatory effects of PAR-2 activation does not appear to be necessary for the expulsion of adult *T. spiralis* from the small intestine. Thus targeting PAR-2 activation may be benefical in Th2 mediated enteropathies without inhibiting protective responses.

# Chapter Seven

## General Discussion

# 7 General Discussion.

This thesis has confirmed the importance of Th2 responses in the development of both pathology and parasite expulsion following infection with *Trichinella spiralis* (Grencis *et al.*, 1991; Lawrence *et al.*, 1998; Vallance *et al.*, 1999). Host strain specific differences were also identified in the role of IL-4, with IL-4 being necessary in C57BL/6 mice for expulsion but not in BALB/c mice, where IL-13 appears to be the more important cytokine. Interestingly although global IL-4Rα -/- mice fail to develop enteropathy a role for IL-4Rα signalling to cells of the macrophage/neutrophil lineage in limiting pathology following *T. spiralis* infection was identified. A role for both ICOS and OX40 co-stimulation was also demonstrated in the development of T helper responses to *T. spiralis* infection. Blockade of ICOS resulted in a reduction in the Th2 response as assessed by reduced levels of IgE, IL-4 and IL-5 production and a corresponding increase in IFN-γ production by MLN cells. Conversely, however, levels of IL-13 and IL-10 were elevated following treatment with anti-ICOS. This elevation in IL-10 production was accompanied by a reduction in enteropathy. Interestingly the expulsion of adult worms from the intestine was unaffected but the accumulation of muscle larvae was reduced. Enhancing OX40 signalling with OX40L-Ig enhanced Th2 responses, while blockade of OX40 signalling with OX40-Ig enhanced mucosal mastocytosis. Neither treatment however, altered the development of enteropathy or protective responses to *T. spiralis*.

Previous studies have shown that the development of enteropathy, but not expulsion, is mediated by signalling via the p55 TNFR1 (Lawrence *et al.*, 1998) suggesting a role for TNFα in the development of pathology. However, in this study we have demonstrated that in the absence of LTα and sTNFα mice show significantly delayed expulsion of *T. spiralis* from the intestine and enhanced enteropathy. Expulsion in tmTNFα-tg mice was further delayed and villus atrophy was significantly enhanced compared to TNFα/LTα-/- mice. The development of the Th2 response following infection with *T. spiralis* was also significantly reduced in the absence of soluble TNFα and LTα, interestingly, however, IL-13 responses were unaffected. Mast cell responses have previously been shown to be important in expulsion and in the development of enteropathy, consistent with delayed expulsion observed in the absence of soluble TNFα and LTα, mast cell responses were virtually absent. Interestingly, enteropathy was enhanced suggesting that enteropathy in this model is developing via a mast cell independent mechanism.

The mast cell derived tryptase mMCP-1 has been shown to play a role in the development of enteropathy (Lawrence *et al.*, 2004) and in the expulsion of *T. spiralis* from the small intestine (Knight *et al.*, 2000). Proteases such as collagenases, elastases, and tryptases (like mMCP-1) are thought to play a role in the development of pathological conditions such as enteropathies by breaking down connective tissues and allowing the remodelling of tissues. This thesis has demonstrated a role for the novel transmembrane protein PAR-2 in the development of enteropathy and in the regulation of mast cells following *T. spiralis* infection. Although PAR-2 deficiency did not affect

mast cell numbers treatment with the synthetic PAR-2 activating peptide ASKH-95 enhanced mastocytosis following infection.

The development of enteropathy following infection with GI parasites has been suggested to be a defence mechanism against the parasite, making the intestinal environment unsuitable and dislodging the parasite from its niche. Evidence supporting this hypothesis has been shown in *T. muris* infection models where the more resistant BALB/c mouse strain develop a more rapid increase in epithelial cell turn over than susceptible AKR strains (Cliffe *et al.*, 2005). In *T. spiralis* infection the development of enteropathy, however, does not coincide with parasite expulsion and the use of TNF-R1 -/- and iNOS -/- mice have shown that parasite expulsion may occur normally in the absence of enteropathy (Lawrence *et al.*, 1998; Lawrence *et al.*, 2000). The studies presented here further demonstrate a disassociation between enteropathy and expulsion in *T. spiralis* infection, with anti-ICOS treatment and PAR-2 deficiency ameliorating pathology without delaying the expulsion of *T. spiralis* from the small intestine. While deficiency in TNFα and LTα and deficiency of IL-4Rα in cells of the macrophage/neutrophil lineage enhanced enteropathy, in TNFα/LTα -/- mice this was associated with delayed expulsion, while in the macrophage/neutrophil IL-4Rα specific -/- mice expulsion was unaffected. If the development of enteropathy is a necessary component of the expulsion of *T. spiralis*, it would be expected that reducing enteropathy would delay expulsion, while enhancing enteropathy would accelerate the expulsion of *T. spiralis*. Mechanisms of expulsion of nematode parasites from the small

intestine are complex, the disassociation of enteropathy from expulsion in *T. spiralis* but not in *T. muris* may relate to differences in the niches occupied by these two parasites, in fact increased epithelial cell turnover ceases to be effective in *T. muris* infection after about day 21 post infection when the parasite has moulted and moved location in the epithelium (Cliffe *et al.*, 2005).

A number of factors are believed to play a role in the development of both enteropathy and protective responses following *T. spiralis* infection such as mast cells and their protease mMCP-1 (Knight *et al.*, 2000; Lawrence *et al.*, 2004), IL-4 and IL-4Rα (Lawrence *et al.*, 1998). In order to further analyse the mechanisms involved in the expulsion of *T. spiralis* and in the development of enteropathy it would be of interest to determine the important sources of different cytokines in the intestine. For example, it has recently been shown that NK cells are an important source of IL-13 during infection with *T. spiralis* and are sufficient to stimulate the development of enteropathy (McDermott *et al.*, 2005). Mast cells are another cell of the innate immune system which have been shown to be important in both the development of enteropathy and in the expulsion of worms from the intestine, expulsion and enteropathy being reduced in the mast cell deficient W/Wv mouse and expulsion delayed following the blockade of stem cell factor in mice, which abrogates the development of intestinal mastocytosis (Donaldson *et al.*, 1996; Grencis *et al.*, 1993). The mice deficient in the mast cell derived protease mMCP-1 have also been shown to develop reduced enteropathy and to be slow in expelling the intestinal phase of *T. spiralis* (Knight *et al.*, 2000; Lawrence *et*

*al.*, 2004). Mast cells have also been shown to produce IL-4 (Frandji *et al.*, 1998), IL-13 (Toru *et al.*, 1998) (Obara *et al.*, 2002) and TNFα (Bischoff *et al.*, 1999). Mast cell derived cytokines have been shown to play important roles in a number of conditions, for example, mast cell derived IL-13 is important in the development of atopic dermatitis (Khan *et al.*, 2003) while mast cell derived TNFα is important in gastric allergic inflammation (Furuta *et al.*, 1997). Thus evaluation the role of mast cell derived cytokines in *T. spiralis* infection, by the reconstitution of mast cell deficient mice with bone marrow from mice deficient in IL-4, IL-13 and TNFα, would be important.

Evidence of interactions between T cells and mast cells has also been shown; *in vitro* activated T cells have been shown to activate mast cells (Bhattacharyya *et al.*, 1998) while mast cells have been shown to be capable of presenting antigen to T cells (Fox *et al.*, 1994; Frandji *et al.*, 1993) and express co-stimulatory molecules including OX40 and CD40 (Kashiwakura *et al.*, 2004). Mast cells and mast cell-derived TNFα have also been shown to be important in driving antigen specific T cell proliferation and cytokine production *in vitro* and *in vivo* in oxazolone induced delayed type hypersensitivity (Nakae *et al.*, 2005). However, the role of mast cells as antigen presenting cells during infection with *T. spiralis* is not yet clear, it would be of interest to analyse the expression of co-stimulatory signals such as ICOSL and OX40L by mucosal mast cells following infection with *T. spiralis*.

In contrast to previous studies using p55 TNFR1 deficient mice where parasite expulsion was normal and enteropathy was reduced (Lawrence *et al.*, 1998), this thesis has shown an interesting if not rather surprising role for TNFα and LTα in both protective responses and in the control of enteropathy following infection with *T. spiralis.* This may indicate a specific protective role for LTα or it may relate to the severe structural abnormalities in the immune system, which occur in the absence of TNFα and LTα (Eugster *et al.*, 1996). To address this, blocking the function of the cytokines using either antibodies directed against the cytokine itself or against their receptors in normal mice would allow the analysis of the function of the cytokine in a mouse with a structurally normal immune system. LTα has been shown to be important in the control of bacterial infections in the intestine (Spahn *et al.*, 2004), so the enhanced enteropathy observed in *T. spiralis* infected TNFα/LTα deficient mice may related to a loss of control of luminal flora which may be carried into the intestinal epithelium by the tissue penetrating *T. spiralis* adults and migrating newborn larvae. This hypothesis may be tested by pre-treating TNFα/LTα -/- mice with antibiotics to remove intestinal flora prior to infection with *T. spiralis.*

This study has also demonstrated a potential role for PAR-2 in the activation of mast cells following infection with *T. spiralis*. It has also previously been shown that the treatment of rat peritoneal mast cells with PAR-2 agonists *in vitro* leads to activation and degranulation (Stenton *et al.*, 2002) and tryptase activates mast cells from human skin via PAR-2 (Steinhoff *et al.*, 1999). However, the *in vivo* function of this receptor on

mucosal mast cells is not clear and thus is of interest, and may be studied by reconstituting W/Wv mice with bone marrow from PAR-2 -/- mice. PAR-2 is expressed on enterocytes through out the intestine and therefore may also signal directly to these cells (Kong *et al.*, 1997), affecting their division and differentiation. This study suggests that the activation of PAR-2 may be involved in the death and loss of enterocytes leading to villus atrophy. This hypothesis may be evaluated the reconstitution of sub-lethally irradiated PAR-2 -/- mice, whose bone marrow has been depleted, with bone marrow from wild type mice, as all non-bone marrow derived cells will be PAR-2 deficient.

This thesis has demonstrated the importance of Th2 cytokines in both the development of enteropathy and the expulsion of *T. spiralis*, the development of Th2 reponses may be modulated by co-stimulatory signals from ICOS and OX40. Th2 responses are also necessary for the development of intestinal mastocytosis, which has been demonstrated to be important in both enteropathy and expulsion. Mast cells have been shown to be major intestinal sources of TNFα (Bischoff *et al.*, 1999; Gordon and Galli, 1990), in contrast to previous studies using TNF-R -/- mice (Lawrence *et al.*, 1998), this thesis has also demonstrated role for TNFα and LTα in parasite expulsion and in protection from enteropathy. Furthermore this thesis has shown that tryptase signalling via PAR-2 is important in both the development of enteropathy and in mast cell degranulation, as assessed by serum mMCP-1 titres, which is a tryptase and may therefore activate PAR-2 (Figure 7.1).

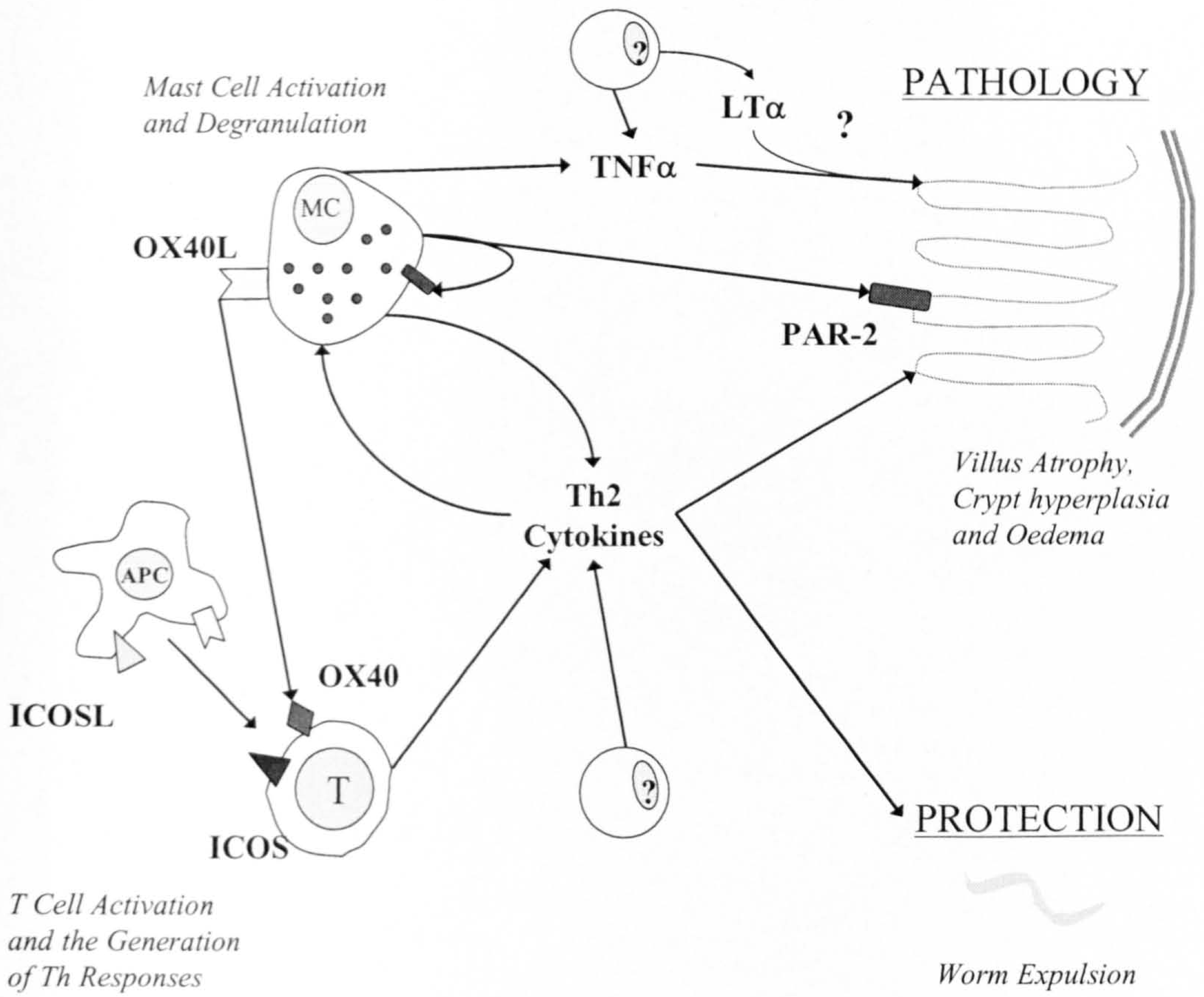



FIGURE 7.1: Overview of the interaction of various factors involved in the generation of pathology and protective responses to *T. spiralis*.

In conclusion, the mechanisms by which enteropathy develops and *T. spiralis* is expelled from the small intestine are complex. The generation of a Th2 response is important, in particular IL-13, and this response may be modulated by co-stimulatory signals such as ICOS and OX40, by cytokines such as TNFα and LTα or by signalling via PAR-2. However, interestingly this may not necessarily alter the rate of expulsion or the development of enteropathy. It has been shown that different strains of mice develop different Th1/Th2 balance following infection with GI nematodes, in infections such as *T. muris* this balance has been shown to determine whether the host is resistant or will develop chronic infection. However in *T. spiralis*, which does not develop into a chronic infection, the Th1/Th2 balance does not appear to directly affect the rate of expulsion. This therefore suggests that other mechanisms are important, a possible mechanism of nematode expulsion from the intestine is the development of enteropathy; however, the data presented in this thesis provides further evidence that expulsion occurs independently of enteropathy. The development of both expulsion and enteropathy is also believed to be at least in part due to the development of mucosal mastocytosis and the release of mMCP-1 (Knight *et al.*, 2000; Lawrence *et al.*, 2004). Combined TNFα and LTα deficiency lead to a failure of the mice to develop mastocytosis and parasite expulsion, enteropathy however, was enhanced suggesting that in this model enteropathy developed by a mast cell independent mechanism. In PAR-2 -/- mice mast cell numbers were normal but serum mMCP-1 titres were significantly reduced but not entirely absent, this was accompanied by a reduction in enteropathy but not by any change in the rate of expulsion of *T. spiralis*. This suggests that PAR-2 may be involved in mast cell

degranulation and that mMCP-1 may be able to mediate expulsion at lower levels than those that result in enteropathy. Further study of the interaction of these molecules during infection with *T. spiralis* and their effects on mast cells is important in both increasing understanding of the mechanisms of nematode immunity and in understanding the role of mast cells in immune responses in general, especially as evidence is emerging that mast cell responses may play a role in the development of conditions such as arthritis (Askenase, 2003), and inflammatory bowel disease (He, 2004) and in protection against bacterial pathogens (Echtenacher *et al.*, 1996).

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# Appendix

## Appendix

**<u>45% Acetic Acid</u>**

450ml Glacial Acetic Acid,

550ml $dH_20$.

**<u>0.1% Agarose/0.9%NaCl Solution</u>**

1g Agarose (VWR International, UK),

9g NaCl (VWR International, UK),

1L $dH_2O$ ,

Heat to dissolve Agarose.

**<u>Carnoy's Fixative</u>**

600ml Absolute Ethanol (VWR International, UK),

300ml Glacial Acetic Acid (VWR International, UK),

100ml Chloroform (Sigma-Aldrich, UK).

**<u>Clark's Fixative</u>**

750ml Absolute ethanol (VWR International, UK),

250ml Glacial Acetic Acid (VWR International, UK).

**50% Ethanol**

500ml Absolute Ethanol (VWR International, UK),

500ml $dH_2O$.

**70% Ethanol**

700ml Absolute Ethanol (VWR International, UK),

300ml $dH_2O$.

**90% Ethanol**

900ml Absolute Ethanol (VWR International, UK),

100ml $dH_2O$.

**50/50 Ethanol/Histoclear**

500ml Absolute Ethanol (VWR International, UK),

500ml Histoclear (National Diagnostics, UK).

**166.67mM 4-(2-Hydroxyethyl)-1-piprazineethansulfonice acid (HEPES) solution**

397.3g HEPES (Sigma-Aldrich, UK),

Make up to 980ml with distilled water ($dH_2O$),

Adjust pH to 7.4,

Make final volume up to 1L and filter sterilise.

**0.7M HCl**

60.06ml concentrated (c)HCl (VWR International, UK),

939.94ml $dH_2O$.

**1M HCl**

85.8ml cHCl (VWR International, UK),

914.2ml $H_20$.

**0.1M β-Mecaptoethanol Solution**

7ml β-Mecaptoethanol (Sigma-Aldrich, UK),

993ml $dH_2O$,

Filter sterilise.

**0.9% NaCl Solution**

9g NaCl (VWR International, UK),

1L $dH_2O$.

**3.5% $NaCO_3$ Solution**

35g $NaCO_3$ (VWR International, UK),

1L $dH_2O$,

Filter sterilise.

**0.1M $Na_2CO_3$ Buffer**

10.58g $Na_2CO_3$(VWR International, UK),

Make up to 980ml $dH_2O$,

Adjust pH to 8.2,

Make final volume up to 1L.

**0.1M $Na_2CO_3$/$NaCO_3$ Buffer**

5,3g $Na_2CO_3$ (VWR International, UK),

4,2g $NaCO_3$(VWR International, UK),

Make up to 980ml with distilled water ($dH_2O$),

Adjust pH to 9.6,

Make final volume up to 1L.

**0.05M $Na_2CO_3$/$NaCO_3$ Buffer**

2.65g $Na_2CO_3$(VWR International, UK),

2.1g $NaCO_3$(VWR International, UK),

Make up to 980ml $dH_2O$,

Adjust pH to 9.6,

Make final volume up to 1L.

**0.5% Pepsin/ 0.9% NaCl/0.5%cHCl**

5g Pepsin A powder (Sigma-Aldrich, UK),

9g NaCl (VWR International, UK),

5ml cHCl (VWR International, UK),

995ml $dH_20$.

**Phosphate Buffered Saline (PBS)**

8g NaCl (VWR International, UK),

2.93g $Na_2HPO4 \cdot 12H_2O$ (VWR International, UK),

0.2g KCl (VWR International, UK),

0.2g $KH_2PO_4$ (VWR International, UK),

Make up to 980ml with distilled water ($dH_2O$),

Adjust pH to 7.4.

Make final volume up to 1L

**PBS/10%FCS**

900ml PBS,

100ml Foetal Calf Serum (Sigma-Aldrich, UK), heat inactivated by incubating at 60°C for 1 hour.

**PBS/0.05% Tween 20**

1L PBS,

500µl Tween 20 (Sigma-Aldrich, UK).

**Complete RPMI 1640**

500ml RMPI 1640 (GIBCO-BRL, UK),

50ml heat inactivated FCS (Sigma-Aldrich, UK) heat inactivated as described above,

5.5ml L-Glutamine 500mM Solution (GIBCO-BRL, UK),

5.5ml Penicillin 10000U/ml/Streptomycin 10000µg/ml Solution (GIBCO-BRL, UK),

11ml Amphotericin B 250pg/ml Solution (GIBCO-BRL, UK),

2.5µl Gentamycin 10mg/ml Solution (GIBCO-BRL, UK),

7.5ml 166.67mM HEPES solution (see above),

2.75ml 3.5% $NaCO_3$ Solution. (see above),

0.275ml 0.1M β-Mecaptoethanol Solution (see above).

**0.4M Sulphuric Acid**

21.8ml $cH_2SO_4$ (VWR International, UK),

978.2ml $dH_2O$.

**0.5% Toluidine Blue (pH0.3)**

5g Toluidine Blue (Sigma-Aldrich, UK),

42.9ml HCl (VWR International, UK),

957.1 $H_20$.

**0.2% Trypan Blue**

2g Trypan Blue (Sigma-Aldrich, UK),

1L PBS.