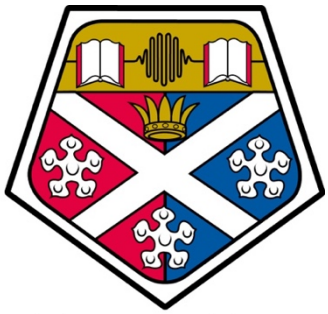

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Evaluation of the role of mast cells in parasitic infection

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"If I have seen further it is by standing on the shoulders of giants."

Sir Isaac Newton

Abstract

Mast cells (MCs) are important for controlling both bacterial and parasitic infections, including those caused by helminths. However, their role in combatting these infections is unclear. MCs have been shown to be essential for the successful expulsion of *Trichinella spiralis* worms and play a role in mounting the immune response towards a T helper 2 type. Intestinal infection with parasitic worms is associated with mastocytosis and the activation and release of specific mediators and cytokines. In this case, MCs play an essential role in the successful expulsion of gastrointestinal worms via their release of mediators that serve the central function of protecting a host from these parasites. Specifically, the functions of MCs in the expulsion of *T. spiralis* were investigated using MC deficient c-kit mutant $Kit^{W/W\text{-}v}$ (W/W-v) models. However, in addition to MC deficiency, these mice have a number of other abnormalities, including anaemia and a lack of interstitial cells of Cajal. Hence, there is a need to identify a model of MC deficiency that is not associated with other abnormalities that could affect the interpretation of results.

The aim of the present study was to investigate the role of MCs in the immune response of mice to a parasitic infection of *T. spiralis*. Immune responses were explored in two recently developed strains of MC-deficient mice, the c-kit model C57BL/6-$Kit^{W\text{-}sh/W\text{-}sh}$ (Wsh/Wsh) mice and an inducible mast cell-deficient model, Mas-TRECK, to determine the role of mast cells in protection against the parasite. These mice were infected with *T. spiralis* larvae, and the progression of the infection and the immune responses generated were examined via the enumeration of worms and the analysis of the associated intestinal pathology, cytokine production and antibody responses.

The results obtained from mast cell deficient Wsh/Wsh mice with low-level infection resulted in a significant worm burden in these mice compared to wild-type mice that showed complete expulsion of the parasite. This suggests that the delay was potentially caused by dose dependent effect as a high dose did not show a significant delay in the expulsion of *T. spiralis* worms. In addition, the development of enteropathy and lengths of both villi and crypts were similar in both the lower and higher infection groups, in both wild-type and Wsh/Wsh mice. The immune responses were similar in wild-type and Wsh/Wsh mice as assessed by antigen-

specific IgG1 levels, the total IgE levels and IL-4 levels. Moreover, Wsh/Wsh mice in both levels of infection were able to induce a significant marked mastocytosis, but they did not have significantly lower levels of mMCP-1 compared to wild-type mice.

The results obtained from Mas-TRECK mice models showed no statistically significant differences between these mice and wild-type mice in the expulsion of *T. spiralis* worms. The enteropathy in Mas-TRECK mice following infection with *T. spiralis* was not significantly improved. In addition, the infection of Mas-TRECK mice did not induce a change in IgG2a levels compared with BALB/c mice, and no significant differences were observed in IgE levels or IL-4 levels in Mas-TRECK mice, compared with wild-type. In addition, Mas-TRECK were able to induce mastocytosis and did not have significantly lower levels of mMCP-1 following infection with *T. spiralis*, although they are considered to be MC-deficient, which suggests that MMCs may not be completely depleted in these mice.

Mast cell activation was assessed using IgE-dependent MC activation to evaluate the ability of helminth antigens to activate mast cells through an immunoglobulin independent mechanism. An *in vitro* culture of bone marrow-derived mast cells (BMMCs) and peritoneal mast cells (PCMCs) used. Although cultured human MCs require stem cell factor (SCF) for growth, the expansion and growth of mouse MCs from bone marrow progenitors in the absence of SCF can be maintained with IL-3. It was found that stimulated PCMCs with *Trichinella spiralis* antigen (*T.* Ag) alone could activate mast cells to release IL-4 in all strains of mice. Moreover, the activation of PCMCs could be observed in the presence and absence of IgE, and C57BL/6 mice showed the greatest response to the stimulation and activation of PCMCs. BMMCs stimulated with helminth antigens led to similar secretions of mediators to those observed in wild-type mice, and all four strains of mice tended to secrete similar levels of mMCP-1.

Overall, the present study concludes that MCs are crucial for protection against and expulsion of *T. spiralis*. However, it is evident that Wsh/Wsh and Mas-TRECK MC-deficient mice are not entirely deficient in mucosal MC. Further studies are required to evaluate the benefits of different MC-deficient strains of mice and, particularly, to determine whether other abnormalities could have potentially affected the results of the present study.

List of abbreviations

-/-	Deficient
AHR	Airway hyper-responsiveness
BMCMC	Bone marrow-derived cultured mast cells
BMMCs	Bone marrow-derived mast cells
BPU	Biological Procedures Unit
CR	Complement receptors
CCL	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
CLP	Cecal ligation and puncture
Con A	Concanalvalin A
CPA3	Carboxypeptidase A3
CTMC	Connective Tissue Mast Cells
CVU	Crypt-Villi unit
CXCR	CXC chemokine receptors
DC	Dendritic cells
DMEM	Dulbecco's Modified Eagle Medium
DT	Diphtheria Toxin
DTR	Diphtheria Toxin Receptor
EAE	Experimental allergic encephalomyelitis
ELISA	Enzyme Linked Immunosorbent Assay
FCS	Fetal Calf Serum
FcεRI	High-affinity IgE receptor
FGF	Fibroblast growth factors
GI	Gastrointestinal
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
HBBS	HBBS Hank's Balanced Salt Solution
HRP	Horse Radish Peroxidase
i.p.	Intraperitneal
ICC	Interstitial Cells of Cajal
MHC	Major histocompatibility complex
IE	Intronic enhancer
IFNγ	Interferon gamma
Ig	Immunoglobulin
IHCs	Innate helper cells
IL	Interleukin
IL-1β	Interleukin 1beta
IL-4Rα	IL-4 receptor α chain
LPS	Lipopolysaccharides
MAdCAM-1	Mucosal addressin cell adhesion molecule-1
Mas-TRECK	Mast cell-specific Toxin Receptor-mediated Conditional Cell Knockout
MC	Mast Cell

MCcps	Mast cell precursor
MC_T	Mast cell tryptases
MC_{TC}	Mast cell tryptase/chymase
MLN	Mesenteric lymph node
MMC	MMC Mucosal Mast Cell
mMCP-1	Mouse Mast Cell Protease – type 1
Muc	Mucus
NK	Natural killer
p.i.	Post infection
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PCMCs	Peritoneal mast cells
PGD2	Prostaglandins D2
PGN	Peptidoglycan
PRR	Pattern-recognition receptors
RPMI	Roswell Park Memorial Institute medium
SCF	Stem Cell Factor
SEM	Standard Error of the Mean
STAT6	Signal transducer and activator of transcription six
***T.* Ag**	*Trichinella* antigen
TGF-β1	Transforming growth factor beta 1
Th1	T helper cell – type 1
Th2	T helper cell – type 2
TLR	Toll-like receptor
TMB	3, 3', 5, 5'-Tetramethylbnzidine
TNFα	Tumor necrosis factor-α
Treg	regulatory T cells
WT	Wild-type mice

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CHAPTER 1. GENERAL INTRODUCTION

1.1. Introduction

Helminths are multicellular organisms that can infect and live inside an array of different host species. Almost one-third of the world population is infected by a parasitic helminth (Al-Riyami and Harnett, 2009, Mcsorley and Maizels, 2012). These parasitic worms can be round, elongated or flat depending on the external shape of its host species (Castro, 1996). These parasites are found in both aquatic and terrestrial environments. Some species of helminths only infect invertebrates.

Helminth diseases are one of the most commonly occurring infections in both animals and humans and are especially prevalent among people in developing countries. Those who live in low-income areas with poor sanitation are often stricken with such infections, and it is estimated that more than one billion people are infected worldwide (Soga *et al.*, 2014). Helminthiasis may lead to health problems, such as iron deficiency, anaemia and chronic diarrhoea (Lustigman *et al.*, 2012). Additionally, the most common concerns in children involve deficiencies in growth and cognitive development as childhood infections with parasitic worms can lead to physical disability and impact children's school attendance (Degarege and Erko, 2013). Helminths are mainly classified into three phyla: Platyhelminthes or flatworms (Trematoda and Cestoda), Nematodes (Roundworms) and Acanthocephalans (thorny-headed worms) (Jackson *et al.*, 2009b). The following discussion will focus on roundworm nematode parasites.

Nematodes are an important phylum of helminth parasite and infect more people than any other type of helminth. Nematodes are mostly free living which feed on organisms in their environment and it is estimated that the phylum comprises more than one million species. They are also important parasites of humans, animals, insects and plants (Yin *et al.*, 2009). Nematodes can be classified into the Secernentea and Adenophorea classes based on various characteristics with their morphology and molecular structure used to classify them into the subdivisions, Enoplea and Chromadorea (Holterman *et al.*, 2006, Schulze and Schierenberg, 2011). The developmental process in nematodes involves egg, larval, and adult stages. Each of four larval stages is followed by a moult in which the cuticle is shed.

The larvae are called second-stage larvae after the first moult a progress through four moults. The nematode formed at the fifth stage is the adult (Castro, 1996). Nematodes are a significant cause of human gastrointestinal diseases. The gastrointestinal nematodes that have the greatest medical impact on the human population are *Ascaris lumbricoides*, *Trichuris trichiura* and the hookworm *(Ancylostoma duodenale, Necator americanus)*. These species of intestinal worms are transmitted through soil and infect three billion people worldwide annually. Gastrointestinal nematodes that infect children are responsible for many diseases, such as anaemia. Infections mostly occur in poverty-stricken tropical and subtropical regions (Tarafder *et al.*, 2010, Stepek *et al.*, 2006) with inadequate sanitation and healthcare.

1.2. Trichinosis

1.2.1. Introduction

Trichinosis is a disease caused by an infection of gastrointestinal nematodes belonging to the genus *Trichinella*. These parasites are usually acquired by consuming raw or contaminated meat (Gottstein *et al.*, 2009). In 1835, Paget and Owen identified the *T. spiralis* parasite in London (Pozio and Darwin Murrell, 2006). This species encapsulates in its host's muscle cells and inhibits muscle function. There are eight different species in the *Trichinella* genus: *T. spiralis, T. britovi, T. murrelli, T. nativa* and *T. nelsoni*, which have larvae that invade and encapsulate the host's muscle cells with collagen to form nurse cells, and *T. papuae*, *T. pseudospiralis* and *T. zimbabwensis,* are non-encapsulated species (Pozio *et al.*, 2009).

Trichinella worms can infect most mammals. Encapsulated species only infect mammals, but the non-encapsulated *T. pseudospiralis* can infect mammals and birds, and other non-encapsulated *Trichinella* species can infect mammals and reptiles (Mitreva *et al.*, 2011). *Trichinella* worms are intracellular and complete their life cycles in a single host while inhabiting the host's intestinal mucosa and skeletal muscle cells (Bruschi and Chiumiento, 2011). The most common *Trichinella* species is *T. spiralis*, which uses pigs as a host reservoir (Mitreva *et al.*, 2011).

1.2.2. Life cycle of T. spiralis

Initially, a helminth infection occurs by ingesting contaminated food that contains nurse cell larvae (Figure.1.1). Secretions from the stomach, such as pepsin, release larvae that then move through the digestive tract to invade the columnar epithelium through the small intestine. Mature adult worms will mate in the small intestine. Female worms can produce between 500–1,500 immature larvae (L1). These new-born larvae are passed into the lymphatic system and bloodstream, and are then distributed into the muscles. An infection can occurs within 15 days and persist for months or years (Mitreva *et al.*, 2011).

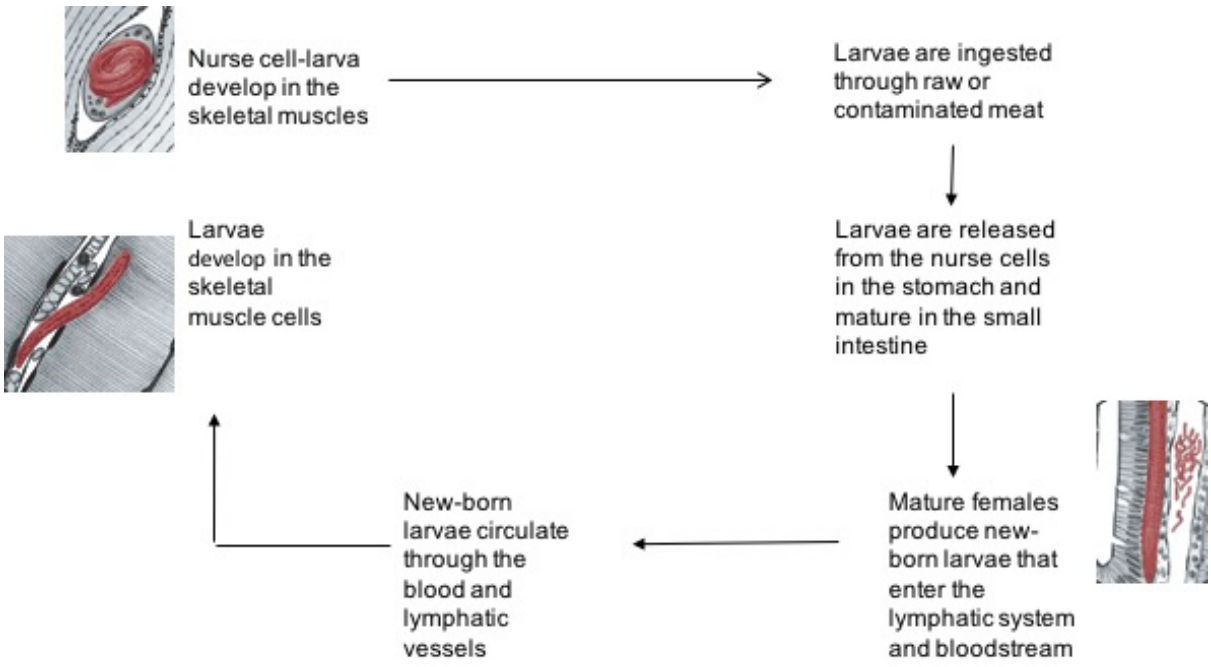


Figure 1.1 Life cycle of *Trichinella spiralis.* Adapted from (Mitreva and Jasmer, 2006)

1.3. The Immune Response to Gastrointestinal Nematodes

1.3.1. *T helper cell type 2 (Th2)*

The host's immune system stimulates different responses to different pathogens, which involves the innate and adaptive immune system. Further, the immune system's defence strategy depends on the specific pathogen causing the infection. The gastrointestinal tract is a major site of infection for helminths and other pathogens (Vallance *et al.*, 1999). The immune responses induced to helminth parasites involves various effector mechanisms to cause its expulsion from the host. The essential mechanisms used to expel helminth species are driven by T helper cells, which are derived from differentiated naïve $CD4^+$ T cells in the mesenteric lymph nodes (MLN) during an infection (King and Mohrs, 2009b). T helper cells are generally divided into the cell phenotypes Th1 and Th2, which are polarised depending on the release of cytokines (Lawrence, 2003).

Each T helper cell type is induced by a specific combination of cytokines and characterized by the expression of a unique transcription factors responsible for the expression of distinct sets of cytokines upon antigen stimulation. Among them, Th2 cells producing IL-4, IL-5, IL-9, and IL-13 are induced by IL-4 (Koyasu *et al.*, 2010). These Th2 cytokines can be produced through bone marrow-derived myeloid cells and are important in promoting the motility of the gastrointestinal tract and mucus hyper secretion to provide protection against gastrointestinal helminth species (Lawrence *et al.*, 2004).

Th2 cells also induce production of antibody isotypes, which include IgG1 and IgE, that contribute to the differentiation and growth of different cells, such as eosinophils, basophils and mast cells (Lawrence, 2003, Jankovic *et al.*, 2001). The binding of IL-4 and IL-13 cytokines to the IL-4 receptor α chain (IL-4Rα) results in the activation of STAT6 which is required for host protection against gastrointestinal nematode parasites. In addition, the induction of multiple effector mechanisms by STAT6 signalling induces the transcription of further cytokine genes associated with a protective response to these parasites. STAT6 is also important in the external muscularis during an infection of *T. spiralis* since it can increase smooth muscle contractility associated with the "weep and sweep" response and associated parasite expulsion (Khan *et al.*, 2001, Akiho *et al.*, 2002). Additionally, STAT6 affects

intestinal epithelial cell function leading to increased intestinal mucus secretion, which is also part of the "weep' response (Urban *et al.*, 2000a, Finkelman *et al.*, 2004).

1.4.1.1 Interleukin (IL) 4

Interleukin 4 (IL-4) is a cytokine first described by Howard *et al.* (1982). IL-4 is produced by the differentiation of Th2 from activated $CD4^+$ T cells and is essential in enhancing the differentiation and regulation of Th2 cells. IL-4 is also important in antibody isotype switching in response to immunoglobulin IgG1 and IgE antibodies (King and Mohrs, 2009b). IL-4 is a dominant mediator in the immune response of Th2 and binds with IL-13 to the IL-4 receptor α chain (IL-4Rα). This is important in the activation of STAT6, which is necessary to expel parasites from their hosts (Marillier *et al.*, 2008).

During an experiment using mice infected with *Heligmosomoides polygyrus* (*H. polygyrus*), IL-4 protected the host from the infection. This experiment showed that the fecundity of the nematode parasite was reduced (Hashimoto *et al.*, 2010). Additionally, an experiment studying the expulsion of *T. spiralis* worms using both wild-type and IL-4 knockout BALB/c mice showed that the parasite expulsion was IL-4 dependent due to the delay in the expulsion of the parasite from the small intestine of IL-4 deficient BALB/c mice (Urban *et al.*, 2000a, Lawrence *et al.*, 1998a). The innate immune response induced by helminth infection also has other cellular components that contribute to producing IL-4 and other Th2 cytokines, such as mast cells, eosinophils, natural killer T cells (NKT) and basophils (Van Panhuys *et al.*, 2011).

1.4.1.2 Interleukin (IL)13 and other Th2 cytokines

IL-13 is a cytokine with similarities with IL-4 (Finkelman *et al.*, 2004) however it has been suggested that IL-13 acts more prominently as a molecular bridge linking Th2 cells to non-immune cells in contact with them, thereby altering physiological function. However, it has been shown that IL-13 can play a role in parasite expulsion in the absence of IL-4 (Finkelman *et al.*, 2004, Urban *et al.*, 2000a). IL-13 is required for developing Th2 responses along with class switching of B cells to IgE

(Finkelman *et al.*, 1999). IL-13 uses different mechanisms to protect a host from various helminth parasites including proliferation of goblet and epithelial cells, production of nitric oxide and activation of fibroblasts. In addition, IL-13 is an important cytokine that is induced by nuocytes to expel parasites (Neill and Mckenzie, 2011). Nuocytes are leukocytes that potentially share a developmental lineage with other cells, such as natural helper cells (NHCs) and innate helper cells (IHCs). Nuocytes have been identified and functionally characterised by using novel *I113eGFP* reporter mice as a new innate type 2 immune effector. These cells can be expanded by stimulation with IL-25 and IL-33, which produces type 2 cytokines, such as IL-3 and IL-5. However, the production of IL-4 by nuocytes is less than 5% (Neill and Mckenzie, 2011, Wong *et al.*, 2012).

IL-25 and IL-33 were shown to be essential initiators of the type 2 immune response since when mice were treated with one of these cytokines to stimulate a potent induction and increase the production of Th2 cytokines (Barlow *et al.*, 2012). Nuocytes can be another source of IL-3, IL-4 and IL-5, which are generally important for the initiation of type 2 responses (Barlow *et al.*, 2012, Neill and Mckenzie, 2011). Moreover, during an infection of *N. brasiliensis,* IL-13 that is secreted from nuocytes is important for the host's immune response. IL-13-deficient mice that have passively transferred wild-type nuocytes can expel worms. Therefore, IL-13 released from nuocytes alone could be sufficient for inducing worm expulsion (Neill and Mckenzie, 2011).

1.3.2. Goblet cells

The secretion of mucus by goblet cells located in the epithelial monolayer, is mainly important in the innate immune response that contributes to expelling helminth parasites (Turner *et al.*, 2013). Goblet cells are derived from the progenitor of epithelial cells. They are the source of many effector molecules, such as antimicrobial proteins and mucins, that are significant in protecting the gut from different infections, including bacteria and parasites (Turner *et al.*, 2013).

Mucins have the potential to be the initial molecules that invasive pathogens interact with at the cell surface. They bind to other glycoproteins and then neutralise the pathogen (Turner *et al.*, 2013, Kim and Khan, 2013). Infection with helminths has

been shown to increase the number of goblet cells. It has been observed that this composition also changes during an infection. The importance of IL-13 is demonstrated by IL-13-deficient mice which when infected with *N. brasiliensis* are unable to induce goblet cell hyperplasia (Mckenzie *et al.*, 1998, Marillier *et al.*, 2008). Muc2 and Muc3 are significant mucin genes upregulated in goblet ells exposed to helminths and contribute to the host's ability to resist parasites. Muc2 has been especially significant in the innate defence against nematode infections (Kim and Khan, 2013, Hasnain *et al.*, 2010). The delay of worm expulsion observed in the absence of IL-22 is strongly correlated to reduced goblet cell hyperplasia and the reduced expression of goblet cell mediators. Goblet cell hyperplasia is also extremely significant to the intestinal epithelium that contributes in the expulsion of helminth parasites (Turner *et al.*, 2013).

IL-22 is a class two, α-helical cytokine from the IL-10 family and was first identified in the year 2000. IL-22 signals through a distinct class 2 receptor (IL-22R) composed of the subunits IL-22R1 (IL-22RA1) and IL-10R2 (IL-10RB2), which are independently shared with IL-20 and IL-24 and with IL-10 and IL-26 (Sonnenberg *et al.*, 2011). IL-22 is functionally important in supporting the barriers of epithelia. It also works as a mediator for promoting the production of mucus. In addition, IL-22 stimulates other cytokines, such as IL-4 and IL-13, which are important for enhancing Th2 response, protecting the immune system and inducing goblet cell hyperplasia (Marillier *et al.*, 2008, Leung and Loke, 2013).

1.3.3. Intestinal epithelial cells

Epithelial cells are fundamentally important in protecting the gastrointestinal (GI) tract from pathogens, such as *Trichuris muris*, and use different mechanisms to maintain the function of barriers, such as mucin secretions. It is suggested that epithelial cells have an impact on the innate and adaptive immune systems (Artis and Grencis, 2008a) by secreting factors that are important for preventing the production of IL-12 by dendritic cells (DC)(Anthony *et al.*, 2007). Epithelial cells also assist in polarising the immune response to helminths by producing cytokines, such as IL-25.

IL-25 is a cytokine which as a member of the IL-17 family is also known as IL-17E, which is a pro-inflammatory cytokine derived from T cells with specific roles in allergic and humoral responses (Nakae *et al.*, 2002). IL-25 has also been shown to

increase eosinophilia and IgE antibodies. These functions of IL-25 are important in the expulsion of worms (Kang *et al.*, 2012).

1.3.4. Mast cells

Mast cells (MCs) are tissue-resident, granule-containing cells derived from haematopoietic progenitor cells. These cells play a key role in the regulation of innate and adaptive immune responses (Bulfone-Paus and Bahri, 2015). Mast cells are found in large numbers in the skin and mucosal linings and may be one of the first immune cells encountered by invading pathogens (Abraham and Malaviya, 1997). MCs have been suggested to participate in protective immunity against bacteria, viruses, fungal, parasitic protozoa and helminth parasites (Okayama and Kawakami, 2006, Saha *et al.*, 2004, Saluja *et al.*, 2012, Abraham and John, 2010). Moreover, MCs are reportedly essential for Th1 response-mediated defence against oral infection with *Toxoplasma gondii* (Cruz *et al.*, 2014).

Some of the earliest observations of MCs evidenced their contribution towards host defence against parasitic infections (Woodbury *et al.*, 1984, Nawa *et al.*, 1985). During parasite nematode infection, a process dependent on IL-3, mucosal mast cells (MMC) were observed to extensively expand (Lantz *et al.*, 1998, Madden *et al.*, 1991). In addition, IgE and mouse MC protease-6 (mMCP-6) were found to be required in chronic immune responses against *T. spiralis* infections (Shin *et al.*, 2008). Finally, MCs contributed to pathogen clearance in a helminth infection by migrating to the draining lymph nodes and producing IL-6 and IL-4 (Liu *et al.*, 2013).

In parasitic infections, MC hyperplasia and release of growth factors (IL-3, SCF, and IL-9) was shown to be associated with parasite loss (Newlands *et al.*, 1995, Faulkner *et al.*, 1998, Lantz *et al.*, 1998). During parasitic infection, mediators released by MCs recruit immune cells and regulate gastrointestinal permeability. Moreover, the microenvironments generated in response to MC mediators lead to suitable conditions for parasite expulsion and chronic infection containment (Knight *et al.*, 2000, Gurish *et al.*, 2004, Abraham and John, 2010). In the early stages of helminthic infection, for example, the degranulation of MCs regulates tissue-derived cytokines IL-25 and IL-33 and thymic stromal lymphopoietin (TSLP) (Hepworth *et al.*, 2012b). Furthermore, mMCP-1 deficient mice (mouse MCPT-1 recombinant protein) showed

a significant delay in the expulsion of *T. spiralis*, which indicates an important contribution of intestinal MMCs and mMCP-1 in the clearance of parasite infections (Knight *et al.*, 2000). In addition, Shin *et al*. (2008) reported that the kinetics of *T. spiralis* expulsion from the small intestine were similar between mice deficient in mMCP-6 and wild-type mice, but eosinophil recruitment in the mMCP-6-deficient mice significantly decreased in skeletal muscles after infection (Shin *et al.*, 2008). Also, the parasite burden was increased in the small intestine of BALB/c-*Cpa3*$^{Cre/+}$ mice at day 6 after infection with *Streptococcus ratti*, indicating that MCs play a role in host defence against *S. ratti* (Blankenhaus *et al.*, 2014).

Infection with *T. spiralis* has been shown to induce physiological changes in the intestine of mice which is associated with the release of MC products (Lawrence *et al.*, 1998a, Garside *et al.*, 2000). Wang *et al*. (1990) suggested that mast cells are apparently not involved in worm expulsion from the intestine to expel a primary infection. However, it has been demonstrated that a temporal correlation is involved between the kinetics of mucosal mastocytosis and expulsion of adult worms (Alizadeh and Wakelin, 1982, Moqbel *et al.*, 1987, Tuohy *et al.*, 1990). In addition, Urban *et al.* (2000) reported that expulsion of worm in mice is mast cell dependent. Moreover, a delayed expulsion of *T. spiralis* is shown in mice deficient in β-chymase mouse mast cell protease-1 (mMCP-1) (Knight *et al.*, 2000). Additionally, the involvement of MC in rats is shown in the intestinal pathophysiology of the infection (Harari *et al.*, 1987).

It has been demonstrated that high responder mice such as NIH and BALB/c mice support lower worm burdens and an infection is rapidly resolved compared to low responder mice such as C57BL/10 (Tuohy *et al.*, 1990). In addition, the level of mMCP-1 in MMC hyperplasia, both local and systemic, is increased, appearing earlier and being importantly more pronounced in high responder mice (Tuohy *et al.*, 1990).

However, MC do not play a role in the expulsion of all GI nematode infections with no evidence for MCs and Th2 antibody responses for the loss of *N. brasiliensis* (Sasaki *et al.*, 2005). However, *Strongyloides venezuelensis* which has a life cycle similar to *N. brasiliensis* showed evidence for the contribution of intestinal MCs and humoral responses (Sasaki *et al.*, 2005, Lantz *et al.*, 1998, Khan *et al.*, 1993, Fukao

et al., 2002) in the rapid expulsion of *S. venezuelensis* from the intestines. It has been demonstrated that IgG/IgE-deficient $AID^{-/-}$ mice delayed expulsion of *S. venezuelensis* and larger numbers of adult worms were maintained in their intestines (Matsumoto *et al.*, 2013). Moreover, administration of exogenous IL-3 (Abe and Nawa, 1988, Abe *et al.*, 1993, Korenaga *et al.*, 1996) or IL-18+IL-2 (Sasaki *et al.*, 2005) in mice infected with *T. spiralis, S. ratti* or *S. venezuelensis* showed evidence that expansion of MMCs can contribute importantly to host defence against these intestinal nematodes. Furthermore, the prevention of MMC expansion and delayed *T. spiralis* expulsion have been reported in mice treated with anti-SCF or anti-c-Kit (Grencis *et al.*, 1993, Donaldson *et al.*, 1996). Increased susceptibility to *S. venezuelensis* infection has been shown in IL-3 deficient mice and these mice are unable to produce an intestinal MMC hyperplasia response (Lantz *et al.*, 1998). In addition, reduce *S. venezuelensis* worm burden by leukotrienes, particularly LTB4, has been shown that possibly through the recruitment of certain inflammatory cells (Machado *et al.*, 2005). Enhanced early phases of Th2 immune responses following infections with *Heligmosomides polygyrus* and *Trichuris muris* has been shown in IgE-independent MC degranulation (Hepworth *et al.*, 2012b, Hepworth *et al.*, 2012a).

MCs may also play a role in the response to parasitic protozoa. MCs are found in large numbers in the skin and possibly respond to mosquito saliva during blood feeding and it has been suggested that MCs may have a gatekeeper effect on the initial stage of malaria transmission (Mukai *et al.*, 2016). IgE-independent and IgE-dependent degranulation of dermal MCs triggered by mosquito saliva has been shown to promote aspects of the ensuing inflammatory response, including local recruitment of granulocytes and induction of hyperplasia of draining lymph nodes (Mecheri, 2012, Demeure *et al.*, 2005). It has been shown that mosquito saliva can trigger IgE-independent and IgE-dependent degranulation of dermal MCs to promote aspects of the resulting inflammatory response which include granulocyte recruitment and induction of hyperplasia of draining lymph nodes (Mecheri, 2012, Demeure *et al.*, 2005). In a mouse model of lethal malaria infection with *Plasmodium berghei* ANKA it has been demonstrated that disease is promote by

MCs through the activation of tissue-damaging $CD8^+$ T cells (Guermonprez *et al.*, 2013).

In healthy individuals, MCs are involved in tissue homeostasis and repair and also play a role in host defence through the release of different pro-inflammatory mediators, proteases and cytokines (Arthur and Bradding, 2016). They are found in large numbers in the skin and, predominantly, in the superficial dermis (Maurer *et al.*, 2006), where *Leishmania* parasites are encountered after infection via sand fly bites. Some evidence has also shown that MCs have crucial roles in combatting parasitic protozoan diseases (Martins *et al.*, 2014, Theoharides, 2015). In this respect, MCs were found to be activated by *Leishmania major*, which induced the release of pro-inflammatory mediators; then, MCs were found to phagocytose this parasite. In addition, during infection with *L. major*, MCs showed evidence of their role in the establishment of a Th2 response (Bidri *et al.*, 1997). During cutaneous granuloma formation, dermal MCs were also required for the recruitment of macrophages (Von Stebut *et al.*, 2003).

It has been demonstrated that both eosinophils and MCs in the initial phase of infection showed a predominance in the primary lesions in footpads and draining lymph nodes after infection of *Leishmania amazonensis* in susceptible (C57BL/10 and CBA) and resistant (C3H.He) mice (De Oliveira Cardoso *et al.*, 2010). In addition, the number of MCs increased significantly in the upper dermis of BALB/c, but not in C57BL/6 mice, after infection with *L. major.* However, CBA/T6T6 mice showed higher MCs degranulation after infected with *L. major* (Lu and Huang, 2017). It has been reported that using Wsh/Wsh mice resulted in worst disease outcome after infected with *L. major* promastigotes in which lesion progression and lesional parasite burdens enhanced significantly, associated with significantly reduced in IFN-γ and IL-17A levels (Lu and Huang, 2017). However, levels of IL-4 and IL-10 significantly increased. Thus, MCs promote Th1 and Th17 responses *in vivo* which indicate that MCs play an important role against *Leishmania* parasites (Dudeck *et al.*, 2011b). Moreover, MC-deficient (W/W-v) mice injected intradermally with metacyclic promastigotes of *L. major* showed that lesion sizes and

lesional parasitic loads were increased significantly associated with reduced locally infiltrating cells in W/W-v mice (Lu and Huang, 2017).

Mast cells may also play an essential role in *Toxoplasma gondii* invasion, a study using a rodent model (*Calomys callosus*), resulted in an increased in the level of mast cells and their activation (Gil *et al.*, 2002). It has been shown that low-virulent ME49 *T. gondii* strain resulted in W/W-v mice succumbing to the infection within 15 days and this was associated with increased levels of IFN-γ and IL-2 (Cruz *et al.*, 2014). It has previously been observed that animals inoculated either (i.p.) or through the conjunctiva with *T. gondii* tachyzoites showed a significant increase in MC numbers (Gil *et al.*, 2002). An *in vitro* study using cultured peritoneal lavage MCs from Sprague-Dawley rats showed that MCs degranulated and released LTB4 after incubation with *T. gondii*, resulting in damage to *T. gondii* tachyzoites (Henderson Jr and Chi, 1998). Further injection of C48/80 which activates MCs in mice resulted in increased inflammatory infiltrate and load of *T. gondii*. However, decreased *T. gondii* load and attenuated inflammatory reaction showed in MC stabilization with disodium cromoglycate (DSCG). These results indicated that MCs play a significant role during infection with *T. gondii* (Huang *et al.*, 2013).

1.4. Mast Cells

1.4.1. Mast cell origin and distribution

Paul Ehrlich first described MCs in 1878. He named these cells ‘Mastzellen’ because of their granules (Ehrlich, 1878). The morphological features of MCs were described in the earliest research on MCs, and the distribution of MCs in distinct physiological and pathological states was subsequently identified (Da Silva *et al.*, 2014). Tissues showing a great number of MCs were first observed by Holmgren and Willander (1937) who found these MCs enriched in heparin. In addition, Riley and West (1952) found that histamine was present in MCs, thereby supporting the establishment of a relationship between MCs, histamine and anaphylaxis, as histamine and heparin are released during anaphylactic shock (Rocha *et al.*, 1947).

Prausnitz and Kustner in (1921) discovered a reaginic agent that was found to be essential in stimulating allergic reactions. Subsequently, in the late 1960s, Kimishige and Ishizaka distinguished this ‘reaginic’ antibody as γE antibodies; later, IgE was

recognised to work as a reaginic agent and cause the activation of MCs (Hershko and Rivera, 2010).

Mice carrying mutations in the white spotting (W) locus, such as *Kit*$^{W/W\text{-}v}$ (W/W-v) and C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ (Wsh/Wsh) mice, or mice carrying mutations in the stem cell factor (SCF) gene, such as Sl/Sl^{d}, have served as useful models for identifying MC functions *in vivo* (Kitamura *et al.*, 1978, Russell, 1979, Grimbaldeston *et al.*, 2005). However, these models (W/W-v and Wsh/Wsh) exhibit MC deficiency and show reduced *c-Kit* tyrosine kinase-dependent signalling as well as abnormalities in erythrocyte, neutrophil and melanocyte phenotypes and cell lineages (Grimbaldeston *et al.*, 2005).

Mast-cell deficient models engrafted with bone marrow or bone marrow-derived mast cells (BMMCs) have helped to clarify and supply additional information about the origin of MCs and to reliably establish connections between the functions of MCs *in vivo* and the participation of MCs in various diseases (Kitamura *et al.*, 1977, Kitamura *et al.*, 1978, Grimbaldeston *et al.*, 2005, Galli and Tsai, 2008, Jamur and Oliver, 2011). Moreover, model strains that were selectively deficient in MCs and that were not dependent on Kit mutations were recently reported (Dudeck *et al.*, 2011a, Feyerabend *et al.*, 2011, Lilla *et al.*, 2011, Otsuka *et al.*, 2011). The work of Kitamura *et al.* (1977) established the hematopoietic origin of adult MCs via the transplantation of bone marrow from beige mice (C57Bl Bg^{J}/ Bg^{J}) to irradiated wild-type (C57Bl) mice. Consequently, the tissue of the recipient mice had MCs with large, abnormal granules from the bone marrow of beige mice. Therefore, it was suggested that MCs were derived from bone marrow precursor cells, which was supported by the reconstitution of MC in MC-deficient (W/W-v) mice by the transfer of bone marrow from wild-type mice (Kitamura *et al.*, 1978). In addition, the hematopoietic origin of human MCs was also confirmed after the transfer of MCs to a leukemic patient by allogeneic bone marrow transplantation. In this case, MCs isolated from the recipient's bone marrow were found in the donor's genotype 198 days after the transplant (Fodinger *et al.*, 1994). Hence, MCs are multifunctional, long-lived cells derived from hematopoietic precursors. Only MC progenitor cells, not mast cell precursor (MCcps), have been observed in the bloodstream; these cells

are responsible for populating peripheral tissues (Jamur *et al.*, 2010).

Mast cells are found in small numbers in the bloodstream but complete their differentiation and maturation in the microenvironments of almost all vascularised tissues (Douaiher *et al.*, 2014, Galli *et al.*, 2008a, Gurish and Austen, 2012, Moon *et al.*, 2010). The migration of MC progenitors is controlled in a tissue-specific manner (Hallgren and Gurish, 2011). In the intestines, for example, dendritic cells regulate MC progenitors through the expression of a transcriptional regulatory protein, T-bet, a T-box transcription factor, in which α4β7 integrin and the chemokine receptor, CXCR2, must be expressed on the surface of MC progenitors, while mucosal addressin cell adhesion molecule-1 (MAdCAM-1) and vascular cell adhesion protein 1 (VCAM-1) must be expressed on the intestinal endothelium (Hallgren and Gurish, 2011). Moreover, the maturation of MC progenitors (As shown in Figure.1.2) and the increased recruitment and survival of MCs may also contribute to the local expansion of MC populations (Galli *et al.*, 2008b, Gurish and Austen, 2012).

As mentioned, MCs distribute throughout nearly all tissues and often near sites that are potential targets of their mediators such as epithelia and glands, smooth and cardiac muscle cells, fibroblasts, blood and lymphatic vessels and nerves. Mature MCs are particularly abundant in tissues and organs and are mainly found at sites exposed to the external environment, such as the skin, the lungs and the gut (Galli *et al.*, 2008b). The c-kit tyrosine-protein kinase receptor is crucial for MC progenitor cells, as their progression to fully mature MCs is dependent on the activation of the KIT as a result of stem cell factor (SCF)-induced KIT dimerisation and auto-phosphorylation (Gilfillan *et al.*, 2011).

MC numbers are substantially reduced in W/W-v and Wsh/Wsh mice wherein surface expression of KIT, or KIT catalytic activity, is defective (Tsai *et al.*, 2011b). Human MCs require SCF for growth. However, in the absence of SCF, growth and expansion of mouse MCs from bone marrow progenitors can be maintained by IL-3 (Kirshenbaum *et al.*, 1999). Stem cell factor, which is produced by MCs and structural cells in the tissues, has a crucial role in the development, survival, migration and function of MCs (Douaiher *et al.*, 2014, Galli *et al.*, 1993b, Gurish and Austen, 2012, Moon *et al.*, 2010). Additionally, interleukin (IL)-3, IL-4, IL-9, IL-10,

IL-33 and TGF-β have been shown to influence MC growth and survival (Galli *et al.*, 2008b, Gurish and Austen, 2012).

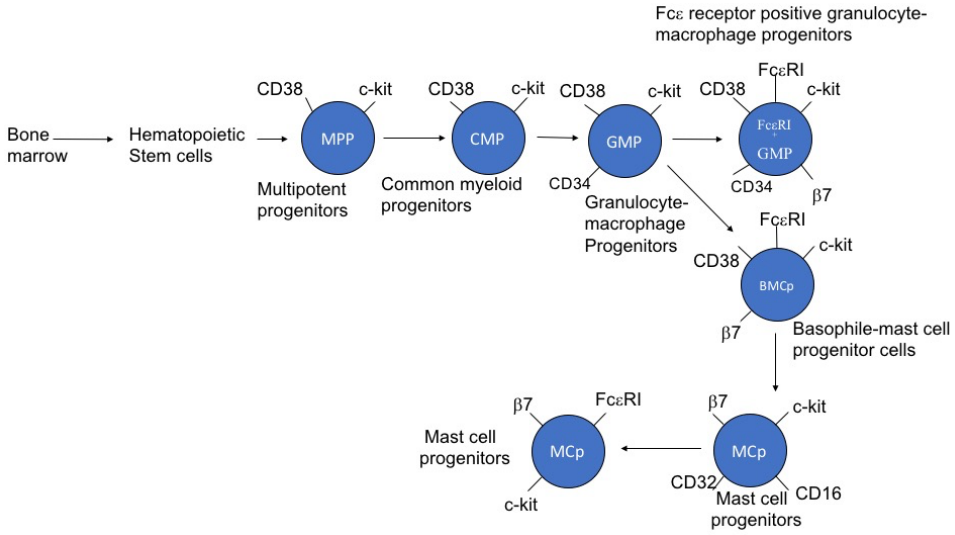


Figure 1.2 Model for MCp development. Adapted from (Dahlin and Hallgren, 2015).

1.4.2. Phenotypic Heterogeneity

As previously mentioned, MC development and maturation is also influenced by other growth factors and cytokines that contribute to the phenotypic expression of MCs (Jamur and Oliver, 2011). Numerous factors can alter the phenotypic expression of MCs during their lifetime; mast cell homeostatic or pathophysiological responses can result from a combination of these factors (Moon *et al.*, 2010). Also, cytokine and the growth factor milieu can shape the phenotypic profile of MCs. Reportedly, IL-4 acts together with IL-3 to promote MC growth and survival (Tsuji *et al.*, 1990, Rennick *et al.*, 1995). It has been demonstrated that changes in proteoglycan expression both *in vitro* and *in vivo* can quickly cause mature mast cells to change staining characteristics (Razin *et al.*, 1982, Sonoda *et al.*, 1984, Nakano *et al.*, 1985, Levi-Schaffer *et al.*, 1986, Sonoda *et al.*, 1986). Moreover, *in vivo* mouse mast cells have been shown to alter serglycin proteoglycans and protease profiles (Friend *et al.*, 1998).

It has been reported that histidine decarboxylase synthesis induced by Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF) and IL-3 leads to an increased

histamine production in rodents (Schneider *et al.*, 1987). In addition, IL-4 has been shown to inhibit the expression of CD117 and FcεRI in mouse BMMCs (Ryan *et al.*, 1998, Mirmonsef *et al.*, 1999). Further, IL-4 in mouse BMMCs in combination with SCF induces differentiation of CTMCs (Karimi *et al.*, 1999). The overexpression of IL-9 in mice leads to increased infiltration of CTMCs and MMCs into the gut, trachea and kidneys (Godfraind *et al.*, 1998). Moreover, the combination of IL-3, IL-4 and IL-10 leads to apoptosis of mouse peritoneal MCs and BMMCs (Yeatman *et al.*, 2000). The number of IL-3-derived BMMCs is also increased by Nerve Growth Factor (NGF) in which a CTMC phenotype is induced and results in increased histamine content and the expression of heparin (Matsuda *et al.*, 1991). Furthermore, NGF has been reported to prevent apoptosis of murine peritoneal mast cells (Kawamoto *et al.*, 1995).

The development of phenotypically distinct populations of MCs results from the varying MC phenotypes in different anatomic sites and animal species. Therefore, during particular biological responses *in vivo*, the differential expression of MC phenotypes can be induced (Galli *et al.*, 2015). As summarized in Table 1.1, two subtypes of mature MCs exist in rodents: mucosal mast cells (MMCs) and connective tissue mast cells (CTMCs) (Enerback, 1966a, Enerback, 1966b). In mice, MCs in the mucosal epithelium of the lungs and the gastrointestinal tract are characterised as MMCs; these cells express protease as chymases, which are mouse MC proteases (mMCP-1 and mMCP-2), although little or no tryptase or carboxypeptidase A3 (CPA3) were bound to chondroitin sulphate chains of serglycin proteoglycans (Pejler *et al.*, 2007). Meanwhile, CTMCs reside in the intestinal submucosa, peritoneum and skin and express the chymase mMCP-4, the tryptases mMCP-5 and mMCP-6 and -7 and the carboxypeptidase A (mCPA), which are bound to heparin chains of serglycin proteoglycans (Yurt *et al.*, 1977, Enerbäck *et al.*, 1986, Metcalfe *et al.*, 1997, Welle, 1997, Miller and Pemberton, 2002, Pejler *et al.*, 2010).

Further, MMCs and CTMCs have been distinguished for their ability to secret histamine and lipid mediators. Upon activation, small amounts of histamine and large quantities of cysteinyl leukotrienes are released by MMCs, whereas higher levels of histamine and prostaglandin D2 are released by CTMCs (Heavey *et al.*,

1988). Additionally, athymic nude mice were found to be devoid of MMCs, as MMCs are more dependent on T-cell-derived factors than CTMCs (Ruitenberg and Elgersma, 1976). The tissue microenvironment may affect different aspects of the phenotypic expression and functioning of MCs, which may be significantly altered during innate or adaptive immune responses or over the course of certain diseases (Galli *et al.*, 2011, Galli *et al.*, 2005b).

In humans, the presence of tryptase and chymase have been used to classified MCs into two large subsets based on their protease content to those containing mainly tryptase and those containing both tryptase and chymase (Craig and Schwartz, 1989). However, classification of MCs in human into subsets is challenging due to the potential plasticity of multiple aspects of MC phenotype in which these cells may have different roles at baseline that opposed to the settings of immune responses or disease. Abundant or negligible amounts of CPA3 have been shown in chymase^{+}/tryptase^{+} human MCs granules, and in the lungs of patients with asthma have been detected high tryptase and CPA3, while low chymase intraepithelial MCs has been reported (Douaiher *et al.*, 2014). Mast cells store tryptases, chymases and carboxypeptidases in granules, which are termed MC tryptase/chymase (MC_{TC}) whereas those containing only tryptases are termed (MC_T) (Irani *et al.*, 1986, Schwartz, 2005, Pejler *et al.*, 2010). MC_T are mainly found in the intestinal and pulmonary mucosa, whereas MC_{TC} reside in the skin, lymph nodes, the lungs and the gut submucosa (Goldstein *et al.*, 1987, Irani *et al.*, 1987). Recently, a third phenotype of MCs that express tryptase and carboxypeptidase A3 but not chymase was described (Abonia *et al.*, 2010, Dougherty *et al.*, 2010). MC maturation and survival is enhanced by treatment of human cultured MCs with IL-4 and IL-4 promotes the expression of FcεRI and chymase in MC_T and downregulates CD117 expression (Sillaber *et al.*, 1991, Yanagida *et al.*, 1995, Toru *et al.*, 1996, Toru *et al.*, 1998).

Table 1.1 Summary of differences between CTMCs and MMCs.

	Connective tissue mast cells (CTMCs)	**Mucosal mast cells (MMCs)**
Location	Reside in the intestinal submucosa, peritoneum and skin	Reside in lungs and the gastrointestinal tract
Stimulation	Stimulated when allergen enters the bloodstream intravenously	Stimulated when infection enters via ingestion or inhalation
Histamine	Releas higher levels of histamine and prostaglandin D2	Release small amounts of histamine and large quantities of cysteinyl leukotrienes
Proteases	Express the chymase mMCP-4	Express chymases, mMCP-1 and mMCP-2
Tryptases	Express tryptases mMCP-5 and mMCP-6 and -7 and carboxypeptidase A (mCPA)	Express little or no tryptase or CPA3
Proteoglycans	Contain heparin chains of serglycin proteoglycans	Contain chondroitin sulphate chains of serglycin proteoglycans

1.4.3. Mast cell mediators

The relative abundance of mast cells in tissues and the extent and nature of the released mediators are important for helping mast cells to function in both health and disease (Gilfillan *et al.*, 2011). As reviewed in Table 1.2, a multitude of pro-inflammatory mediators have been shown to be released by mast cells (Metcalfe *et al.*, 1997), and it has been demonstrated that the granules of mast cells are rich in proteases, bioactive amines and proteoglycans (Metcalfe *et al.*, 1997). Histamine is the most important bioactive amine present in the granules (Gilfillan *et al.*, 2011).

Mast cell granules are also especially rich in proteases, which constitute the major proteins present in mast cells. These proteases represent up to 50% of the total protein content of the granule (Stevens and Adachi, 2007). β-tryptase is the major protease present in human mast cells (Schwartz, 1994). Substantial amounts of chymase are present in particular sub-populations of mast cells. In human mast cells, the relative abundance of tryptase and chymase has been utilised to determine particular mast cell phenotypes (Irani and Schwartz, 1994). It has also been reported

that the rapid generation of lipid-derived inflammatory mediators is a result of mast cell activation. The substrate sources for lipid derived pro-inflammatory mediators are provided by both membrane-associated phospholipids and sphingolipids (Boyce, 2007).

Following initial activation of mast cells, gene expression is enhanced and an array of cytokines (including GM-CSF, TNFα, IL-3, IL-4, IL-5, IL-6, IL-10 and IL-13), chemokines (including CCL2, CCL3, CCL5 and CXCL8) and growth factors (including SCF, FGF, VEGF and angiogenin) are generated (Zhang *et al.*, 1998, Okayama, 2005, Katsanos *et al.*, 2008, Kulka *et al.*, 2009, Freeman *et al.*, 2010, Theoharides *et al.*, 2010, Stone *et al.*, 2010).

Mast cell mediators have a profound influence on the surrounding tissues causing an extensive array of cellular responses, including immune induction, inflammatory responses, cellular hyperplasia, angiogenesis and tumorigenesis (Shelburne and Abraham, 2011, Tsai *et al.*, 2011b, Moiseeva and Bradding, 2011, Ribatti and Crivellato, 2011).

Depletion of different MC-specific proteases has been used to investigate the roles of these proteases in both host defence and inflammation. For example, decreasing intestinal permeability and delayed expulsion of adult *T. spiralis* have been observed in mice lacking the chymase mMCP-1 (Knight *et al.*, 2000, Mcdermott *et al.*, 2003). In addition, protection against a bullous pemphigoid-like lesion induced in neonates with a rabbit antibody directed to mouse-type XVII collagen was found in a mouse lacking the chymase mMCP-4 (Lin *et al.*, 2011). Moreover, protection against scald burn was also reported in a mouse lacking the chymase mMCP-4, whereas the application of human MC chymase led to full injury with ulceration and down-stream fibrosis at the scald site in the first hour post burn (Younan *et al.*, 2010). Mouse strains lacking the MC-restricted chymase, mouse MC protease (mMCP)-4, or elastase, mMCP-5, show decreased injury after a second-degree scald burn, whereas mice lacking the MC-restricted tryptases, mMCP-6 and mMCP-7, or MC-specific carboxypeptidase A3 activity are not protected. (Gurish and Austen, 2012). Mice lacking the tryptase mMCP-6 showed a reduction in the number of swollen joints and in the thickness of ankle joints following the administration of an autoantibody to glucose-6 phosphate isomerase via an immune complex-mediated

reaction (Shin *et al.*, 2009). Administration of human β-tryptase to the lungs of sheep was shown to induce airway hyper-responsiveness (Molinari *et al.*, 1996). Similarity, γ-tryptase induced airway hyper-responsiveness following its administration to the lungs of mice (Wong *et al.*, 2002). In addition, the administration of a mouse chymase (mMCP-4) was found to induce substantial inflammation in mice (Watanabe *et al.*, 2002).

Table 1.2 Mast cell mediators. Adapted from (Da Silva *et al*., 2014).

Mediator	Role
Phospolipid Metabolites (Prostaglandins D2, Leukotrienes and Platelet-activating factor)	Pro-inflammatory, vasodilation, pyrogenic mediator
Biogenic Amines (Histamine)	Vasodilation, increased capillary permeability, smooth muscle constriction and neural influence
Serglycin (Heparin, Chondroitin sulphate)	Anticoagulation, anti-inflammatory activity
Proteases (e.g. mMCP-1)	Antimicrobial mediators, regulation of epithelial permeability
IL-33	Earlier expression of tryptase, increased expression of mMCP-6
IL-3	Hematopoietic stem cell differentiation and development
IL-4	Th2 cytokine, induces B cell class switching to IgE
IL-5	Th2 cytokine, stimulates B cell growth and Ig secretion
lL-6	Th2 cytokine, pro-inflammatory, reduced SCF-dependent development, increased expression of chymase and histamine production
IL-13	Th2 cytokine, stimulates Ig secretion, triggers mast cell degranulation
Nerve growth factor	Increased maturation, histamine content and heparin expression, reduced Apoptosis
IFNγ	Th1 cytokine, macrophage activator
Tumor necrosis factor (TNF) α	Pro-inflammatory mediator

1.4.4. Mast cell activation

The surface of mast cells contains a range of receptors that when engaged by ligands result in mast cell activation. Mast cells can be activated directly by antigens or indirectly by pattern-recognition receptors (PRR), high-affinity FcεR I receptors (FcεRI), or complement receptors (CR) (reviewed in Table 1.3).

FcεRI contains three main parts: an α-binding chain for IgE, a β subunit known as a tetraspanin and two disulfide γ chains bound together. These components are also found on basophils (Huber, 2013). Another immunoglobulin Fc receptor, FcγRIII,

binds to IgG on mast cell surfaces at a low affinity, similar to other immunoglobulins (Urb and Sheppard, 2012). PAMPS can also binds directly to PRR present on mast cell surfaces, such as a toll-like receptor (TLR), a signal is triggered that leads to the secretion of active mediators (Da Silva *et al.*, 2014). Indirect activation can be enacted by crosslinking an antigen to IgE bound to FcεRI (Marshall *et al.*, 2003). During the phase of sensitization, IgE is synthesised to an antigen and binds to the FcεRI. Upon a following exposure to the same antigen, IgE-cross-linking will stimulate degranulation of the mast cell. This activation is particularly important in allergies. C3R and C5aR are complementary receptors that react to complement and lead to degranulation (Marshall *et al.*, 2003). Other forms of stimulation involved in mast cell activation include cytokines, growth factors, toxins and drugs (Da Silva *et al.*, 2014).

The mast cells' functional flexibility allows them to respond to a range of immunological challenges. This depends on the mast cells' location, subtype and activation (Galli *et al.*, 2005b). In addition, mast cells perform their function by synthesizing and releasing mediators that are classified as preformed mediators, neoformed or lipid mediators, and neosynthesised mediators (Da Silva *et al.*, 2014). The preformed mediators are the largest group and include biogenic amines such as histamine, lysosomal enzymes such as β-hexosaminidase, proteases such as chymase, and several other compounds (Lundequist and Pejler, 2011). These mediators are stored within secretory granules and released upon degranulation.

MC can be activated by TLR ligands and bacterial products can activate mast cells (Malaviya *et al.*, 1999, Leal-Berumen *et al.*, 1996, Leal-Berumen *et al.*, 1994, Ackermann *et al.*, 1998). Mast cell-deficient W/W-v mice showed significantly increased mortality in a model of acute septic peritonitis (Echtenacher *et al.*, 1996) and engraftment with MC restored survival to almost the level observed in wild-type mice (Echtenacher *et al.*, 1996), demonstrating their protective role in a bacterial infection.

The expression of TLR4 by MC, but not TLR2, was required to enhance MC survival in a cecal ligation and puncture (CLP) experiment in W/W-v mice (Supajatura *et al.*, 2002). Moreover, low doses of recombinant tumour necrosis factor-**α** (TNF-**α**) injected in W/W-v mice led to an increase in survival in the CLP

experiment, whereas injection of high doses of TNF worsened the rate of survival (Echtenacher *et al.*, 1996). Mice deficient in the complement products C4 or C3 were significantly more sensitive to caecal ligation and puncture than controls, showing that complement is also involved in mast cell activation. C3-deficient mice also exhibited reductions in peritoneal mast cell degranulation, production of TNF-**α**, neutrophil infiltration and clearance of bacteria. The important role of complement was confirmed when treating C3-deficient mice with purified C3 protein enhanced activation of peritoneal mast cells, TNF-**α** production, neutrophil recruitment, opsonophagocytosis of bacteria and resistance to caecal ligation and puncture (Debus *et al.*, 1984, Tohyama *et al.*, 1992, Prodeus *et al.*, 1997).

It has been suggested that during the expulsion of *T. spiralis* MC activation was normally considered to occur through the cross-linking of FcεRI (Bell, 1998). However, mice deficient in the common γ chain of Fc receptors infected with *T. spiralis* efficiently expelled worms, and a strong intestinal MC response developed which included production of mMCP-1 (Grencis, 1997). Therefore, it has been suggested that MC can be activated via an Ig-independent mechanism and participate in expulsion of worm (Yépez-Mulia *et al.*, 2009).

Table 1.3 Mechanisms of activation of mast cells. Adapted from (Yu *et al.,* 2016)

Stimuli	**Receptor**	**Mast cell response**
IgE	FcεRI	Degranulation
IgG	FcγRI	Degranulation
C3a	C3aR	Degranulation, chemotaxis, CCL2, CCL5
C5a	C5aR	Degranulation, chemotaxis
Lipopolysaccharides (LPS)	TLR4	TNFα, IL-5, IL-10, IL-13 TNFα, CCL1, IL-5
Peptidoglycan (PGN)	TLR2	Histamine; TNFα, IL-5, IL-10, IL-13, GM-CSF, IL-1β; CysLT
Stem cell factor (SCF)	C-kit	Proliferation, differentiation, histamine survival; CysLTs; migration of PGD2
IL-4	IL-4 R	Induced production of Th2 cytokine, maturation

1.4.5. Role of mast cells in disease

A variety of physiological functions are regulated by MCs, including vasodilation, angiogenesis and elimination of bacteria and parasites (reviewed in Table 1.4) (Krystel-Whittemore *et al.*, 2016). In addition, MCs appear to interact with antigens, toxins and pathogens. Therefore, various receptors are expressed on the surface of MCs, enabling these cells to detect potentially harmful signals and to respond rapidly and appropriately via the release of a number of preformed, pre-stored and neo-synthesised mediators (Da Silva *et al.*, 2014). Different mechanisms enable MCs to recognise pathogens through direct binding, for example, or to detect pathogen components via pathogen-associated molecular pattern (PAMP) receptors on the MC surface. Other possible mechanisms include the binding of antibodies or complement-coated bacteria to immunoglobulin receptors or the recognition of endogenous peptides produced by infected or injured cells (Hofmann and Abraham, 2009).

1.5.5.1 Allergic inflammation models

Allergic airway inflammation has been studied in mouse models of antigen (Ag) sensitisation through the administration of artificial adjuvants (Nakae *et al.*, 2007, Reuter *et al.*, 2008, Taube *et al.*, 2004, Williams and Galli, 2000, Yu *et al.*, 2006, Yu *et al.*, 2011) or relatively low doses of Ag (Kobayashi *et al.*, 2000b, Kung *et al.*, 1995). In addition, MC-associated TNF-**α** has been identified as a mediator that contributes to airway hyper-responsiveness (AHR) and airway inflammation (Kim *et al.*, 2007, Nakae *et al.*, 2007, Reuter *et al.*, 2008).

The activation of MCs through the FcR γ chain and the IFN-γ receptor 1 in allergic airway inflammation is required for the full development of many features of allergic airway responsiveness, inflammation and tissue remodelling (Yu *et al.*, 2006, Yu *et al.*, 2011). Sawaguchi *et al*. (2012) reported that MCs played an important role in ovalbumin OVA-induced AHR in Mas-TRECK mice. A20 is a key negative regulator of mast cell activation and the effects of MC-specific A20 deficiency in mouse asthma models was investigated. The results showed significant enhanced airway inflammation following the loss of MC-specific A20 in c-*kit* normal *Mcpt5-Cre;A20*$^{fl/fl}$ mice when these mice were immunised with an intranasal administration

of house dust mite (HDM) extracts or i.p. injections of OVA without alum Ag-specific serum IgE (Heger *et al.*, 2014). These findings indicate that MCs can contribute to asthma pathology in mice models (Galli *et al.*, 2015). Gri *et al.,* (2008) demonstrated that the increase in MC responsiveness *in vivo* resulted from a decrease in regulatory T cells (Treg) cells and/or their loss of function. These results indicate that the Treg cells contribute towards the allergy reduction which is possibly mediated not only by inhibition of T cell-driven inflammation, but also by direct regulation of the release of preformed pro-inflammatory mediators by mast cells (Gri *et al.*, 2008). It has been shown that the blockade of OX40L by using an OX40–Ig fusion protein caused the enhanced expulsion of *T. spiralis* from the small intestine, which corresponds with the amplification of mucosal mastocytosis (Ierna *et al.*, 2006).

1.5.5.2 Arthritis models

In a mouse model of human inflammatory arthritis, Lee *et al.* (2002) reported a substantial reduction in joint inflammation and destruction in MC-deficient (W/W-v and Sl/Sl^{d}) mice induced with arthritis via injection of K/BxN serum. Moreover, restored disease susceptibility in W/W-v mice was found after local engraftment of wild-type MCs but not *Il-1*$^{-/-}$ MCs (Lee *et al.*, 2002, Nigrovic *et al.*, 2008). In addition, injection of anti-type II collagen antibody in Wsh/Wsh mice strongly induced proliferative arthritis (Zhou *et al.*, 2007). However, W/W-v mice were protected in this arthritis model (Zhou *et al.*, 2007). Meanwhile, full susceptibility to developing joint pathology was observed in *Cpa3*$^{Cre/+}$ mice in a K/BxN serum transfer model (Feyerabend *et al.*, 2011).

1.5.5.3 Autoimmune encephalomyelitis (EAE) models

Induction of autoimmune encephalomyelitis (EAE) in MC-deficient mice showed inconsistent induction of this disease (Galli *et al.*, 2015). Delayed onset and milder severity of disease were reported in W/W-v mice immunised with myelin oligodendrocyte glycoprotein $(MOG)_{35–55}$ peptide (Secor *et al.*, 2000). Similarly, disease severity was significantly reduced in SJL-*Kit*$^{W/W-v}$ mice when immunised with proteolipid protein $(PLP)_{131–159}$ peptide (Sayed *et al.*, 2011). Further, mast cell-deficient W/W-v mice that showed disease susceptibility were restored by MC

engraftment to wild-type levels, showing the contribution of MCs to the EAE-associated pathology in these models (Galli *et al.*, 2015). W/W-v and Wsh/Wsh mice were both shown to be fully susceptible to EAE (Bennett *et al.*, 2009), and more severe EAE has been reported in Wsh/Wsh mice in comparison to corresponding $Kit^{+/+}$ mice (Li *et al.*, 2011). In one experiment, three different immunisation conditions were used to induce EAE; disease was exacerbated in Wsh/Wsh mice in comparison to the corresponding wild-type mice (Piconese *et al.*, 2011).

In addition, Piconese *et al.* (2011) demonstrated that W/W-v mice immunised with lower doses of MOG and adjuvants showed more severe disease than wild-type mice, similar to the disease phenotype observed in Wsh/Wsh mice., It was confirmed that induction of EAE under a strong immunisation protocol in W/W-v mice had milder EAE compared to $Kit^{+/+}$ mice ((Piconese *et al.*, 2011) similar to that demonstrated by Secor *et al.* (2000). Feyerabend *et al.* (2011) reported that EAE disease severity did not differ in $Cpa3^{Cre/+}$ or W/W-v mice or in the respective MC-containing control mice. This finding led to the conclusion that, at least in the tested models, MCs are unnecessary for the development of EAE (Galli *et al.*, 2015).

1.5.5.4 Bacterial infection models

MCs are protective against bacterial infections, as demonstrated in studies using the CLP model of sepsis (Echtenacher *et al.*, 1996). Also, MCs were crucial for protection against enterobacteria and i.p. injected *Klebsiella pneumoniae* and *Escherichia coli* (Malaviya *et al.*, 1996). Activation of MCs by lipopolysaccharide (LPS) via gram-negative bacteria and TLR-4 did not induce degranulation of MCs but did result in the production of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 as well as anti-inflammatory IL-13 (Supajatura *et al.*, 2001). Mast cell deficient models (W/W-v and Wsh/Wsh mice) have been used to support the findings that MCs are protective in CLP models (Supajatura *et al.*, 2001, Nakano *et al.*, 2007) and also protect against infection by a variety of bacterial pathogens, including *Citrobacter rodentium* (Wei *et al.*, 2005), *Pseudomonas aeruginosa* (Siebenhaar *et al.*, 2007), *Listeria monocytogenes* (Edelson *et al.*, 2004), *Streptococcus pyogenes* (Matsui *et al.*, 2011), *Helicobacter pylori* (Velin *et al.*,

2005) and *Francisella tularensis* (Ketavarapu *et al.*, 2008). In addition, the important role of MCs in protection against *Mycoplasma pneumoniae* has been studied using Wsh/Wsh mice (Xu *et al.*, 2006).

MCs are also capable of presenting bacterial antigens to T-cell hybridomas via class I major histocompatibility complex molecules (Malaviya *et al.*, 1996). Additionally, the antibody response to *E. coli* has been shown to be promoted by MCs (Shelburne *et al.*, 2009).

Moreover, MCs have reportedly played a critical role in anti-*Helicobacter* vaccination (Velin *et al.*, 2005). The direct action of MCs on bacteria has been shown in several studies; however, *in vivo* evidence to support these findings is limited (Johnzon *et al.*, 2016). *In vitro*, MCs phagocytosed FimH-expressing enterobacteria (*E. coli, Klebsiella pneumoniae* and *Enterobacter cloacae*) and killed them via acidic vacuoles (Malaviya *et al.*, 1994). The killing of group A *Streptococcus* led to a 50% reduction in MC deficient mice in the antimicrobial peptide cathelicidin in comparison with MCs in wild-type mice (Di Nardo *et al.*, 2003). Similar to neutrophils, MCs can exert antibacterial activities through the formation of extracellular traps in response to *S. pyogenes* (Von Köckritz-Blickwede *et al.*, 2008) and *Staphylococcus aureus* (Von Köckritz-Blickwede *et al.*, 2008, Abel *et al.*, 2011).

1.5.5.5 Viral infection models

The role of MCs is less well characterised in viral infections (Graham *et al.*, 2013). MC have been shown to play a role in the immune response to several viruses, including human immunodeficiency virus (HIV), dengue, cytomegalovirus, adenoviruses and influenza A (IAV), wherein MCs are activated by viral products or induced by the production of cytokines and chemokines, including IL-1β, IL-6, CCL3, CCL4, CCL5 and CCL8 (Marshall *et al.*, 2003, Dawicki and Marshall, 2007, Burke *et al.*, 2008). For instance, the recruitment of $CD8^+$ T lymphocytes to the site of infection and the production of IFN-γ during viral challenge were promoted by MCs, indicating that MCs encourage viral recognition in cellular responses directed towards viral clearance (Kulka *et al.*, 2009, Orinska *et al.*, 2005). Within draining lymph nodes, an increased viral burden in MC-deficient mice was observed following infection by the dengue virus, which was likely due to deficient NK and

NK T-cell recruitment to the site of infection (John *et al.*, 2011). The herpes simplex virus 2 (HSV-2), when injected intradermally into W/W-v mice, was also found to result in increased clinical severity and mortality and was associated with raised virus titres in HSV-infected skin.

Moreover, HIV was found to be capable of infecting human MC progenitors; during latent infection, long-lived viral reservoirs can mature and develop (Sundstrom *et al*. 2007). Additionally, Graham *et al.* (2013) reported that the establishment of IAV-induced inflammatory response and lung damage were associated with the contribution of MCs. In addition, MCs were shown to be important in inflammatory cell infiltration in a TLR3-dependent manner in a peritonitis model of Newcastle disease virus infection (Orinska *et al.*, 2005). Also, MCs play an important role in immunosurveillance through RIG-I- and Mda5-dependent recognition of the dengue virus following cutaneous infection (John *et al.*, 2011, Brown *et al.*, 2012).

1.5. Models for Investigations into Mast Cell Function

The role of mast cells in the immune response has been elucidated by the use of various mouse models. Investigating the potential roles of mast cells in biological responses would be simplest if mast cells could be selectively or genetically ablated *in vivo*. It would also be ideal to be able to selectively ablate either all mast cells or only the mast cell of interest. Once the depletion is established, it would be useful to define how the role of the mast cell is expressed in that setting. There are different ways by which mast cells might express a function, so it would be helpful to be able to selectively delete elements of mast cell activation pathways or products, or to specifically block mast cell-derived products. For this reason, genetic approaches may provide a more definitive way to identify and characterise mast cell functions in mice *in vivo* (Reber *et al.*, 2012a).

Mast/stem cell growth factor receptor (SCFR), also known as proto-oncogene c-Kit or tyrosine-protein kinase Kit or CD117, is a receptor tyrosine kinase protein that in humans is encoded by the KIT gene. Multiple transcript variants encoding different isoforms have been found for this gene. The investigation of mast cell function began with the use of mast cell-deficient murine models with mutations in the KIT gene. Mice with mutations affecting the function of this gene are known as c-kit mutants (Kitamura *et al.*, 1978).

1.5.1. c-Kit mutants

Mast cell-deficient W/W-v and Wsh/Wsh mice were most commonly used for such studies (Reber *et al.*, 2012a). Kit^{W} is a point mutation that results in production of a truncated KIT protein that is not expressed on the cell surface (Hayashi *et al.*, 1991). In addition, in the c-kit tyrosine kinase domain, $Kit^{W\text{-}v}$ is a mutation that substantially reduced the kinase activity of the receptor (Nocka *et al.*, 1990), whereas $Kit^{W\text{-}sh}$ is an inversion mutation that affected the transcriptional regulatory elements upstream of the c-kit transcription start site on mouse chromosome five (Nagle *et al.*, 1995, Nigrovic *et al.*, 2008).

W/W-v and Wsh/Wsh mice are both extremely deficient in mast cells and melanocytes. Additionally, these mice were found to have several other phenotypic abnormalities that reflected the KIT biological distribution and the functions of the

cells both inside and outside of the immune system. These included some abnormalities affecting hematopoietic cells other than mast cells that contributed to innate or adaptive immune responses (Grimbaldeston *et al.*, 2005, Piliponsky *et al.*, 2010, Zhou *et al.*, 2007, Nigrovic *et al.*, 2008, Tsai *et al.*, 2005, Chervenick and Boggs, 1969). However, some of these phenotypic abnormalities in the two types of kit mutant mast cell-deficient mice differed. For example, W/W-v mice are anaemic and had reduced neutrophil (Piliponsky *et al.*, 2010, Zhou *et al.*, 2007, Nigrovic *et al.*, 2008, Chervenick and Boggs, 1969) and basophil numbers (Piliponsky *et al.*, 2010, Feyerabend *et al.*, 2011, Lantz *et al.*, 1998). These mice were also sterile (Galli *et al.*, 2005a, Grimbaldeston *et al.*, 2005). In contrast to W/W-v mice, Wsh/Wsh mice were neither anaemic nor sterile and had increased neutrophil (Grimbaldeston *et al.*, 2005, Piliponsky *et al.*, 2010, Zhou *et al.*, 2007, Nigrovic *et al.*, 2008) and basophil numbers (Piliponsky *et al.*, 2010).

The biological responses obtained from Kit mutant mice, which were different when compared to the responses of wild-type (WT) mice, may represent one or more of the abnormalities that resulted from the KIT alteration structure or expression in these mice. These abnormalities may have directly or indirectly affected cell lineages that may not be, even partly, due to their deficiency in mast cells. At many anatomical sites, the deficiency in MCs in kit mutant mice can be selectively "repaired" by the adoptive transfer of genetically-compatible, *in vitro*-derived WT or mutant MCs (Kitamura, 1989, Galli *et al.*, 2005a, Grimbaldeston *et al.*, 2005, Grimbaldeston *et al.*, 2006, Nakano *et al.*, 1985). For example, since their description in 1985, bone marrow-derived cultured mast cells (BMCMCs) have allowed for bone-marrow to be administrated intravenously (i.v.), intraperitoneally (i.p.) or intradermally (i.d.) to create mast cell knock-in mice (Nakano *et al.*, 1985). Mast cell-deficient W/W-v and Wsh/Wsh mice presented with additional abnormalities, particularly the lack of intestinal pacemaker cells of Cajal (ICC), which are electrical pacemakers generated in the GI tract that produce slow electrical waves that are important for GI motility (Sanders *et al.*, 2006). The ICC may be an affecting factor in expelling helminths from the gut in c-Kit mutated mice.

Another MC-deficient mouse model that has been identified is WCB6F1-Mgf$^{Sl/Sl\text{-}d}$ mice, which have two loss-of-function mutations in the *Scf* gene, which has the gene sequence for the KIT ligand (Yu *et al.*, 2015). Removing the coding region of the *Scf* gene results in the generation of the steel (*Sl*) mutation (Bedell *et al.*, 1995), while the SCF molecule is encoded by the steel-Dicke (*Sl-d*) mutation without its transmembrane and cytoplasmic domain (Brannan *et al.*, 1991). Therefore, the production or function of SCF is dramatically impaired as a result of the two loss-of-function mutations which lead to the deficiency in the KIT signalling (Yu *et al.*, 2015).

1.5.2. Constitutive knockout models

Several attempts have been made to develop mast cell-deficient mice due to the potential complexities in finding kit mutants that lack abnormalities related to KIT gene structure or expression (Reber *et al.*, 2012a). Cre-recombinase (Cre) is a common approach to generate mice that are expressed under the control of promoters thought to be mast cell-specific or at least mast cell-associated (Scholten *et al.*, 2008, Feyerabend *et al.*, 2009, Lilla *et al.*, 2011, Müsch *et al.*, 2008). There are currently number of mouse models which have been developed as having constitutive mast cell deficiencies.

1.6.2.1 Mcpt5-Cre; R-DTA mice

Mast cell protease (Mcpt)5-Cre transgenic mice have been crossed with R-DTA$^{fl/fl}$ mice (Voehringer *et al.*, 2008) to generate a mouse strain that can produce the diphtheria toxin alpha chain (DTA) in only Cre-expressing cells. This led to the Cre-specific ablation (Dudeck *et al.*, 2011a). Naïve Mcpt5-Cre; R-DTA have a constitutive lack in mast cells in peritoneal and ear skin. In addition, the number of abdominal and back skin mast cells had been reduced in comparison to the Cre^{-} counterparts (Dudeck *et al.*, 2011a). This resulted in the depletion of mast cells in specific sites. Moreover, Mcpt5-Cre; R-DTA mice are deficient only in connective tissue mast cells (CTMCs), whereas mucosal mast cells (MMCs) were unaffected, which led to the belief that MCPT5 were not expressed in MMCs. Other abnormalities in the Mcpt5-Cre;R-DTA model have not yet been assessed (Dudeck *et al.*, 2011a).

1.6.2.2 Cpa3$^{Cre/+}$-'Cre-Master' mice

Cre-mediated mast cell eradication (Cre-Master, Cpa3$^{Cre/+}$) mice are another model of constitutive mast cell-deficient mice. Feyerabend *et al*. (2011) produced a Cre-recombinase expression on the mast cell carboxypeptidase A (Cpa3) locus, which led to the ablation of mast cells through Cre-mediated genotoxicity and encoded the mast cell-associated protease carboxypeptidase A3 (CPA3). Heterozygous Cpa3$^{Cre/+}$ mice unexpectedly display a complete lack of mast cells. Mast cells in the skin are not detected, even under inflammatory conditions that are associated with the development of skin mast cells in WBB6F$_1$-Kit$^{W/W-v}$ mice (Gordon and Galli, 1990b). Similarly, following infection with the helminth *N. brasiliensis*, MMCs were not found in the intestine (Feyerabend *et al.*, 2011).

The extreme mast cell depletion appeared to be mediated by Cre-induced genotoxicity (Schmidt-Supprian and Rajewsky, 2007). However, it has been demonstrated that CPA3 was expressed in basophils (Voehringer *et al.*, 2004) and some populations of T cell progenitors and thymic T cells (Feyerabend *et al.*, 2009, Taghon *et al.*, 2007). In addition, certain hematopoietic progenitor cells were also shown to express CPA3. Basophils in the Cpa3$^{Cre/+}$ model were not completely depleted but did show a 60% reduction in spleen basophil numbers (Feyerabend *et al.*, 2011).

1.6.2.3 Cpa3-Cre; Mcl-1$^{fl/fl}$-'Hello Kitty' mice

Cpa3-Cre; Mcl-1$^{fl/fl}$ was another transgenic mast cell-deficient mouse model that was generated from insertion of Cre-recombinase into the mouse genome (Lilla *et al.*, 2011). Mcl-1 was the gene coding for the anti-apoptotic factor and its deletion resulted in mast cell eradication (Rodewald and Feyerabend, 2012). Mice with floxed myeloid cell leukaemia sequence 1 (Mcl-1) alleles were crossed with Cpa3-Cre transgenic mice which resulted in the specific deletion of the Mcl-1 gene in mast cells (Lilla *et al.*, 2011). Thus, Cpa3-Cre transgenic mice showed reduced numbers of mast cells. In addition, the number of basophils in Cpa3-Cre; Mcl-1$^{fl/fl}$ mice was also substantially reduced in the bone marrow, blood and spleen (Lilla *et al.*, 2011). Cpa3-Cre; Mcl-1$^{fl/fl}$ transgenic mice, due the lack of c-Kit mutation and the reduced basophil numbers, were informally called 'Hello Kitty' mast cell- and basophil-deficient mice.

1.5.3. Inducible mast cell-deficient models

Investigating the importance of mast cells in a therapeutic target should hypothetically be performed using mice in which mast cell ablation can be selectively or genetically achieved. The diphtheria toxin (DT) injection into transgenic mice bearing the DT receptor (DTR) in only one particular cell type (Buch *et al.*, 2005) is a promising model for a more successful and efficient depletion of a particular cell population. This approach was recently used by two different groups to deplete mast cells in adult mice (Dudeck *et al.*, 2011a, Sawaguchi *et al.*, 2012, Otsuka *et al.*, 2011).

1.6.3.1 Mcpt5-Cre; iDTR mice

Mcpt5-Cre mice crossed with iDTR$^{fl/fl}$ mice expressed a floxed simian DTR transgene that was inserted into the *Gt(ROSA)26Sor* (ROSA26) locus. This was used to achieve a Cre-dependent expression of DTR in mast cells (Dudeck *et al.*, 2011a). Dudeck *et al*., (2011) demonstrated that a single i.p. injection of DT in Mcpt5-Cre; iDTR mice led to a nearly complete ablation of peritoneal mast cells after 24 hours of injection. In addition, it has been demonstrated that repeated DT treatments in the peritoneal cavity and abdominal skin of Mcpt5-Cre; iDTR mice (once a week for four weeks) led to the complete ablation of mast cells when compared to Mcpt5-Cre^{-} mice. This was assessed one week after the last DT injection (Dudeck *et al.*, 2011a). Additionally, DT-treated Mcpt5-Cre; iDTR mice exhibited a depletion of subepithelial CTMCs in the small intestine and stomach, whereas intraepithelial MMCs were not depleted. This was most likely due to a lack of Mcpt5-Cre transgene expression in MMCs (Dudeck *et al.*, 2011a).

1.6.3.2 Mas-TRECK mice

Sawaguchi *et al*. (2012) generated an additional transgenic model, named Mast cell-specific enhancer-mediated Toxin Receptor-mediated Conditional Cell Knock-out (Mas-TRECK), in which the ablation of mast cells could be specifically stimulated. Mas-TRECK mice models were based on the expression of the human diphtheria toxin receptor (DTR). The expression of the interleukin (IL)-4 gene was under the control of the intronic enhancer element (IE). DTR was inserted, which led to the specific depletion of IL-4 expressing cells. This resulted in the depletion of mast

cells and basophils (Yu *et al.*, 2015). Otsuka *et al*., (2011) and Sawaguchi *et al*. (2012) previously demonstrated that the IE element was essential for IL-4 expression in mast cells but not for basophils, natural killer (NK) T cells or Th2 cells (Yagi *et al.*, 2007). They also reported that five days of repeated i.p. injections of DT in the skin, peritoneal cavity, stomach and mesenteric windows of Mas-TRECK mice completely depleted mast cells after three days from the last injection (Sawaguchi *et al.*, 2012).

Otsuka *et al*. (2011) and Sawaguchi *et al*. (2012) also showed that mast cells in the skin remained depleted for at least 12 days after ending the treatment of DT. However, blood basophils in Mas-TRECK mice were temporarily depleted after the DT treatment. The DT treatment also completely inhibited the development of a type of basophil-dependent and IgE-mediated chronic allergic inflammation of the skin (Mukai *et al.*, 2005, Sawaguchi *et al.*, 2012). Moreover, other major types of leukocytes (dendritic cells, B, T, NKT cells, eosinophils and neutrophils) which did not express DTR mRNA were not affected by DT treatment, although numbers of these cells were only reported for analyses performed 12 days after the end of DT treatment (Sawaguchi *et al.*, 2012, Otsuka *et al.*, 2011).

1.6. Aims and Objectives

Immune responses to helminth parasites require various effector mechanisms, and one of the essential mechanisms is the stimulation of T helper 2 cells. The adaptive immune response can be amplified by mast cells that function as a modulator response to the T helper cells (Hershko and Rivera, 2010). Mast cells have also proven to be important in the expulsion of *Trichinella spiralis* from the gut (Hepworth *et al.*, 2012b). These observations had previously been shown using the mast cell deficient c-Kit W/W-v mice (Ha *et al.*, 1983, Lawrence *et al.*, 2004). The development of a range of mast cell-deficient mice has provided an understanding of the importance of mast cells in the immune response to parasitic infection.

Mast cells participate significantly in the immune defence system and the expulsion of *T. spiralis* worms. Therefore, it is essential to identify the activation mechanisms that mast cells utilise during an infection of *T. spiralis* to determine whether helminthic products alone would be significant in inducing cytokine responses from the mast cells.

In the present study, we aimed to assess and confirm the importance of mast cells in the immune response to *T. spiralis*. The investigation was carried out on Wsh/Wsh mice and C57BL/6 mice. The effect of *T. spiralis* on Mas-TRECK mice in comparison to BALB/c mice was also estimated to compare c-Kit mutant models against inducible mast cell-deficient models and to evaluate how helminthic products might activate MCs. The ability of mice to expel worms was then estimated via worm establishment at two different time points after infection. The progression of infection and generated immune system responses were examined by counting the number of worms and analysing the intestinal pathology, cytokine and antibody responses to assess the use of Wsh/Wsh mice and Mas-TRECK mice as mast cell-deficient models for the investigation of the function of mast cells during an infection of gastrointestinal nematodes.

Table 1.4 The importance of MCs in different types of biological responses

MC-deficient mice	Construct/deletion	Model	Main findings	Reference
C-Kit mutants (W/W-v and Wsh/Wsh)	Mutations in the Kit gene	IgE-dependent passive anaphylaxis	Strong PCA response restored in W/W-v mice by WT BMCMCs	(Wershil *et al.*, 1987)
Cre-Master	A Cre-recombinase expression on the MC carboxypeptidase A (Cpa3) locus under control of the Cpa3 promoter along with the deletion of 28 nucleotides of the first exon of the Cpa3 locus		KIT mutant mice could not elicit PCA nor PSA; the ability of MC-deficient mice to express PSA responses was restored by WT BMCMCs	(Feyerabend *et al.*, 2011)
Cpa3-Cre; Mcl-1$^{fl/fl}$ (Hello Kitty)	Under a Cpa3 promoter fragment; cross between Mcl-1-floxed mice and transgenic mice expressing Cre		Ability of MC-deficient mice to express strong PCA responses were restored by WT BMCMCs	(Lilla *et al.*, 2011)
Mas-TRECK	Expressing human DTR under an intronic enhancer of the IL-4 gene (DTR-IL-4)		MC-deficient mice were unable to induce PCA and PSA responses	(Sawaguchi *et al.*, 2012)
Kit$^{W/W\text{-}v}$		Asthma (allergic inflammation and hyperresponsiveness of the airways)	Sensitisation with alum for 17 days showed the possibility of MCs to contribute to asthma	(Kung *et al.*, 1995)
Kit$^{W/W\text{-}v}$			Sensitisation with adjuvant for 30 days showed that MCs have unnecessary or no roles	(Takeda *et al.*, 1997)
MC knock-in Kit$^{W/W\text{-}v}$			Sensitisation with adjuvant for 20 days showed roles for MCs with low-dose antigen	(Kobayashi *et al.*, 2000b)

Kit$^{W\text{-}sh/W\text{-}sh}$, C57BL/6 background			Sensitisation for 47 days without adjuvant indicated that MCs may have roles	(Becker *et al.*, 2011)
C.B6-Kit$^{W\text{-}sh/W\text{-}sh}$, BALB/c background			Sensitisation for 47 days without adjuvant showed the full development of asthma	(Becker *et al.*, 2011)
Mas-TRECK			Sensitisation for 28 days without adjuvant induced the depletion of MCs prior to reduced airway hyperreactivity	(Sawaguchi *et al.*, 2012)
MC knock-in Kit$^{W/W\text{-}v}$		Antibody-dependent arthritis	Testing of K/BxN (Nakae *et al.*, 2007) serum transfer model indicated the important role of MCs in inducing arthritis	(Lee *et al.*, 2002)
Kit$^{W/W\text{-}v}$			Testing of anti-collagen antibodies indicated the important role of MCs in inducing arthritis	(Zhou *et al.*, 2007)
Kit$^{W\text{-}sh/W\text{-}sh}$			Testing of K/BxN (Nakae *et al.*, 2007) serum transfer model and anti-collagen antibodies showed that arthritis fully developed in MC-deficient mice	(Elliott *et al.*, 2011, Mancardi *et al.*, 2011, Zhou *et al.*, 2007)
Cre-Master			Testing of K/BxN (Nakae *et al.*, 2007) serum transfer model showed that arthritis fully developed in MC-deficient mice	(Feyerabend *et al.*, 2011)

CHAPTER 2. MATERIALS AND METHODS

2.1. Mice

Male C57BL/6 and BALB/c mice were originally sourced from Harlan (Indiana, USA) and then bred in-house. Male and female Wsh/Wsh mice were sourced from Jackson Labs (Maine, USA). The Mas-TRECK (*Il*)-4 gene was inserted at the intronic enhancer control element with a human DTR gene (Otsuka *et al.,* 2011). These transgenic mice were originally obtained from Dr Minoru Sawaguchi and colleagues (Research Centre for Allergy and Immunology, RIKEN, Yokohama Institute, Japan), and were developed through a BALB/c background (Sawaguchi *et al*., 2012). All mice were maintained in the BPU laboratory for the duration of the experiment. They were matched with their controls, with regard to age, gender and background. The guidelines of the UK Animals (Scientific Procedures) Act 1986 were adhered to, such that animal safety and well-being was maintained throughout all *in vivo* experiments. The mice were weighed every day, ensuring that their weight did not drop below 20% of their initial body weight, as required under Home Office regulations. DT-treated mice were also graded on degree of hump, hair stagnancy, lethargy and diarrhoea for Home Office records.

2.1.1. Diphtheria toxin treatment

BALB/c and Mas-TRECK mice were pre-treated with diphtheria toxin (250 ng in 250 µl) (Sigma-Aldrich, UK) via an intraperitoneal injection as described by (Sawaguchi *et al.*, 2012). The toxin was diluted in sterile phosphate buffered saline (PBS) as follows: 7.5 µl of 1mg/ml toxin in 7.5 ml PBS (Tablets sourced from Oxoid, UK). The mice were treated five times over a 2-week course, with a 2-day minimum gap between each treatment. The mice were then given a 72-hour break to allow basophils to recover to normal levels before infection with *Trichinella spiralis (T. spiralis*).

2.2. Infection of mice

T. spiralis parasites were maintained by serial passage, and L3 larvae were obtained from infected wild-type C57BL/6 mice, as described by Wakelin and Wilson (1977). The larvae were obtained by sacrificing the mice (C57BL/6) and removing their eyes, ears, upper and lower jaw, tail, skin and abdominal organs. Next, the carcasses

were cut into pieces and placed in a blender. The tissue was then placed in 500 ml of digestion buffer (0.9% sodium chloride, 0.5% pepsin from Sigma-Aldrich, UK and 0.5% Hydrochloric Acid (Acros Chemicals, UK), and kept in a conical flask in a water bath at 37°C under agitation for 90 minutes. After the larvae were settled, they were washed with 0.9% saline solution until the supernatant was clear. They were then re-suspended in 0.1% agarose (Bioline, UK) in 50 ml 0.9% saline and the total number was counted. The mice were orally infected with 400 *T. spiralis* larvae/0.2 ml 0.1% agarose by gavage unless otherwise stated. Infected mice were sacrificed at various times on days 7 and 14 post infection (p.i.) with carbon dioxide inhalation (CO_2), which is important in collecting blood samples (Lawrence *et al.*, 1998a).

2.3. Blood samples

Blood samples were obtained from uninfected and infected mice by cardiac puncture, after which the samples were incubated overnight at 4°C. After incubation, clotting occurred and the serum was removed from the samples after centrifugation at 16000g for 5 minutes. The samples were then stored at -20°C.

2.4. *Trichinella* antigen

Trichinella antigen (*T.* Ag) was removed from muscle larvae (L3) as described by (Lee *et al.*, 1982),washed with PBS and centrifuged for 5 minutes at 16000g. The supernatant was discarded, replaced with more PBS and then centrifuged. The worms were then homogenised on ice. The *T.* Ag concentration was determined by Coomassie assay, using the Coomassie (Bradford) Protein Assay Kit with Albumin (BSA) standards. The plates were then read at 540nm on a plate reader (Molecular Devices Corporation, California. USA). The protein concentration of the antigen was calculated from the standard curve. The antigen was stored at -20°C, and was used for evaluation of T-cell proliferation, *Trichinella*-specific immunoglobulin IgG1/IgG2a ELISAs and IL-4 cytokine production.

2.5. Intestinal pathology

The pathology of the small intestine was assessed in all mice. The small intestinal was taken from jejunum samples, approximately 10 cm below the pylorus, and then all samples (gut) were individually weighed as described by (Mowat and Ferguson, 1982). The gut samples were longitudinally opened and divided into two groups. An

approximately 1 cm length of small intestine was placed on cardboard and then fixed in 5–10 ml of Clarke's fixative. After 24 hours, the tissue was transferred into 70% ethanol (EtOH), (Sigma-Aldrich, UK), and stored until it was to be used for measurement of villus and crypts. Prior to using these samples, the tissue was stained by incubating the samples in 5–10 ml 50% EtOH for 10 minutes. The EtOH was then poured off, after which the samples were filled with tap water and left for 10 minutes. They were then placed in 5–10 ml of 1 M hydrochloric acid (HCL) at 60°C for 7.5 minutes. Following completion of the protocol, the samples were washed in tap water for 10 minutes. The sections were removed from the cardboard and stained with 1–2 ml of Schiff's reagent (Fischer Scientific) for 20–30 minutes. In order to evaluate the intestinal pathology, the muscularis layer was removed under an inverted microscope, and one section of thickly cut villi was then placed on a slide in a drop of 45% acetic acid (Sigma-Aldrich, UK). An eyepiece micrometre was used to measure 10 villi and 10 crypts per sample (Lawrence *et al.*, 1998a).

2.6. Worm burden count

The small intestine was removed and longitudinally opened to assess worm burden at days 7 and 14 p.i. The samples were placed in a mesh and suspended in 50 ml Hank's solution (Sigma-Aldrich, UK) for 3 hours at 37°C, allowing parasites (worms) to migrate from the small intestine to the solution. After 3 hours, the mesh was removed and the total number of worms was counted under an inverted microscope via scored Petri dish (Thermo Scientific, MA, USA), with approximately 1 cm margins (Lawrence *et al.*, 1998a).

2.7. T-cell proliferation

The mice were sacrificed via euthanasia with CO_2 inhalation. The skin was opened to collect the mesenteric lymph nodes (MLN) from uninfected and infected mice at day 7 p.i. under sterile conditions, as this the suitable time to assess cytokine production as described by (Lawrence *et al.*, 1998a). A single-cell suspension of the MLN was prepared in complete Roswell Park Memorial Institute medium (RPMI 1640, Gibco™) supplemented with 10% heat inactivated foetal bovine serum (FCS) (Biosera. Boussens, France), L-glutamine (200mM), amphotericin B (2.5µg/ml), penicillin/streptomycin (100U/100µg/ml) (Sigma-Aldrich, UK) for 48 hours. The cells were transferred into complete RPMI 1640 and a syringe plunger was used to

force them into suspension. The suspension was then centrifuged at 400g for 5 minutes and re-suspended in 5 ml of media, and the number of cells were counted using a haemocytometer. Microtiter™ 96-well plates were used to incubate $1x10^7$ cells per ml at 37°C in 5% CO_2 for 48 hours in triplicate, supplemented with media only (control), concanavalin A (100 µl; final concentration 5 µg/ml) or *T.* Ag (100 µl; final concentration 5 µg/ml). Following the incubation, 25 µl Alamar Blue (Resazurin) (Bioscience, USA) was added to each well of one set of the plates and incubated for 5 hours (37°C, 5% CO_2). The plates were then read in a fluorescence reader at 550 nm. The remaining plates were stored at –20°C for analysing cytokine producing source.

2.8. ELISA assays

2.8.1. Measurement of parasite specific IgG1 and IgG2a

Antigen specific IgG assays were carried out using a direct ELISA (Lawrence *et al*., 1998). 96 well ELISA plates (Greiner Bio-One, UK) were coated with 2 µg/ml *T.* Ag in 50 µl coating buffer (0.05 M Na_2CO_3/$NaHCO_3$ coating buffer pH 9.6) (Sigma-Aldrich, UK); and incubated overnight at 4°C. After incubation, plated were knocked out and blocked with 200 µl blocking buffer (10% v/v FCS in PBS pH 7.4) per well for one hour at 37°C. Serum samples were added in triplicate and doubling dilutions carried out down the plate (50 µl per well), with a starting dilution of 1/40 in blocking buffer. Plates were incubated for a further hour at 37°C and then washed three times with wash buffer (0.05% v/v Tween-PBS) (Calbiochem, Darmstadt, Germany). HRP-conjugated IgG1 and IgG2a secondary antibodies (Goat anti- mouse IgG1-HRP and Goat anti-mouse IgG2a-HRP) (Southern Biotechnology Associates, Birmingham, AL, USA) were diluted to 1/10,000 in blocking buffer and 50 µl added to each well. After a one hour incubation at 37°C, the plates were washed three times and the substrate tetramethylbenzidine TMB (Calbiochem, Darmstadt, Germany) added at 50 µl per well. The reaction was left to occur at room temperature, when the colour gradient had developed the reaction was stopped by the addition of ELISA stop solution (0.4 M H_2SO_4) (Riedel-de Haen, Hannover, Germany), and the absorbance of samples was measured on a plate reader at 450 nm (Molecular Devices Corporation, California. USA).

2.8.2. Measurement of total IgE

A sandwich ELISA (indirect) was used to measure total serum IgE levels (Lawrence *et al*., 1998). A total of 2 µg/ml of IgE capture antibody (purified anti-mouse IgE, 0.5mg/ml) (BD Pharmingen, Oxford, UK) was coated in coating buffer (0.1 M Na_2CO_3 pH 8.2) and 50 µl/well was added. The plates were then incubated in a refrigerator overnight, after which they were blocked with 200 µl/well of blocking buffer (10% v/v FCS in PBS) for 2 hours in an incubator at 37°C. The plates were then knocked out, the samples were diluted to 1/100 and 50 µl/well was added in triplicate. IgE standard (purified mouse IgE, 0.5mg/ml) (BD Pharmingen, Oxford, UK) was prepared, and 50 µl/well was added to the plates in triplicate for six concentration-doubling dilutions, starting from 8 µg/ml in blocking buffer. The plates were then incubated for 1 hour and 30 minutes at 37°C and washed four times in wash buffer. A 2 µg/ml concentration of 100 µl/well of secondary Biotin anti-mouse Ab (0.5 mg/ml) (Biolegend, Cambridge, UK) was added and incubated at 37°C for 1 hour. The plates were then washed four times in wash buffer, streptavidin HRP was diluted at 1/1,000 (Biolegend, UK) with blocking buffer and 50 µl/well was added, followed by incubation at 37°C for 1 hour. The plates were then washed four times in wash buffer and TMB substrate was added at 50 µl/well to develop colour at room temperature. The reaction was then stopped by adding stop solution (0.4 M H_2SO_4) at 50 µl/well, and the plates were read at 450 nm.

2.9. Measurement of cytokine (IL-4)

IL-4 was measured in sera and supernatant samples by sandwich ELISA, according to the manufacturer's instructions. ELISA plates were coated overnight in the refrigerator with 50 µg/well of IL-4 capture antibody (0.5mg/ml, purified rat anti-mouse IL-4) (BD Pharmingen, Oxford, UK at 2 µg/ml in coating buffer (0.05 M $Na_2CO_3/NaHCO_3$ pH 9.6). Next, the plates were knocked out, and then blocking buffer (10% v/v FCS in PBS pH 7.4) was used to coat the plates with 200 µl/well for 2 hours in an incubator at 37°C. Cytokine (standard IL-4 Recombinant Protein 0.1mg/ml) (eBioscience, Hatfield, UK) was made up, and 50 µl/well was added to the plates in triplicate for six concentration-doubling dilutions, starting at 10 ng/ml. Blocking buffer was used as a blank, which was included in triplicate. A total of 50 µl/well of cultured MLN cells cultured was added to the 96-well plates in triplicate

and re-stimulated with different treatment medium only, Con A or *T.* Ag. The plates were then incubated at 37°C for 2 hours. Following incubation, the plates were washed with wash buffer, after which 50 µl/well of biotinylated secondary antibody (0.5mg/ml, eBioscience, Hatfield, UK) was added at 2 µg/ml and then incubated at 37°C for 1 hour. The plates were washed in wash buffer and 50 µl/well of streptavidin-HRP, diluted at 1/1,000 in blocking buffer, was added, after which they were incubated at 37°C for 1 hour. After incubation, the plates were washed, 50 µl/well of the TMB substrate was added to each well, and the colour developed at room temperature. The reaction was then stopped using stop solution (0.4 M H_2SO_4), and the plates were read on a plate reader at 450 nm.

2.10. Mouse mast cell protease-1 (mMCP-1)

Sandwich ELISA was used to measure mMCP-1 in sera and supernatant samples, and the eBioscience mouse MCPT-1 ELISA kit was used according to the manufacturer's instructions. Mouse mast-cell protease-1 was coated overnight (10x Coating buffer, eBioscience, Hatfield, UK) in 96-well ELISA plates in a refrigerator with 50 µl/well of capture antibody (Anti-mouse MCPT-1 Capture Ab 0.5mg/ml, eBioscience, Hatfield, UK). The plates were knocked out and blocked with 200 µl/well assay diluents (5x ELISA/ELISPOT diluent, eBioscience, Hatfield, UK) and then incubated at 37°C for 1 hour. The standard (1 µg/ml mouse MCPT-1 recombinant protein, eBioscience, Hatfield, UK) was prepared and added at 50 µl/well in triplicate to the plates for six concentration-doubling dilutions, starting at 15 µg/ml. Blocking buffer (10% v/v FCS in PBS pH 7.4) was used as a blank, which was included in triplicate. After dilution of sera to 1/10 in blocking buffer, 50 µl/well was added to the plates in triplicate, following which the plates were incubated at 37°C. for 2 hours. A total of 50 µl/well of detection antibody (0.5 mg/ml anti-mouse MCPT-1 Biotin Ab, eBioscience, Hatfield, UK) was added, after which the plates were washed with wash buffer and incubated at 37°C for 1 hour. Following incubation, the plates were washed with wash buffer. Avidin-HRP (0.5mg/ml avidin HRP 250x, eBioscience, Hatfield, UK) was added at 50 µl/well, and the plates were then incubated at 37°C for 1 hour. Following completion of the protocol, the plates were washed, 50 µl/well of the TMB substrate (TMB Substrate Solution) (eBioscience, Hatfield, UK) was added and the colour was developed at room

temperature. The reaction was then stopped using stop solution (0.4 M H_2SO_4), and the plates were read on a plate reader at 450 nm.

2.11. Enumeration of mucosal mast cells

Approximately 4-5 cm of gut sample was taken from the jejunum samples and opened to count the mucosal mast cells, as described previously (Scales *et al*., 2007). The samples were placed around a cocktail stick and then transferred into Carnoy's fixative. These samples were kept in fixative for histological preparation, and then removed from the cocktail stick and placed into labelled histocassettes (VWR, USA) before being transferred to 70% v/v aqueous EtOH and placed in a ThermoShandon Citadel 1000 tissue processor overnight. The tissue was then embedded in wax blocks and a 5 nm-thick section was cut on a microtome to make three slides. These slides were then allowed to dry for 2-3 hours at 40°C and were then used to stain mast cells by rehydrating through 100% Histoclear (Histoclear II National Diagnostics, Hessle, UK) for 5 minutes (twice), 50/50 Histoclear/ EtOH for 5 minutes and 100% EtOH (twice) for 5 minutes, followed by 90%, 70% and 50% v/v/ aqueous EtOH for 2 minutes each and, finally, distilled water for 2 minutes. The slides were then stained overnight in 0.5% Toluidine blue (Sigma-Aldrich, UK) in 0.5 M HCL for visualisation of mast cells, after which they were rinsed in 0.7 M HCL and then stained for 2 minutes in 0.5% Safranin "O" (EMP Millipore Corp, MA, USA) in 0.125 M (HCL). These slides were then dehydrated and mounted with DPX, and mucosal mast cells were counted in 10 villus-crypt units.

2.12. Isolation of peritoneal mast cells

Peritoneal mast cells (PCMC) were removed by peritoneal lavage with complete Dulbecco's Modified Eagle Medium (DMEM), prepared using DMEM from Sigma (Poole, UK), supplemented with heat-inactivated FBS (10%), L-glutamine (200 mM), penicillin/streptomycin (100 U/100 μg/ml) and Amphotericin B (2.5 μg/ml) from Sigma-Aldrich (Irvine, UK), as well as β-mercaptoethanol (0.1 M) from Gibco™ (Irvine, UK). Mast cell culture medium was prepared by adding IL-3 (1 ng/ml), IL-9 (5 ng/ml), both from Pepro Tech Inc. (London, UK), and stem cell factor (50 ng/ml) from Miltenyi Biotech (Woking, UK) to complete DMEM. Isolates were centrifuged at 500g for 5 minutes at 20°C. The pellets were re-suspended in 10

ml culture medium and transferred to tissue culture flasks (25cm^2). The PCMC were cultured for 7–10 days at 37°C.

2.13. Generation of bone marrow mast cells

Bone marrow mast cells (BMMC) were generated as previously described (Galli *et al.,* 2005). Bone marrow was extracted from femurs and tibias using complete DMEM, and extracts were centrifuged at 500g for 5 minutes at 20°C. The pellets were re-suspended in 10 ml culture medium and transferred to tissue culture flasks. Cells were cultured at 37°C for 23–30 days and passaged weekly by centrifuging at 500g for 5 minutes at 20°C and re-suspending the pellets in 10 ml cell culture medium.

2.14. Mast cell growth and maturity

The samples were mixed 1:1 with toluidine blue and the cells were counted using a haemocytometer. 40 ml of toluidine blue stock solution was diluted in 360 ml of 1% sodium chloride solution (Chieco *et al.*, 1993). Mast cell maturity was measured by smearing samples onto glass slides. The slides were fixed in 75% ethanol for 3 minutes, stained in 10% toluidine blue/1% NaCL for 90 minutes and counted under a microscope.

2.15. Detection of β-hexosaminidase secretion

β-hexosaminidase was detected as previously described (Galli *et al.,*2005). 10µl of supernatant samples were added to an ELISA plate along with 50µl of Triton-X/Tyrode's (0.5%) to lyse the cells. Then 10µl of the lysate was added to a fresh ELISA plate along with 50µl of Poly-N- acetylglucosamine (p-NAG) (1.3 mg/ml) in p-NAG buffer (Na_2HPO_4, 29 mg/ml, citric acid 84 mg/ml, pH 4.5). was added to supernatant samples, and the plates were incubated at 37°C for 24 hours. After the incubation, 150 µl of Glycine (0.2M, pH 10.7) was added to each well, and the absorbance was read at a wavelength of 405nm. The percentage degranulation of the cells was calculated as follows:

$$\%\, degraulation = \left(\frac{ODsupernatant}{ODsupernatant + ODpellet}\right) x\ 100$$

2.16. Statistical Analysis

Results are represented as the mean ± SEM and analysed using GraphPad Prism Version 6.00 for Mac, GraphPad Software, La Jolla California USA, www.graphpad.com. Non-parametric data from *in vitro* or *in vivo* studies were analysed using a Mann Whitney U test to compare two treatments, or a Kruskal Wallis test for 3 or more treatments followed by a Dunn's ad hoc test to identify whether there was a significant difference between groups. Results ($P < 0.05$) were considered to be statistically significant.

CHAPTER 3. HELMINTH INFECTIONS IN C57BL/6-KIT W^{SH}/W^{SH} MICE

3.1. Introduction

The host immune system stimulates multiple responses against pathogens, including innate and adaptive immune responses. The defence strategy employed depends on specific infections by different pathogens; among others, the gastrointestinal (GI) tract is infected by helminths (Vallance *et al.*, 1999). Immune responses to helminth parasites require various effector mechanisms, and one such essential mechanism is the induction of T-helper (Th)2 cells. The adaptive immune response can be amplified by mast cells (MCs), which function as modulators of the Th2 cell response (Hershko and Rivera, 2010). When these cells are induced during infection by helminth species, they stimulate the production of cytokines, interleukin (IL)-4, IL-13, and IL-9, chemokines, and inflammatory mediators, such as histamine, which can amplify and maintain the immune response (Crivellato *et al.*, 2004).

It has previously been demonstrated that mastocytosis plays a crucial role in the expulsion of *T. spiralis* from the gut (Lawrence *et al.*, 2004), and the contribution of MCs to the biological responses to pathogens has been investigated *in vivo* using mouse models (Reber *et al.*, 2012b). Mice carrying mutations in the white spotting (W) locus, such as $Kit^{W/W\text{-}v}$ (W/W-v) and C57BL/6-$Kit^{W\text{-}sh/W\text{-}sh}$ (Wsh/Wsh) mice, have been used to identify MC functions (Becker *et al.*, 2011). These models result in MC deficiency, as they show reduced *c-Kit* tyrosine kinase-dependent signalling and abnormalities of other phenotypes (Grimbaldeston *et al.*, 2005). Mast/stem cell growth factor receptor (SCFR), also known as proto-oncogene c-Kit or tyrosine-protein kinase Kit or CD117, is a receptor tyrosine kinase protein that in humans is encoded by the KIT gene. Multiple transcript variants encoding different isoforms have been found for this gene (Andre *et al.*, 1997).

Mutations of the W locus in mice have been investigated in detail due to their marked effects on hematopoiesis, melanogenesis, and spermatogenesis (Becker *et al.*, 2011). This is due to stem cell factor signalling and Kit playing an essential role in MC function, evolution, gametogenesis, haematopoiesis, and melanogenesis. Additionally, Kit transcripts are expressed in embryonic stem cells of humans and

mice. Stem cell factor (SCF) occurs as both a soluble and a membrane-bound glycoprotein. However, Kit is a glycoprotein receptor protein-tyrosine kinase (Roskoski, 2005). Ultimately, it has been shown that the mutated alleles of the gene encoding c-Kit (CD117) are responsible for the observed phenotypes (Geissler *et al.*, 1988). A non-functional soluble receptor has been shown to be encoded by Kit^{W}(Hayashi *et al.*, 1991), and the $Kit^{W\text{-}v}$ allele shows a mutation in the kinase domain of c-Kit, resulting in reduced activity (Nocka *et al.*, 1990).

W/W-v mice have a mutation in one allele of the W locus, (W^{V}). This point mutation results in the production of a defective c-Kit protein which is not expressed on the cell's surface. Expression of a functional c-Kit protein is important for cell growth and survival, and its activation plays a significant role in haematopoiesis, fertility, and intestinal movement, and mice with this deficiency have microcytic anaemia and are infertile (Lennartsson and Rönnstrand, 2012). Another c-Kit mutant model is that of the Wsh/Wsh mouse (Lyon and Glenister, 1982). This model has an inversion mutation in the W locus, which results in the dysfunctional regulation of *Kit* transcription (Nigrovic *et al.,* 2008). One major advantage of this model over the earlier W/W-v model is that Wsh/Wsh mice are fully fertile and not anaemic, and can, therefore, be crossed with other strains.

The roles of the mast cell in helminth immunity have been reported using the gastrointestinal nematode parasite *T. spiralis* (Anthony *et al.*, 2007). It has been demonstrated that intestinal helminthiasis is commonly associated with intestinal mastocytosis (Miller, 1984). Consequently, mucosal mast cells (MMC) have possibility regular or effector against parasites in the local defence mechanisms of the gut (Bienenstock *et al.*, 1982, Woodbury *et al.*, 1984). In addition, resistance to intestinal nematode infection has been shown to be mediated via the action of $CD4^{+}$ T cell-derived cytokines (Grencis *et al.*, 1991, Finkelman *et al.*, 1991).

Following such infections, a variety of immunological changes that are controlled by Th2 cells have been described, including hyperplasia of the mast cell, eosinophilia, and increased IgE serum levels. Although the mechanisms for nematode parasite expulsion remain to be fully determined, these mechanisms seem to differ between species of parasite (Donaldson *et al.*, 1996). Additionally, elimination of nematode

parasites is thought to be mediated by mast cells, and these cells are considered to be pivotal effector cells. The role of mast cells has been established in infections with *Nippostrongylus brasiliensis*, *Strongyloides venezuelensis* and *T. spiralis* (Befus and Bienenstock, 1979, Abe and Nawa, 1988, Donaldson *et al.*, 1996, Khan *et al.*, 1993, Lantz *et al.*, 1998).

However, it has been suggested that mast cells are not necessary for the expulsion of worms, even in some of these species, and seems insignificant in responses against other species such as *Trichuris muris* (Betts and Else, 1999).
The hypothesis of leak lesion, proposed by Murray *et al*. (1971), suggests that mast cells release amines which induce mucosal permeability changes. These changes encourage the release of macromolecules and an anti-worm antibody into the lumen of the parasitized gut. However, it has been claimed that mast cells accumulate in the small intestine only after *N. brasiliensis* had been expelled (Keller, 1971). In addition, it has been proposed that detrimental mechanical effects or toxic damage of the host intestinal tissue caused by parasites might lead to the increased permeability (Yukifumi, 1979). Although Kojima *et al*. (1980), Crowle and Reed (1981) and Mitchell *et al.* (1983) demonstrated that, in the late stages of *N. brasiliensis* infection in W/W-v mice, large worm burden and prolonged faecal egg are relatively production. Additionally, it has been demonstrated that bone marrow or spleen cells of control mice injected into W/W-v mice had no effect on *N. brasiliensis* expulsion (Crowle, 1983). However, Oku *et al*. (1984) suggested that haematopoietic cells in W/W-v mice are involved in *T. spiralis* expulsion. Moreover, it has been reported that transfer of bone marrow from wild type (WT) mice into W/W-v mice accelerated *T. spiralis* expulsion (Ha *et al.*, 1983).

Furthermore, it has been observed that infection of W/W-v mice with *T. spiralis* induced a defective intestinal mastocytosis and delayed expulsion of the parasite (Alizadeh and Murrell, 1984). In addition, an initial report has suggested that regulation response of the mast cell and the parasite expulsion was dependent upon the activity of stem cell factor (SCF) during infection (Grencis *et al.*, 1993). Donaldson *et al.* (1996) extended the preliminary observation by blocking SCF activity in infected mice *in vivo* by administration of anti-SCF or anti-SCF receptor *(c-kit)* mAb, and demonstrated that SCF was involved in the generation of helminth-

induced mastocytosis, and that this mastocytosis participates in resistance to infection (Donaldson *et al.*, 1996). Moreover, it has been reported that there is no detectable effect on the antigen-induced production of Th2 cytokine or the response of parasite-specific antibody, suggesting an essential role for the SCF/c-kit system in the host protective response to helminth infection (Donaldson *et al.*, 1996).

MMC is derived from bone marrow cells (Kitamura *et al.*, 1977) and depend on the mast cell-growth factor, SCF for their growth/proliferation, which is produced by T lymphocytes (Ihle and Weinstein, 1986). It has been reported that worm expulsion and the simultaneous response of MMC are both dependent on T cells during intestinal helminthiases, such as *N. brasiliensis* or *T. spiralis* infection in rodents. These can be accelerated by adoptive transfer of immune T cells (Nawa and Miller, 1979, Alizadeh and Wakelin, 1981). On the other hand, it has been demonstrated that athymic nude mice are incompetent in the expulsion of intestinal parasites nor are they able to develop a MMC response. Defects in nu/nu mice are restored by grafting of thymus or thymocyte (Ruitenberg and Elgersma, 1976, Olson and Levy, 1976). Abe and Nawa (1988) showed that expulsion of worms and simultaneous responses of MMC in KSN (nu/nu) mice infected with *S. ratti* is dependent on T cells (Abe and Nawa, 1988). Additionally, it has been demonstrated that the defects in the expulsion of worm and responses of MMC of *S.ratti*- infected nu/nu mice are almost completely restored by repeated injection with semi-purified IL-3 (Abe and Nawa, 1988). Infection with *S. ratti* showed that W/W-v mice were more susceptible and slower to expel the worm. However, the defective protective capacity of W/W-v mice was restored by bone marrow grafting (Nawa *et al.*, 1985).

Bone marrow restoration of mast cell–deficient c-kit mutant mice (Ha *et al.*, 1983), as well as mice treated with antibodies against c-kit (Donaldson *et al.*, 1996) and stem cell factor (Faulkner *et al.*, 1997) have been used as evidence for the involvement of mast cells in the rejection of nematode parasites. The functions of MMC-specific β-chymases have been studied in transgenic mMCP-1$^{-/-}$ mice (Knight *et al.*, 2000). It has been demonstrated that highly soluble MMC β-chymase is involved in the expulsion of nematode parasites from the intestine (Knight *et al.*, 2000). In addition, Knight *et al.* (2000) reported that mMCP-1 is necessary for the

effective expulsion of *T. spiralis* (initial and challenge infections), but not for the expulsion of *N. brasiliensis.* It has been reported that expulsion of *N. brasiliensis* has failed in infected W/W-v mice or the use of antibodies against IL-3 or IL-4 (Madden *et al.*, 1991, Crowle and Reed, 1981). On the other hand, although mucosal mastocytosis is generated in STAT6 knockout mice they fail to expel *N. brasiliensis* (Urban *et al.*, 1998).

Mast cells have been shown to be an influential source of pro-inflammatory cytokines, including tumour necrosis factor (TNF-α) (Bradding *et al.*, 1993, Burd *et al.*, 1989, Brown *et al.*, 1987, Gordon and Galli, 1990a). It has been shown that expulsion of *T. spiralis* in TNF-R1-deficient mice occurred with minimal pathology in the obvious absence of an intestinal mastocytosis and a relatively decreased response of IgG1 and IgG2b (Garside *et al.*, 2000). As discussed, it is believed that MCs play a crucial role in the expulsion of, and protection against, *T. spiralis* infection, and this has been previously shown in MC-deficient W/W-v mice (Ha *et al.*, 1983, Lawrence *et al.*, 2004). Mast cell-deficient W/W-v mice and W/W-v mice made mast cell sufficient by bone marrow grafts (W/W-v-BM) have been used to assess the function of mast cells in the expulsion of adult *T. spiralis* from the intestines (Ha *et al.*, 1983).

It has been demonstrated that adult *T. spiralis* are expelled by W/W-v-BM mice more rapidly than by mast cell-deficient W/W-v mice, and that W/W-v mice are normal or enhanced in their ability to produce antigen-specific immunoglobulins, such as M, G, and E antibody responses (Ha *et al.*, 1983). It has been reported that W/W-v mice unsuccessfully generated a mucosal mastocytosis and that *T. spiralis* expulsion from these mice was significantly delayed compared with that of controls after infected with 400 *T. spiralis* larvae (Lawrence *et al.*, 2004). Additionally, W/W-v mice showed significantly lower levels of antigen-specific IgG1 and total IgE responses than wild-type mice. However, antigen-specific IgG2a levels were significantly increased in W/W-v mice (Lawrence *et al.*, 2004).

As mentioned previously, it has been shown that MCs play a significant role in the innate immunity and host defence. It has been well documented that W/W-v MC-

deficient mice have an impaired ability to expel *T. spiralis* worms (Ierna *et al.*, 2008). However, the mutation means that W/W-v mice have additional abnormalities, including reduced numbers of basophils, neutrophils, and intestinal pacemaker cells [the interstitial cells of Cajal (ICCs)]. Therefore, the aim of the present study was to examine whether these observations in W/W-v mice could be replicated in other MC-deficient models, such as the C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ (Wsh/Wsh) mouse. Thus, the present study was conducted on the MC-deficient Wsh/Wsh mouse strain. Wsh/Wsh mice are profoundly MC-deficient and have fewer *c-Kit*-associated abnormalities have been observed in W/W-v mice (Grimbaldeston *et al.*, 2005).

A comparison was made of the immunological responses on a strain of MC-deficient mice, namely Wsh/Wsh mice, to investigate the ability of Wsh/Wsh mice to expel *T. spiralis* using multiple levels of infection. The intestinal pathology, antigen-specific IgE, IgG and IgG2a antibody responses, mucosal mastocytosis, and levels of cytokines IL-4 produced by the mesenteric lymph node (MLN) cells *in vitro* were assessed to provide information about the relative activities of Th2 T-helper (Th2) lymphocyte subsets.

3.2. Results

3.2.1. Worm burden in Wsh/Wsh mice is higher than in controls

The function of MCs in the expulsion of *T. spiralis* was investigated in MC-deficient W/W-v mice, and it was shown that the expulsion of worms was delayed and Th2 immune responses were impaired. Therefore, we examined whether these observations in c-Kit models could be replicated in other MC-deficient mice models, such as that of the Wsh/Wsh mouse model, which has fewer phenotypic abnormalities compared to the W/W-v model.

In the present study, initial experiments were carried out to determine the ability of mast cell deficient mice to expel worms and if this was dependent on the infective dose. To this end wild-type C57BL/6 and MC-deficient Wsh/Wsh mice were infected with either 160 or 400 freshly isolated *T. spiralis* larvae, and worm burdens assessed at 7 and 14 days post infection (p.i.).

On day 7 p.i., a high level of infection was established in the group given 400 larvae, and a low level of infection was established in the mice given 160 larvae (Figure. 3.1). In addition, at a high level of infection, no significant difference in the establishment of *T. spiralis* in the gut was observed between the mouse strains at day 7 p.i. ($p=0.99$). However, in the lower infection group at day 7 p.i., we detected a significant increase in the establishment of *T. spiralis* in the gut of Wsh/Wsh compared to wild-type C57BL/6 ($p=0.017$).

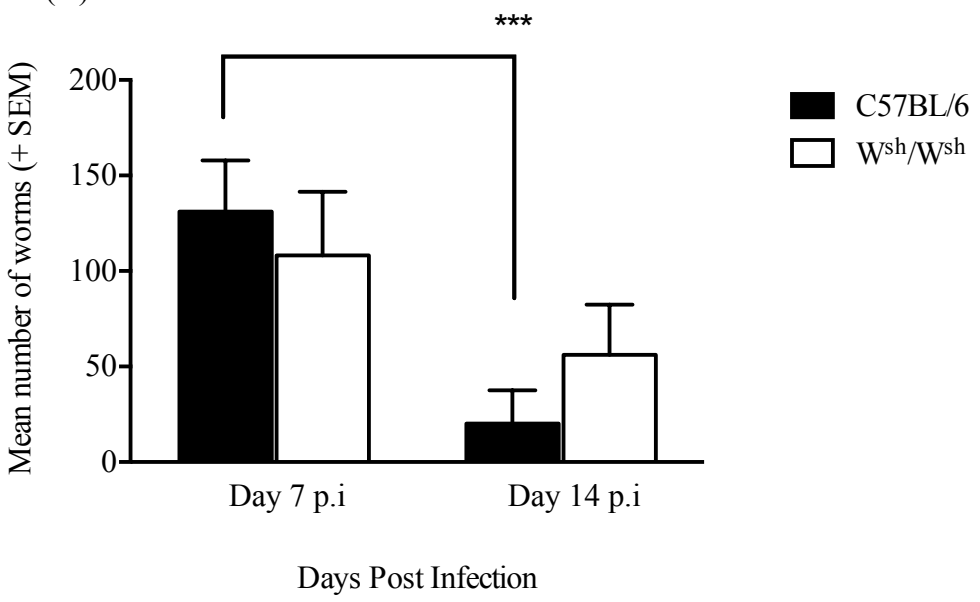
By day 14 p.i, the majority of parasites in C57BL/6 mice had been expelled whereas W-sh/W-sh still harboured worms. In the high level of infection condition, there was a significant reduction in the number of worms in wild-type mice at day 14 p.i. compared to day 7 p.i. ($p=0.0024$). In addition, no significant decreased in the number of worms was observed in Wsh/Wsh mice at day 14 p.i. compared to infected mice at day 7 p.i. ($p=0.586$). In addition, in the lower infection groups resulted in a significantly decreased number of worms in C57BL/6 mice at day 14 p.i. compared to infected mice at day 7 p.i. ($p=0.0001$). In the lower infection groups resulted in a significantly decreased number of worms in Wsh/Wsh mice at day 14 p.i. compared to infected mice at day 7 p.i. ($p=0.0012$). Mice infected with low-level

infection showed statistically significant differences in worm numbers between each strain at day 14 p.i.

These experiments were repeated five separate occasions with 5-6 animals per group. The numbers of worms and the values of other measurements were not significantly different between the repeats, therefore results from just one of the experiments is shown.

Overall, the result obtained from mast cell deficient Wsh/Wsh mice with low-level infection resulted in a significant worm expulsion compared to high-level infection. This suggests that the delay in expulsion of *T. spiralis* from Wsh/Wsh was dose dependent.

High dose (A)



Low dose (B)

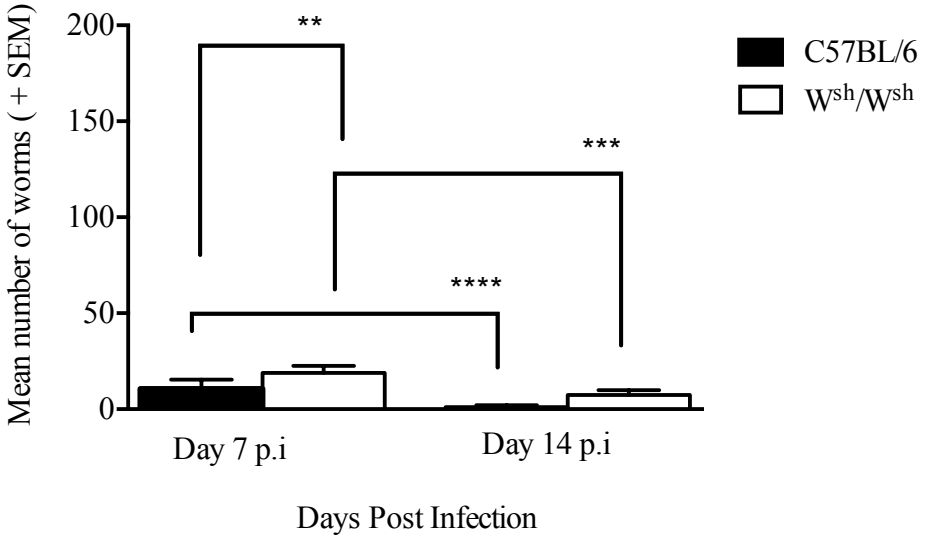


Figure 3.1 Worm burden in Wsh/Wsh mice. Mice were orally infected with 400 (A) and 160 (B) *Trichinella spiralis* larvae, and worm burdens evaluated at 7 and 14 days post infection (p.i.). Worms were removed from small intestine samples and counted under an inverted microscope. The data are expressed as mean + S.E.M. (n=5-6). This experiment was repeated five times and similar results were obtained. Data were analysed using a Kruskal Wallis test followed by a Dunn's ad hoc test to identify differences between treatments. ** $P<0.01$ between Wsh/Wsh and C57BL/6 mice day 7 p.i.; *** $P<0.001$ between C57BL/6 mice day 14 and day 7 p.i.; *** $P<0.001$ between Wsh/Wsh mice day 14 and day 7 p.i.; **** $P<0.0001$ between C57BL/6 mice day 14 and day 7 p.i.

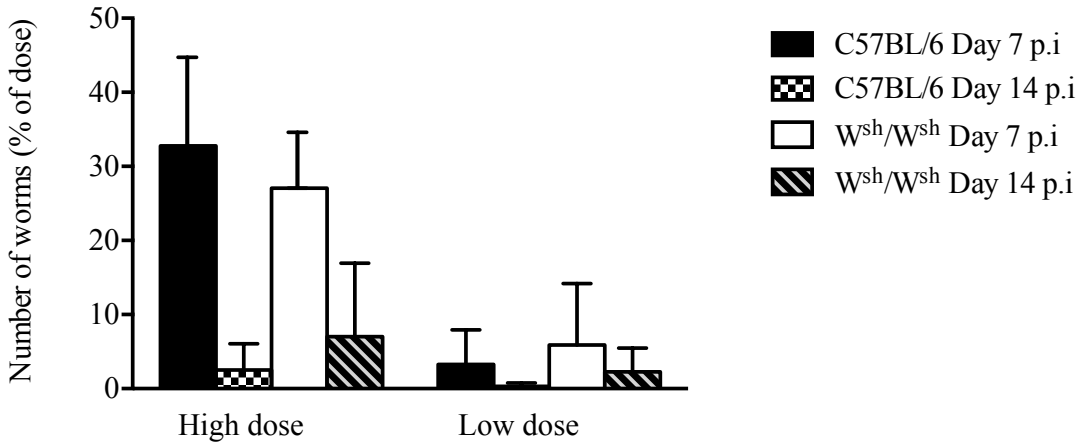


Figure 3.2 Worm burden in Wsh/Wsh mice. Mice were orally infected with 400 (High dose) and 160 (Low dose) *Trichinella spiralis* larvae, and worm burdens evaluated at 7 and 14 days post infection (p.i.). Worms were removed from small intestine samples and counted under an inverted microscope. The data are expressed as the mean percentage of dose + S.E.M (n=5-6). This experiment was repeated five times and similar results were obtained. Analysis of the data using a Kruskal Wallis test followed by a Dunn's ad hoc test.

3.2.2. The development of enteropathy in MC-deficient models is not different to that in C57BL/6 mice

Infection with *T. spiralis* is generally associated with intestinal inflammation, possibly due to the release of MC mediators (Knight *et al.*, 2000). To assess the development of intestinal pathology, the small intestine was weighed, and the intestinal architecture was examined.

We found that a high level of infection lead to the developed of enteropathy in the group given 400 larvae, which was indicated by a statistically significant increase in the weight of the small intestine in wild-type mice at day 14 p.i. compared to uninfected mice ($p=0.0104$). Similarly, Wsh/Wsh mice showed an increase in gut weight at day 14 p.i. following infection with *T. spiralis*, and this increase was significant compared to uninfected mice of the same strain ($p=0.0057$). Moreover, intestinal oedema was also evident in mice given 160 larvae. However, no statistical significance was observed between the different strains of mice (Figure. 3.3).

The development of enteropathy was determined by measuring villus lengths and crypt depth (Figure. 3.4). Villous atrophy (villus length) was not significant in either

strain. The C57BL/6 mice had shorter villi than the Wsh/Wsh mice at day 14 p.i., but this difference was not significant ($p > 0.99$).

The crypt hyperplasia (crypt length) of the two strains of mice were not significantly different at day 14 p.i in either uninfected or infected mice. The weight in the higher infection group at day 7 was higher compared with the uninfected mice. However, the lower infection group had a similar gut size compared with uninfected mice. No significant difference was seen in either the lower or higher infection groups when comparing day 7 or day 14 models against each other. These results demonstrate that the development of villous atrophy and crypt hyperplasia was not significantly different between mouse strains at either day 7 or day 14 p.i. This might be due to the resistance of these mice to *T. spiralis* infection.

Overall, similar responses were observed in wild-type mice and mast cell deficiency Wsh/Wsh mice given equal doses, and pathological responses occurred in both strains. Wsh/Wsh mice showed an increase in gut weight following infection with *T. spiralis.* These results show that an overall increase in the size of the small intestine causes a subsequent *T. spiralis* infection. Lengths of both villi and crypts were similar in both the lower and higher infection groups in which the dose of parasite did not make any differences in the development of enteropathy between wild-type mice and mast cell deficiency Wsh/Wsh.

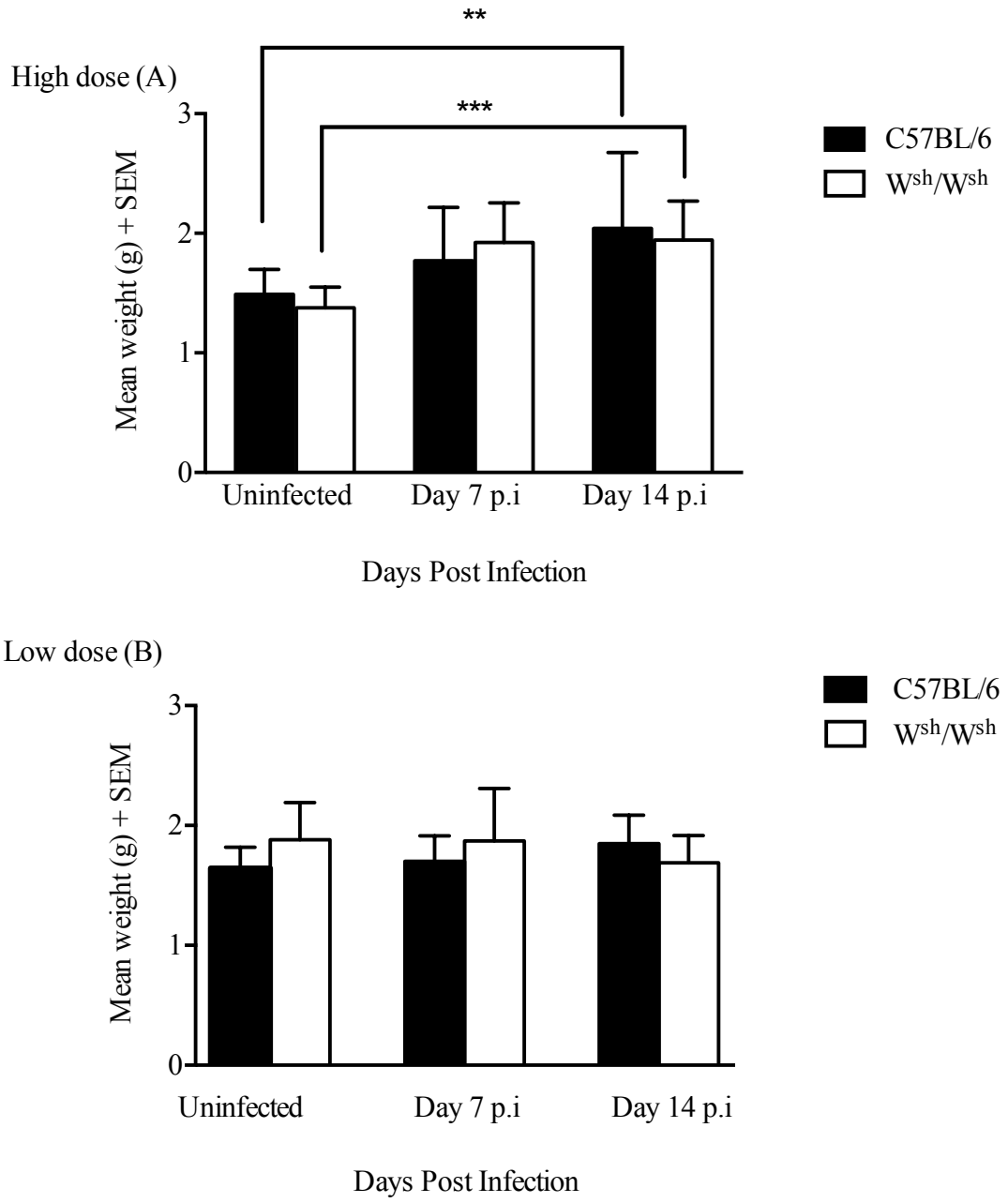


Figure 3.3 Development of intestinal oedema in the Wsh/Wsh mice. The gut was weighed in all mice orally infected with 400 (A) and 160 (B) *Trichinella spiralis* larvae, as well as all uninfected mice, at 7 and 14 days post infection. The data presented are mean + S.E.M. (n=5–6) and the experiment repeated five times with similar results. Data were analysed by Kruskal Wallis test followed by a Dunn's ad hoc test to identify differences between treatments. ** $P<0.01$ between uninfected C57BL/6 mice and infected mice at day 14 p.i; *** $P<0.001$ between uninfected Wsh/Wsh mice and infected mice at day 14 p.i.

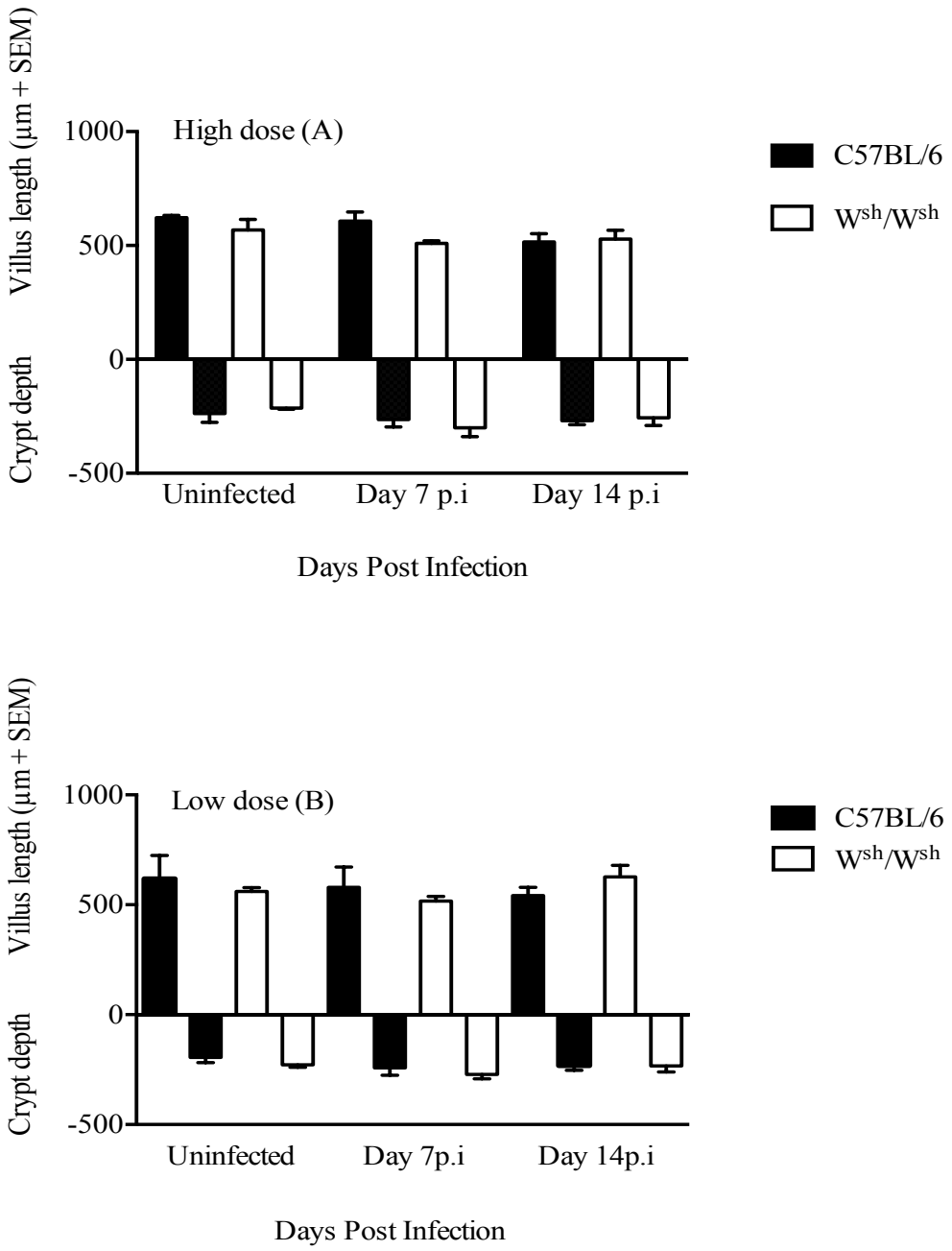


Figure 3.4 The intestinal architecture of Wsh/Wsh mice. Gut sections were fixed in Clark's fixative and stained with Schiff's reagent. The tissues were microdissected, and ten villi and crypts per mouse were measured. Villous atrophy and crypt hyperplasia were measured in uninfected mice and in those infected orally with 400 (A) and 160 (B) *Trichinella spiralis* larvae, and the mice were sacrificed at days 7 and 14 post infection. The data are presented as mean + S.E.M. (n=5-6) Data from one of 5 experiments with similar results are shown. Analysis of the data using a Kruskal Wallis test followed by a Dunn's ad hoc test showed there was no significant difference between treatments.

3.2.3. The proliferative capacity of mesenteric lymph node cells from MC-deficient mice is not higher than that of controls

Infection with *T. spiralis* is associated with stimulation of Th2 immune responses (Turner *et al.*, 2003). The MLN cells were cultured in medium only, Concanavalin A (Con A), or *T. spiralis* antigen (*T.* Ag).

We established the proliferative capacity of MLN cells in the mice at the high (400 larvae) and low (160) larvae levels of infection 7 days post infection since we have previously shown that this time point is the peak for MLN cell proliferation and cytokine production (Lawrence *et al.*, 1998a)(Figure. 3.5). Overall, the proliferative capacity of MLN cells in both higher and lower level infections in response to either Con A or *T.* Ag did not appear to be affected by a deficient mast cell response. The dose of parasite did not show any differences in the proliferative capacity of both wild-type and Wsh/Wsh mice.

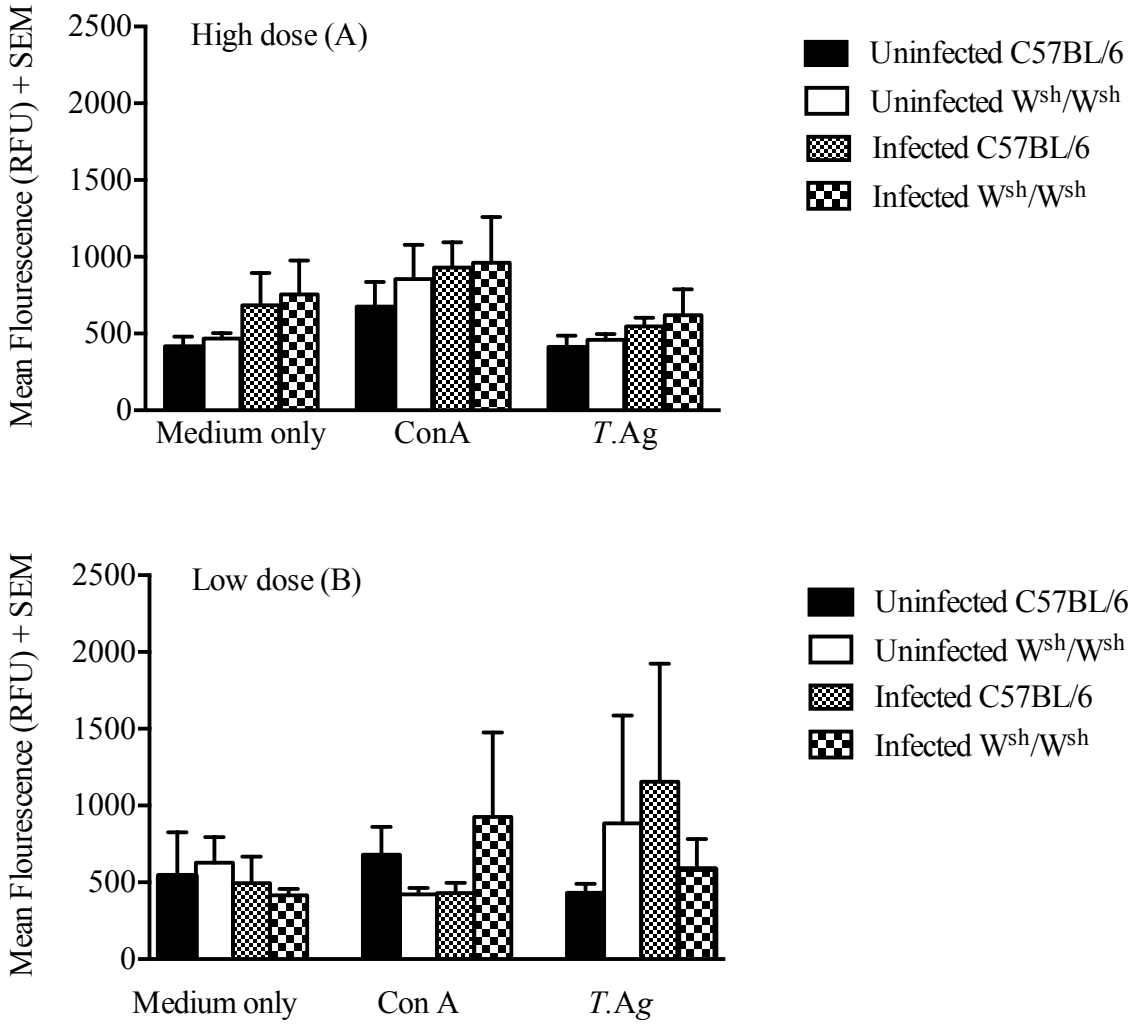


Figure 3.5 The proliferative responses of Wsh/Wsh mice. T cell proliferation was assessed after mesenteric lymph node cells were cultured in complete RPMI 1640 from uninfected mice and those infected orally with 400 (A) and 160 (B) *Trichinella spiralis* larvae for 7 days. MLN were cultured in medium only, Con A (10 μg/ml) or *T*. Ag (10 μg/ml) for 48 h at 37°C. The proliferative capacity was evaluated using Alamar Blue (Resazurin). The data are expressed as mean + S.E.M. (n=5-6). Data from one of 5 experiments with similar results are shown.

3.2.4. Antibody responses in Wsh/Wsh MC-deficient mice are decreased in low dose

Following infection with *T. spiralis,* Th2 responses are elicited to induce antibody responses for IgG1 and IgE (Wakelin *et al.*, 1994). It has been suggested that MC stimulation is required for sufficient activation of Th2 responses, and MC-deficient W/W-v mice have been investigated to assess the immune responses to *T. spiralis* (Lawrence *et al.*, 2004). W/W-v mice infected with *T. spiralis* had increased IgG2a

levels, demonstrating that W/W-v mice prompt immune responses toward Th type 1 (Th1). However, the levels of antigen-specific IgG1 and total IgE were significantly lower in W/W-v than in wild-type mice (Lawrence *et al.*, 2004). The development of Th1/Th2 immune responses to *T. spiralis* was assessed, and total serum IgE and antigen-specific IgG1and IgG2a levels were measured in uninfected and infected C57BL/6 and Wsh/Wsh mice at all time points.

We detected a difference in antibody production between the two levels of infection. Interestingly, parasite-specific IgG1 levels measured in serum from mice given a low level of infection or a high-level infection were similar (Figure. 3.6). This may indicate that low-level infected mice produce low levels of Th2 cytokines but this is sufficient to induce production of IgE.

Following infection with high levels of *T. spiralis* larvae, we detected no statistically significant difference in antigen-specific IgG1 levels between infected wild-type C57BL/6 mice at day 7 p.i and uninfected ($p>0.99$). In contrast, there was a significant increase in wild-type C57BL/6 mice at day 14 p.i. compared to uninfected mice ($p=0.0001$). Additionally, statistically significant differences between C57BL/6 mice at days 7 and 14 p.i were recorded ($p=0.0001$). A significant increase in antigen-specific IgG1 levels was shown in infected Wsh/Wsh mice at day 14 p.i. compared to uninfected mice ($p=0.0021$). However, there was no significant difference in IgG1 levels between infected mice of both strains at day 14 p.i.

Following infection with low levels of *T. spiralis* larvae, we observed that the antigen-specific IgG1 with low level infection were not significantly different to those from mice with high levels of infection, however this is likely due to the high variability of the data. There were no significant differences the antigen-specific IgG1 between either strain or between uninfected and mice infected with a low parasite dose. Similar to uninfected control mice, only negligible levels of IgG2a were produced by mice given high and low levels of infection (Figure. 3.7); no significant differences were detected. And no significant increase between infected Wsh/Wsh mice occurred at days 7 and 14 p.i. In addition, there was no significant difference between infected mice of both strains at day 14 p.i.

Although low-level infected mice produced less IgE than high-level infected mice, we detected no significant differences in IgE levels between mouse strains at days 7 and 14 p.i. ($p > 0.05$) (Figure. 3.8). However, for the high level of infection, we noted a significant increase in total IgE levels in infected wild-type C57BL/6 mice at day 14 p.i compared to uninfected mice ($p=0.0043$). In addition, total IgE levels in C57BL/6 mice were increased significantly at day 14 p.i. compared to day 7 p.i. ($p=0.0006$). In Wsh/Wsh mice, IgE levels were significantly increased in mice at day 14 p.i. compared to uninfected mice ($p=0.048$). Total IgE levels in Wsh/Wsh mice were increased significantly at day 14 p.i compared to day 7 p.i. ($p=0.0001$). In addition, in contrast with W/W-v mice, no significant differences in IgE levels were observed in Wsh/Wsh mice compared to C57BL/6 mice.

For the low-level infected mice, total IgE levels were significantly higher in wild-type C57BL/6 mice at day 14 p.i compared to mice at both uninfected and 7 days p.i ($p=0.015$ and 0.0043, respectively). A significant increase in total IgE levels in infected Wsh/Wsh mice at day 14 p.i compared to uninfected mice ($p=0.001$). Additionally, total IgE levels were significantly increased in Wsh/Wsh mice at 14 days p.i. compared to day 7 p.i ($p=0.0012$). In Wsh/Wsh mice, total IgE levels were significantly increased in the high-level infected mice at day 7 p.i. compared to the low-level infected mice at day 7 p.i. ($p=0.0018$) (Figure. 3.9).

Overall, a rapid dominant Th2 response was not observed in those mice infected with a low-level infection, which might account for why these mice are slow to expel the parasite. However, similar responses were observed in wild-type mice and mast cell deficiency Wsh/Wsh mice which showed that the dose of parasite did not make any differences in antigen-specific IgG1 levels and in the total IgE levels. The elevated antigen-specific IgG1 levels observed in Wsh/Wsh mice suggest that this model did not have impaired Th2 immune responses. This contrasts with the results of previous studies in W/W-v mice, which found that the production of Th2 antibodies was reduced in comparison to wild-type mice (Lawrence *et al.*, 2004).

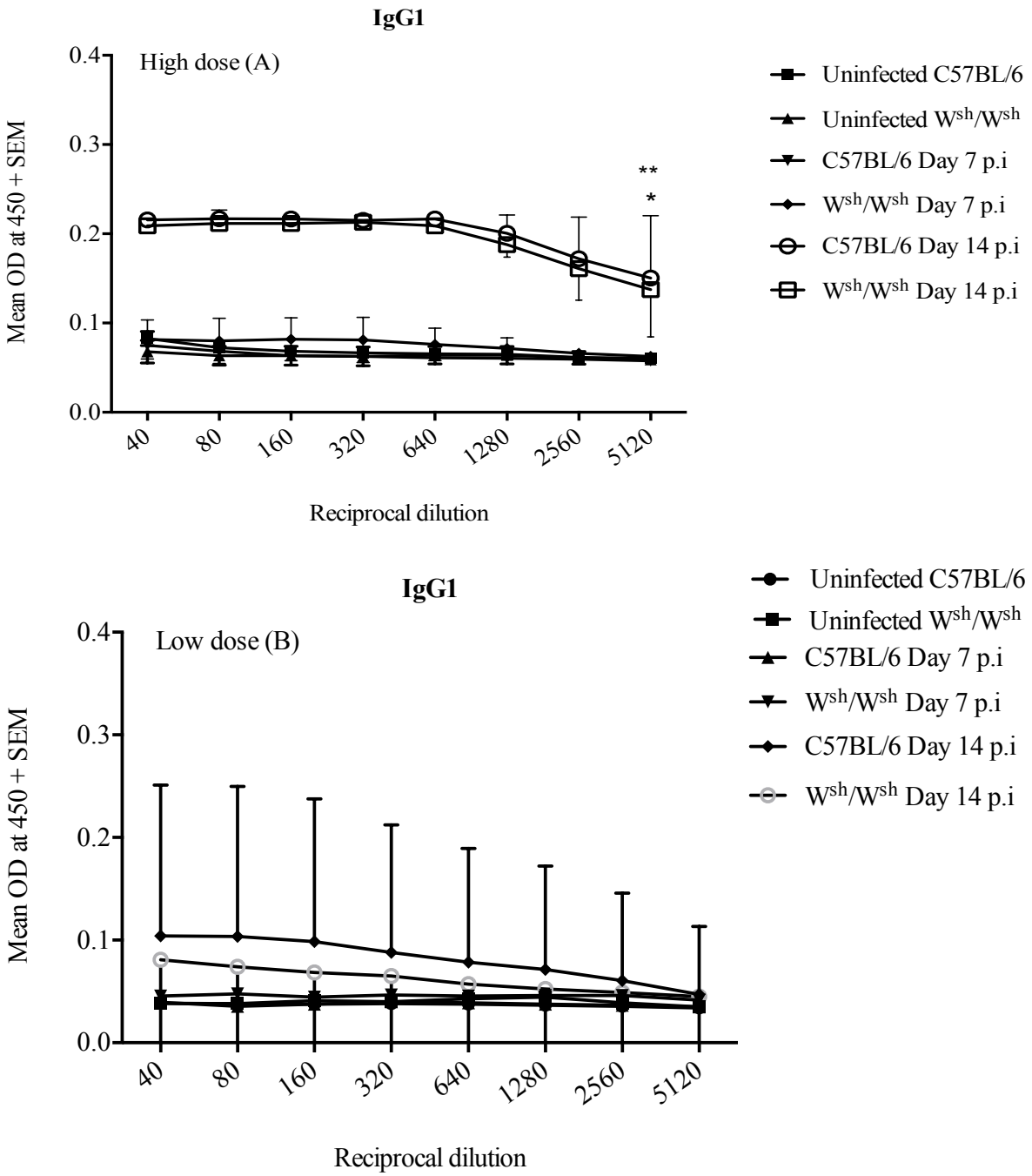


Figure 3.6 IgG responses in Wsh/Wsh mice. Serum was obtained from all mice. Wild-type C57BL/6 mice and Wsh/Wsh mice were infected orally with 400 (A) and 160 (B) *T. spiralis* larvae and sacrificed at day 7 or day 14 p.i. A directed enzyme-linked immunosorbent assay was used to measure the IgG levels. The data are expressed as mean + S.E.M. (n=5-6). Data from one of 5 experiments with similar results are shown. Data were analysed by Kruskal Wallis test followed by a Dunn's ad hoc test to identify differences between treatments. * represents significant increases compared with uninfected mice. ** represent significant increases compared to infected mice at day 7 p.i. ($p<0.05$).

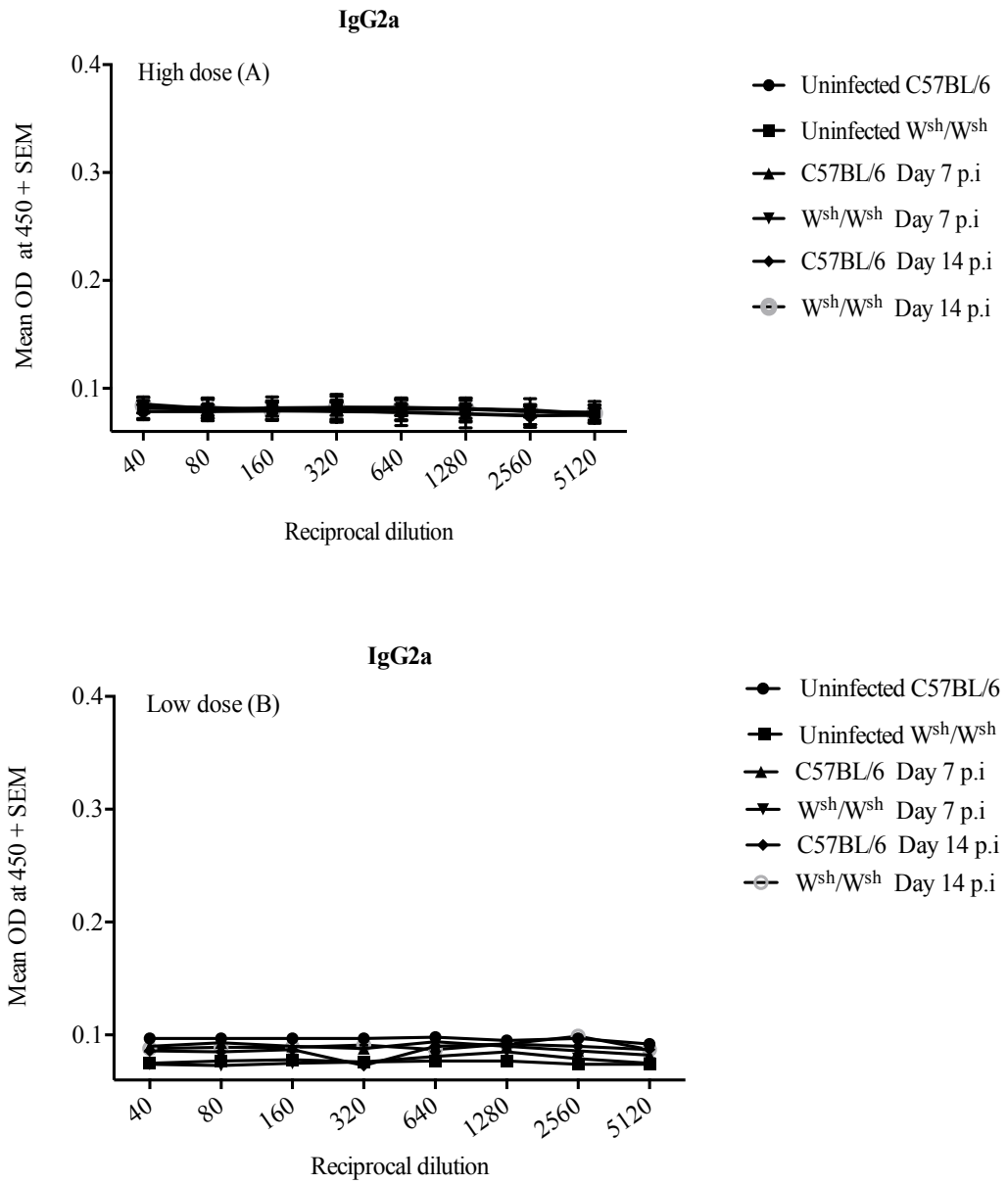


Figure 3.7 IgG responses in Wsh/Wsh mice. Serum was obtained from all mice. Wild-type C57BL/6 mice and Wsh/Wsh mice were infected orally with 400 (A) and 160 (B) *T. spiralis* larvae and sacrificed at day 7 or day 14 p.i. Levels of *Trichinella* antigen specific IgG was measured by ELISA are expressed as mean + S.E.M. (n=5-6). Data from one of 5 experiments with similar results are shown.

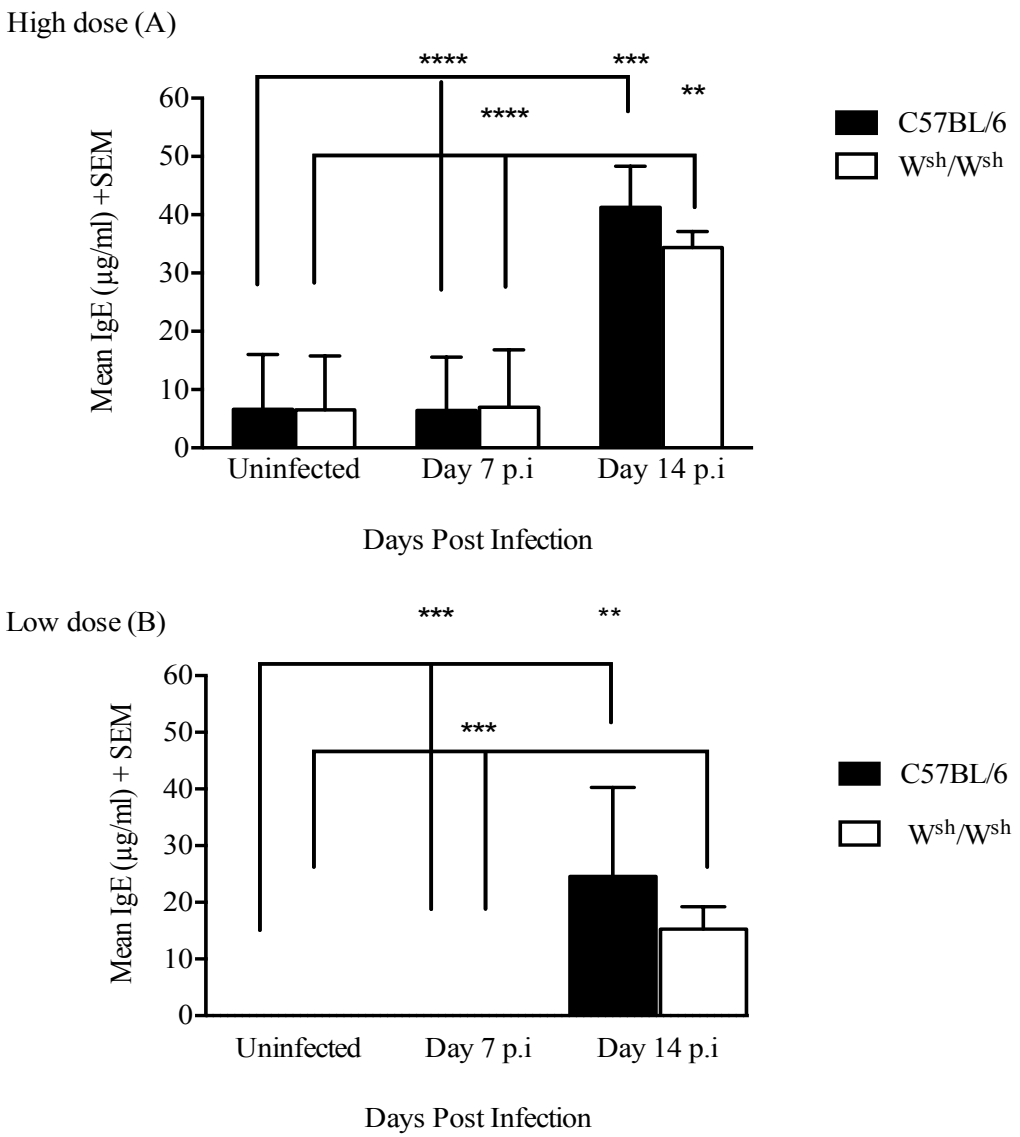


Figure 3.8 IgE levels in Wsh/Wsh mice. Serum samples were obtained from uninfected and infected C57BL/6 and Wsh/Wsh mice infected orally with 400 (A) and 160 (B) *T. spiralis* larvae. Total IgE concentration was measured using the sandwich enzyme-linked immunosorbent assay, together with a purified IgE standard. The data were expressed as mean µg/ml + S.E.M. (n=5-6). The experiment repeated five times with similar results. Data were analysed by Kruskal Wallis test followed by a Dunn's ad hoc test to identify differences between treatments. High dose, *** P<0.001 between uninfected C57BL/6 mice and infected mice at day 14 p.i; **** P<0.0001 between C57BL/6 mice day 14 and day 7 p.i. ** P<0.01 between uninfected Wsh/Wsh mice and infected mice at day 14 p.i; **** P<0.0001 between Wsh/Wsh mice day 14 and day 7 p.i. Low dose, ** P<0.01 between uninfected C57BL/6 mice and infected mice at day 14 p.i; *** P<0.01 between C57BL/6 mice day 14 and day 7 p.i. *** P<0.01 between uninfected Wsh/Wsh mice and infected mice at day 14 p.i; *** P<0.01 between Wsh/Wsh mice day 14 and day 7 p.i.

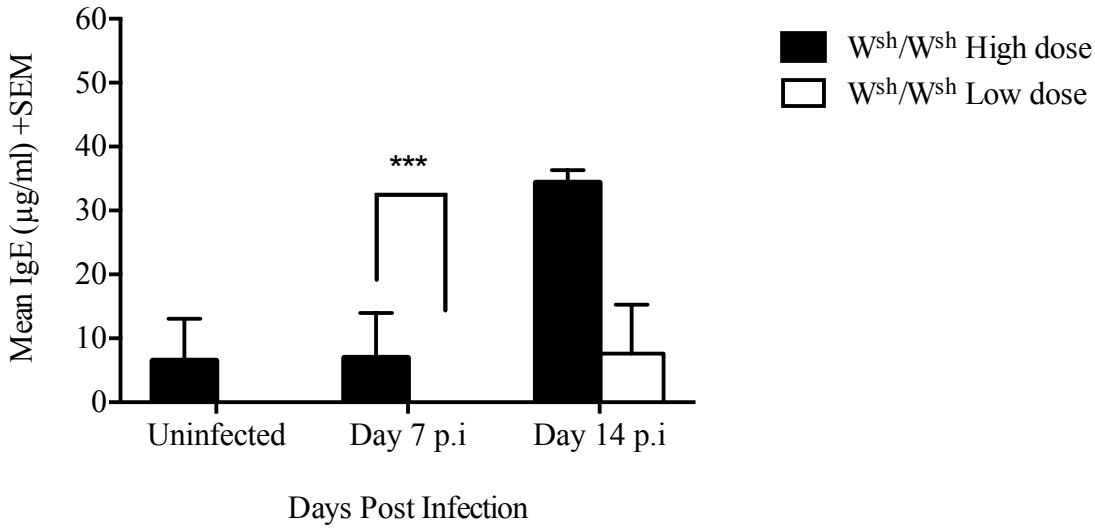


Figure 3.9 IgE levels in Wsh/Wsh mice. Serum samples were obtained from uninfected and infected C57BL/6 and Wsh/Wsh mice infected orally with 400 (A) and 160 (B) *T. spiralis* larvae. Total IgE concentration was measured using the sandwich enzyme-linked immunosorbent assay, together with a purified IgE standard. The data were expressed as mean µg/ml + S.E.M. (n=5-6). The experiment repeated five times with similar results. Data were analysed by Kruskal Wallis test followed by a Dunn's ad hoc test to identify differences between treatments. *** $P<0.001$ between high and low dose Wsh/Wsh mice at day 7 p.i.

3.2.5. MC-deficient models induced lower responses that are sufficient for generation of Th2 responses

The response characteristics of Th cells and cytokines (e.g., IL-4) have been investigated and found to be essential in protective immunity against *T. spiralis* (Lawrence *et al.*, 1998b, Scales *et al.*, 2007b). Here, IL-4 concentrations of MLN cells were measured using a sandwich enzyme-linked immunosorbent assay (ELISA). Levels of IL-4 were measured in MLN cells stimulated with medium only, Con A, and or *T.* Ag. MLN cells from uninfected or mice infected with a high dose of the parasite showed no signs of cytokine release when cells were stimulated with medium or antigen. However, levels of IL-4 produced by MLN cells from mice infected with *T. spiralis* after stimulating with Con A, were significantly increased. Important differences were observed between a high and a low level of infection. The low level of infection resulted in a reduction in the level of cytokine produced. We initially hypothesised that the low levels of Th2 cytokines in the low-level

infected mice was because of a lower stimulus of antigen. It can be seen that a high level of infection higher production of IL-4 was found in cells from the infected wild-type C57BL/6 and Wsh/Wsh mice that were stimulated by Con A, compared to uninfected mice of both strains. However, no statistically significant difference was observed (Figure. 3.10). It was observed that MLN cells from infected C57BL/6 mice produced higher concentrations of IL-4 when cultured in Con A than when cultured in medium only ($p=0.010$). Similarly, MLN cells from infected Wsh/Wsh mice showed significant increases in IL-4 responses when stimulated with Con A compared to cells that were stimulated with medium only ($p=0.0009$).

No IL-4 was produced by MLN cells from either mouse strain in response to *T.* Ag. MLN cells from Wsh/Wsh mice appeared to produce higher concentrations of IL-4 when stimulated with Con A than did MLN cells from C57BL/6 mice; however, no statistically significant difference was observed ($p=0.9$). In addition, unlike with W/W-v mice, no significant differences were observed in IL-4 levels in Wsh/Wsh mice compared to C57BL/6 mice. Mice infected with a low-level infection show negligible levels of IL-4 production. In contrast, mice given high-level infections produce high amounts of IL-4 cytokine when stimulated with Con A. Overall, similar responses were observed in wild-type and mast cell-deficient Wsh/Wsh mice with no differences effect of the dose between both of strains. The IL-4 levels in mice with a high dose were greater than in the low dose infected mice.

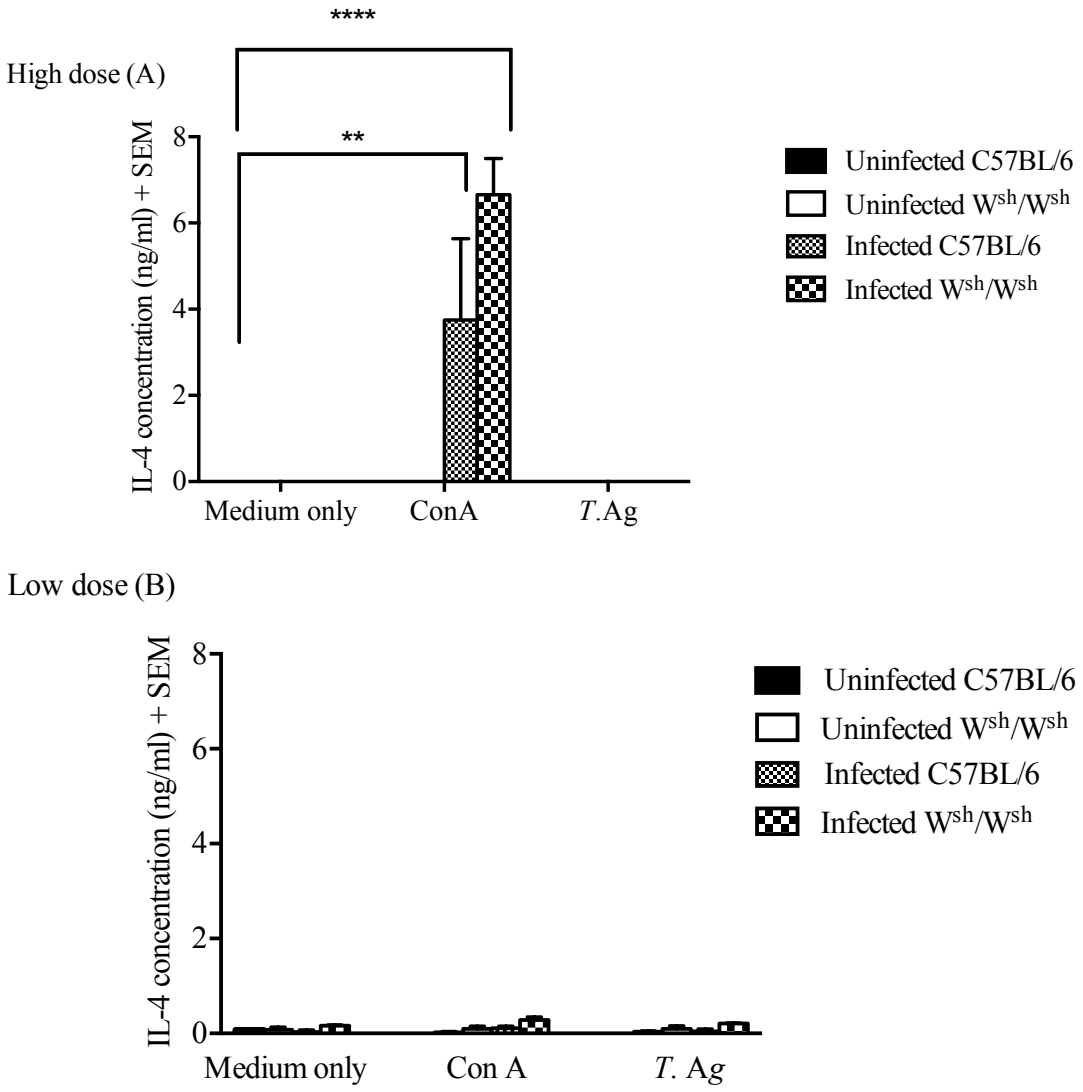


Figure 3.10 Cytokine levels in Wsh/Wsh mice. MLN cells were obtained from C57BL/6 and Wsh/Wsh mice infected orally with 400 (A) and 160 (B) *T. spiralis* larvae and incubated with medium, (ConA) (10 μg/ml) or *Trichinella* antigen (*T*. Ag) (10 μg/ml) for 48 h and IL-4 levels were assessed by ELISA. The data are expressed as mean + S.E.M. (n=5–6), and the experiment repeated five times with similar results. Data were analysed by Kruskal Wallis test followed by a Dunn's ad hoc test to identify differences between treatments. ** $P<0.01$ represents a significant increase in Con A from infected C57BL/6 mice compared to medium only; **** $P<0.0001$ represents a significant increase in Con A from infected Wsh/Wsh mice compared to medium only.

3.2.6. W-sh/W-sh mice induced MCs following infection with T. spiralis

Successful expulsion of *T. spiralis* is associated with mastocytosis (Lawrence *et al.*, 2004). Therefore, enumerated MMCs were measured following infection with *T. spiralis.*

We found that a significant mastocytosis was induced in both the group given 400 larvae and the group given 160 larvae. However, we detected no difference in mastocytosis between the tested mouse strains at these infection levels (Figure. 3.11).

At day 14 p.i., mast cell numbers in mice infected with a high dose were increased in comparison to uninfected mice ($p=0.0015$) and there wassignificant increase at day 14 p.i compared with day 7 p.i ($p=0.0021$). In contrast, no statistically significant differences were observed between uninfected and infected Wsh/Wsh mice infected with a high dose of parasites. However, at low level of infection there was a significant increase in the number of MMCs in infected Wsh/Wsh mice at day 14 p.i compared with day 7 p.i ($p=0.0317$). In addition, at day 14 p.i., mast cell numbers in low dose infected mice were higher in infected wild-type mice than uninfected mice ($p=0.001$).

T. spiralis infection in both levels of infection resulted in significantly increased the number of MMCs in wild-type mice at day 14 p.i, as a significant difference was observed between wild-type and Wsh/Wsh mice ($p=0.0043$).

Overall, Wsh/Wsh mice at day 14 p.i. showed the presence of MMC in the mucosa, albeit at lower levels than WT mice, which means that these mice may not have be completely deficient in MMCs. WT mice infected with both levels of infections were able to induce a marked mastocytosis, although numbers of mast cells at the high and low levels of infection were significantly lower compared to control mice.

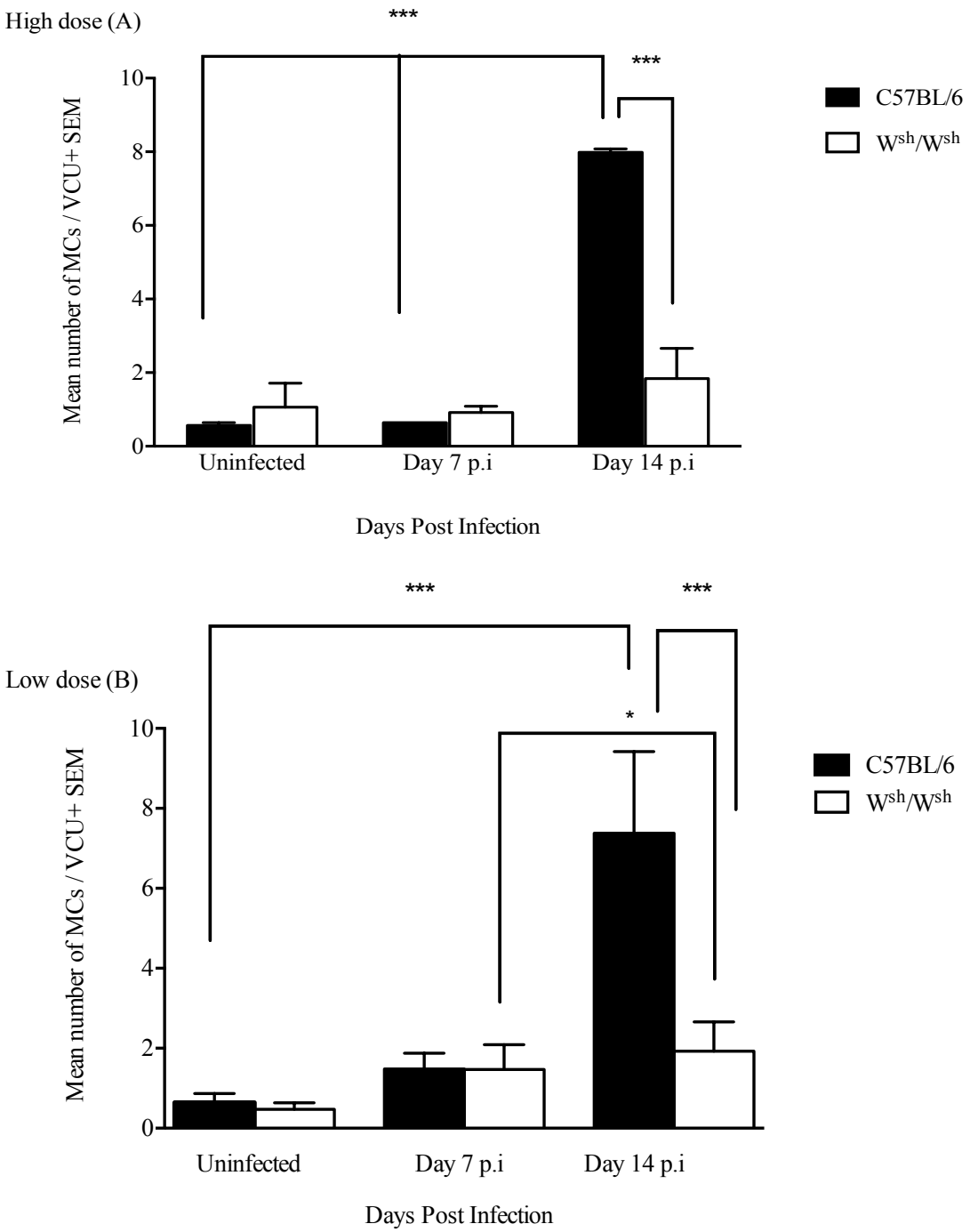


Figure 3.11 Mast cell (MC) numbers in Wsh/Wsh mice. Sections of small intestine were obtained from uninfected and mice infected orally with 400 (A) and 160 (B) T. spiralis larvae. The samples were processed using histological techniques and stained with Toluidine Blue for 24 h to visualise MCs, and the number of mucosal MCs was counted per 10 villus-crypt units. The data are expressed as mean + S.E.M. (n=5-6) and this data is representative of 5 separate experiments, and the experiment repeated five times with similar results. Data were analysed by Kruskal Wallis test followed by a Dunn's ad hoc test to identify differences between treatments. *** $P<0.001$ for infected C57BL/6 mice at day 14 p.i against infected at day 7 p.i and uninfected mice. *** $P<0.001$ between C57BL/6 mice day 14 and Wsh/Wsh mice day 14. *$P<0.05$ between Wsh/Wsh mice day 14 and day 7 p.i.

3.2.7. Production of Mouse Mast Cell Protease-1 is not significantly higher in MC-deficient models

It has previously been shown that mucosal mast cell protease-1 (mMCP-1) is associated with the development of pathology and is necessary for successful *T. spiralis* expulsion (Lawrence *et al.*, 2004). MC degranulation was evaluated by analysing the levels of mMCP-1 in the sera of uninfected and infected mice by ELISA.

It can be seen that a high level of infection induced a significant increase in mMCP-1 levels, which is observed in infected wild-type mice at day 14 p.i. compared to uninfected mice ($p=0.0058$). In addition, significant increases in mMCP-1 levels were observed in C57BL/6 mice at day 7 p.i when compared to uninfected mice ($p=0.007$).

In Wsh/Wsh mice infected with a high dose, the mMCP-1 level was increased at day 14 p.i. compared to uninfected or infected mice at day 7 p.i. (0.007 and 0.004 respectively, Figure 3.12). Moreover, the mMCP-1 levels were not significantly higher in wild-type C57BL/6 mice in comparison to Wsh/Wsh mice at day 14 p.i. A low level of infection lead to a lower level mMCP-1 response; a significant increase was observed in wild-type mice at day 7 p.i compared to uninfected mice ($p=0.0043$, Figure 3.12). Whereas a significant reduction was recorded between the higher and lower does in Wsh/Wsh at day 14 p. I (Figure 3.13).

Overall, these results are in contrast to the number of MMCs, whereby Wsh/Wsh mice did not have significantly lower levels of mMCP-1 and produced MMC that was significantly different from that of wild-type C57BL/6. Similar responses were observed in wild-type and mast cell-deficient mice in which the high dose, the level of mMCP-1 production in Wsh/Wsh were induced at day 14 p.i.

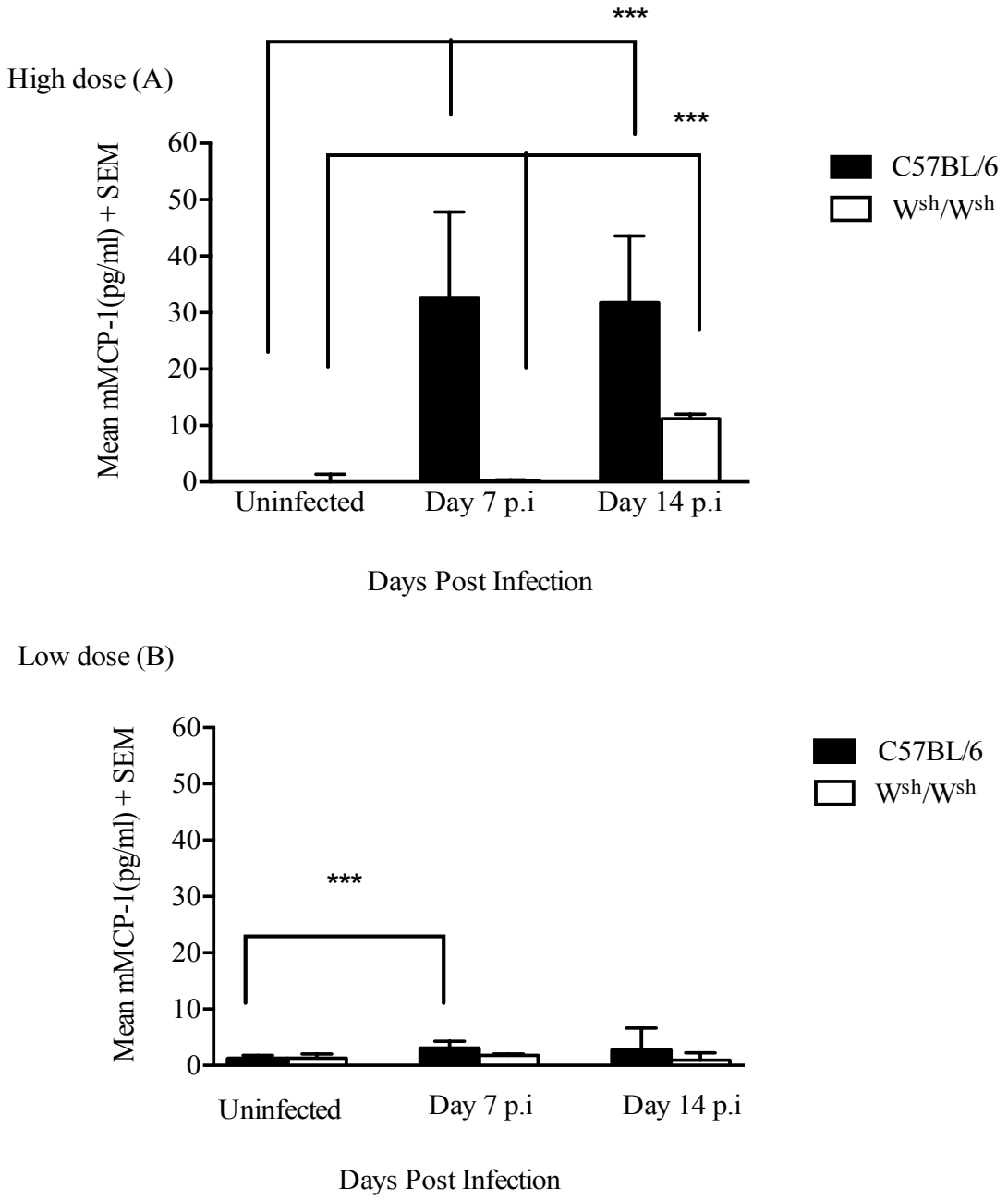


Figure 3.12 Mucosal Mast Cell Protease-1 (mMCP-1) levels in Wsh/Wsh mice. A sandwich enzyme-linked immunosorbent assay was used to measure mMCP-1 levels in serum samples obtained from uninfected mice and mice infected orally with 400 (A) and 160 (B) *T. spiralis* larvae. The data are expressed as mean + S.E.M. (n=5–6), and the experiment repeated five times with similar results. Data were analysed by Kruskal Wallis test followed by a Dunn's ad hoc test to identify differences between treatments. (A) *** $P<0.001$ for infected C57BL/6 mice at day 14 p.i against infected and uninfected mice. *** $P<0.001$ for infected Wsh/Wsh mice at day 14 p.i against infected and uninfected mice. (B) *** $P<0.001$ between uninfected C57BL/6 mice and infected mice at day 7 p.i.

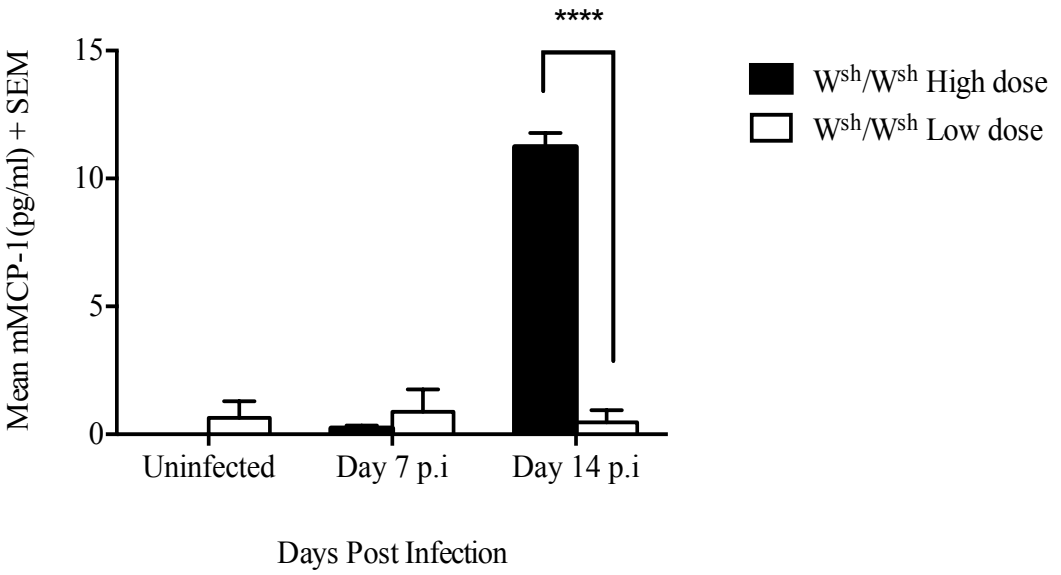


Figure 3.13 Mucosal Mast Cell Protease-1 (mMCP-1) levels in Wsh/Wsh mice. A sandwich enzyme-linked immunosorbent assay was used to measure mMCP-1 levels in serum samples obtained from uninfected mice and mice infected orally with 400 (A) and 160 (B) *T. spiralis* larvae. The data are expressed as mean + S.E.M. (n=5-6), and the experiment repeated five times with similar results. Data were analysed by Kruskal Wallis test followed by a Dunn's ad hoc test to identify differences between treatments. **** P<0.0001 between high and low dose Wsh/Wsh mice at day 14 p.i.

3.3. Discussion

Multiple mechanisms of the host immune system are stimulated to protect against *T. spiralis* and other GI nematode species, via the activation of Th cells (King and Mohrs, 2009a). Th2 immune responses are induced and release cytokines, such as IL-4, which results in mastocytosis and the production of parasite-specific IgG1 and IgE antibodies (Artis and Grencis, 2008b). MCs play a key role in the expulsion of *T. spiralis*; this expulsion is significantly delayed in the absence of MCs and their protease (Knight *et al.*, 2000, Lawrence *et al.*, 2004). MC-deficient c-Kit mutant mice have been widely used to investigate the role of MCs in different biological responses (Grimbaldeston *et al.*, 2005). In addition, the role of Wsh/Wsh mice in the expulsion of GI nematodes, such as *N. brasiliensis*, *H. polygyrus bakeri* and *T. muris*, has also previously been investigated (Saleem *et al.*, 2012, Hepworth *et al.*, 2012b).

The purpose of the present study was to investigate the function of MCs in the expulsion of *T. spiralis,* and it was, therefore, conducted in mice with mutations affecting c-Kit: Wsh/Wsh mice in a C57BL/6 background (Wolters *et al.*, 2005). However, as previously discussed, it is possible that W/W-v and Wsh/Wsh mice have other abnormalities due to reduced c-Kit functioning, although Wsh/Wsh mice have shown fewer problems, which may influence the results of investigations into MC function (Nigrovic *et al.*, 2008). In addition, it is considered that Wsh/Wsh mice exhibit fewer phenotypic abnormalities than do W/W-v mice (Zhou *et al.*, 2007, Nigrovic *et al.*, 2008). Thus, Wsh/Wsh mice could be appropriate for use in determining MC mechanisms that influence adaptive immunity or other biological responses. Although Wsh/Wsh mice have advantages, they also have fewer phenotypic abnormalities that may influence the expulsion of parasites. These abnormalities are associated with the lack of ICCs, which are important for GI motility. Therefore, the absence of these cells leads to abnormalities in intestinal motility, which results in delayed intestinal transit (Vallance *et al.*, 2001).

Previous studies have used W/W-v mice, which are frequently associated with phenotypic abnormalities that can affect the interpretation of results. The Wsh/Wsh adult mice that were used in the present study could easily be cross-bred, and they

are fertile with no recorded anaemia, in comparison with W/W-v mice. In addition, adult Wsh/Wsh mice have normal levels of haematopoietic cells, such as neutrophils and lymphoid cells (Grimbaldeston *et al.*, 2005, Nigrovic *et al.*, 2008). Therefore, transgenic mice models that lack the abnormalities observed in c-Kit mutants, such as *Mcpt5-Cre; iDTR* mice and Mas-TRECK mice, are becoming more appropriate models for MC investigations.

Following infection with *T. spiralis*, a high dose infection resulted in significantly greater parasite numbers than the low-level infection in Wsh/Wsh mice. It has been shown that *T. pseudospiralis* at high levels of infection (2000 larvae) induced significantly less intestinal inflammation than *T. spiralis* (Stewart *et al.*, 1988). In addition, previous studies have reported that low-level infections (< 40 eggs) with *T. muris* are not expelled as rapidly as normal infection levels (400 eggs) (Bancroft *et al.*, 1994).

In the present study, at low levels of infection, the proliferative responses to *T. spiralis* were somewhat lower and generated more slowly. Although the levels of serum antibody were low during initial infection, the results indicate an obvious increase in responses, which leads to an earlier Th2 response. Mice infected with *T. spiralis* in both the higher and lower infection treatments produced IgG1 responses and showed baseline levels of IgG2a in all strains.

Following infection with *T. spiralis*, mucosal mastocytosis was elicited in both the higher and lower infection treatments. We initially assumed that Wsh/Wsh mice would have delayed expulsion of worms, similar to W/W-v mice, (Lawrence *et al.*, 2004). Indeed, we found that expulsion of *T. spiralis* from MC-deficient Wsh/Wsh mice was significantly delayed compared to controls in mice with low-level of infection, suggesting that Wsh/Wsh mice were not able to stimulate sufficient immune responses. However, in the high level of infection condition, there was not a significant reduction in the number of worms in Wsh/Wsh mice at day 14 p.i. compared to controls which showed that the delayed expulsion of worms may due to dose-dependent. In addition, it has been demonstrated that in Wsh/Wsh mice infected with *N. brasiliensis*, the worm burden remained at day 14 p.i. However, wild-type mice showed complete expulsion by day 10 p.i. (Ohnmacht and Voehringer, 2010).

Although expulsion of *T. spiralis* from Wsh/Wsh mice was delayed relative to the control mice, it was not as substantial as the delay observed in previous studies in W/W-v or in IL-4-deficient mice.

Alternatively, the difference between Wsh/Wsh and C57BL/6 mice could be the result of other genetic differences that affect resistance to *T. spiralis*. Moreover, during primary infection with *T. spiralis*, intestinal mastocytosis occurs simultaneously with a marked tissue eosinophilia (Basten *et al.*, 1970). The capability of eosinophils for adhering to and killing several species of parasitic worms has been shown *in vitro* studies. However, *T. spiralis* requires the presence of specific antibodies to promote killing (Capron *et al.*, 1979, Bass and Szejda, 1979). Mice deficient in eosinophils have shown that eosinophils are unnecessary in immune responses to primary infections with intestinal worms (Swartz *et al.*, 2006, Svensson *et al.*, 2011, Fabre *et al.*, 2009, Knott *et al.*, 2007, O'connell *et al.*, 2011). Infection with *N. brasiliensis* in either primary or secondary infection has shown that eosinophils in the gut are not required for worm expulsion (Beiting *et al.*, 2007).

In addition, primary infection in *Trichuris muris* has shown that eosinophils were unsuccessfully influenced either worm expulsion or Th2 immunity (Svensson *et al.*, 2011). However, infections with *T. spiralis* showed that eosinophils are beneficial to the parasite during primary and secondary infection (Fabre *et al.*, 2009, Gebreselassie *et al.*, 2012, Babayan *et al.*, 2010, Vallance *et al.*, 2000) which has been demonstrated that eosinophils are required for parasite survival in *T. spiralis* infection (Fabre *et al.*, 2009). Therefore, Wsh/Wsh mice may have abnormalities in the levels of eosinophils.

Additionally, it has been previously demonstrated that some of the non-MC phenotypic abnormalities differ between W/W-v and Wsh/Wsh mice in which W/W-v had reduced numbers of neutrophils (Piliponsky *et al.*, 2010, Zhou *et al.*, 2007, Nigrovic *et al.*, 2008, Chervenick and Boggs, 1969) and basophils (Piliponsky *et al.*, 2010, Feyerabend *et al.*, 2011, Lantz *et al.*, 1998). By contrast, Wsh/Wsh mice have increased numbers of neutrophils (Grimbaldeston *et al.*, 2005, Piliponsky *et al.*, 2010, Zhou *et al.*, 2007, Nigrovic *et al.*, 2008) and basophils (Piliponsky *et al.*, 2010). Grimbaldeston *et al*. (2005) reported that Wsh/Wsh mice have normal levels

of basophils in the bone marrow and spleen similar to that found in W/W-v mice. Thus c-Kit mice may not an appropriate model for investigation of MCs in helminth infection. In addition, a full assessment of all haematopoietic cell lines, such as eosinophils, affected by this c-Kit mutation should be carried out to assess the potential effects of any differences in MCs or any alterations of the immune response to helminth infection. Therefore, an assessment of the benefit of Wsh/Wsh mice for this purpose is required in larger studies in order to obtain significant results that may help to overcome the effect of variance that occurs between mice. It has been shown that the development of intestinal pathology is crucial for *T. spiralis* expulsion, which is associated with the release of mediators from MCs (Knight *et al.*, 2000). We observed no significant differences in intestinal pathology in Wsh/Wsh mice that could possibly be described as potential resistance to this infection. This contrasts with observations in MC-deficient W/W-v mice, as villous atrophy and crypt hyperplasia was significantly lower than in infected wild-type controls (Lawrence *et al.*, 2004).

Previous research on *T. spiralis* infection has shown that this parasite is associated with Th2 immune responses. Lawrence *et al*. (2004) reported that IgG1 and IgE Th2 responses were reduced in W/W-v mice compared to wild-type mice. Consequently, an increase in Th1 responses, such as IgG2a, has been observed, which indicates that the lack of MCs resulted in a skewing of immune responses toward Th1 (Lawrence *et al.*, 2004). In contrast with previous findings in W/W-v mice, in which Th2 responses were reduced, and Th1 responses were increased in MC-deficient mice, the Wsh/Wsh mice in our study stimulated higher levels of IgG1 compared to those observed in the C57BL/6 mice.

Furthermore, the Wsh/Wsh mice showed low levels of IgG2a compared to background strains, following infection with *T. spiralis*. Taken together, these results may indicate that these mice are not completely deficient in MMCs. It has previously been shown that total IgE levels are reduced in W/W-v mice (Lawrence *et al.*, 2004). However, this observation was not replicated in Wsh/Wsh mice in the present study; the IgE levels were significantly increased at day 14 p.i. compared to those found at day 7 p.i., which is consistent with the results obtained from the Th2 responses.

Our IL-4 results showed that infected Wsh/Wsh mice appeared to be unable to induce IL-4 levels, which contrasts with the result relating to the MC number, as they exhibited sufficiently high responses following infection with *T. spiralis.* It has been shown that mastocytosis is an essential element in *T. spiralis* expulsion from the gut (Lawrence *et al.*, 2004). In addition, β-chymase mouse mMCP-1 secreted from MMCs in the gut via the use of mMCP-1$^{-/-}$ mice, showed that mMCP-1 was required for successful expulsion of this parasite (Lawrence *et al.*, 2004, Knight *et al.*, 2000). This was assisted by an increase in epithelial permeability in the gut and in blood vessels, which helps immune cells to migrate to the site of infection (Mcdermott *et al.*, 2003). In the present study, mMCP-1 levels were not significantly lower in MC-deficient Wsh/Wsh mice compared to C57BL/6 mice. These results also contrasted when compared to MMC numbers, in that Wsh/Wsh mice that were unable to produce significant levels of mMCP-1 had an MC number that was significantly different from the number of MCs observed in C57BL/6 mice. The disparity seen between MC numbers and levels of mMCP-1 could possibly be as a resulted of the high levels of variance in MC-deficient Wsh/Wsh mice. Therefore, additional research is required to confirm whether these observed can be replicated, or if these a result of human error while carrying out experimental analyses.

MC-deficient c-Kit mutant mice have contributed to the investigation and understanding of the function of MCs in a variety of immune responses, including autoimmune disease (Suurmond *et al.*, 2016). Autoantibodies and inflammatory arthritis were studied in the serum of K/BxN mice (Lee *et al.*, 2002); however, this study did not reveal which cellular populations were reactive to inflammatory signals. Therefore, two MC-deficient mouse models, WCB6F-*Kitl*^{Sl}/*Kitl*$^{Sl\text{-}d}$ (Sl/Sl^{d}) and W/W-v , were then utilised to estimate whether MCs provided an important cellular link between soluble mediators and synovial pathology, and to investigate the function of MCs during the effector phase of inflammatory arthritis (Lee *et al.*, 2002). These MC-deficient models showed no clinical or histological evidence of arthritis compared to wild-type mice.

Therefore, MCs constitute a possible cellular link between autoantibodies, soluble factors and other effector populations in inflammatory arthritis (Lee *et al.*, 2002). In

addition, MCs have been investigated in graft-versus-host disease (GVHD), using MC-deficient Wsh/Wsh mice and C57BL/6-*Cpa3-Cre; Mcl-1* mice, and it was shown that they play an immunomodulatory role (Leveson-Gower *et al.*, 2013). In addition, an increase in GVHD-associated mortality was observed when these mice were given allogeneic T-cells. This is in contrast with a previous study, in which W/W-v mice had improved survival in the absence of MCs (Leveson-Gower *et al.*, 2013). Furthermore, MCs have a potentially significant role in protecting the host from acute septic peritonitis and pneumonia (Maurer *et al.*, 1998). This has, therefore, been examined using MC-deficient Wsh/Wsh mice infected with a small, free-living, self-replicating bacterium, *Mycoplasma pneumoniae*, which differs from other microbes due to, for example, lacking a cell wall. Pathogens are primarily mucosal and live as extracellular parasites in close association with host epithelial cells, typically in the respiratory and urogenital tracts (Xu *et al.*, 2006). Moreover, C3H/HeSnJ-$^{scid/scid}$, C3H/HeSnJ and C57BL/6J-$^{scid/scid}$ mice were utilised to investigate the role of host defence in respiratory mycoplasmosis, and it was found that MCs are important for the innate immune containment of, and recovery from, respiratory mycoplasma infection (Xu *et al.*, 2006).

Allergen-driven airway hyper-responsiveness (AHR) has been investigated in MC-deficient c-Kit mutant mice to understand the function of MCs. This has been evaluated for the physiological response to sensitization and challenge to ovalbumin (OVA) in W/W-v mice (Takeda *et al.*, 1997). The mouse model was challenged with OVA for a 30-day-long sensitization with adjuvant (alum). The finding in the W/W-v model suggested that the function of MCs is unnecessary or has no role in the development of an allergic inflammatory reaction (Takeda *et al.*, 1997). However, conflicting results have been reported by Kobayashi et al. (2000), who demonstrated that MC knock-in W/W-v mice were immunised with alum-adsorbed OVA for a 20-day-long sensitization and showed contribution roles for MCs with low dose antigen challenge, but a high dose was not recorded (Kobayashi *et al.*, 2000b). From previous findings in the W/W-v model, the significance of MC roles may differ based on Ag dose differences used for sensitization. Moreover, the amount of adjuvant might potentially affect the development of Ag-induced AHR.

Williams and Galli (2000) showed the possible importance of MCs in the development of AHR and inflammation associated with asthma. This study was carried out on W/W-v mice that were sensitised to OVA with adjuvant for 31 days and concluded that MCs were redundant or that there is no role for these cells in a high Ag dose challenge (Williams and Galli, 2000). In addition, Williams and Galli (2000) reported that MC knock-in W/W-v mice were sensitised to OVA without adjuvant for 44 days, and found a contributing role for MCs in airway hyper-reactivity and airway inflammation. These results showed differences in the models used to investigate the importance of MCs in AHR, such as sensitization to OVA with or without the use of an adjuvant, which may influence the involvement and activation of immune cells in addition to MCs.

The previous studies mentioned above have been conducted in MC-deficient W/W-v mice to investigate whether MCs contribute to the development of airway hyper-responsiveness and airway inflammation. However, Becker *et al.* (2011) examined MC roles in AHR using Wsh/Wsh mice in different genetic backgrounds: sensitization and challenge in MC-deficient mice with $Kit^{W\text{-}sh/W\text{-}sh}$ C57BL/6 background and CB6-$Kit^{W\text{-}sh/W\text{-}sh}$ BALB/c background. This study by Becker *et al.* (2011) showed that after sensitization without adjuvant for 47 days, $Kit^{W\text{-}sh/W\text{-}sh}$ C57BL/6 background showed potential contribution for MCs. However, CB6-$Kit^{W\text{-}sh/W\text{-}sh}$ BALB/c background was capable of developing asthmatic features in the MC-deficient mice (Becker *et al.*, 2011). Previous studies have used Wsh/Wsh mice to show that MCs have an important role, which can be impacted by differences in genetic backgrounds.

Inflammatory arthritis has been intensively studied using the K/BxN serum transfer mouse model, which presents many of the pathological hallmarks of human rheumatoid arthritis (Elliott *et al.*, 2011). Therefore, the functional role for MCs in MC knock-in W/W-v was studied to assess the effector phase of inflammatory arthritis in the serum transfer model of K/BxN mice. This studied showed an important function for MCs in enhancing inflammatory arthritis (Lee *et al.*, 2002). Moreover, Zhou *et al*. (2007) reported similar results by injecting W/W-v mice with anti-collagen antibodies, and they found that MCs have an important role in induced

arthritis. However, the full development of features of arthritis was reported in Wsh/Wsh mice in the K/BxN serum transfer model, which was contrary to expectations, as W/W-v mice and Wsh/Wsh mice strains are both MC deficient (Elliott *et al.*, 2011, Mancardi *et al.*, 2011). Additionally, Zhou *et al*. (2007) demonstrated that full arthritis had been reported in Wsh/Wsh mice after injection with anti-collagen antibodies. In addition, the direct participation of MC functions in obesity has been assessed in MC-deficient mice, since Wsh/Wsh mice were studied in diet-induced obesity (Liu *et al.*, 2009).

Mice were fed a Western diet for 12 weeks; consequently, MCs showed evidence of a contributory role in diet-induced obesity and diabetes (Liu *et al.*, 2009). Liu *et al*. (2009) confirmed their observation from Wsh/Wsh mice in the additional use of MC-deficient W/W-v mice. It has been demonstrated that W/W-v mice reduced body weight gain and improved glucose tolerance (Liu *et al.*, 2009).

MC roles in type 1 diabetes (T1D), induced by multiple low-dose streptozotocin (MLD-STZ) treatments, have been estimated in two strains of MC-deficient mice, W/W-v or Wsh/Wsh mice, and the adoptive transfer of MCs was investigated (Carlos *et al.*, 2015). These studies demonstrated that W/W-v and Wsh/Wsh mice were more susceptible to T1D, with the development of raised levels of hyperglycaemia and more severe disease (Carlos *et al.*, 2015).

As previously mentioned, mast cells have been extensive studies to investigate their roles in the successful expulsion of *T. spiralis.* In addition, much has still to be confirmed to entirely clarify and understand the multiple mechanisms of action that have been determined to assist the immune response for the elimination of *T. spiralis*.

The aim of this study was to assess the significance of mast cells in the generation of immune responses to expel gastrointestinal nematode that the ablation of MCs was shown that the expulsion of worms was delayed and Th2 immune responses were impaired.

Overall, the present study is inconsistent with previous work on W/W-v mice that MC-deficient Wsh/Wsh mice were not significantly delayed compared to controls. Low-level infection was delayed expelling the parasite which the number of larvae used for infection was significantly lower in mice with 160 *T. spiralis* larvae. In addition, MMCs and levels of mMCP-1 were observed in Wsh/Wsh mice, which these results show that MC-deficient might be ablated in connective tissue mast cells (CTMCs). However, due to such a strong Th2 response induced by helminth infection, they are may not completely deplete in mucosal mast cells (MMCs).

Further studies of MMCs are required to illuminate their influence in models deficient in c-Kit. Moreover, additional studies of other cytokine responses, such as IL-13 and Th1 cytokine interferon gamma (IFN-γ), in Wsh/Wsh, should be conducted to provide further explanation of the change in immune responses that result from *T. spiralis* infection. Overall, the present study in MC-deficient Wsh/Wsh mice highlights the importance of MCs in eliminating infections by stimulating the release of specific cytokines through binding to their cell surface receptors. The study also provides opportunities for researching MCs that could assist in increasing the consideration of the role of MCs in a number of protective and pathological functions.

CHAPTER 4. HELMINTH INFECTIONS IN MAS-TRECK MICE

4.1. Introduction

Mast cells have essential functions that are important in the defence of the host during innate and adaptive immune responses (Galli *et al.*, 2008b). These functions include killing pathogens and assisting in the degradation of poison components. In addition, MCs play an important role in the regulatory functions of structural cells, such as fibroblasts and vascular endothelial cells, contributing to the regulation of the distribution and viability of these cells (Galli *et al.*, 2008b). The location of MCs makes a significant contribution to the first-line protection of the host immune system. Moreover, the adaptive immune response can be amplified by MCs and can function as a modulator response of T-helper cells (Hershko and Rivera, 2010). Furthermore, decreased inflammation and an induced immune system in allergic or autoimmune disorders can be affected by MCs as mediators (Galli *et al.*, 2008b).

Although W/W-v and Wsh/Wsh deficient mice have been extensively used to investigate the role of MCs *in vivo*, these mice have additional abnormalities related to their c-Kit mutation, which makes it difficult to identify the role of MCs in different diseases. Therefore, several attempts have been made to generate strain models that are selectively deficient in MCs, without affecting the immune system (Yu *et al.*, 2015). In addition, in the previous chapter, our observations in these mice, indicate they are not an appropriate model for investigation of MCs in helminth infection. Therefore, we decided to induce different models for the reason that we outlined.

Sawaguchi *et al*. (2012) generated an additional transgenic model, named Mast cell-specific enhancer-mediated Toxin Receptor-mediated Conditional Cell Knock-out (Mas-TRECK), in which the ablation of MCs can be specifically stimulated. Mas-TRECK mice models are based on the expression of human diphtheria toxin receptor (DTR). The expression of the interleukin (*Il*)4 gene is expressed under the control of the intronic enhancer element, and DTR is consequently inserted, which leads to the specific depletion of IL-4 expressing cells. This results in depletion of MCs and basophils (Yu *et al.*, 2015).

The role of MCs as a therapeutic target can be investigated using mouse strains in which there is selective ablation of MCs (Reber *et al.*, 2012a). In addition, the function of MCs and basophils has presented similar features to immune responses with T- helper 2 (Th2) cells (Otsuka and Kabashima, 2015). Therefore, mouse models with selective depletion are beneficial in considering which cells play a role during immune responses and in preventing additional abnormalities that are associated with c-Kit mutation models (Otsuka and Kabashima, 2015).

Transgenic Mas-TRECK mice have contributed to the investigation and understanding of the function of MCs in a variety of immune responses. Contact hypersensitivity (CHS) has been investigated using W/W-v mice, and this revealed attenuated CHS responses. Other studies have simultaneously shown that use of W/W-v mice does not impair CHS. Therefore, Mas-TRECK mice have been utilised to re-evaluate the significance of MCs in CHS (Otsuka *et al.*, 2011). It has been suggested that the functions of dendritic cells are enhanced by MCs during CHS, such that MCs interact with dendritic cells to establish its sensitisation phase (Otsuka *et al.*, 2011). Transgenic mouse models lack other abnormalities that have been observed in c-Kit mutants, such as W/W-v and Wsh/Wsh mice. In addition, MCs exert their natural function on Mas-TRECK mice before they are treated with diphtheria toxin (DT). This treatment permits the depletion of MCs at different time points during infection.

It has been shown that repeated intraperitoneal DT treatment for 5 consecutive days completely depleted MCs in Mas-TRECK mice. This was assessed 3 days after the last injection, and it was found that MCs were depleted in the skin, peritoneal cavity, stomach and mesentery. In addition, IgE-dependent passive cutaneous anaphylaxis (PCA) and passive systemic anaphylaxis (PSA) reactions were revoked (Sawaguchi *et al.*, 2012). Otsuka et al. showed that after the end of DT treatment, mast cells in the skin remain depleted for at least 12 days (Otsuka *et al.*, 2011). However, if Mas-TRECK mice are treated with DT it also leads to a transient depletion of blood basophils and it effectively completely inhibited a model of basophil-dependent development (Mukai *et al.*, 2005, Sawaguchi *et al.*, 2012). Other major types of leukocytes that did not express *DTR* mRNA, which include dendritic cells, B-cells, T-cells, natural killer T-cells, eosinophils and neutrophils, were not affected by DT

treatment, although the number of these cells was only reported in analyses that were carried out 12 days after cessation of DT treatment (Sawaguchi *et al.*, 2012, Otsuka *et al.*, 2011).

In the present study, our aim was to evaluate the utility of Mas-TRECK mice as MC-deficient models for a more precise role of mast cell in gastrointestinal nematode infection. The ability of mice to expel worms was estimated via worm establishment at two different time points after infection. Therefore, wild-type BALB/c and MC-deficient Mas-TRECK mice were orally infected with 400 freshly isolated *T. spiralis* larvae, and the mice were then sacrificed on days 7 and 14 post infection (p.i.) to assess the worm burden. All samples obtained from the mice were examined for their ability to expel the parasite, and the intestinal pathology, immune responses and mucosal MCs (MMCs) were assessed.

4.2. Results

4.2.1. Worm burdens in Mas-TRECK mice are not statistically significantly different

In the present study, MCs were depleted in Mas-TRECK mice prior to infection. In order to clarify the role of MCs in helminth infections using mouse models that lack the abnormalities that have been observed in c-Kit mutants, Mas-TRECK and BALB/c mice prior to infected with *Trichinella spiralis* was received intraperitoneal injections of 250 ng DT for 5 alternate days recording to the schedule below (Figure 4.1). There was then a 72-hour rest period to allow basophils to recover to normal levels before infection with *Trichinella spiralis*.

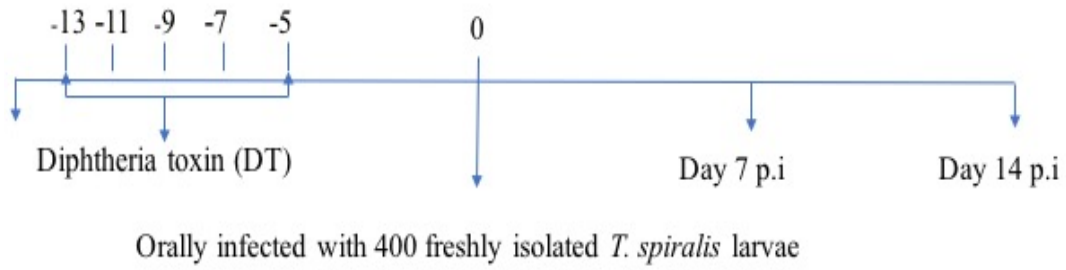


Figure 4.1 A diagram showing treatment protocol used in studies.

Following infection with 400 *T. spiralis* the number of worms was significantly reduced in wild-type BALB/c mice at day 14 p.i compared with day 7 p.i (p=0.0091).

Infected Mas-TRECK mice had reduced worm burdens at day 14 p.i compared to day 7 p.i, but this difference was not significant (p=0.001). The number of worms was reduced in both wild-type BALB/c and Mas-TRECK mice at day 14 p.i. However, no statistically significant differences in the worm numbers between the two strains were observed at day 14 p.i (p=0.99) (Figure 4.2). This suggests unexpectedly that Mas-TRECK mice were not impaired in their ability to expel *T. spiralis*.

These experiments were repeated on five separate occasions with 5-6 animals per group. The numbers of worms and the values of other measurements were not

significantly different between the repeats. A representative experiment is presented in the data given below.

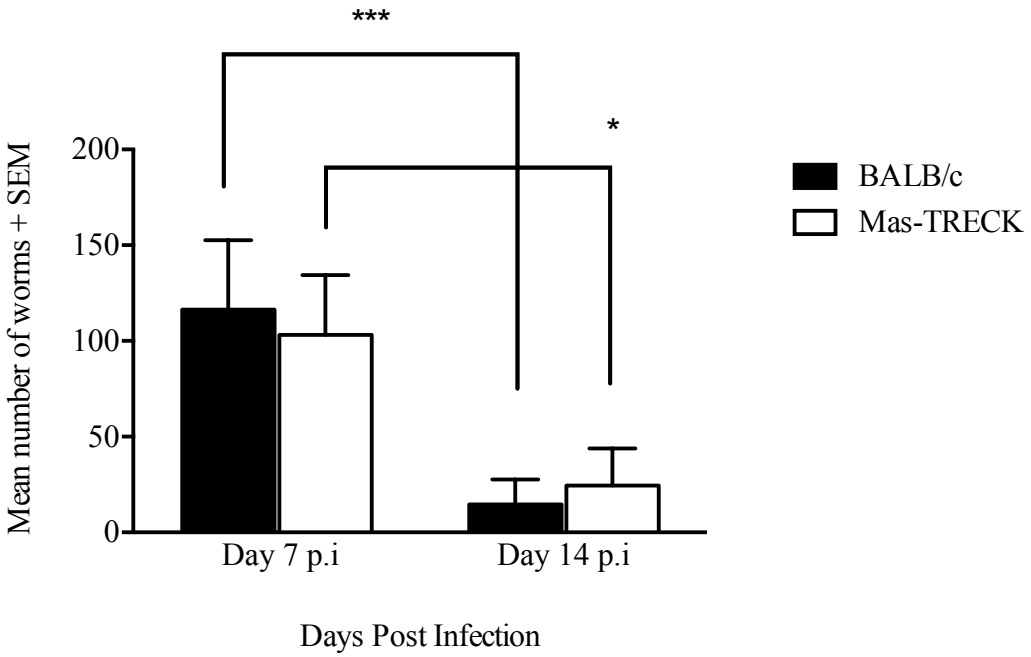


Figure 4.2 Worm burdens in Mas-TRECK mice. Mice were injected with 250 ng of diphtheria toxin on 5 alternate days. After a rest period, the mice were orally infected with 400 *T. spiralis* larvae, and the number of worms assessed at days 7 and 14 p.i. Worms were removed from small intestine samples and counted under an inverted microscope. The data are expressed as mean + S.E.M. (n=5-6), and the experiment repeated five times with similar results. Data were analysed using a Kruskal Wallis test followed by a Dunn's ad hoc test to identify differences between treatments. ***P<0.001 between BALB/c mice day 14 and day 7 p.i.; *P<0.05 between Mas-TRECK mice day 14 and day 7 p.i.

4.2.2. Intestinal oedema and pathology in MC-deficient models are not statistically significant

The small intestine was weighed as a representation of oedema to determine the pathological responses in uninfected and infected mice of each strain. Moreover, enteropathy in mice infected with *T. spiralis* results in increased luminal fluid as part of the 'weep and sweep' function, which assists in making the intestinal lumen an unsuitable environment for parasites (Anthony *et al.*, 2007).

Following infection with *T. spiralis*, infected and uninfected wild-type BALB/c mice did not show a significant increase in gut weight on day 7 p.i. or day 14 p.i ($p=0.99$ and $p=0.934$, respectively). In addition, the weight of the small intestine in BALB/c mice was not significantly different at day 14 p.i compared to day 7 p.i. At both days 7 and 14 p.i., Mas-TRECK mice showed a significant increase in their gut weight compared to uninfected mice ($p=0.0001$and $p=0.019$, respectively).

No statistically significant difference was observed between BALB/c and Mas-TRECK mice at both days 7 and 14 (Figure 4.3).

The intestinal architecture was determined by measuring villi lengths and crypt depths. Development of villus atrophy and crypt hyperplasia was observed in both infected wild-type and Mas-TRECK mice at days 7 and 14 p.i compared with uninfected mice of both strains. However, no significant difference in villi lengths between uninfected wild-type and infected wild-type mice at day 14 p.i was observed.

In addition, no significant difference in villus length was observed in Mas-TRECK mice infected with *T. spiralis* compared with uninfected mice at day 14 p.i. Crypt hyperplasia did not appear to be significant in the different groups of mice. Although crypt depths were no greater in uninfected Mas-TRECK mice than they were in uninfected wild-type mice, no significant difference was observed in either strain. Although infected mice of both strains showed greater crypt depths compared with uninfected mice, the difference was not significant (Figure 4.4).

T. spiralis infection of wild-type mice was associated with the development of intestinal inflammation. In contrast, the enteropathy that was induced following infection of Mas-TRECK mice was not significantly improved with the deficiency in MMCs. These results contrast with those previously described by Lawrence *et al.* (2004) using W/W-v mice, which showed that MC-deficient mice were significantly different compared with WT mice.

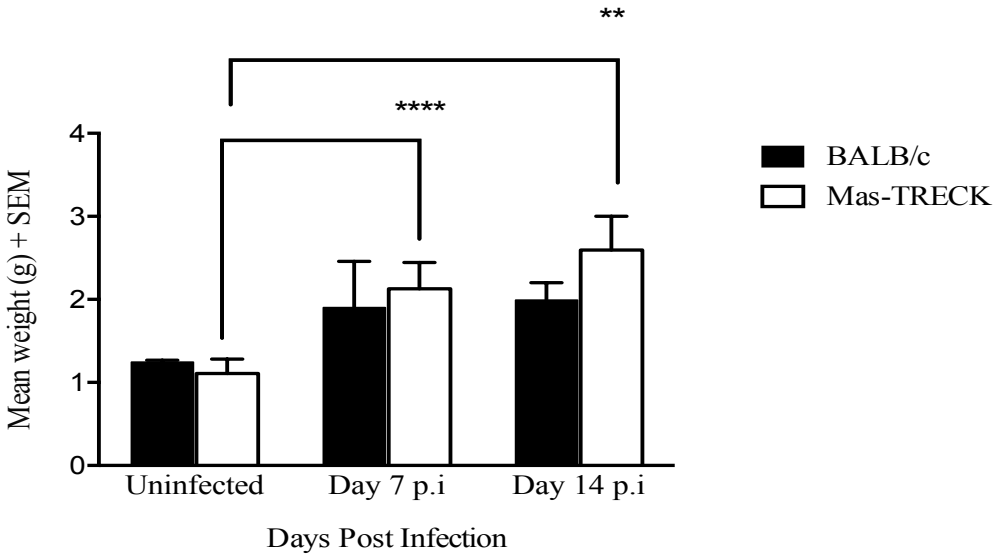


Figure 4.3 Development of enteropathy in Mas-TRECK mice. The weight of the small intestine was measured to determine the pathological responses in uninfected mice of both strains and mice of both strains infected with 400 *T. spiralis* larvae at days 7 and 14 p.i. The data presented are mean + S.E.M. (n=5-6), and the experiment repeated five times with similar results. Data were analysed by Kruskal Wallis test followed by a Dunn's ad hoc test to identify differences between treatments. *** $P<0.001$ between uninfected Mas-TRECK mice and infected mice at day 7 p.i; ** $P<0.01$ between uninfected Mas-TRECK mice and infected mice at day 14 p.i.

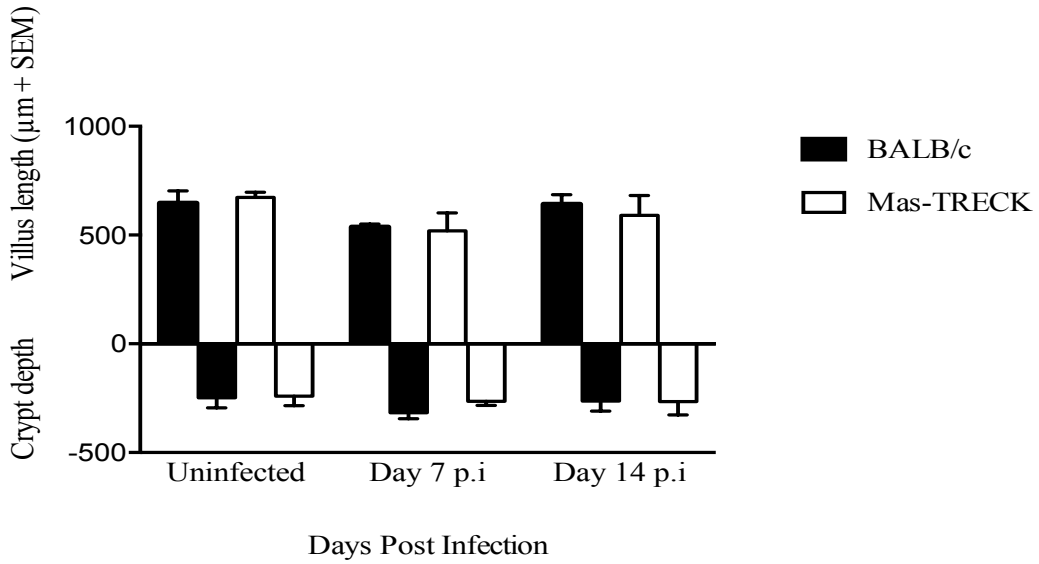


Figure 4.4 The intestinal architecture in of Mas-TRECK mice. Gut sections were fixed in Clark's fixative and stained with Schiff's reagent. The tissues were microdissected, and 10 villi and crypts per mice were measured. Villus atrophy and crypt hyperplasia were measured in uninfected mice and mice infected with 400 *T. spiralis* larvae. The data presented are mean + S.E.M. (n=5-6), and the experiment repeated five times with similar results. Analysis of the data using a Kruskal Wallis test followed by a Dunn's ad hoc test showed there was no significant difference between treatments.

4.2.3. Proliferative capacity in MC-deficient models is unaffected

In the present study, the activation of Th2 cells was assessed to evaluate the effect of MC-deficiency on the proliferation and production of Th2 cells in the mesenteric lymph node (MLN). MLN cells were removed from uninfected and infected wild-type BALB/c and Mas-TRECK mice, re-stimulated with different stimuli *in vitro* and then incubated with medium, Concanavalin A (Con A) or *T.* Ag for 48 hours. The proliferative responses of the MLN cells rapidly increased following stimulation with Con A in uninfected and infected mice of both strains (Figure 4.5). It was observed that there was a significant increase in proliferation in comparison to medium only in all strains when stimulated with Con A but not *T.* Ag ($P<0.01$). Mas-TRECK mice showed a lower proliferative capacity than wild-type mice; however, the difference was not statistically significant ($p>0.05$) (Figure 4.6). MLN cells from wild-type BALB/c mice had a higher proliferative rate than MLN cells from Mas-TRECK mice.

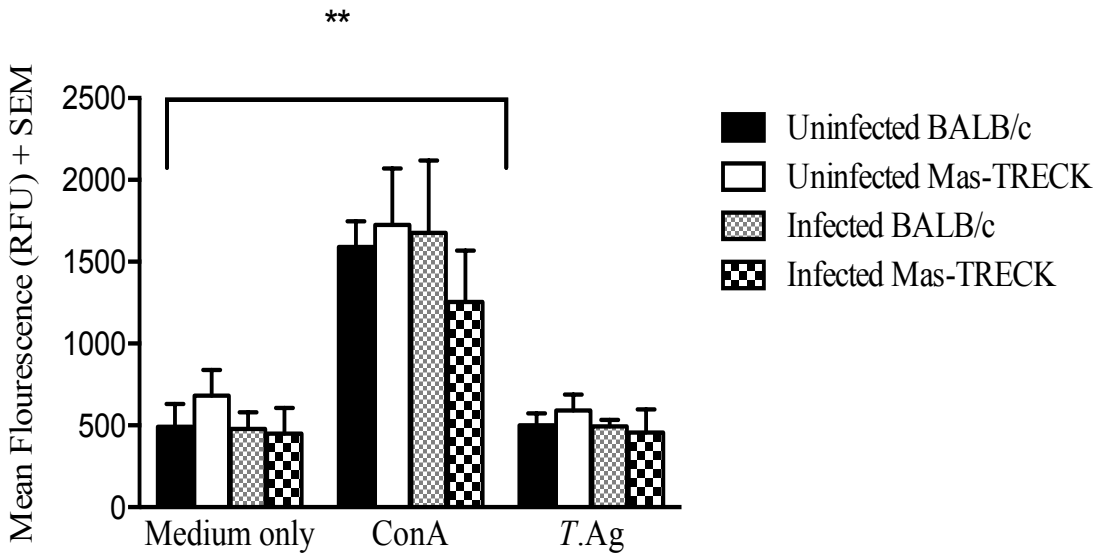


Figure 4.5 Proliferative responses of Mas-TRECK mice. T-cell proliferation was assessed after culturing mesenteric lymph node cells in complete RPMI 1640 and incubating them in medium, Concanavalin A, (10 μg/ml) or *T.* Ag (10 μg/ml) for 48 hours. Proliferation was evaluated using Alamar Blue (resazurin) and mice were infected with 400 *T. spiralis* larvae. The data are expressed as mean + S.E.M. (n = 5-6), and the experiment repeated five times with similar results. Data were analysed by Kruskal Wallis test followed by a Dunn's ad hoc test to identify differences between treatments. ** $P<0.01$ represents a significant increase between Con A and medium in all strains.

4.2.4. Antibody responses in MC-deficient models are significantly increased

The development of significant IgE and antigen-specific IgG1 has previously been observed following infection with *T. spiralis;* however, IgG2a did not develop (Lawrence *et al.*, 1998a). In the present study, total serum IgE, antigen-specific IgG1, and IgG2a levels were determined in the serum of uninfected and infected BALB/c and Mas-TRECK mice at days 7 and 14 p.i.

Our results show that antigen-specific IgG1 levels were significantly increased in infected wild-type BALB/c mice at day 14 p.i compared with uninfected or infected mice at day 7 p.i mice ($p= 0.0005$ and $p=0.0001$, respectively).

Mas-TRECK mice at day 14 p.i showed a significant increase in antigen-specific IgG1 levels compared with uninfected mice ($p= 0.0001$). Additionally, statistically significant differences between Mas-TRECK mice at days 7 and 14 p.i were

recorded (p=0.0001). In addition, it was observed that the IgG1 levels of infected mice at day 14 p.i were significantly different between strains at day 14 p.i (p=0.0001) (Figure. 4.6A).

Negligible levels of IgG2a were produced by the BALB/c and Mas-TRECK mice, which were similar to uninfected control mice (Figure. 4.6B). In addition, low levels of IgG2a were measured in infected Mas-TRECK mice at day 14 p.i, and no significant differences were observed in either strain at day 14 p.i (p=0.0758).

Following infection with *T. spiralis*, the levels of total IgE in the sera were measured by ELISA. This infection resulted in significantly increased levels of total IgE in wild-type mice at day 14 p.i, compared with infected mice at day 7 p.i (p=0.0144). However, no significant difference between uninfected and infected BALB/c mice at day 14 p.i was observed (p=0.681).

In addition, the total IgE level was increased in Mas-TRECK mice at day 14 p.i, but was not statistically significant compared with that found in uninfected mice (p=0.0883). However, the increase that was observed at day 14 p.i was statistically significant compared with day 7 p.i (p=0.023). In both strains, no statistically significant differences between the infected and uninfected mice were observed at day 14 p.i (Figure 4.7).

The elevated antigen-specific IgG1 levels in Mas-TRECK mice suggest that this model did not display impaired Th2 immune responses. This is similar to our previous finding in Wsh/Wsh mice, whereby the production of IgG1 and IgE were increased to comparable levels to that observed in wild-type mice infectd with the parasite.

The results of the present study contrasted with finding in a previous study in W/W-v mice, in which the infection of Mas-TRECK mice with *T. spiralis* did not induce a change in IgG2a levels compared with BALB/c mice. In addition, in contrast with W/W-v mice, no significant differences were observed in IgE levels in Mas-TRECK mice, compared with wild-type BALB/c mice.

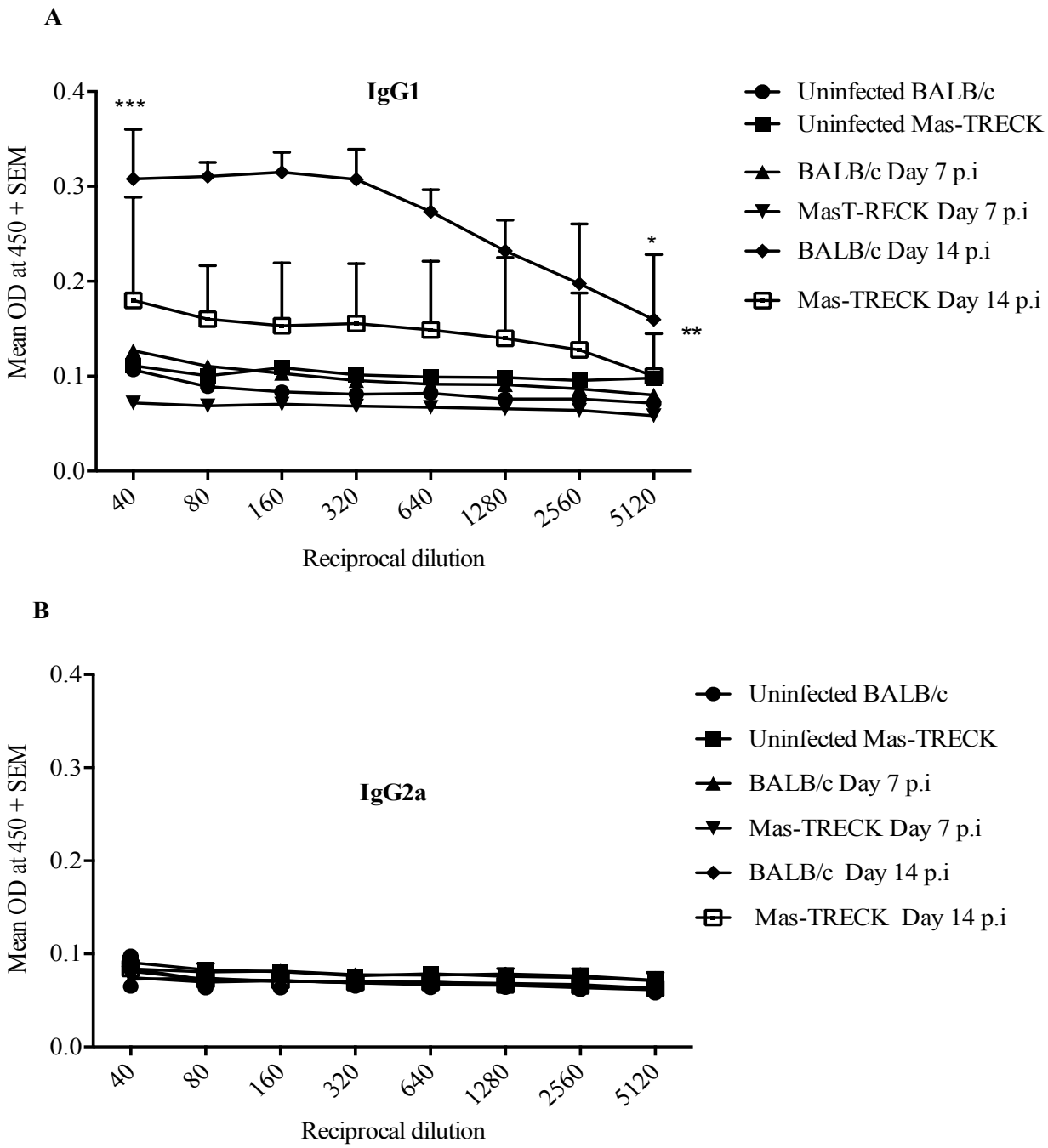


Figure 4.6 Immunoglobulin (Ig)G responses in Mas-TRECK mice. Serum was obtained from all mice. Wild-type C57BL/6 and Mas-TRECK mice were infected with 400 *T. spiralis* larvae and sacrificed at days 7 and 14 p.i. Levels of *Trichinella* antigen specific IgG was measured by ELISA. The data are expressed as mean ± S.E.M. (n=5–6), and the experiment repeated five times with similar results. Data were analysed by Kruskal Wallis test followed by a Dunn's ad hoc test to identify differences between treatments. * represents significant increases compared with uninfected mice. ** represents significant increases compared to infected mice at day 7 p.i. *** represents a significant increase differences between each strain at day 14 p.i. ($p<0.05$).

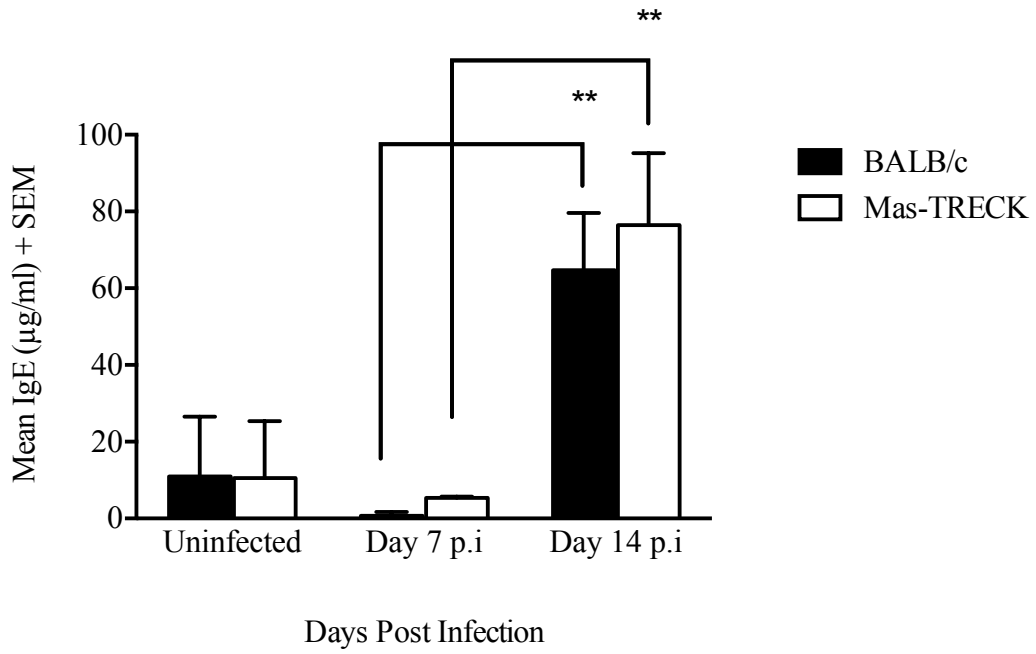


Figure 4.7 The levels of Immunoglobulin (Ig)E in Mas-TRECK mice. Serum samples were obtained from uninfected BALB/c and Mas-TRECK mice and mice of both strains infected with 400 *T. spiralis* larvae. Total IgE concentration was measured by sandwich ELISA. The data are expressed as mean µg/ml + S.E.M. (n=5-6), and the experiment repeated five times with similar results. Data were analysed by Kruskal Wallis test followed by a Dunn's ad hoc test to identify differences between treatments. ** $P<0.01$ between BALB/c mice day 14 and day 7 p.i.; ** $P<0.01$ between Mas-TRECK mice day 14 and day 7 p.i.

4.2.5. MC-deficient models induce responses that are not sufficient to generate Th2 responses.

IL4 is a cytokine that is considered to be a fundamental regulator of Th2 responses in the development of enteropathy associated with *T. spiralis* infection (Lawrence *et al.*, 1998a). A previous study showed that MC-deficient W/W-v mice were impaired in their ability to induce responses to Th2 during infection (Lawrence *et al.*, 2004).

In the present study, we aimed to discover whether or not inducible models, such as Mas-TRECK mice, have similar responses following infection with *T. spiralis*. IL-4 concentrations were measured by conducting sandwich ELISA on MLN cells.

Following infection with *T. spiralis,* IL-4 production by MLN cells which had been stimulated with Con A, was not significantly higher in comparison to uninfected wild-type BALB/c mice. Moreover, IL-4 levels produced by MLN cells obtained from uninfected wild-type mice in response to *T.* Ag compared was not significantly different to unstimulated cells or cells stimulated with Con A (Figure 4.8). No statistically significant differences were observed in infected wild-type BALB/c and Mas-TRECK mice that were stimulated by Con A, compared to uninfected mice of both strains.

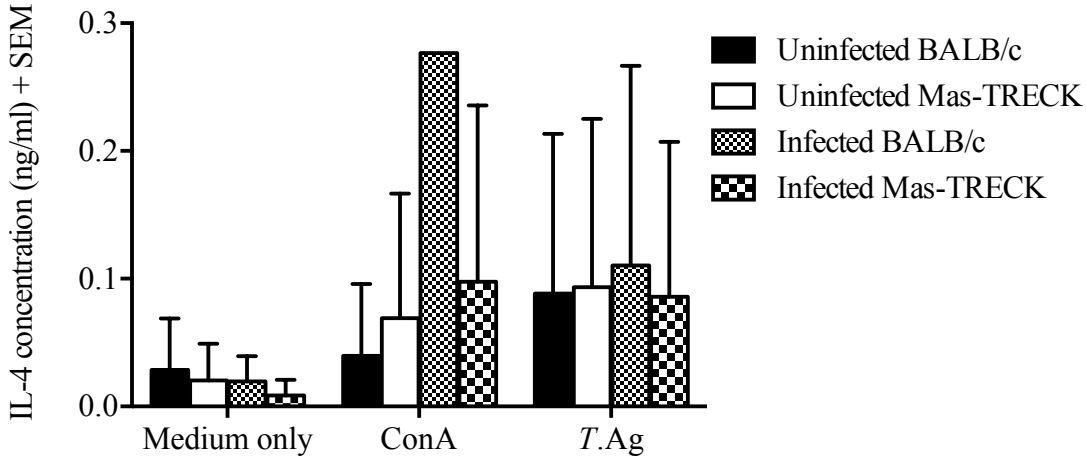


Figure 4.8 Cytokine levels in Mas-TRECK mice. Wild-type BALB/c and Mas-TRECK mice were infected with 400 *T. spiralis* larvae and isolated MLN cells (1 x 10^7/ml) cultured cells in the presence of medium, Con A (10 μg/ml) and *Trichinella* antigen (10 μg/ml) for 48 hours, and IL-4 levels assessed by ELISA. The data are expressed as mean + S.E.M. (n=5-6). The experiment repeated five times with similar results. Analysis of the data using a Kruskal Wallis test followed by a Dunn's ad hoc test showed there was no significant difference between treatments.

4.2.6. Mas-TRECK mice produced MCs following infection with T. spiralis.

The development of enteropathy and expulsion of *T. spiralis* worms from the small intestine are associated with infiltration of MCs into the mucosa (Lawrence *et al.*, 2004). Therefore, the numbers of MMCs were measured following infection with *T. spiralis* in the present study. MMC numbers were increased significantly in infected wild-type BALB/c mice at day 14 p.i compared to uninfected mice (p=0.0065) (Figure. 4.9). In addition, a rapid increase in the number of MMCS was observed in Mas-TRECK mice, with a statistically significant difference at day 14 p.i compared with the number of MMCs counted in uninfected mice (p=0.0414). There was no statistically significant difference between infected wild-type BALB/c mice and infected Mas-TRECK mice at day 14 p.i (p>0.99). Mas-TRECK mice were able to induce mastocytosis following infection with *T. spiralis*, although they are considered to be MC-deficient, which suggests that MMCs may not be completely depleted in these mice.

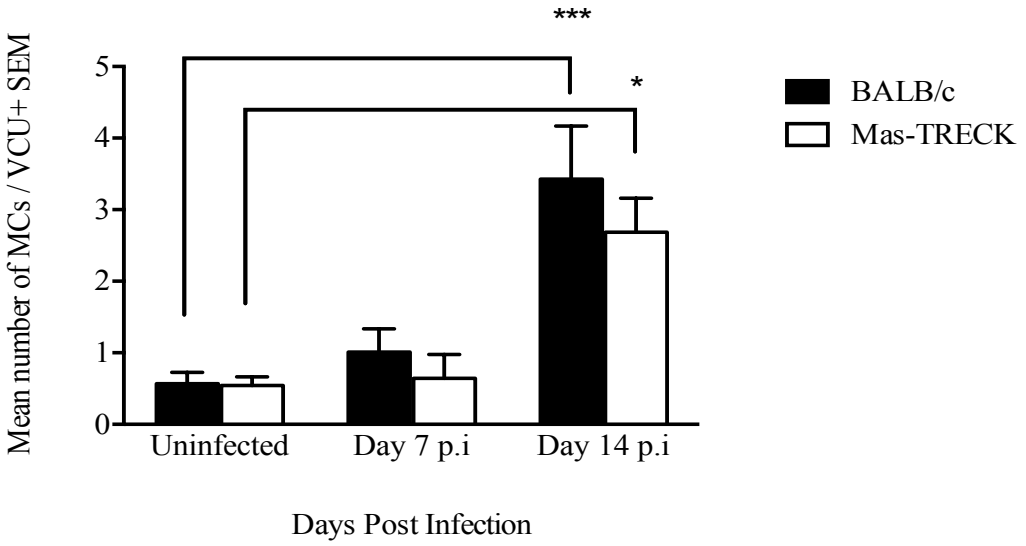


Figure 4.9 Mast cell numbers in Mas-TRECK mice. Sections of small intestine were obtained from uninfected and infected mice. The samples were processed using histological techniques and stained with Toluidine Blue for 24 hours for visualisation of mast cells, and the number of mucosal mast cells was counted per 10 villus-crypt units. BALB/c and Mas-TRECK mice were infected with 400 *T. spiralis* larvae and then sacrificed at days 7 and 14 p.i. The data are expressed as mean + S.E.M. (n=5–6), and the experiment repeated five times with similar results. Data were analysed by Kruskal Wallis test followed by a Dunn's ad hoc test to identify differences between treatments. *** P<0.001 between uninfected BALB/c mice and infected mice at day 14 p.i; *P<0.05 between uninfected Mas-TRECK mice and infected mice at day 14 p.i.

4.2.7. Production of Mouse Mast Cell Protease-1 did not indicate a significant difference

It can be seen that both mice that given infection had induced significant increase in mMCP-1 levels compared to uninfected mice. Levels of MMC β chymases mouse mast cell protease (mMCP)-1 serum were significantly increased in infected BALB/c mice at day 7 and 14 p.i compared to uninfected mice ($p=0.0079$ and 0.0034, respectively). However, these increases did not indicate a significant difference between infected mice of both strains (Figure 4.10). In addition, in Mas-TRECK mice, the mMCP-1 level was increased at day 14 p.i. compared to uninfected or infected mice at day 7 p.i, and significant differences were observed ($p=0.014$ and 0.035, respectively). Additionally, there was significant difference in mMCP-1 levels in infected Mas-TRECK mice on both days 7 and 14 p.i ($p=0.0012$). A comparison at day 7 p.i in both strains showed no statistically significant difference ($p>0.99$); an increase in mMCP-1 levels was observed in both mice strains in animals that were sacrificed at day 14 p.i, but no statistically significant difference between BALB/c and Mas-TRECK mice was observed ($p>0.99$).

Overall, these results are correlated when compared to the number of MMCs, whereby Mas-TRECK mice did not have significantly lower levels of mMCP-1, and had an MC number that was not significantly different from that of wild-type BALB/c. In Mas-TRECK mice, the level of production remained almost similar from day 7 p.i.

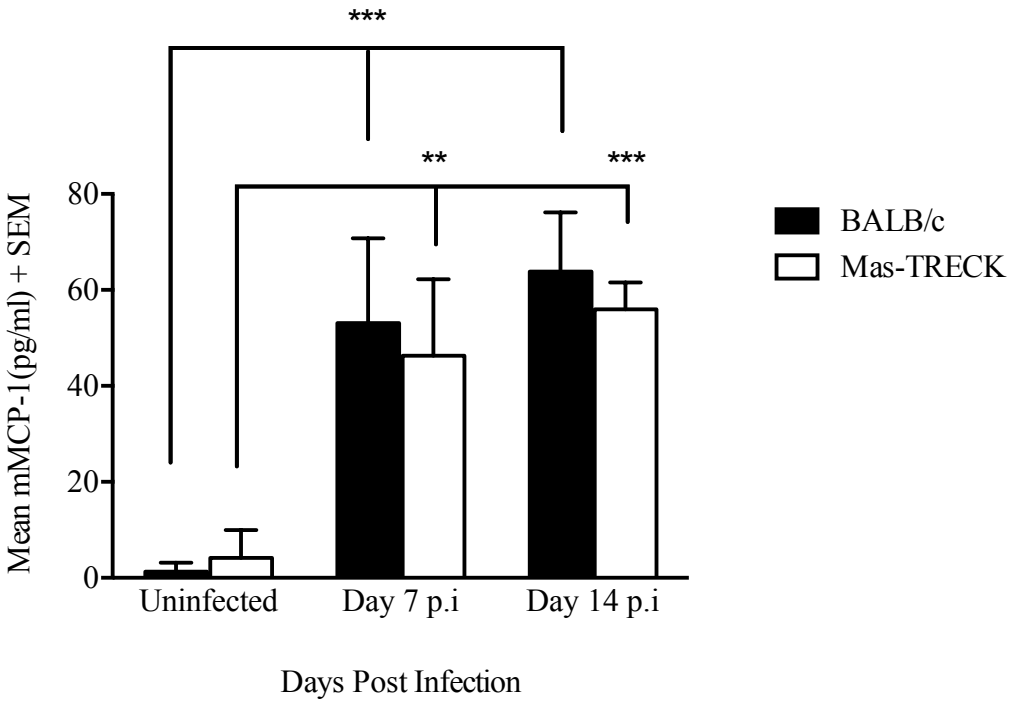


Figure 4.10 Mouse Mast Cell Protease (mMCP)-1 levels in Mas-TRECK mice. Sandwich enzyme-linked immunosorbent assay was used to measure levels of mMCP-1 in serum samples obtained from uninfected mice and mice infected with 400 *T. spiralis* larvae. The data are expressed as mean + S.E.M. (n=5–6), and the experiment repeated five times with similar results. Data were analysed by Kruskal Wallis test followed by a Dunn's ad hoc test to identify differences between treatments. *** $P<0.001$ for infected BALB/c mice at day 14 p.i against infected at day 7 p.i and uninfected mice; ** $P<0.01$ for infected Mas-TRECK mice at day 14 p.i against infected at day 7 p.i and uninfected mice; *** $P<0.001$ between Mas-TRECK mice day 7 p.i and day 14 p.i.

4.3. Discussion

MC functions have been investigated as a basic factor in the expulsion of nematodes, using mice with mutations affecting c-kit or anti-stem cell factor, which are essential for the survival of MCs. In the present study, we attempted to investigate the role of MCs in different mouse models that lack the additional abnormalities that are related to c-Kit mutation.

The Mas-TRECK transgenic mouse model has been utilized in the identification of appropriate models that could contribute to *in vivo* experiments to investigate the role of MCs in helminth infection. The study of pathological responses is important in determining the function of MCs, as they are indicators of the severity of the infection, and the number of worms is expected to decrease from day 7 p.i to day 14 p.i (Michels *et al.*, 2009). It has previously been found that Mas-TRECK mice are completely MC-deficient following treatment with DT (Sawaguchi *et al.*, 2012).

These experiments were repeated on five separate occasions with 5-6 animals per group. The number of worms and the values of other measurements were not significantly different between the repeats. The results shown are of one representative experiment. Our finding regarding the numbers of worm contrasts with the results of a study by Ierna *et al*. (2008), in which MC-deficient mice had a significantly higher number of worms at day 14 p.i, such that they had reduced intestinal pathology and were impaired in their ability to expel the parasite. In addition, the weight of the small intestine is important as an indicator of inflammation, which demands the recruitment of inflammatory lymphocytes and other mediators to the site of infection (Ierna *et al.*, 2008).

It has been shown that the development of intestinal pathology is significant for *T. spiralis* expulsion (Knight *et al*., 2000). Moreover, at low levels of infection, inflammatory changes in the gut have been observed (Dehlawi and Wakelin, 2002).

Our results showed an increase in the gut weight of Mas-TRECK mice compared to BALB/c mice. In addition, infection with *T. spiralis* encouraged the development of villus atrophy and crypt hyperplasia. This modification in the intestinal epithelium is associated with extreme apoptosis of enteropathy in the villus, leading to its shortening; the crypts then compensate for the loss of enteropathy, resulting in their

lengthening (Lawrence *et al.*, 1998a). Lawrence *et al*. (2004) showed contrasting results whereby the animals had shorter villi at day 14 p.i than the wild-type mice. Therefore, DT treatment of Mas-TRECK mice may potentially directly cause intestinal pathology and oedema rather than MC mediated effects induced by parasite infection.

The T-cell proliferation response in MLN cells was measured by *in vitro* stimulation of medium only, Con A, and *T.*Ag. Our findings showed that *T. spiralis* infection induced proliferation in response to Con A. However, there were no significant differences between the two tested mice strains. In addition, *T.* Ag induced responses in infected Mas-TRECK mice, so it is suggested that these mice may not be completely MC-deficient.

It has been shown that infection with a helminth induces a strong Th2 response. This response corresponds to a release of cytokines, such as IL-4 and IL-13, accompanied by a class switching of B-cells to IgE, in which mast cells are released; IL-5 induces eosinophils and basophils are also released (Maizels *et al.*, 2004, Jackson *et al.*, 2009a). Moreover, *T. spiralis* infection is associated with increased serum levels of parasite-specific IgG1 and total IgE. B-cell activation is an essential promoter, via IL4, in releasing IgE antibody during Th2 response (Anthony *et al.*, 2007, Suzuki *et al.*, 2008, Van Panhuys *et al.*, 2011). Thus, IL-4 deficiency results in decreased Th2 responses of antibodies that encourage MC activation and degranulation (Lawrence *et al.*, 1998a). The IgG2a response is Th1-associated, and it is expected that this will be impaired in the response, while the Th2 response is induced (Lawrence *et al.*, 1998a).

Our study showed that Mas-TRECK mice were able to induce high levels of IgG1 and low levels of IgG2a compared with wild-type BALB/c mice, which means that these mice induced a sufficient Th2 response and may not be completely MC-deficient. In accordance with our previous findings in Wsh/Wsh mice, it was found that infection of Mas-TRECK mice induced higher levels of IgG1 in comparison with the background strain in the present study. In addition, levels of specific IgG2a were reduced in Mas-TRECK mice compared with the background strain BALB/c, which is consistent with our observations in Wsh/Wsh mice.

The IgE antibody is a Th2 hallmark that is induced by *T. spiralis*. It has been found that total levels of IgE are reduced in W/W-v mice (Lawrence *et al.*, 2004). However, this observation was not replicated in Mas-TRECK mice in the present study, such that the IgE levels at day 14 p.i were significantly increased following infection with *T. spiralis* compared with day 7 p.i,, which is consistent with the results obtained from the Th2 responses.

Our results showed that level of IL-4 were not affected in Mas-TRECK mice. It has been reported that *Mcpt5-Cre; iDTR* mice showed nearly complete ablation of peritoneal MCs when assessed 24 h after a single intraperitoneal injection. However, effects on other cell types and other organs were not described (Dudeck *et al.*, 2011a). In addition, repeated once-weekly injections of *Mcpt5-Cre; iDTR* mice for 4 weeks led to complete ablation of MCs in the peritoneal cavity and abdominal skin. This was compared with *Mcpt5-Cre*$^{-}$ mice which were assessed 1 week after the last DT injection (Dudeck *et al.*, 2011a).

In addition, the effect of DT treatment in the small intestine and stomach in *Mcpt5-Cre; iDTR* mice has been analyzed, and it was shown that the depletion was not of intraepithelial MMCs, but rather of subepithelial CTMCs. The mucosal type of MCs lack *Mcpt5-Cre* transgene expression, which is the most likely reason for the absence of depletion (Dudeck *et al.*, 2011a). Therefore, the *Mcpt5-Cre; iDTR* mice utilized in the investigation of MCs in gastrointestinal helminth infection have the disadvantage of only being deficient in subepithelial CTMCs, making them unsuitable for the present study, as MMCs are crucial for protection against *T. spiralis*.

An increase of MCs in the intestine has previously been shown, with high levels of IgE and eosinophils that help to expel parasitic infection (Morimoto and Utsumiya, 2011). It has been shown that, in the deficiency observed in mastocytosis in IgE$^{-/-}$ mice, even eosinophils remained, which suggests the importance of an IgE response and cross-linking to generate mastocytosis and rapid expulsion of parasitic infection (Gurish *et al.*, 2004).

MMCs make a significant contribution to protection against the parasite by releasing mMPC-1 (Knight *et al.*, 2000). The mMCP-1 levels are at their highest during expulsion, and studies have shown an increase in the time taken for the worm to be

expelled from the host when mMCP-1 is knocked out prior to *T. spiralis* infection (Knight *et al.*, 2000).

Similar to what was observed for presence of mast cells these mice had similar levels of mMPC-1 during infection with *T. spiralis* compared to BALB/c mice, and it was also observed that they had increased MC numbers. This may be sufficient to induce lower responses, meaning that Mas-TRECK mice may not be completely MC-deficient. These results were consistent with those found in our investigation using Wsh/Wsh mice, in which it was shown that the mMCP-1 levels were not statistically significant in comparison with wild-type mice.

Sawaguchi *et al.* (2012) demonstrated that treated Mas-TRECK mice with 250 ng DT via intraperitoneal injection showed that mast cells would fully ablate without any evident disadvantageous effects. However, in the present study, side effects of diphtheria toxin treatment were detected, which encountered with survival rates of mice treated with DT. In our experiments, several mice were lost unexpectedly throughout the study, including wild-type BALB/c, and Mas-TRECK deficient mice, both uninfected and infected with *T. spiralis*.

Previous studies have shown that DT treatment may not be an effective approach for ablation. It has been reported that significant neutrophilia showed in systemic administration of DTx to $CD11c^{DTR}$ mice which resulted in increased clearance of bacteria in a mouse pyelonephritis model (Tittel *et al.*, 2012). However, Jung *et al.* (2002) demonstrated that repeated intraperitoneal administration DTx to $CD11c^{DTR}$ mice leads to toxicity that is probably due to a non-hematopoietic radioresistant cell type.

$Foxp3^{DTR}$ mice have been used to investigate local depletion of lung Foxp3+ cells, although strong depletion of Foxp3+ cells in the lung has been reported after Intratracheal injection of DTx, the administration of DTx was shown to produce crucial local inflammation, even in control mice (Chapman and Georas, 2013). In addition, Chapman and Georas (2013) have proposed that studies of DTx-mediated cell depletion are unsuitable for investigating mucosal immune responses in the lung.

It has been demonstrated that CD11c.DOG mice treated with DT are effectively depleted of alveolar macrophages from the airway, while dendritic cells in both pulmonary and systemic are protected (Roberts *et al.*, 2015). However, after 24 h post-DT treatment of inoculated mice with *Francisella tularensis* live vaccine strain, the mice died rapidly of neutrophilic pneumonia (Roberts *et al.*, 2015), even before infected MyD88 knockout mice with similar doses of *Francisella* succumb to infection (Abplanalp *et al.*, 2009, Collazo *et al.*, 2006).

The role of plasmacytoid DCs (pDCs) in atherosclerosis has been assessed in BDCA-2 DTR mice treated with DT. However, pDCs occurred following the end of treatment, limiting the assessment to acute atherosclerosis (Mandl *et al.*, 2015). In addition, evaluation of the role of dendritic cells (DCs) in innate and adaptive immunity, have shown that the results are inconclusive due to the lymph node difficulties resulting from the DT treatment. Moreover, it has been suggested that DTR may be present in other cells which is the immune environment may be affected ,thus the results are not specifically DC related (Van Blijswijk *et al.*, 2015).

Along with our finding in the present study, we conclude that DT treatment is not the most appropriate method for investigating the role of an immune response and that genetic manipulation for mast cells might be more suitable. In addition, further research is required to confirm that Mas-TRECK mice can be used to investigate the role of MCs in the expulsion of *T. spiralis.*

Overall, our findings indicate that the use of DT does not induce changes between wild-type BALB/c and Mas-TRECK mice, which may occur due to differences between connective tissue mast cells (CTMCs) and mucosal mast cells (MMCs), which perhaps the other models of inducible depletion of MCs are focusing on CTMCs rather than MMCs as we cannot be certain these are completely ablated. Our evidence suggests that MMCs are not completely depleted in Mas-TRECK mice and that these mice are not a suitable model for the evaluation of mast cell responses to gastrointestinal helminths infection, and the type of these mice should be use with caution.

CHAPTER 5. ACTIVATION OF MC BY HELMINTH PRODUCTS

5.1. Introduction

Mast cells are an adaptable cell type with essential roles in the morphogenesis of tissue and host defence against parasites and bacteria. Mast cells are developed from the myeloid lineage and show similarities to basophilic granulocytes. Mast cells and basophils both contain large amounts of histamine (Meurer *et al.*, 2016). It is believed that histamine plays role in allergies, but its involvement in a variety of immunopathologies has also been shown (Marshall *et al.*, 2003). Vasodilation, capillary permeability, bronchoconstriction and smooth muscle contraction have been observed as a result of releasing histamine (Lundequist and Pejler, 2011).

In the destination tissue, mast cells are mature and adopt either the connective tissue (CTMC) or mucosal (MMC) type. The activation or degranulation of mast cells induces different effector functions, which lead to the production of a typical set of MC proteases (MCPT) and secretion of preformed or newly synthesised mediators from granules into the local microenvironment. Mast cell lines can have mutations in key signalling pathway elements which make cultured mast cells preferable for the study of general functions and features of mast cell biology in human disease (Meurer *et al.*, 2016).

Tissue mast cells have been shown to occur widely as two distinct phenotypes; these phenotypes are derived from bone marrow precursors. In the mucosal layer of the gastrointestinal tract, the granule proteoglycan of mucosal type mast cells (MMC) presented low levels of histamine and expressed chondroitin sulphate (Galli, 1993, Galli, 1990). In addition, an intact thymus and T cell-derived IL-3 are important for MMC differentiation (Haig *et al.*, 1982, Ihle *et al.*, 1983).

In contrast, connective tissue mast cells (CTMCs) dominate in the skin, and CTMCs predominate in the peritoneal cavity in rodents. The granule proteoglycan of connective tissue mast cells contains high levels of histamine and express heparin (Galli, 1990, Galli, 1993). Moreover, stem cell factor (SCF), which is the ligand for tyrosine kinase encoded by the proto-oncogene c-*kit,* is required for CTMC differentiation (Wershil *et al.*, 1992, Tsai *et al.*, 1991, Galli *et al.*, 1993a). However, it has been shown that CTMC growth and secretory functions *in vitro* can be affected

by T cell-derived cytokines. It has been determined that the fully differentiated mouse peritoneal CTMCs in clonal proliferation are stimulated by the incorporation of IL-3 and IL-4 (Hamaguchi *et al.*, 1987, Tsuji *et al.*, 1990) an effect that is antagonised by IFN-γ (Takagi *et al.*, 1990). In addition, these cytokines have been shown to influence the secretory behaviour of CTMCs, whereby IgE/antigen-induced mediator release from mouse peritoneal mast cells is enhanced by IL-3 and/or IL-4 (Coleman *et al.*, 1993) and inhibited by IFN-γ (Coleman *et al.*, 1991).

5.1.1. MCs activation via FcεRI

An abundance of cell surface receptors is expressed by mast cells which have the ability to influence responses of mast cells via the regulation of proliferation, migration and activation. However, KIT and FcεRI, the high affinity receptors for IgE, are the two major receptors responsible for regulating functions of mast cells (Okayama and Kawakami, 2006, Kraft and Kinet, 2007). KIT is a crucial anti-apoptotic molecule and growth factor for mast cell generation from progenitors and also for the successive survival of mature mast cells and tissue-resident mast cells. However, stem cell factor (SCF)-dependent KIT activation also amplify antigen-mediated degranulation of mast cells and the production of cytokines (Tkaczyk *et al.*, 2004, Hundley *et al.*, 2004). Also, it has been shown that KIT and FcεRI-initiated signalling events interacted together to enhance mast cell chemotaxis (Kuehn *et al.*, 2010). In addition, these responses have been previously observed with a number of GPCR agonists including PGE_2 and adenosine (Kuehn *et al.*, 2010).

Mast cell characterisation is crucial for the assessment of mast cell differentiation or isolation (Kovarova, 2013). FcεRI expression on the surface of cell is not only pivotal for the function of mast cells in allergic reactions, but together with *c-Kit* expression, they can also be utilised as a milestone for the successful maturation and differentiation of bone marrow-derived mast cell cultures (Kovarova, 2013).

Moreover, it has been determined that the expression of FcεRI receptor in rodents is limited to mast cells and basophils (Kovarova, 2013). Mast cells have been extensively studied using bone marrow-derived mast cells (BMMCs) as a mast cell model. These cells are immature cells with no known physiological equivalent in tissues, and do not respond to IgG immune complexes. Although resident peritoneal

mast cells (PCMC) are not easily extracted as they are a minor population of differentiated cells, these cells can be expanded in culture to generate large numbers of homogeneous cells (Malbec *et al.*, 2007). However, it has been demonstrated that in the absence of antigen, FcεRI combined with IgE will induce degranulation of mast cells (Kashiwakura *et al.*, 2011, Gilfillan and Metcalfe, 2011).

This results in the induction of chemokine production, chemotaxis of mast cells and the inhibition of apoptosis (Kawakami and Galli, 2002). Nevertheless, the requirement for FcεRI crosslinking to provoke the essential signalling events and range of antigen-induced release of inflammatory mediators has been shown (Kawakami and Kitaura, 2005). Low airway inflammation showed in mice lacking the FcεRI α chain compared to wild-type mice in which decreased concentrations of eosinophil have been reported in an asthma model (Mayr *et al.*, 2002). Studies in wild-type, genetically mast-cell–deficient and mast-cell–knockin mice showed that the activation of MCs via FcRγ-dependent mechanisms is a key promoter of many of the clinical features of chronic asthma model (Yu *et al.*, 2006, Yu *et al.*, 2011).

5.1.2. MCs activation via Toll-Like Receptor (TLR)

Mast cells have been previously examined in studies to investigate responses of these cells to other stimuli, such as bacterial and viral products. These studies have demonstrated that mast cells are capable of producing very different types of mediators, and their production is dependent upon the mechanism used to activate cells (Marshall *et al.*, 2003).

Agonists of Toll-Like Receptors (TLR) including bacterial, viral, parasitic, and host defence products can induce activation of mast cell. These studies showed that mast cells are a significant part of innate immune responses to a variety of pathogens (Shelburne and Abraham, 2011). However, it has been suggested that unlike antigen-, KIT-, and GPCR-mediated responses, these are probably limited to the induction of the production of cytokines and chemokines. Moreover, it has been demonstrated that when mast cells are co-activated through the TLRs and FcεRI, the responses of KIT and GPCRs are cooperatively improved (Qiao *et al.*, 2006).

A number of pro-inflammatory MC responses are induced in Gram-positive or Gram-negative bacteria and cell wall components such as peptidoglycan (PGN) from Gram-positive or lipopolysaccharide (LPS) from Gram-negative bacteria are potent activators (Mccurdy *et al.*, 2001). CD14 on macrophages binds erythrocytes coated with LPS and LBP, but not erythrocytes coated with LPS or LBP alone (Wright *et al.*, 1989, Wright *et al.*, 1990). LPS binds to the serum protein, LPS-binding protein (LBP), and is subsequently transferred to the glycosylphosphatidylinositol-anchored membrane protein (CD14) (Hailman *et al.*, 1994). However, activation of endothelial and epithelial cells is mediated by soluble CD14 (sCD14) as these cells lack membrane expression of CD14 and an intracellular signalling domain (Haziot *et al.*, 1993).

Therefore, additional molecules with intracellular-signalling capacity are required for cells that lack membrane expression of CD14, including endothelial and epithelial cells. Members of the Toll-like receptor (TLR) superfamily have been demonstrated to serve this function (Mccurdy *et al.*, 2001). It has been suggested that in macrophages that TLR2 was the signalling receptor for LPS (Kirschning *et al.*, 1998, Tang *et al.*, 1998). However, there is evidence that mutations in the *Lps* gene map to TLR4 (Poltorak *et al.*, 1998, Hoshino *et al.*, 1999, Qureshi *et al.*, 1999). It has been shown that mast cells are activated by LPS, without degranulation, to produce IL-6 and TNF-α (Leal-Berumen *et al.*, 1994, Marshall *et al.*, 1996).

Additionally, McCurdy *et al* (2001) reported that mRNA is expressed by mast cells for several TLRs including TLR2, TLR4, and TLR6 but not TLR5 which suggests that through TLR ligation mast cells have the possible direct response to a variety of invading pathogens (Mccurdy *et al.*, 2001).

Activation of murine mast cells TLR2 and TLR4 by LPS stimulation results in production of cytokines (Mccurdy *et al.*, 2001) and stimulation of rat peritoneal mast cells stimulated with LPS and PGE_2 showed that there was not a direct relationship between mast cell degranulation and IL-6 production (Leal-Berumen *et al.*, 1995, Leal-Berumen *et al.*, 1994). C3H/HeJ and C57Bl/10ScNCr mice contain a point mutation and a null mutation, respectively, in TLR4. Using these models, it has been

demonstrated that IL-6 and TNF-α responses by BMMC derived from these mice stimulated with LPS were completely dependent on having a functional TLR4 as LPS failed to induce PGN (Mccurdy *et al.*, 2001, Leal-Berumen *et al.*, 1996). Moreover, rat peritoneal MC treated with IL-12 demonstrated the induced production of IFN-γ (Gupta *et al.*, 1996). Additionally, it has been shown that mouse mast cells treated with CpG-containing oligonucleotides can induce the production of TNF-α without degranulation (Marshall *et al.*, 2003).

The role of TLR4 in mast cell innate immunity *in vivo* has been studied W/W-v mice given in derived BMMC to correct their MC deficiency in an acute septic peritonitis model induced by caecal ligation and puncture. Reconstituted W/W-v mice given TLR4-mutated BMMCs (C3H/HeJ) had a significantly higher mortality than W/W-v mice reconstituted with TLR4-intact BMMCs (C3H/HeN). These studies of the reconstitution of W/W-v mice with BMMC from C3H/HeN but not C3H/HeJ mice showed that host survival was significantly enhanced compared to W/W-v mice (Supajatura *et al.*, 2001).

5.1.3. MCs activation via complement system

Different methods for the activation of mast cells have been determined which include the complement system, which is widely considered to be necessary for normal inflammation, which has the ability to activate mast cells (Altman, 1997, Akazawa *et al.*, 1995, Ben-Arie *et al.*, 1996, Jarman *et al.*, 1993, Hatten and Heintz, 1995). Mast cells can be activated through complement pathways. Immune complexes activate receptors on the mast cell, for C3a and C5a. An experiment using C3-deficient mice found that treating the mice with C3 protein showed that peritoneal mast cells were reduced (Prodeus *et al.*, 1997).

The complement role in mast cell-dependent natural immunity has been investigated by studying the complement-deficient mouse responses (Debus *et al.*, 1984, Tohyama *et al.*, 1992) to caecal ligation and puncture (Smeyne and Goldowitz, 1989). In addition, reductions in peritoneal mast cell degranulation have been observed in C3-deficient mice with TNF-production, neutrophil infiltration and expulsion of bacteria. Consequently, the complement activation presented in these results provided evidence that complement is crucial for the complete expression of

innate immunity in the mast cell-dependent model used for bacterial infection (Prodeus *et al.*, 1997).

Human skin mast cell chemotaxis can be stimulated by C3a or C5a (Hartmann *et al.*, 1997) as well as degranulation *in vitro* (Nigrovic *et al.*, 2010, Kubota, 1992). In addition, wheal and flare reactions have been shown in normal mouse or human skin after injection of C3a or C5a, associated with dermal degranulation of mast cell and polymorphonuclear leukocyte rich inflammatory infiltrates (Lim *et al.*, 1991, Lepow *et al.*, 1970, Yancey *et al.*, 1985, Swerlick *et al.*, 1989). It has been demonstrated that the intradermal injection of C3a or C5a induced much of the local inflammation in mast cell-engrafted KitWsh/Wsh mice in which mast cells at the site of the reaction are required to express the appropriate anaphylatoxin receptors (Schäfer *et al.*, 2013). Impaired neutrophil recruitment in a model of immune complex arthritis has been shown recently in $C5aR^{-/-}$ mice in which mast cells also have been implicated (Nigrovic *et al.*, 2010).

5.1.4. MCs activation via an Ig-independent pathway

In addition, activation of MC has been reported with other helminth extracts through the binding of non-specific IgE (Schramm *et al.*, 2007). It has been demonstrated that freshly isolated rat peritoneal MC can be activated by *T. spiralis* muscle larval (TSL-1) antigens via an Ig-independent mechanism resulting in histamine, mMCP-5 and TNF production (Arizmendi *et al.*, 1997). Arizmendi-Puga *et al*. (2006) reported that TSL-1 antigens induced MC which release fast of histamine is similar to the release of Substance P histamine which occurs via a Ca^{2+} independent pathway which propose that activation of MC is induced by TSL-1 antigens through an IgE-independent pathway. Moreover, it has been demonstrated that unsensitised MC that directly bind to TSL-1 antigens resulting in MC activation and histamine production, and increased IL-4 and TNF at the mRNA and levels of protein (Arizmendi *et al.*, 2001, Niborski *et al.*, 2004).

It has been shown that rat peritoneal mast cells released histamine following exposure to the muscle larvae (ML) stage of *T. spiralis* (TSL-1 antigens) glycoproteins (Arizmendi-Puga *et al.*, 2006). In addition degranulation of MC in

neonates and adults during parasite expulsion was associated with the release of rat mast cell protease-II (RMCPII), which was detected in the sera at 3 hours post-challenge (Blum *et al.*, 2009). Therefore, the *in vitro* models of the rat mucosal mast cell, the rat basophilic leukemia cell line (RBL-2H3) and bone marrow-derived mast cells (BMMC), have been evaluated to compare BMMC and RBL-2H3 cells as models for antibody-mediated mast cell activation (Thrasher *et al.*, 2013). It has been demonstrated that a strong mucosal phenotype exhibited by BMMC are phenotypically apparent from RBL-2H3 cells, which confirms that in the context of IgG-mediated activation and parasitic worm infection BMMC and RBL-2H3 cells are beneficial models for mucosal mast cells (Thrasher *et al.*, 2013).

Mast cells have showed to be activated in various T cell-mediated inflammatory processes (Kashiwakura *et al.*, 2004, Nakae *et al.*, 2006). Gri *et al.,* (2008) have reported that mast cell degranulation can be inhibited by T regulatory (T reg) cells via cell-cell contact. In addition, it has been demonstrated that the interaction of OX40-OX40L drives the inhibition of mast cell degranulation of the extent of MC degranulation *in vitro* and of the immediate hypersensitivity response *in vivo* (Gri *et al.*, 2008).

Mast cells have been studied in BMMC models in which they are phenotypically different from PCMC populations. These differences are associated with the maturation level and the expression level of mast cell protease (Kovarova, 2013). It has shown that BMMCs are similar to mucosal mast cells *in vivo*, as they contain high amounts of chondroitin sulphate and small amounts of heparin (Humphries *et al.*, 1999). In contrast PCMCs have a phenotype that is more similar to connective tissue mast cells as they contain small amounts of chondroitin sulphate and large amounts of heparin (Humphries *et al.*, 1999). The glycosaminoglycan (GAG) attached to serglycin differ in the two populations of mast cells (Razin *et al.*, 1982).

From our previously described results, it is evident that Mas-TRECK and Wsh/Wsh MC deficient mice do appear to have the capacity to produce mast cells. W/W-v and Wsh/Wsh mice, in which KIT is expressed on the surface, or KIT catalytic activity is seen, have considerably reduced mast cell numbers (Tsai *et al.*, 2011a). Although cultured human mast cells require SCF for growth, the expansion and growth of

mouse mast cells from bone marrow progenitors can be maintained by IL-3 in the absence of SCF (Kirshenbaum *et al.*, 1999). This may therefore suggest that in certain circumstances mast cells may develop without the requirement for SCF signalling.

If mast cells play a role in the induction and initiation of Th2 immune responses associated with nematode infection, prior to the induction of a specific IgE response, then their ability to be activated via IgE independent mechanisms needs to be evaluated. Therefore, the aims of these studies were firstly to determine if parasite antigens could activate mast cells in the presence and absence of IgE.

Further, we sought to determine if BMMC could be generated *in vitro* from bone marrow from mast cell deficient mice. Thus, bone marrow-derived mast cells (BMMCs) and peritoneal mast cells (PCMCs) obtained from Wsh/Wsh and Mas-TRECK and their corresponding wild types (C57BL/6 and BLAB/c respectively) were cultured *in vitro* in the presence of different growth factors i.e. SCF, IL-3, and IL-9.

The cells were then stimulated with various ligands known to activate mast cells as well as helminth antigens in the presence and absence of IgE and MC and activation was assessed by determining production of hexosaminadase, IL-4 and mMCP-1.

5.2. Results

5.2.1. Peritoneal Mast Cell (PCMC) numbers and maturity

In order to determine if PCMCs were present in MC-deficient mice or if they responded to growth factors, PCMCs were removed from the peritoneal cavity and cultured for 7-12 days in Dulbecco's Modified Eagle Medium (DMEM) medium in a culture medium. Overall, cell numbers dropped after the first day of culture (day 0), and each strain presented similar growth. However, the growth was not significantly different between mast cell-deficient mice and wild-type mice. Mast cell-deficient mice presented similar cell numbers compared to wild-type mice (Figure 5.1).

To determine the maturity of cultured PCMC cells, samples of cell suspensions were stained using toluidine blue in 1% NaCl solution. Progenitor mast cells were distinguished from mature mast cells. Mast cells of all strains presented a similar percentage of maturity after 7-12 days (Figure 5.2).

These experiments were repeated on two separate occasions using 2-3 mice/treatment. Similar results were obtained in repeat experiments therefore data from one representative experiment is shown**.**

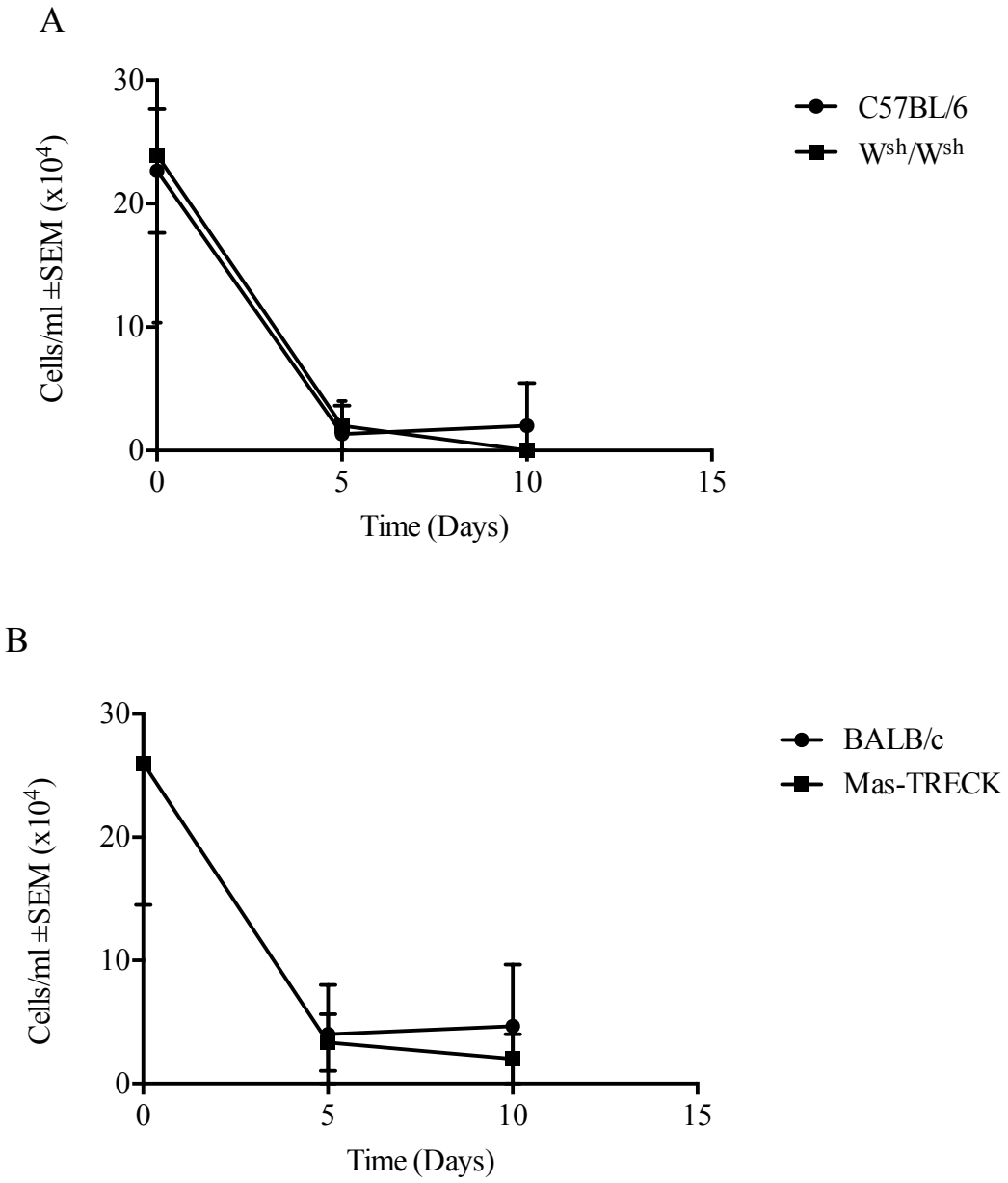


Figure 5.1 Growth of PCMCs cultured for different time periods. PCMCs were removed at day 0 and cultured for 7-12 days in 40 ml complete Dulbecco's Modified Eagle Medium (DMEM) supplemented with growth factors IL-3 (1ng/ml), IL-9 (5ng/ml) and SCF (50ng/ml). Trypan blue was used to assess cell growth. (A) Mast cell growth of C57BL/6 and Wsh/Wsh mice. (B) Mast cell growth of BALB/c and Mas-TRECK mice. Data are expressed as mean ± S.E.M. (n=3). The experiment repeated twice with similar results.

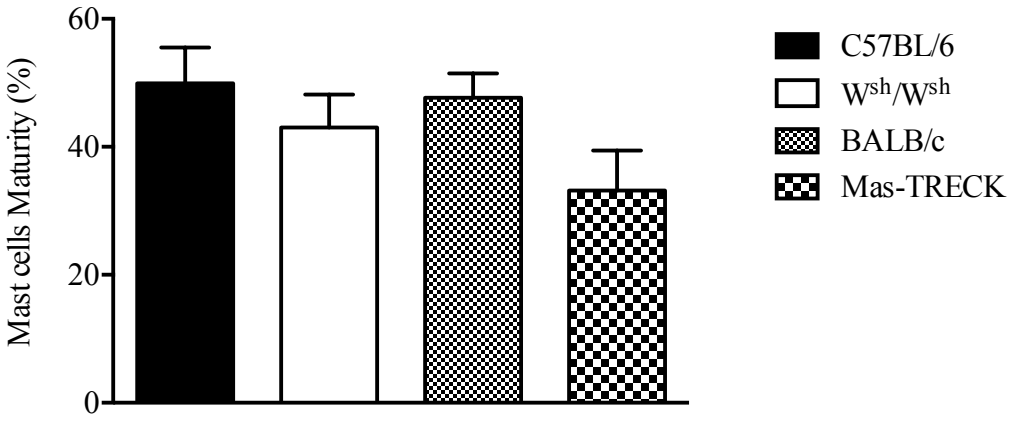


Figure 5.2 PCMC maturity in vitro after 7-12 days from different mouse strains. Samples were mixed 1:1 with Trypan blue and cells were counted using a haemocytometer. The maturity of mast cells was measured by smearing samples onto glass slides. The slides were fixed in 75% ethanol for 3 min, stained in 10% Toluidine blue in a 1% NaCl solution for 90 min and counted under a microscope. Data are expressed as percentage of positively stained cells+ S.E.M (n=3). Mast cell maturity was estimated by toluidine blue staining. The experiment repeated twice with similar results. Analysis of the data using a Kruskal Wallis test followed by a Dunn's ad hoc test showed there was no significant difference between treatments.

5.2.1.1 β-hexosaminidase was not released in the stimulation of PCMCs in MC-deficient mice

To assess mast cell degranulation with different stimulants, β-hexosaminidase was measured as an indicator of activated mast cell degranulation after stimulation of mast cells with medium, lipopolysaccharide (LPS), *Trichinella spiralis* antigen (*T.* Ag), *Trichinella spiralis* positive serum or normal mice serum (NMS).

The release of β-hexosaminidase was observed in stimulated PCMC obtained from C57BL/6 wild-type mice which resulted in the greatest release that can be observed with both sensitised and unsensitised sera at a concentration of (*T.* Ag 0.2 μg/ml). In addition, a comparison to controls and stimuli result in detectable releases ($P < 0.001$). A significant difference between C57BL/6 and Wsh/Wsh mice was observed ($P<0.01$, Figure. 5.3).

Additionally, stimulation with LPS did not show any significant differences between BALB/c and Mas-TRECK mice. Moreover, stimulation with specific antigen did cause a significant increase in the release of β-hexosaminidase by PCMC from all strains of mice compared to unstimulated controls and β-hexosaminidase released by PCMC stimulated with *T. spiralis* positive serum was significantly increased between strains of mice (Figure. 5.4).

PCMC from WT mice released β-hexosaminidase after stimulation with parasite antigen in the absence of IgE, and the effect was increased in the presence of IgE, suggesting that mast cells have IgE independent degranulation mechanisms.

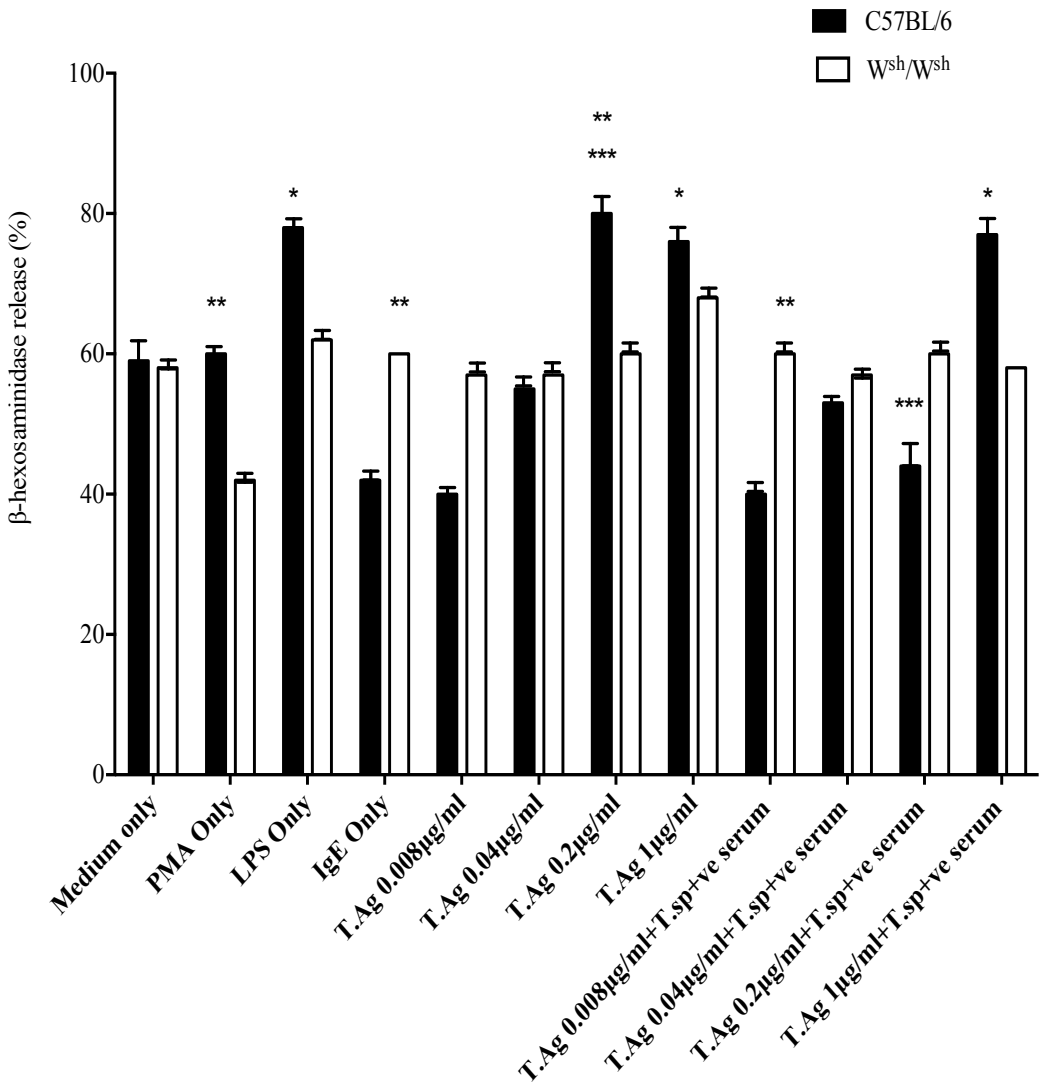


Figure 5.3 Release of β-hexosaminidase after stimulation of PCMC from C57BL/6 and Wsh/Wsh mice. Cell cultures were either stimulated with medium only, or stimulated with PMA (0.5µg/ml), *T. spiralis* specific IgE (10µg/ml), LPS (10µg/ml), wells contained $5x10^5$ mast cells. Cell culture assays were incubated at 37^oC and 5% CO_2 for 1 hour. The absorbance was read at 405nm wavelength. Data represents mean (%) degranulation + SEM (n=3), and the experiment was repeated twice with similar results. Data were analysed by Kruskal Wallis test followed by a Dunn's ad hoc test to identify differences between treatments. * represents $P<0.05$ and ** $P<0.01$ represent significant comparing WT vs Wsh/Wsh mice. *** represents $P<0.001$ comparing unstimulated vs stimulated.

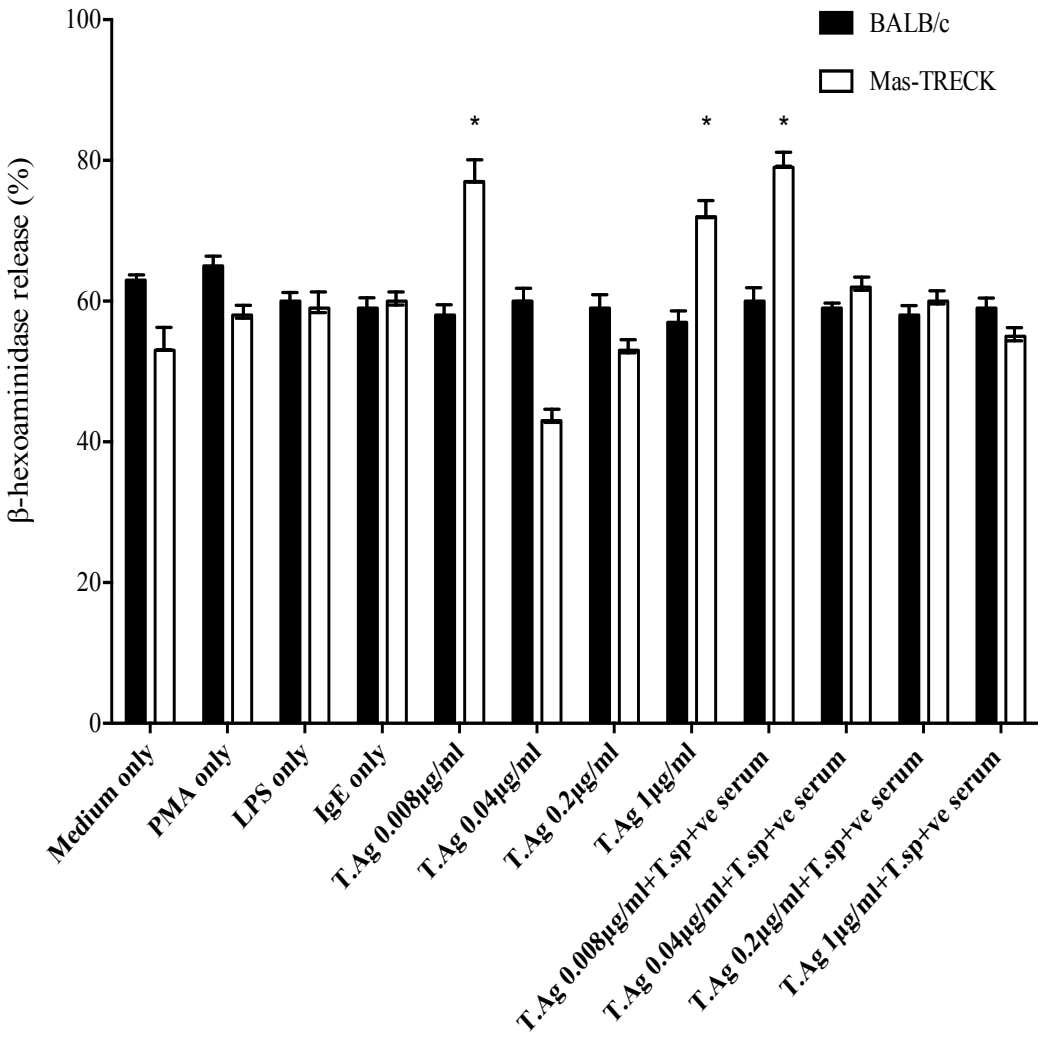


Figure 5.4 Release of β-hexosaminidase after stimulation of PCMC from BALB/c and Mas-TRECK mice. Cell cultures were either stimulated with medium only, or stimulated with PMA (0.5µg/ml), *T. spiralis* specific IgE (10µg/ml), LPS (10µg/ml), wells contained $5x10^5$ mast cells. Cell culture assays were incubated at 37^oC and 5% CO_2 for 1 hour. The absorbance was read at 405nm wavelength. Data represents mean (%) degranulation + SEM (n=3). *P<0.05 significant comparing WT vs Mas-TRECK mice. The experiment was repeated twice with similar results. Data were analysed by Kruskal Wallis test followed by a Dunn's ad hoc test to identify differences between treatments.

5.2.1.2 IL-4 was released after stimulation of PCMC cells

Levels of IL-4 were measured as an indicator of the capacity of MC to initiate and amplify a Th2 response. PCMC cells were stimulated, and IL-4 was measured using an ELISA.

Under stimulation the highest release of IL-4 was observed in stimulated PCMC obtained from C57BL/6 mice at (0.008µg/ml *T.* Ag) for both sensitised and unsensitised sera and with sensitised sera alone at (1µg/ml *T.* Ag +*T. spiralis* positive serum) with significance values of ($P<0.05$). In addition, both (0.008µg/ml *T.* Ag) stimulants were significantly higher when compared to sensitised (1µg/ml *T.* Ag + *T. spiralis* positive serum) and controls. However, no significant difference between both sensitised and unsensitised sera at (0.008µg/ml *T.* Ag) was observed.

Under stimulation of sensitised and unsensitised sera with *T.* Ag at 0.008µg/ml in stimulated PCMC obtained from Wsh/Wsh mice showed a significant increased release of IL-4 ($P<0.05$) in comparison to control stimulation. However, positive stimulation results did not show significance differences (Figure. 5.5).
PCMC cells from BALB/c resulted in significantly higher release of IL-4 at a concentration of (0.008µg/ml *T.* Ag) under stimulation of sensitised and unsensitised sera ($P<0.05$). However, no significant difference between these results was observed (Figure. 5.6).

Additionally, stimulation of both sensitised and unsensitised sera at a concentration of (0.008µg/ml *T.* Ag) show significant release of IL-4 in Mas-TRECK mice ($P<0.05$) in comparison to control stimulation. However, no statistical difference was observed between mast cell stimulants (Figure 5.6).

In this study, it was found that *T. spiralis* antigen (*T.* Ag) alone was able to activate mast cells from WT, Mas-TRECK and Wsh/Wsh mice to release IL-4 in all strain of mice. In addition, the findings show that the host would have the ability to mount a Th2 response and amplify it against *T. spiralis* infection.

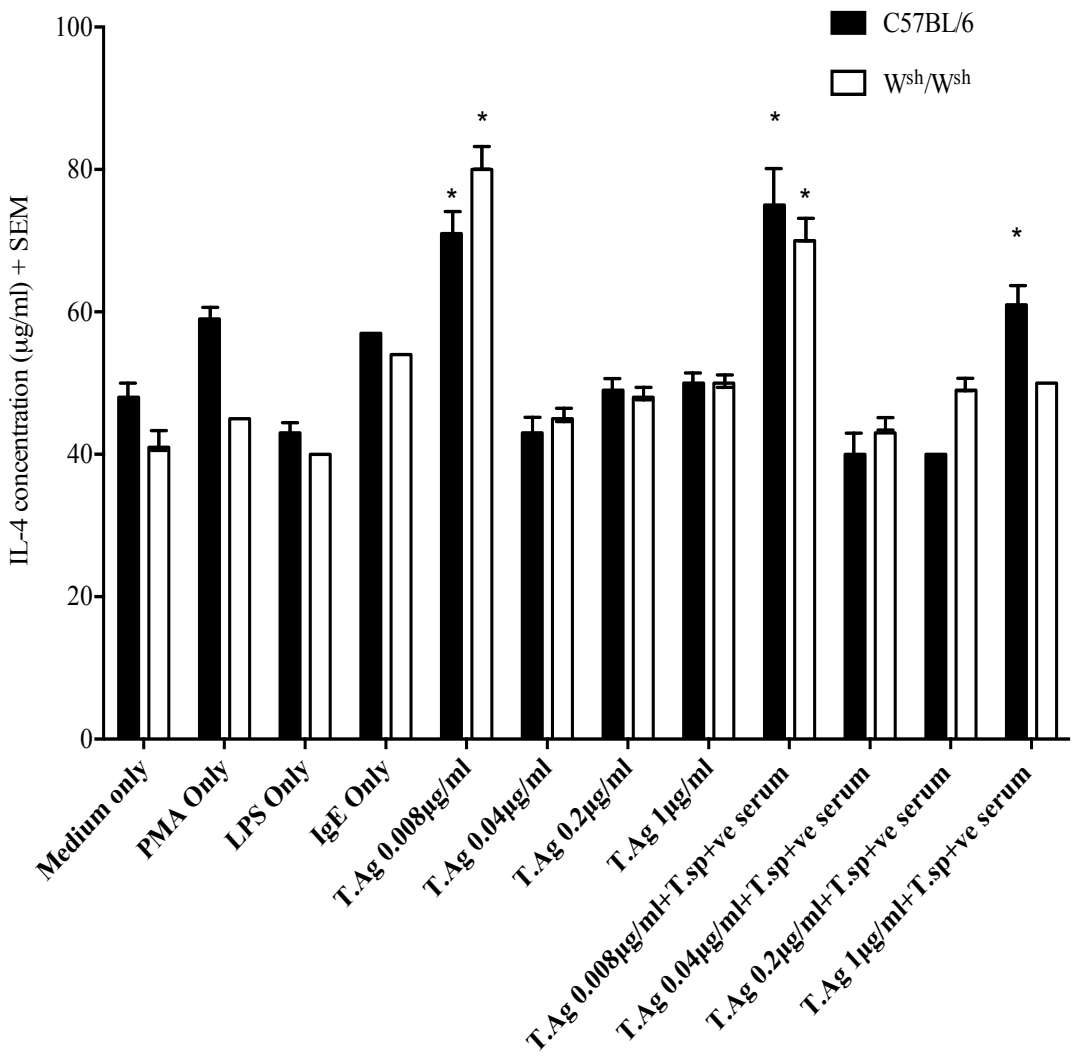


Figure 5.5 Release of IL-4 after stimulation of PCMC from C57BL/6 and Wsh/Wsh mice. Cell cultures were either stimulated with medium only, or stimulated with PMA (0.5µg/ml), *T. spiralis* specific IgE (10µg/ml), LPS (10µg/ml), wells contained $5x10^5$ mast cells. Standard dilutions (mouse IL-4 standard 150 pg/ml) and stimulated cell samples were added to each plate. Plates were incubated for 2h at 37°C. Absorbance was read at 450 nm wavelength. Data are expressed as mean + S.E.M. (n=3). The experiment was repeated twice with similar results. Data were analysed by Kruskal Wallis test followed by a Dunn's ad hoc test to identify differences between treatments. * represents $P<0.05$ comparing unstimulated vs stimulated.

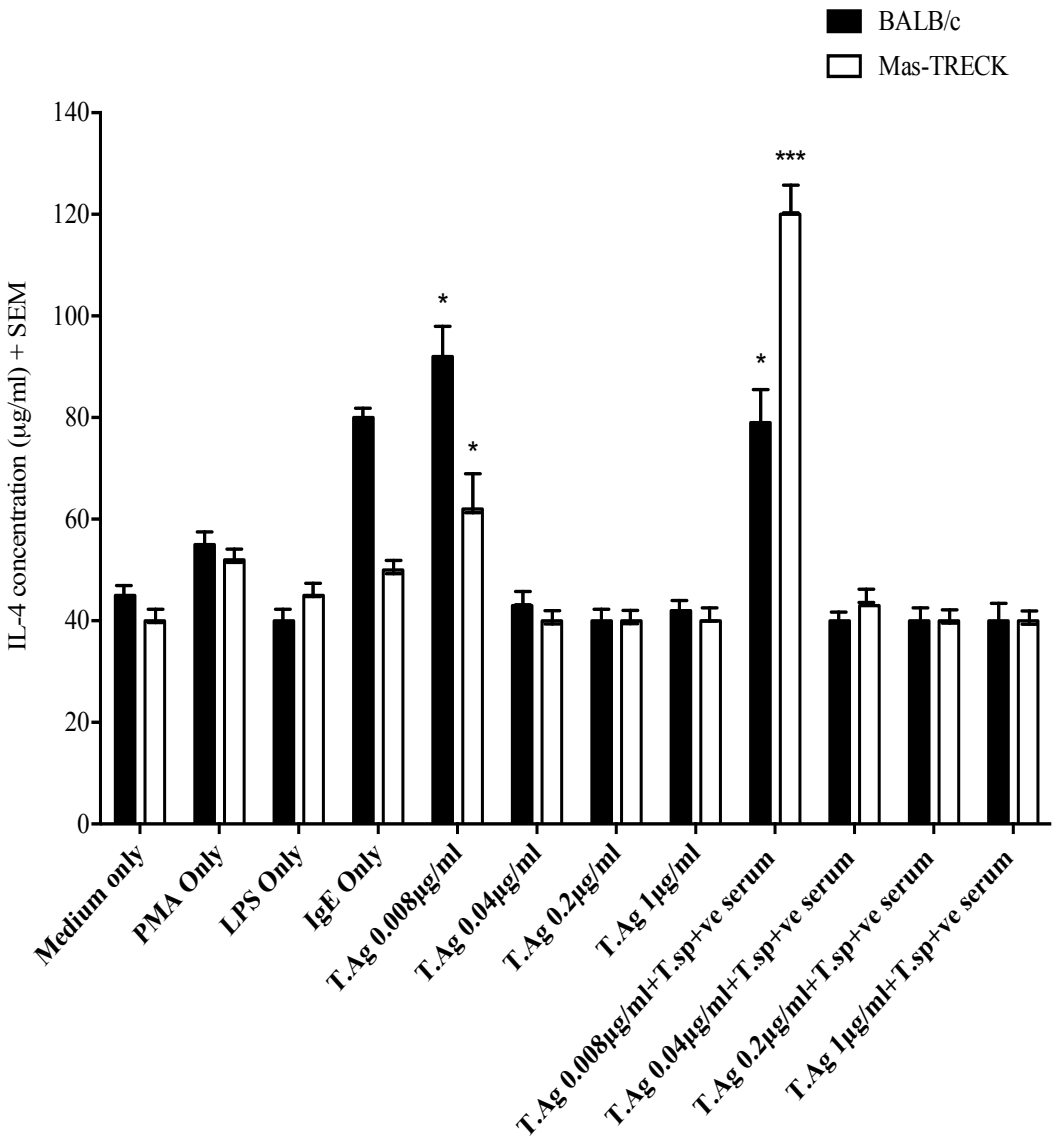


Figure 5.6 Release of IL-4 after stimulation of PCMC from BALB/c and Mas-TRECK mice. Cell cultures were either stimulated with medium only, or stimulated with PMA (0.5µg/ml), *T. spiralis* specific IgE (10µg/ml), LPS (10µg/ml), wells contained $5x10^5$ mast cells. Standard dilutions (mouse IL-4 standard 150 pg/ml) and stimulated cell samples were added to each plate. Plates were incubated for 2h at 37°C. Absorbance was read at 450 nm wavelength. Data are expressed as mean + S.E.M. (n=3). The experiment was repeated twice with similar results. Data were analysed by Kruskal Wallis test followed by a Dunn's ad hoc test to identify differences between treatments. * represents $P<0.05$ and *** represents $P<0.001$ comparing unstimulated vs stimulated.

5.2.1.3 Levels of mMCP-1 were not induced in PCMC cells of all strains

Mucosal mast cell protease-1 (mMCP-1) has previously been shown to be significant in the induction of pathology and necessary for the successful expulsion of *T. spiralis* (Lawrence *et al.*, 2004). PCMC cells from all strains did not show significant secretions of mMCP-1 from cells stimulated with either stimulated *T. spiralis* positive serum alone or in combination with *T.* A*g*.

Stimulation of mast cells with medium or LPS was not able to activate mast cells to produce detectable levels of mMCP-1 in all strains. However, *T. spiralis* antigen combination with *T. spiralis* positive serum resulted in the productions of significant levels of mMCP-1 in PCMC from C57BL/6, these cells also showed significantly elevated secretions of mMCP-1 when compared with medium only (p=0.02) (Figure. 5.7). In addition, a significant difference between C57BL/6 and Wsh/Wsh mice were observed (P<0.01). PCMCs from Wsh/Wsh mice did not show a significant rise in the level of mMCP-1 was observed compared to the medium control.

PCMCs from both BALB/c and Mas-TRECK showed significantly (p=0.0001-p=0.04) raised secretions of mMCP-1 compared to medium or LPS by cells stimulated with either *T. spiralis* positive serum alone or in combination with *T.* Ag (Figure. 5.8).

Overall, the findings show that *T. spiralis* antigen alone is not able to stimulate PCMC cells to release levels of mMCP-1. PCMC cells from Mast cell-deficient Wsh/Wsh and Mas-TRECK were shown to release similar levels of mMCP-1 compared to wild-type.

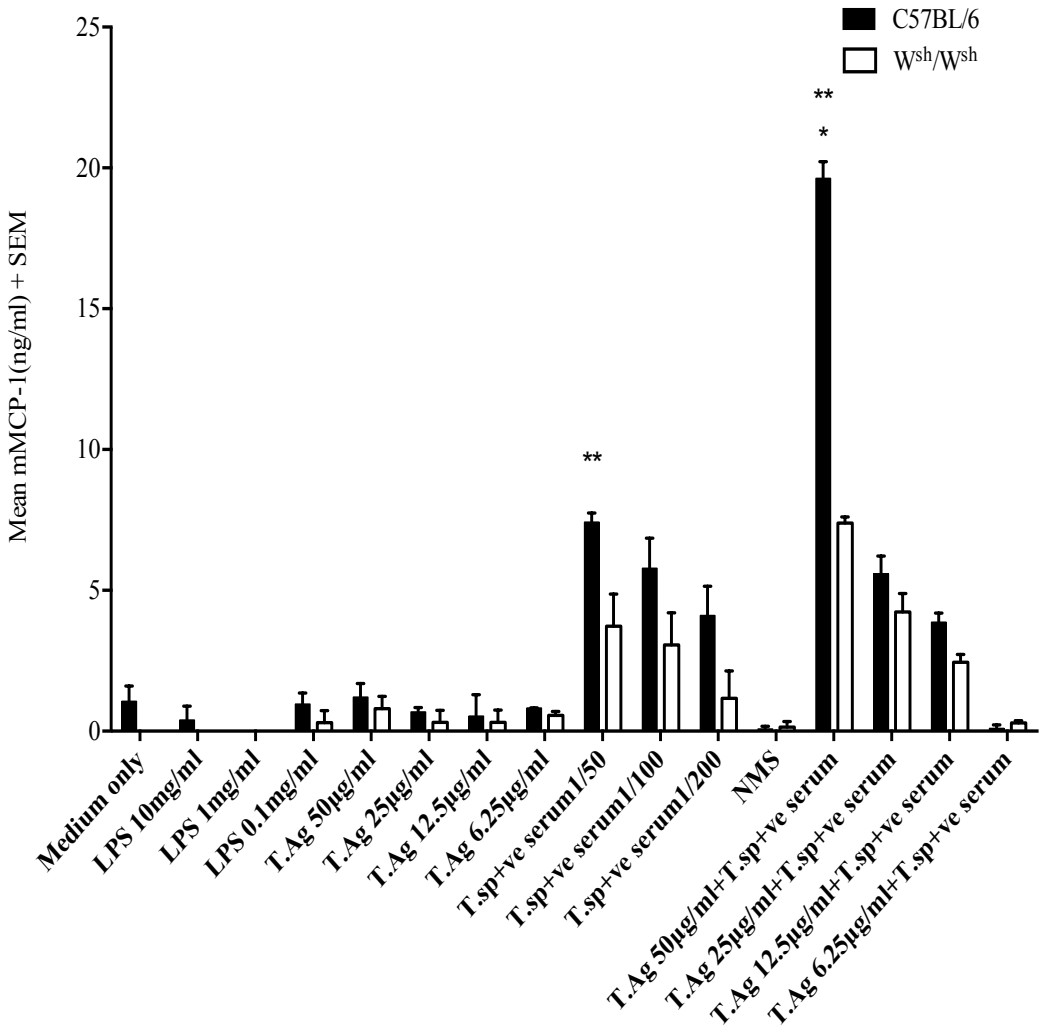


Figure 5.7 Release of mMCP-1 after stimulation of PCMC from C57BL/6 and Wsh/Wsh mice. Mast cells (5 x 10^5) were cultured and subsequently stimulated with medium, lipopolysaccharide (LPS), *Trichinella spiralis* antigen (*T.* Ag) or *Trichinella spiralis* positive serum or normal mice serum (NMS). Production of mMCP-1 was quantified using ELISA. Plates were incubated for 2h at 37°C. Data are expressed as mean + S.E.M. (n=3), and the experiment was repeated twice with similar results. Data were analysed by Kruskal Wallis test followed by a Dunn's ad hoc test to identify differences between treatments. * $P<0.05$ for significant increases comparing to medium only. ** $P<0.01$ represent significant comparing WT vs Wsh/Wsh mice.

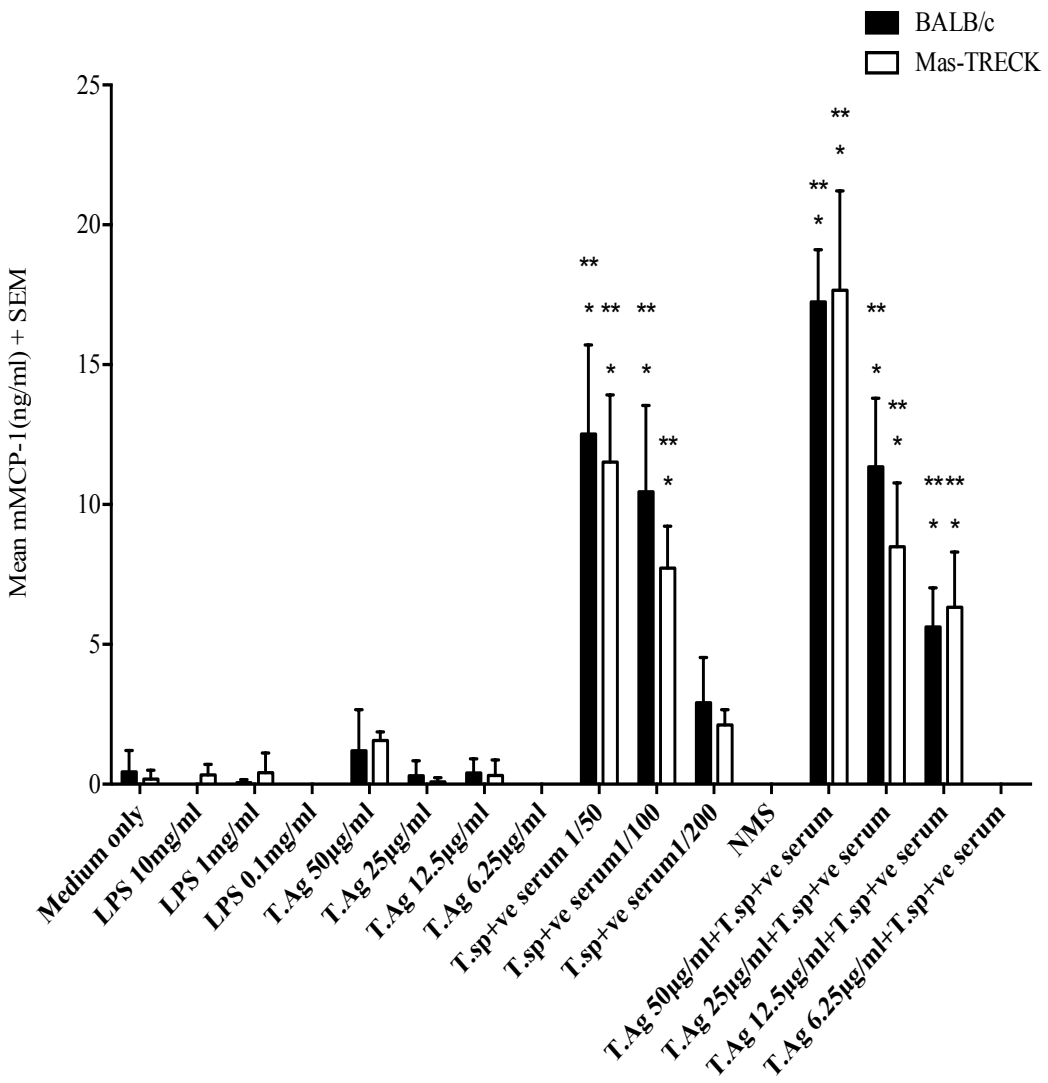


Figure 5.8 Release of mMCP-1after stimulation of PCMC from BALB/c and Mas-TRECK mice. Mast cells ($5x10^5$) were cultured and subsequently stimulated with medium, lipopolysaccharide (LPS), *Trichinella spiralis* antigen (*T.* Ag) or *T. spiralis* positive serum or normal mice serum (NMS). mMCP-1 production was quantified using ELISA. Plates were incubated for 2h at 37°C. Absorbance was read at 450nm wavelength. Data are expressed as mean + S.E.M. (n=3), and the experiment was repeated twice with similar results. Data were analysed by Kruskal Wallis test followed by a Dunn's ad hoc test to identify differences between treatments. * $P<0.05$ for significant increases comparing unstimulated vs stimulated. ** $P<0.01$ for significant increases comparing unstimulated vs stimulated.

5.2.2. Production of mast cells from bone marrow from mast cell deficient mice

MC-deficient mice have been shown to be a beneficial tool for understanding and defining functions of mast cells *in vivo* due to mutations in the Kit or Stem Cell Factor (SCF) genes ($Kit^{W/W\text{-}v}$, $Kit^{W\text{-}sh/W\text{-}sh}$, and Sl/Sl^{d}) (Kitamura *et al.*, 1978, Russell, 1979, Grimbaldeston *et al.*, 2005). However, MC-deficient mice have been shown to express other abnormalities in other cells due to the role of Kit, which include erythrocytes, neutrophils and melanocytes, as well as other cell lineages. Bone marrow or bone marrow-derived mast cells (BMMCs) that integrate into MC-deficient strains have assisted to clarify the origin of the mast cell and to consistently initiate connections between functions of mast cells *in vivo* and their involvement in various diseases (Kitamura *et al.*, 1977, Kitamura *et al.*, 1978, Grimbaldeston *et al.*, 2005, Galli and Tsai, 2008, Jamur and Oliver, 2011).

Differentiation and culture of mast cells from bone marrow mast cell progenitors has been studied in a mouse system, whereby protocols were created using mast cell growth factors and cytokines. In addition, the impact of mutation on mast cell function can be easily determined, and could be obtained by culturing bone marrow (Kovarova, 2013).

Bone marrow was obtained and cultured in a mast cell-specific culture DMEM medium with growth factors IL-3, IL-9 and SCF for 30 days. Although the number of BMMCs was not significant ($p > 0.05$), the total number of cells dropped after the first day of cell culture (day 0), and each strain presented a similar growth pattern (Figure. 5.9).

BMMC numbers plateaued, and no significant differences were observed between mast cell-deficient and wild-type mice ($p > 0.05$).

To assess the development of mature mast cells from mast cell deficient models and wild-type mice, BMMC maturity was measured by staining cells with toluidine blue in 1% NaCl solution. No significant difference between the strains was detected ($p > 0.05$). BMMCs matured as cultures progressed (Figure. 5.10). C57BL/6 BMMC maturation showed a reduced rate of maturity after two weeks of culturing the cells; but this was most likely due to a counting error or insufficient culture medium.

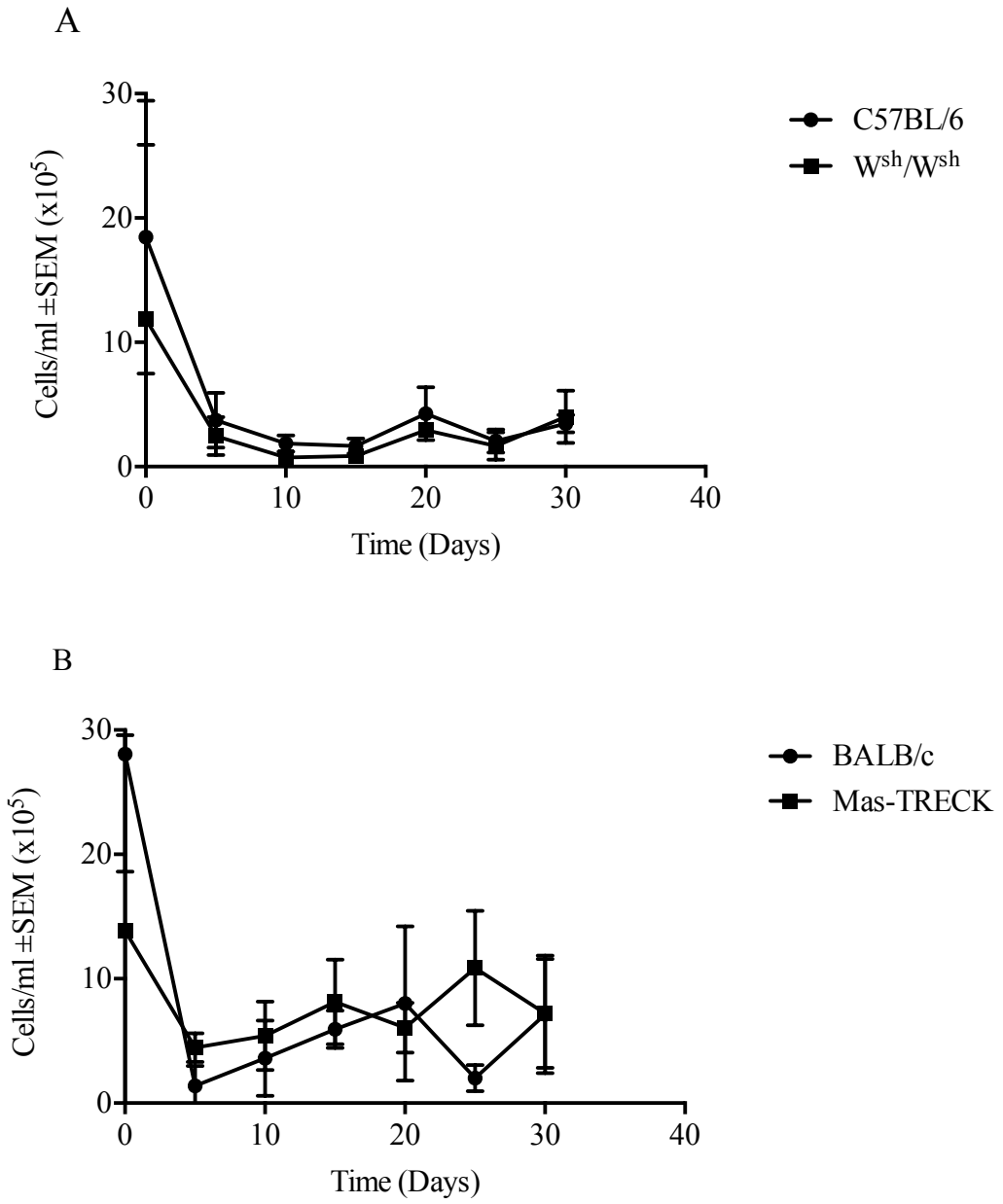


Figure 5.9 Growth of BMMC cells *in vitro* cultured at different time periods. BMMC cells were removed at day 0 and cultured for 30 days in 40 ml complete Dulbecco's Modified Eagle Medium (DMEM) supplemented with growth factors IL-3 (1ng/ml), IL-9 (5ng/ml) and SCF (50ng/ml). Trypan blue was used to assess cells growth. (A) Mast cell growth of C57BL/6 and Wsh/Wsh mice. (B) Mast cell growth of BALB/c and Mas-TRECK mice. Data are expressed as mean ± S.E.M. (n=3). The experiment was repeated twice with similar results. Analysis of the data using a Kruskal Wallis test followed by a Dunn's ad hoc test showed there was no significant difference between treatments.

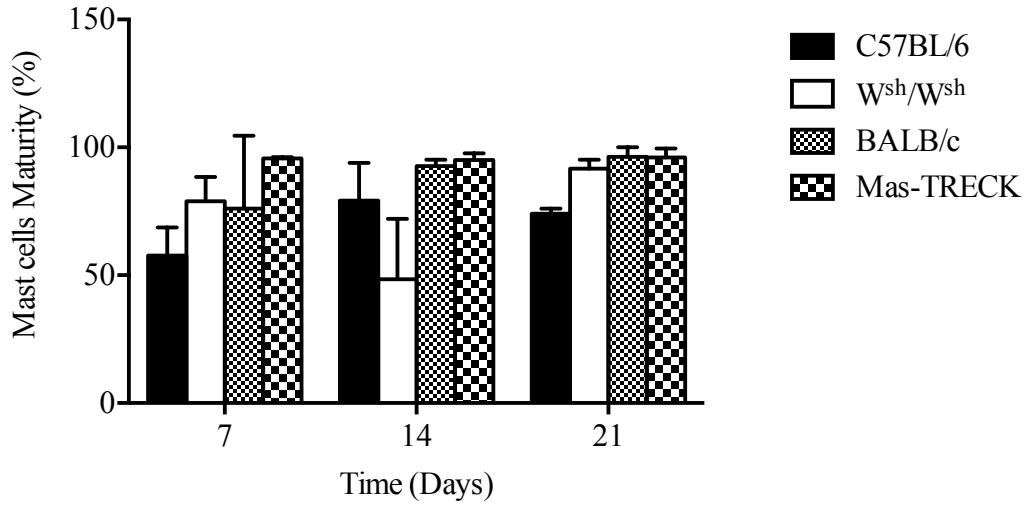


Figure 5.10 Percentage of BMMC maturity *in vitro* after 30 days from different mouse strains. Samples were mixed 1:1 with Trypan blue and counted using a haemocytometer. The maturity of mast cells was measured by smearing samples onto glass slides. The slides were fixed in 75% ethanol and stained with 10% Toluidine blue in 1% NaCl solution for 90 min and counted under a microscope. Data are expressed as percentage of positively stained cells+ S.E.M (n=3). The experiment was repeated twice with similar results. Analysis of the data using a Kruskal Wallis test followed by a Dunn's ad hoc test showed there was no significant difference between treatments.

5.2.3. β-hexosaminidase was released following stimulation of BMMCs.

The ability of BMMCs to degranulate after stimulation with helminth products was determined, and the absorbance of β-hexosaminidase levels was measured using an enzyme assay.

After stimulation of mast cells with medium, LPS, *T.* Ag or *T. spiralis* positive serum or normal mice serum (NMS), β-hexosaminidase levels were detectable in BMMC supernatants, significant rise in the level was observed compared to the medium control ($p < 0.05$). Significant release of β-hexosaminidase was observed in LPS stimulated BMMC obtained from Wsh/Wsh mice ($p<0.05$) (Figure. 5.11).

Moreover, stimulation with *T.* Ag resulted in significant increase in β-hexosaminidase production by BMMC from C57BL/6 compared to Wsh/Wsh mice ($p<0.01$). BMMC stimulated with *T. spiralis* positive serum significantly increased production of β-hexosaminidase in C57BL/6 and Wsh/Wsh mice compared to the medium control ($p < 0.05$). After stimulation of mast cells with LPS, there is a significant increased between C57BL/6 and Wsh/Wsh mice ($p=0.001$).

Similarly, significant rise in the level of β-hexosaminidase was observed in LPS 0.1mg/ml from Mas-TRECK mice compared to the medium control ($p = 0.004$). Moreover, significant rise in the level of β-hexosaminidase was observed in LPS 0.1mg/ml between BALB/c and Mas-TRECK mice ($p=0.0005$). Stimulation with (0.008µg/ml *T.* Ag) from Mas-TRECK mice detected significant increase in the level of β-hexosaminidase of to activate BMMCs comparing to medium only (0.01) (Figure. 5.12).

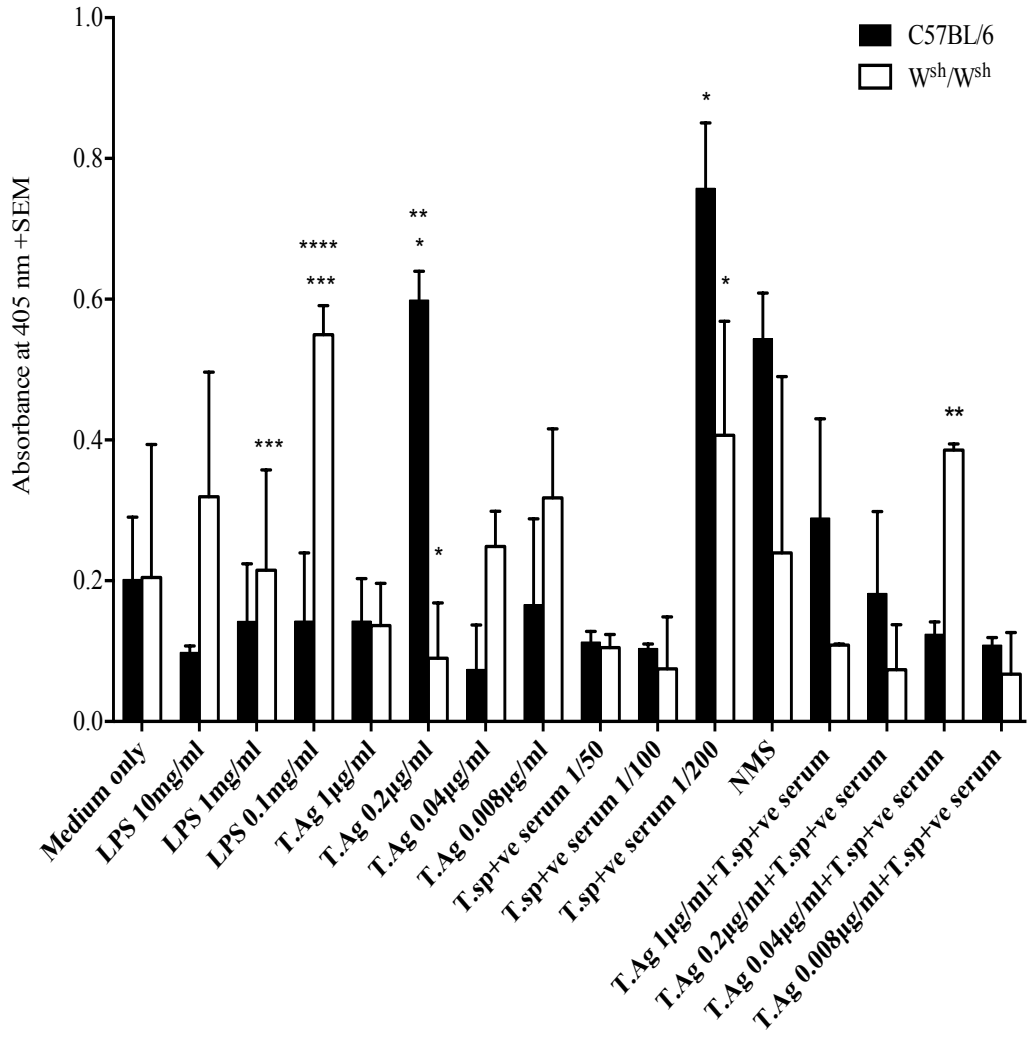


Figure 5.11 Release of β-hexsoaminidase after stimulation of BMMCs from C57BL/6 and Wsh/Wsh mice. Mast cells ($5x10^5$) were cultured and subsequently stimulated with medium, lipopolysaccharide (LPS), *Trichinella spiralis* antigen (*T.* Ag) or *T. spiralis* positive serum or normal mice serum (NMS). Plates were incubated for 1h at 37°C. Data are expressed as mean + S.E.M. (n=3). The experiment was repeated twice with similar results. Data were analysed by Kruskal Wallis test followed by a Dunn's ad hoc test to identify differences between treatments. * P<0.05 for statistically significant difference comparing unstimulated vs stimulated. ** P<0.01 represent significant comparing WT vs Wsh/Wsh mice. *** P<0.001 for statistically significant difference comparing unstimulated vs stimulated. **** P<0.0001 for statistically significant difference between C57BL/6 and Wsh/Wsh.

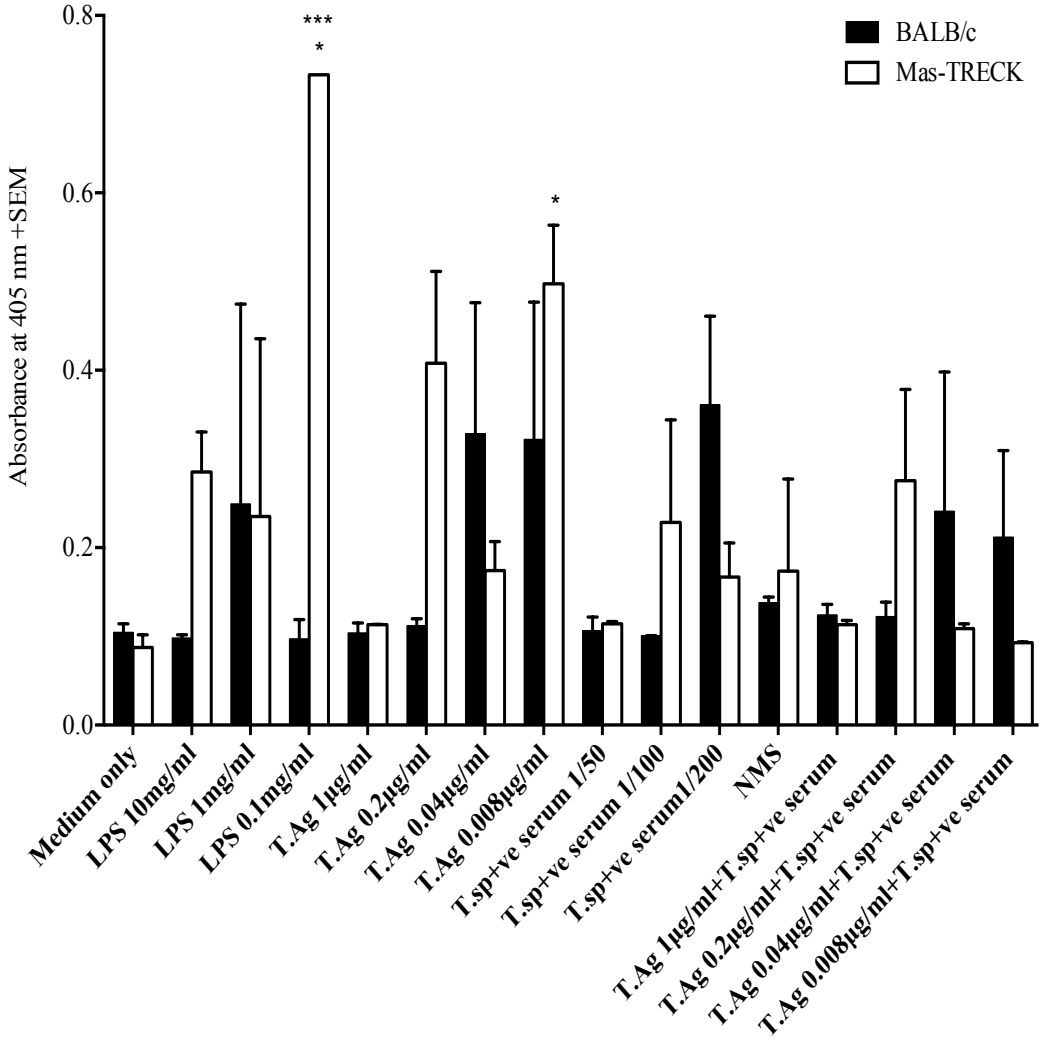


Figure 5.12 Release of β-hexsoaminidase after stimulation of BMMCs from BALB/c and Mas-TRECK mice. Mast cells ($5x10^5$) were cultured and subsequently stimulated with medium, lipopolysaccharide (LPS), *Trichinella spiralis* antigen (*T.* Ag) or *Trichinella spiralis* positive serum or normal mice serum (NMS). Plates were incubated for 1h at 37°C. Data are expressed as mean + S.E.M. (n=3). The experiment was repeated twice with similar results. Data were analysed by Kruskal Wallis test followed by a Dunn's ad hoc test to identify differences between treatments. * $P<0.05$ for statistically significant difference comparing to medium only. *** $P<0.001$ for statistically significant difference between BALB/c and Mas-TRECK.

5.2.4. BMMCs stimulated by Trichinella spiralis antigen produced IL-4.

BMMCs were stimulated with different mast cell stimuli, and IL-4 was measured using ELISA. BMMCs from C57BL/6 did not produce significantly different levels of IL-4 after stimulation ($p > 0.05$). Further Wsh/Wsh mice did not show a significant release of IL-4 compared to C57BL/6 mice (Figure. 5.13).

In contrast, stimulation of BMMC with medium, LPS and *T*. Ag showed significant increases ($p<0.05$) in the levels of IL-4 obtained from Mas-TRECK mice compared to BALB/c mice (Figure. 5.14).

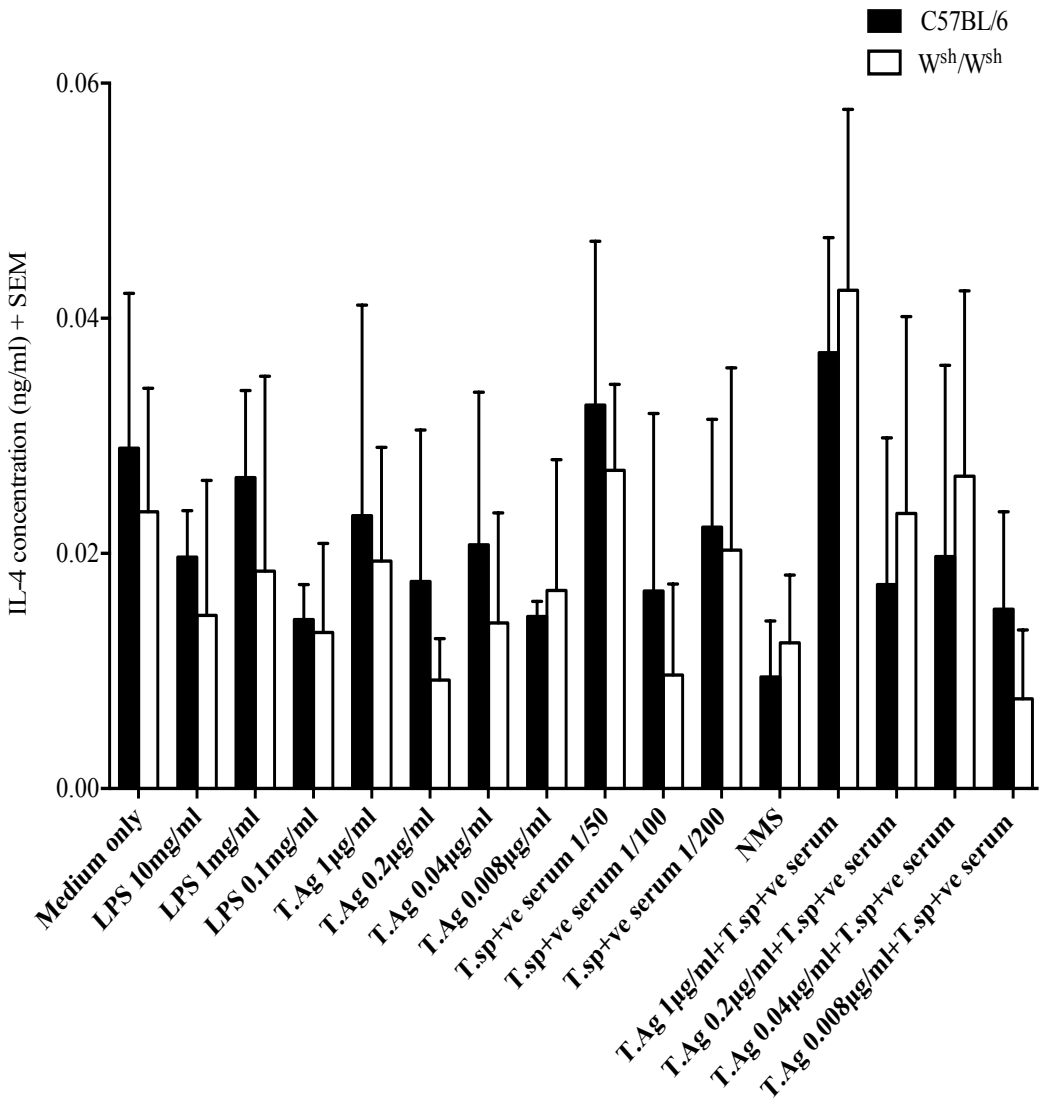


Figure 5.13 Release of IL-4 after stimulation of BMMCs from C57BL/6 and Wsh/Wsh mice. Mast cells ($5x10^5$) were cultured and subsequently stimulated with medium, lipopolysaccharide (LPS), *Trichinella spiralis* antigen (*T*. Ag) or *T. spiralis* positive serum or normal mice serum (NMS) mice. Plates were incubated for 2h at 37°C. IL-4 production was quantified using ELISA assay. Data are expressed as mean + S.E.M. (n=3). The experiment was repeated twice with similar results. Analysis of the data using a Kruskal Wallis test followed by a Dunn's ad hoc test showed there was no significant difference between treatments.

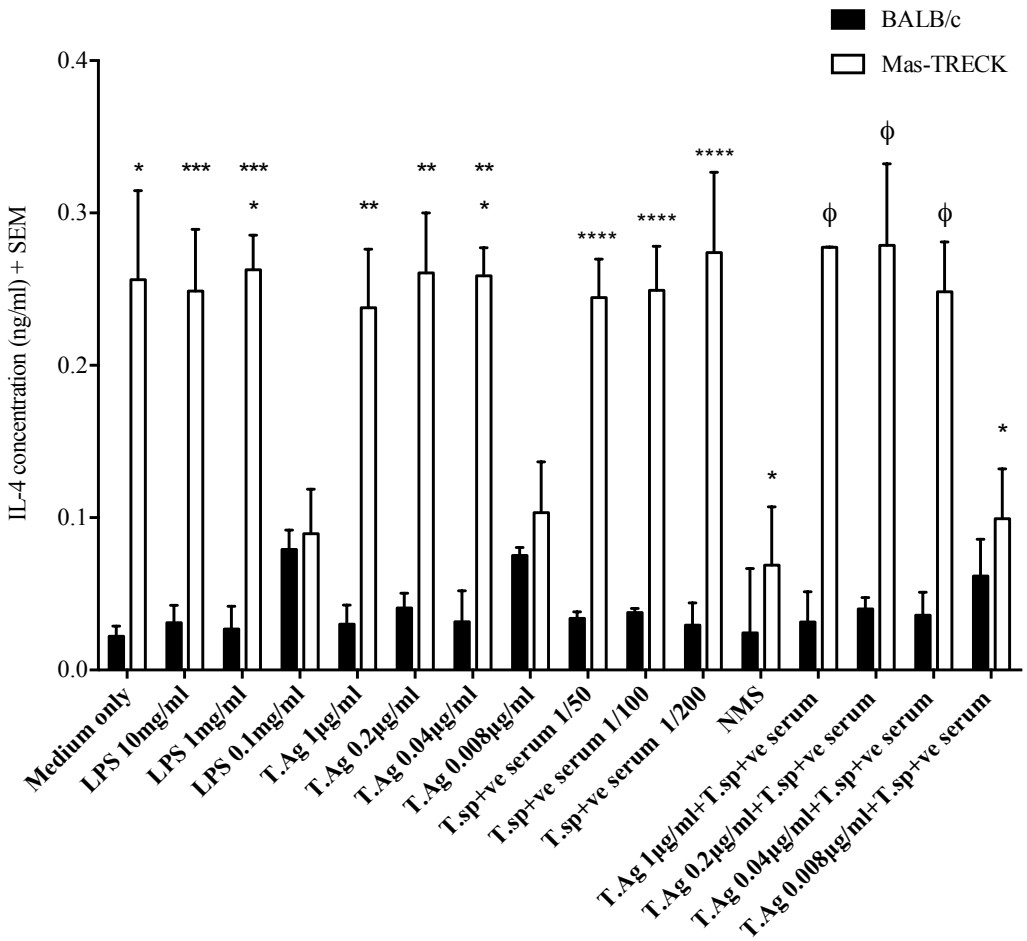


Figure 5.14 Release of IL-4 after stimulation of BMMCs from BALB/c and Mas-TRECK mice. Mast cells ($5x10^5$) were cultured and subsequently stimulated with medium, lipopolysaccharide (LPS), *Trichinella spiralis* antigen (*T*. Ag) or *T. spiralis* positive serum or normal mice serum (NMS) mice. IL-4 production was quantified using ELISA assay. Plates were incubated for 2h at 37°C. Absorbance was read at 450nm wavelength. Data are expressed as mean + S.E.M. (n=3). The experiment was repeated twice with similar results. Data were analysed by Kruskal Wallis test followed by a Dunn's ad hoc test to identify differences between treatments. * $P<0.05$ for statistically significant difference comparing unstimulated vs stimulated. ** $P<0.01$ for significant difference between BALB/c and Mas-TRECK mice. *** $P<0.001$ for statistically significant difference between BALB/c and Mas-TRECK mice. **** $P<0.0001$ statistically significant difference between BALB/c and Mas-TRECK mice. Φ represents statistically significant difference between BALB/c and Mas-TRECK mice ($p<0.05$).

5.2.5. mMCP-1 was produced by BMMC following stimulation with parasite antigen

BMMCs were stimulated with medium, LPS, *T*. Ag, *T. spiralis* positive serum or normal serum mice (NMS) and mMCP-1 level were measured by ELISA.

Trichinella spiralis antigen and (*T*. Ag combined with *T. spiralis* positive serum) produced significant levels of mMCP-1 in C57BL/6, BMMC showed significantly ($p=0.001$-$p=0.04$) elevated secretions of mMCP-1 following stimulation with medium, LPS or *T*. Ag (Figure. 5.15). Similarly, BMMC obtained from Wsh/Wsh mice stimulated with medium or LPS showed significant secrete significant amounts of mMCP-1 ($p < 0.05$). Moreover, stimulation of BMMCs from BALB/c and Mas-TRECK mice showed significantly raised mMCP-1 levels comparing unstimulated vs stimulated cells (Figure. 5.16).

Overall, the findings show that *T. spiralis* antigen alone or in combination with *T. spiralis* positive serum is capable of stimulating BMMCs cells to release mMCP-1. BMMCs cells from all four strains tended to secrete similar levels of mMCP-1. However, immune serum on its own did not elicit activation of MCs

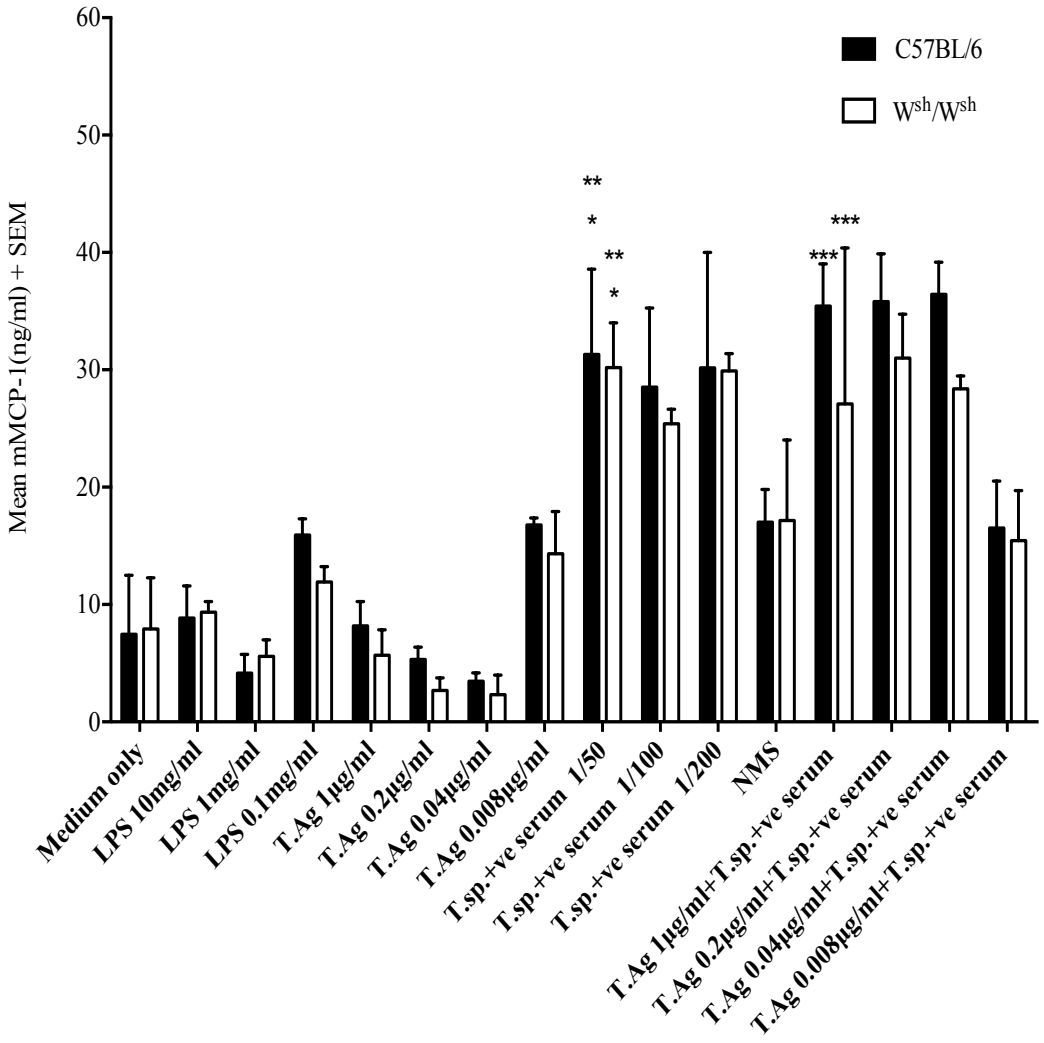


Figure 5.15 Release of mMCP-1after stimulation of BMMCs from C57BL/6 and Wsh/Wsh mice. Mast cells (5-10x10^7) were cultured and subsequently stimulated with medium, lipopolysaccharide (LPS), *Trichinella spiralis* antigen (*T.* Ag) or *T. spiralis* positive serum or normal mice serum (NMS) mice. MMCP-1 production was quantified using ELISA. Plates were incubated for 2h at 37°C. Absorbance was read at 450nm wavelength. Data are expressed as mean + S.E.M. (n=3). The experiment was repeated twice with similar results. Data were analysed by Kruskal Wallis test followed by a Dunn's ad hoc test to identify differences between treatments. *P<0.05 for statistically significant difference comparing unstimulated vs stimulated. **P<0.01 for statistically significant difference comparing unstimulated vs stimulated. ***P<0.001 for statistically significant difference comparing unstimulated vs stimulated.

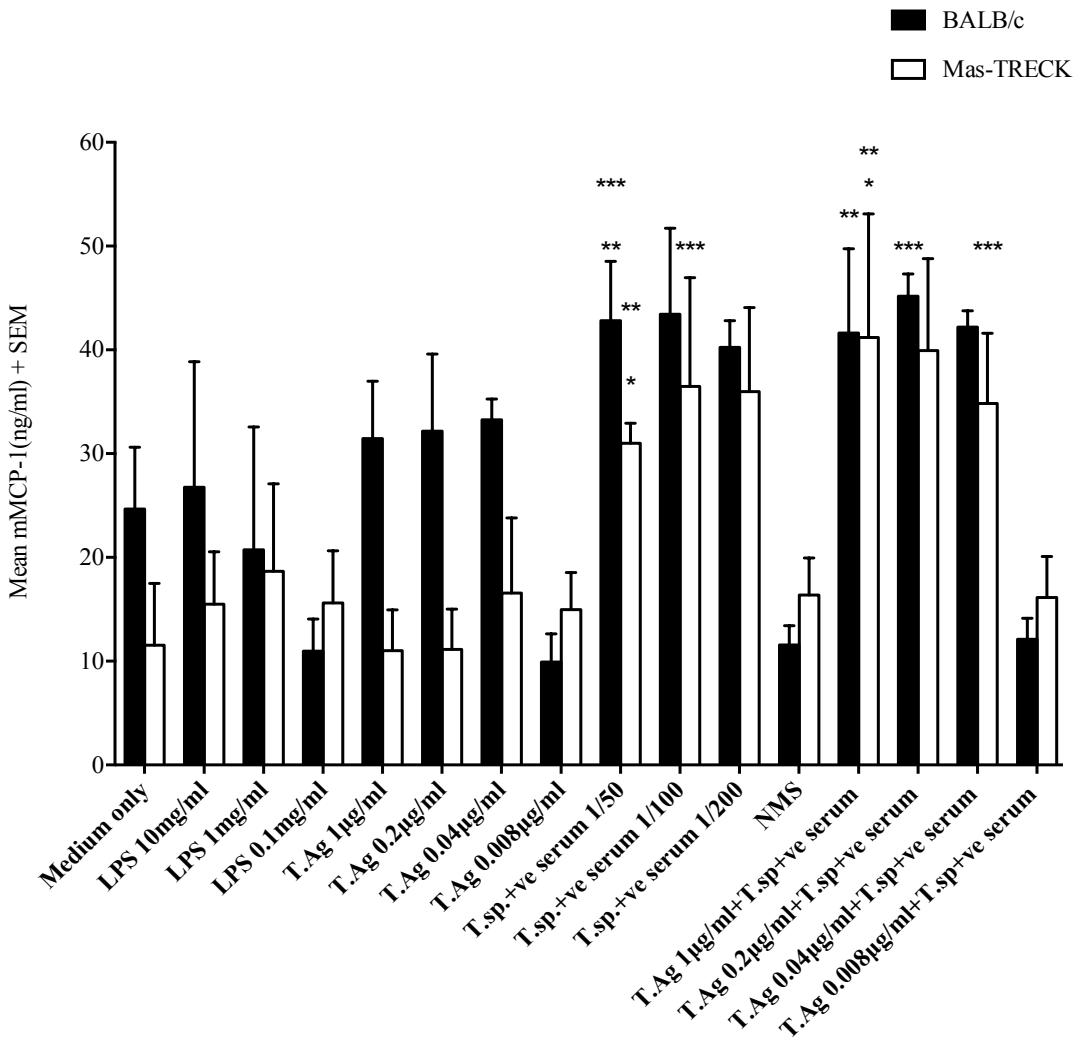


Figure 5.16 Release of mMCP-1after stimulation of BMMCs from BALB/c and Mas-TRECK mice. Mast cells (5-10x10^7) were cultured and subsequently stimulated with medium, lipopolysaccharide (LPS), *Trichinella spiralis* antigen (*T.* Ag) or *T. spiralis* positive serum or normal mice serum (NMS) mice. MMCP-1 production was quantified using ELISA. Plates were incubated for 2h at 37°C. Absorbance was read at 450nm wavelength. Data are expressed as mean + S.E.M. (n=3). The experiment was repeated twice with similar results. Data were analysed by Kruskal Wallis test followed by a Dunn's ad hoc test to identify differences between treatments. *P<0.05 for statistically significant difference comparing unstimulated vs stimulated. **P<0.01 for statistically significant difference comparing unstimulated vs stimulated. ***P<0.001 for statistically significant difference comparing unstimulated vs stimulated.

5.3. Discussion

Mast cells play a central role in the initiation of inflammatory reaction associated with allergic diseases such as asthma, rhinitis and anaphylaxis (Metcalfe, 2008). The physiological responses, which are a consequence of inflammatory reaction, are due to various classes of biologically active compounds released from mast cells. The functions of these compounds probably initially developed to participate in innate immune defence of the body to invading pathogens (Mekori and Metcalfe, 2000, Marshall, 2004, Galli *et al.*, 2005b, Tkaczyk *et al.*, 2006). Moreover, in the expression of allergic responses, mast cells are essential effector cells (Metcalfe *et al.*, 1997, Schroeder *et al.*, 2001).

These cells have been shown to produce preformed mediators such as histamine, serotonin, and proteoglycans after the cross-linking of Fc receptors for immunoglobulin E antibody and antigen. These mediators are stored in secretory granules and secrete lipid mediators such as prostaglandins (PGs), leukotrienes (LTs), and platelet-activating factors (Serafin and Austen, 1987, Hart, 2001). In addition, various cytokines and chemokines have been synthesised by mast cells as effector cells for innate immunity and regulator cells for acquired immunity (Kobayashi *et al.*, 2000a, Okayama *et al.*, 2001). Protective type 2 immune responses have long been associated with mast cells, basophils, and eosinophils (Finkelman *et al.*, 1997, Kawakami and Galli, 2002, Dombrowicz and Capron, 2001), as well as allergy and asthma immunopathologies (Wills-Karp, 1999).

These cells are bone marrow-derived myeloid populations which are preferentially located in peripheral sites to be recruited into; due to their innate response to local stimuli, these cells are considered lead effector cells (Galli *et al.*, 1999). In addition, the expression of the high affinity IgE receptor (FcεRI) has been used to distinguish mast cells from basophils. However, FcεRI is absent from murine eosinophils (De Andres *et al.*, 1997). Upon stimulation, a widely, but not similar, spectrum of biologically-active mediators, such as histamine, arachidonic acid metabolites, proteases, chemokines, and cytokines, is rapidly generated (Kawakami and Galli, 2002, Dombrowicz and Capron, 2001, Walsh, 2001, Voehringer *et al.*, 2004). Upon stimulation, there is rapid development of IL-4 and IL-13 which is common to these cells (Gessner *et al.*, 2005).

In the present study, helminth products were used to determine the mechanisms by which MCs can be activated. The ability of MCs to be activated in the presence and absence of IgE in an *in vitro* culture has been confirmed. Four strains of mouse models, C57BL/6, Wsh/Wsh, BALB/c and Mas-TREK, were used for comparing the stimulation of MCs and were chosen due to the genetic backgrounds of the strains, C57BL/6 mice used as a control for the Wsh/Wsh mice and BALB/c mice used as a control for the Mas-TREK mice.

These studies demonstrated that mast cells could be cultured from both wild type and mast cell deficient strains *in vitro* at similar levels. The release of β-hexosaminidase has been used to determine the degranulation of cell culture samples (Macdonald *et al.*, 1989). In this study, degranulation in PCMC cells obtained from C57BL/6 increased compared to other strains. The degranulation also increased in the BALB/c cultures, though not to the same degree as those from C57BL/6. A negligible response to antigen and IgE stimulation was observed in PCMC cell cultures in both of the MC-deficient mouse strains in that there was no significance between the presence and absence of IgE in the promotion of degranulation. Because the IgE levels did not promote degranulation, the immune system was not involved, meaning that direct activation may occur by parasite surface antigens.

In the present study, the release of IL-4 from PCMCs was observed in wild-type mice with no significant differences seen between the sensitised and unsensitised stimulations, while MC-deficient mice showed lower levels of IL-4 with different stimulations. Additionally, PCMCs from MC-deficient mice were shown to release similar levels of mMCP-1 compared to the wild-type.

Our results showed that PCMCs produced of mMCP-1 in cells stimulated with *T. Ag* combined with *T. spiralis*-positive serum, however, the *T. spiralis* antigen alone was not able to stimulate this release in all four mouse strains. Although MMCs contribute significantly to protecting against the parasite by releasing mMPC-1 (Knight *et al.*, 2000), this result was unexpected because PCMCs are predominantly connective tissue MCs that lack mMCP-1 (Da Silva *et al.*, 2014). Therefore, it is important to note that MC proteases can vary substantially between various strains and conditions of infection. Moreover, mature MCs have been shown to change their

phenotype as a result of environmental changes (da Silva *et al.*, 2014). Thus, PCMCs not in contact with helminths *in vivo* might be able to produce and secrete mMCP-1 in an *in vitro* micro-environment. Upon activation of the PCMC cells, levels of mediators secreted from the MC-deficient mice were similar to the wild-type BALB/c mice. This is in line with findings by Sawaguchi *et al.* (2012), who reported that detectable PCMCs were present in Mas-TREK in the peritoneum after treatment with diphtheria toxin.

From these results, it could be proven that MCs could be stimulated in both the presence and absence of IgE and that β-hexosaminidase, IL-4 and mMCP-1 expression were detectable at lower levels of stimulation. Overall, the activation of PCMCs could be observed in the presence and absence of IgE, and C57BL/6 mice showed the greatest response to the stimulation and activation of PCMCs.

The ability of BMMCs to degranulate after stimulation with helminth products was measured. The protein β-hexosaminidase was detectable in BMMC supernatants of all of the strains, and a significant rise observed in this level was observed compared to the media control. This was in contrast to previous studies where Noguchi *et al.* (2005) reported that the percent release of β-hexosaminidase from both BALB/c and C57BL/6 mice was similar. However, they also reported that the total content of β-hexosaminidase was obviously different between the two strains. Therefore, BMMCs from C57BL/6 released higher amounts of β-hexosaminidase than BMMCs from BALB/c. These findings were similar to our findings that showed that the BMMCs from all of the strains of mice were different. In addition, the secretion of β-hexosaminidase from the BMMCs was not significant after the stimulation with helminth products.

In the present study, we determined that the different mouse models displayed different responses, and that mature MCs from the bone marrow of Wsh/Wsh mice could be cultured and detected in the peritoneal cavity. BMMCs stimulated with helminth antigens had similar secretions of mediators to those of wild-type C57BL/6. This contrasts with the findings by Grimbaldeston *et al.* (2005) who demonstrated that MC levels were undetectable in Wsh/Wsh mice. Although Wsh/Wsh mice have

been extensively used to investigate the role of MCs *in vivo*, these mice have additional abnormalities related to their *c-Kit* mutation (Reber *et al.*, 2012a).

The release of IL-4 from BMMCs was observed in wild-type mice with no significant difference between sensitised and unsensitised stimulation. The MC-deficient mice showed lower levels of IL-4 with different stimulations. BMMCs showed significant evidence that the IgE-independent activation of these cells leads to IL-4 secretion. In addition, it has been demonstrated that IL-6 was induced by IL-1 without degranulation from human MCs (Kandere-Grzybowska *et al.*, 2003). Moreover, it has been reported that in the absence of histamine, IL-6 was induced by SCF (Gagari *et al.*, 1997). From these studies and the reports by Noguchi *et al.* (2005), it was suggested that the degranulation and production of newly synthesized mediators were different occurrences and were controlled by different mechanisms (Noguchi *et al.*, 2005).

Our findings are in contrast with the results of Banks and Coleman (1996) that used mice carrying mutations in the *IL-4* gene so that the content of the peritoneal MCs would not influence their numbers, size, granularity or histamine content. Therefore, they demonstrated that *in vivo,* IL-4 is not a necessary growth and differentiation factor for CTMC. Consequently, this finding does not definitely mean that IL-4 in normal mice is not a CTMC growth factor (Banks and Coleman, 1996). In addition, it has been shown *in vitro* that when combining IL-4 with other cytokines, such as IL- 3, IL-10 or SCF, IL-4 functions as a CTMC growth-promoting factor (Hamaguchi *et al.*, 1987, Tsuji *et al.*, 1990, Rennick *et al.*, 1995). Furthermore, peritoneal MCs from mice deficient in IL-4 failed to respond to stimulation with an anti-IgE antibody, and surface FcεRI-bound IgE was not expressed, which reflected the known IgE deficiency of these mice (Kuhn *et al.*, 1991).

The present study showed that BMMCs cells produced mMCP-1 in cells stimulated with *T. spiralis* antigen or stimulated with *T. Ag* combined with *T. spiralis*-positive serum. Overall, the activation of BMMCs can be observed in the presence and absence of IgE, and all four strains of mice tended to secrete similar levels of mMCP-1. This is in agreement with observations in previous studies showing that mMCP-1 expression is regulated strictly by the multifunctional cytokine,

transforming growth factor (TGF)-β1. Moreover, it has been determined that mouse BMMCs in TGF- β1 cultured simultaneously with IL-3, IL-9 and SCF are highly homologous to MMCs (Brown *et al.*, 2004).

Overall, the activation of MCs by *T. spiralis* antigens independent of IgE has been demonstrated. Although an immunoglobulin-dependent pathway is considered the classical way to activate MCs, the use of four mouse strains in this study showed that MCs can be activated by independent pathways in which TLR receptors may be involved. This has been shown after MCs were activated by nematode antigens. Therefore, further research is required to determine the specific parasite antigens and their methods of MC degranulation via the immunoglobulin-independent pathway, and further research into the cytokine response could help to explain the immune responses resulting from infection.

CHAPTER 6. GENERAL DISCUSSION AND FUTURE WORK

GI-dwelling nematodes may have a very broad host range, such as *T. spiralis*, whilst others display host species specificity such as *H. polygyrus*. Some GI nematodes have an invasive phase in their life cycle such as *N. brasiliensis*; however, development strictly within the intestine has been shown in *T. muris*. *H. polygyrus* inhabits the lumen of the gut, while others such as *T. spiralis* and *T. muris* are intraepithelial. *N. brasiliensis* and *H. polygyrus* inhabit the small intestine, while *T. muris* is found in the large intestine (Lawrence, 2003, Grencis, 2015). Different species of gastrointestinal helminths also induce different immune responses, which is potentially due to differences in the gut location of these parasites (Lawrence, 2003).

An example of this is *T. spiralis* which lives within the small intestine and elicits a $CD4^+$ T cell response, and this is assocaited with the induction of intestinal inflammation. This is accompanied by the accumulation of mast cells and this prompt mastocytosis is required for the effective expulsion of the *T. spiralis* worm (Maizels *et al.*, 1993). In contrast *H. polygyrus* larvae develop in the gut submucosa, and the immune response is characterized by an influx of neutrophils, immediately surrounding the invading larva. Alternatively, activated macrophages, which are present in larger numbers than neutrophils, enclose the parasite and neutrophils forming a granulomatous structure (Anthony *et al.*, 2007). There is an accumulation of $CD4^+$ T cells, $CD11c^+$ dendritic cells (DCs) and eosinophils; however, basophils, mast cells, $CD8^+$ T cells, γδT cells and B cells are absent (Anthony *et al.*, 2006, Morimoto *et al.*, 2004, Liu *et al.*, 2004). It has been shown that production of IL-4, IL-5 and IL-13 cytokines by $CD4^+$ Th2 cells is the key immune response for controlling gastrointestinal helminth infection (Angkasekwinai *et al.*, 2017).

These differences in the mechanisms of parasite elimination may explain why infection by GI nematodes is generally chronic with some parasites, where infections can last months or years, while others are expelled in a matter of days e.g. *N. brasiliensis* and *T. spiralis* (Lawrence, 2003, Grencis, 2015). These differences have been examined in *T. muris*; where a comparison of mouse strains resistant or susceptible to infection showed that the induction of a Th1 response was associated

with susceptibility, whereas resistance was correlated with Th2 responses (Else and Grencis, 1991, Else *et al.*, 1992).

Following intestinal helminth infection, the initiation of a Th2 response has been identified in intestinal epithelial cells (IECs) (Zaph *et al.*, 2007). It has been demonstrated that transgenic mice with an IEC-specific activation of NF-$_k$B (*Ikk*β^{DIEC} mice) in which IKKβ was specifically deleted in IECs are susceptible to infection with *T. muris*; levels of IL-4, IL-5 and IL-13 were decreased, and levels of IFN-γ were increased, resulting in a failure of worm expulsion (Zaph *et al.*, 2007). In resistant mouse strains, the blocking of IL-4 or administration of IFN-γ rendered the mice susceptible. However, GI helminths in susceptible strains were expelled after blocking IFN-γ or giving IL-4 (Else *et al.*, 1994, Urban *et al.*, 1993, Urban *et al.*, 1995). It has been shown that expulsion of *T. muris* required both IL-4 and IL-13 (Else *et al.*, 1994, Bancroft *et al.*, 1998). A critical component of the protective response to *N. brasiliensis* is IL-13, but IL-4 appears to be insignificant (Mckenzie *et al.*, 1998, Urban *et al.*, 1998). In contrast, although the expulsion of *H. polygyrus* required IL-4, IL-13 does not appear to be important (Finkelman *et al.*, 1997).

Moreover, *N. brasiliensis* following infection of IL-4/IL-5/IL-9/IL-13 knock-out (KO) mice demonstrated a significant delay in the parasite expulsion, which was associated with the induction of Th1 cytokines (Fallon *et al.*, 2002). Infection with *T. spiralis* or *N. brasiliensis* in an experiment using IL-4Rα and Stat6 KO mice resulted in expulsion of these parasites after activation of Stat6 by the binding of IL-13 and/or IL-4 to IL-4Rα (Urban *et al.*, 1998, Urban *et al.*, 2000b). The expulsion of *N. brasiliensis* requiring Stat6 was not related to Stat6 effects on Th2 responses, secretion of IgG1 or eosinophilia, where these were normal or increased; furthermore, great increases were shown in mast cell responses (Lawrence, 2003).

The expulsion of *T. spiralis* is different to *N. brasiliensis,* which is dependent on mast cells, suggesting that parasite loss would be independent of Stat6 (Lawrence, 2003). However, it has been reported that IL-4/IL-13-activated Stat6 was required for both mast cell and cytokine responses to induce intestinal mastocytosis (Urban *et al.*, 2001). In addition, induction of *T. spiralis* expulsion required both mast cells and T cells (Lawrence, 2003).

$CD4^+$ Th2 immune response is mediated goblet cell hyperplasia, which also appears to be IL-4 independent (Lawrence, 2003). Infection with *N. brasiliensis* showed the potent role of goblet cells in the expulsion of parasites (De'broski *et al.*, 2009). It has been demonstrated that *N. brasiliensis* was not expelled efficiently in IL-13 KO mice; despite developing a Th2 response, goblet cell hyperplasia related to the expulsion of the worms failed to be generated in these mice (Lawrence, 2003). Therefore, it was suggested that IL-13 may induce the expulsion of *N. brasiliensis* through the production of goblet cell hyperplasia and intestinal mucus (Mckenzie *et al.*, 1998). However, the expulsion of *T. spiralis* does not appear to require goblet cells where the expulsion of the parasite does not require IL-13 (Lawrence, 2003).

In addition, degraded mucin Muc2 can be caused by *T. muris* produces secretions, but these secretions do not produce degraded Muc5Ac (Hasnain *et al.*, 2012). The protective role of Muc5Ac following infection with *N. brasiliensis* and *T. spiralis* has been demonstrated, suggesting that it is a broad-acting effector mechanism against GI nematodes (Grencis, 2015).

Various effects of both cytokines and effector cells on the intestine have been suggested to create an inhospitable environment for the parasite; increased fluid and mucus secretion and increased accelerative activity of the gut result in expulsion of the parasite, termed the 'weep and sweep' response (Shea-Donohue *et al.*, 2001). Gastrointestinal worm expulsion may be partly induced by IL-4 and IL-13 via effects on non- lymphoid cells, such as intestinal epithelial cells. However, a primary infection with *H. polygyrus* showed only minor changes in epithelial cell function, while more dramatic changes were observed in treatment with an IL-4 complex or secondary *H. polygyrus* infections (Shea-Donohue *et al.*, 2001).

Nematode infection-induced inflammation of the gastrointestinal tract is followed by increased contractility of the intestinal muscles, propulsive activity and accelerated intestinal transit (Castro *et al.*, 1976, Vallance *et al.*, 1997, Vermillion and Collins, 1988). These responses have been suggested to serve parasite expulsion that has been damaged by the effector immune response (Urban *et al.*, 1995, Finkelman *et al.*, 1997).

Previous work on MCs suggested that these cells can help to expel *T. spiralis*, but most of these studies were done with MC-deficient W/W-v mice (Ha *et al.*, 1983, Lawrence *et al.*, 2004), which have a profound MC deficiency but also express other Kit-related abnormalities (Piliponsky *et al.*, 2010, Zhou *et al.*, 2007, Nigrovic *et al.*, 2008, Chervenick and Boggs, 1969, Feyerabend *et al.*, 2011, Lantz *et al.*, 1998). As previously discussed, Wsh/Wsh are c-kit mutants, which are constitutively deficient in terms of mast cells; Mas-TRECK mice are an inducible model that is mast-cell deficient, with diphtheria toxin (DT) administered to abolish mast-cell populations. Therefore, by studying two types of MC-deficient mice (Wsh/Wsh and Mas-TRECK), this study confirmed the importance of evaluating different models of MC-deficiency in the study of both the pathology and parasite expulsion of *T. spiralis.*

Our study contradicts the previous report (Lawrence *et al.*, 2004) which indicated that *T. spiralis* expulsion was significantly delayed in MC-deficient W/W-v mice as well as contradicts to prior reports that repeated intraperitoneal DT treatment for 5 consecutive days completely depleted MCs in Mas-TRECK mice (Sawaguchi *et al.*, 2012). We demonstrated that expulsion in Mas-TRECK mice on day 14 p.i was not significantly different to that of control mice with the same background. Wsh/Wsh mice with low-level infections resulted in a significantly increased worm burden compared to the wild-type, but a high dose did not result in a significant delay in expulsion. These contradictory observations in our results could be due to Wsh/Wsh mice not being completely MC-deficient in comparison to the W/W-v mouse model, which has more significant phenotypic abnormalities.

In another study which is in contrast with our results in which delayed cessation of egg excretion in Wsh/Wsh mice was shown when they were infected with *Strongyloides venezuelensis,* with an increased worm burden remaining at day 12 in comparison to the corresponding wild-type mice (Mukai *et al.*, 2017). From these results, in both primary and secondary infections, they demonstrated that $CD4^+$ T cells contributed importantly to cessation of *S. venezuelensis* egg excretion resulting in the expansion of mucosal MCs and blood basophils in the primary infection, and also the development of an IgE response has been shown (Mukai *et al.*, 2017). Moreover, a 7-day delay in the cessation of egg excretion was reported by Mukai *et al.* (2017) in Mas-TRECK mice infected with *S. venezuelensis* in comparison to DT-

treated littermate wild-type controls or to phosphate-buffered saline (PBS)-treated Mas-TRECK mice. In addition, delayed expulsion in *N. brasiliensis* infection with Wsh/Wsh mice was previously demonstrated, with the worm burdens persisting at day 14, while wild-type mice at day 10 showed complete expulsion (Ohnmacht and Voehringer, 2010). In addition, other strains of knockout mice, due to the low production of MMC in the response (which includes IL-$4^{-/-}$, IL-$13^{-/-}$, and STAT-$6^{-/-}$ mice), exhibited a delayed expulsion of the parasites upon infection with *N. brasiliensis* and *T. spiralis* (Finkelman *et al.,* 2004). These results support the argument that mast cells are essential and that a complete immune response is required for successful expulsion of intestinal helminths. Our mice did not show a significant delay, which could be due to different species of gastrointestinal helminths having different immune responses or due to our mice not being completely MC-deficient.

The expulsion of *T. spiralis* worms is associated with the development of intestinal pathology and the release of a number of mediators from mast cells (Knight *et al*., 2000). The inflammatory reactions have a direct effect on the mucosal structure that was generated to prevent the worms from penetrating the intestinal epithelium (Lawrence *et al.*, 1998a). Consequently, intestinal pathology was measured by an increase of the gut weight, whilst observation of villous atrophy and crypt hyperplasia were used to assess enteropathy. The changes that were observed in the inflammatory responses are dependent on the IL-4 generation (Lawrence *et al.*, 1998a) and mast-cell (Lawrence *et al.*, 2004) responses to *T. spiralis* infection.

MC-deficient mice that were treated with bone marrow from a wild-type mouse showed more significant levels of pathology in comparison to MC-deficient mice that were treated with IL-4 and TNF-α KO bone marrow; this latter treatment with IL-4 and TNF-α KO showed significantly lowered pathology (Ierna *et al.,* 2008). In the present study, intestinal pathology occurred in MC-deficient mice. However, the development of enteropathy was shown to be reduced in MC-deficient mice, which is associated with the remaining of *T. spiralis* in the gut. These results contrast with those of previous studies, where improvement in enteropathy following the expulsion of *T. spiralis* was shown in W/W-v, TNFR1- and mMCP-1 MC-deficient mice. The improvement was associated with the reduced levels of IL-4, TNF-α and mMCP-1

following infection with *T. spiralis* (Knight *et al.*, 2000, Lawrence *et al.*, 2004, Ierna *et al.*, 2008). In addition, no difference in intestinal pathology was observed in Wsh/Wsh mice, which could potentially be explained by a potential resistance to this infection, while the enteropathy in Mas-TRECK mice following infection with *T. spiralis* was not significantly improved in those deficient in MMCs. It has previously been established that *T. spiralis* infection is associated with Th2 immune responses (Lawrence *et al.*, 2004).

In contrast to the findings reported by Lawrence *et al*. (2004), who showed that production of Th2 antibodies, IgG1 and IgE were reduced in W/W-v mice (associated with a lack of mast cells and with an increase in Th1 antibodies, such as IgG2a), we found that the infection of MC-deficient mice induced higher levels of IgG1 in comparison to the background strain, but baseline levels of IgG2a production was observed throughout, which indicates that this marker for a Th1 response was not induced until post day 14. Again, these results may indicate that these mice may not be fully deficient in MMCs, as these models did not have impaired Th2 immune responses. However, while MC-deficient mice have shown a decrease in the number of mucosal mast cells and a reduction in IL-4 cytokines, they generated similar levels of IgG1 in comparison to control mice, particularly at day 14, which shows that the Th2 immune response is still active. On mast-cell surfaces, Dectin-1 is present, and it is also present on B cells upon class switching. It can stimulate the production of multiple immunoglobulins when it is stimulated. It has been demonstrated that Dectin-1 directly enhances IgG1 on B cells, with TLR-4 also playing a role (Seo *et al.*, 2013). Moreover, it has been shown that an increased IgG1 response can be induced by other parasites, including *N. brasiliensis* and *H. polygyrus,* that infect the small intestines, along with an overall Th2 expressed response (Gause *et al.*, 2003).

In addition, in the present study, both Wsh/Wsh and Mas-TRECK mice saw the expansion of mucosal MCs and the development of an IgE response, as inferred based on the increased levels of IgE. Moreover, the IgE levels were highest at day 14 in the MC-deficient mice. In contrast with the W/W-v strain, no significant differences were observed in the IgE levels of MC-deficient mice compared to wild-type mice. It has been shown that IgE-deficient mice cannot expel worms from the

gut following infection with *T. spiralis* due to reductions in MMCs and mMCP-1, which suggests the protective role of IgE in worm expulsion (Gurish *et al.*, 2004). Rats infected with *T. spiralis* have shown declined levels of IgE, which supports the idea that IgE plays a significant role in the expulsion of parasites (Negrao-Correa *et al.*, 1999). The present study shows that IgE is important for mast-cell development and the expulsion of *T. spiralis*. However, neither IgE nor mast cells are required for expelling *N. brasiliensis* or *H. polygyrus* (Anthony *et al.,* 2007). Therefore, it would be beneficial to have more sensitive assays to detect *T. spiralis*-specific IgE. Clearly, more research is required to better understand the roles of parasite-specific antibodies, as IgE during primary infections with *S. venezuelensis* has contributed to the cessation of egg excretion (Mukai *et al.*, 2017).

The Th2 response following infection with *T. spiralis* was not significantly reduced in MC-deficient mice, and interestingly, the IL-4 responses were unaffected. IL-4 KO mice have been observed to have low levels of IgE, IgG1 and mMCP-1, which indicates the importance of IL-4 in amplifying Th2 responses and assisting in parasite expulsion (Ierna *et al.*, 2008). In the present study, the IL-4 responses in MC-deficient mice were essentially absent following infection with *T. spiralis*; however, IL-4 is necessary to produce the Th2 responses that combat *T. spiralis*. This suggests that MC-deficient mice lack a potent population of Th2 cells capable of releasing IL-4 but do not lack intestinal muscle hypercontractility; this combination leads to a delay in the expulsion of adult worms. These results are consistent with previous studies on $IL4^{-/-}$-deficient mice. *T. spiralis* expulsion from the gut is delayed and significantly impaired in mastocytosis, which suggests that $IL4^{-/-}$-deficient mice have a decreased ability to induce protective Th2 responses (Lawrence *et al.*, 1998a, Scales *et al.*, 2007a).

MMCs have been demonstrated to provide protective responses to inflammation through the release of a wide range of inflammatory mediators, and the expulsion of nematode parasites is associated with the release of the MMC granule chymase, mMCP-1 (Lawrence *et al.*, 2004). The increased release of mMCP-1 into the intestinal tissue is connected to *T. spiralis* infection and has been shown to aid in worm expulsion (Knight *et al.*, 2000, Mcdermott *et al.*, 2003). An experiment using mMCP-1-deficient mice showed that these mice significantly delayed worm

expulsion from the intestine, this demonstrates that MCs are crucial for increasing mucosal permeability during *T. spiralis* infection (Mcdermott *et al.*, 2003, Knight *et al.*, 2000).

In the present study, the mMCP-1 levels were not significantly lower in MC-deficient Wsh/Wsh mice than in C57BL/6 mice. Contrasting results were found when comparing MMC numbers, as Wsh/Wsh mice that were unable to produce significant levels of mMCP-1 had MC numbers that differed significantly from those observed in C57BL/6 mice. The disparity between the MC numbers and the levels of mMCP-1 could be due to the high levels of variance in the MC-deficient Wsh/Wsh mice. In addition, similar to the Wsh/Wsh and C57BL/6 mouse comparison, Mas-TRECK mice had similar levels of mMPC-1 as BALB/c mice during infection, though the Mas-TRECK mice had increased MC numbers. This may be sufficient to induce a lower infection response, which means that Mas-TRECK mice may not be completely MC deficient. In the present study, however, we detected the recovery of MMCs, and Mas-TRECK mice produced higher numbers of MMCs than Wsh/Wsh mice. This may suggest that, following ablation with DT, the recovery of MMCs occurs rapidly (Otsuka *et al.*, 2011). Another method of DT administration to mice, such as treating mice with 100 ng of DT at intervals of seven days following the initial five-day treatment period, could be more effective in ablating MCs and could obtain more accurate results. Previous studies have shown that no detectable mast cells from CTMCs were observed in Wsh/Wsh mice (Grimbaldeston *et al.*, 2005).

MCs play a significant role in immune defence and the expulsion of *T. spiralis* worms. It has been shown that MC activation occurs when a ligand binds to a surface receptor in the presence of IL-4, after which B cells are stimulated to produce IgE. Consequently, the degranulation of MCs is stimulated through the activation of FcεRI (Finkelman *et al.*, 2004). A previous study by Grencis (1997) utilised Fcγ KO mice and demonstrated that the activation of MCs during intestinal nematode infection can occur by mechanisms that do not involve FcεRI. *T. spiralis* was expelled successfully through a strong intestinal MC response that involved mMCP-1 (Grencis, 1997). This suggests that, in the absence of IgE, MCs showed activated MC degranulation and the ability to perform immune actions. It has also been demonstrated that the degranulation of cell culture samples occurred through the

production of β-hexosaminidase (Macdonald *et al.*, 1989).

This thesis has shown that PCMC cells release β-hexosaminidase and that the level of IgE did not affect the percentage of β-hexosaminidase in stimulated PCMCs obtained from the C57BL/6 mice, which suggests that MCs may have other mechanisms for degranulation independent of IgE. This also suggests that surface antigens may induce MC activation. In addition, significantly different responses were detected in IgE-independent MCs and IgE-dependent MCs. The amount of released β-hexosaminidase increased in MC-deficient mice stimulated with LPS, which suggests that MC degranulation occurred through an IgE-independent stimulation via the activation of TLR-4. Other receptors, such as C3a and C5a, may also be able to induce activation, as MCs can be activated through complement pathways (Prodeus *et al.*, 1997). Immune complexes activate certain receptors on MCs, including C3a and C5a. An experiment using C3-deficient mice found that treating these mice with C3 reduced the number of peritoneal MCs (Prodeus *et al.*, 1997).

This study also demonstrated that after stimulation, the PCMCs of all the strains showed detectable levels of IL-4. This suggests that either the *T.* Ag alone or sensitised with IgE were able to activate MCs to release IL-4 in the different strains. MC-deficient mice did not release higher quantities of mMCP-1 in IgE-independent or IgE-dependent MCs. The findings show that the *T.* Ag alone was unable to stimulate PCMCs to release mMCP-1. The PCMCs from the MC-deficient mice were shown to release similar levels of mMCP-1 as the wild-type mice.

The ability of BMMCs to degranulate after stimulation with helminth products was measured. The levels of β-hexosaminidase were detectable in the BMMC supernatants of all the strains, and no significant change in this level was observed in comparison to the media control. It has been suggested that complement pathway products and TLRs could assist in the activation of MCs, either through the direct activation of MCs or through indirect activation via an interaction with the FcεRI receptors (Gilfillan and Tkaczyk, 2006). The studies presented herein could find no significant evidence that the IgE-independent activation of BMMCs led to IL-4 secretion. Moreover, the findings show that the *T. spiralis* antigen, alone or in

combination with *T. spiralis*-positive serum, was unable to stimulate BMMCs to release mMCP-1. The BMMCs from all four strains secreted similar levels of mMCP-1.

MCs have been investigated extensively in previous studies; it has been concluded that they are essential for the successful expulsion of intestinal helminths. Multiple mechanisms have been discovered that assist in the immune response to and the expulsion of *T. spiralis*. The results in this thesis showed that MC-deficient mice did not experience delays in the expulsion of *T. spiralis*. MMCs did not appear to be absent in Wsh/Wsh mice, and these mice did have levels of mMCP-1. Similarly, Mas-TRECK mice also have MMCs and high levels of mMCP-1. This study demonstrated a potential role for MMCs; while MC-deficient mice are potentially deficient in CTMCs due to the Th2 responses induced by a helminth infection, they might not be deficient in MMCs. Further research is required to fully elucidate the extent of MMC deficiency in these mice models, particularly in Wsh/Wsh mice.

Wsh/Wsh mice in this study may not be completely mast cell deficient as W/W-v mice, it is considered that Wsh/Wsh mice exhibit fewer phenotypic abnormalities than do W/W-v mice (Zhou *et al.*, 2007, Nigrovic *et al.*, 2008). Thus, Wsh/Wsh mice could be appropriate for use in determining MC mechanisms that influence adaptive immunity or other biological responses. Although Wsh/Wsh mice have advantages, they also have fewer phenotypic abnormalities that may influence the expulsion of parasites. These abnormalities are associated with the lack of ICCs, which are important for GI motility. Therefore, the absence of these cells leads to abnormalities in intestinal motility, which results in delayed intestinal transit (Vallance *et al.*, 2001).Therefore, in future studies, it would be of interest to further evaluate the role of mast cells and expand our knowledge by reconstituted Wsh/Wsh mice with bone marrow mast cell (BMMC) cultured from mice having the gene of interest deleted. Such mice can be created by reconstitution of Wsh/Wsh mice with BMMC from IL-4-deficient mice and/or TNF-deficient mice during infection with *T. spiralis*. These mice can be used for a number of new approaches for the *in vivo* study of mast cells and their mediators.

Using larger groups of mice would potentially overcome the disparity in results caused by the natural variance between mice, as this would ensure better understanding and interpretation of the data. In addition, further investigation in these models is required to evaluate the method of mast-cell deficiency, and other cell types, including basophils, eosinophils, neutrophils and dendritic cells, need to be fully evaluated to determine the effect of mutations on these cells which may alter the immune response to helminth infection. Further analysis of the phenotype of BBMC and PCMC cells from the different strains could also be analysed by flow cytomtery.

The role of mast cells in *T. spiralis* infection also requires additional analysis of the immune responses to this gastrointestinal nematode, such as studying other cytokine responses, including IL-13 and IFN-γ in Wsh/Wsh and Mas-TRECK mice, to further explain the skewing of the immune responses as a result of *T. spiralis* infection. 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, and this response may be modulated by cytokines, such as IL-4.

MMCs also play a vital role in the intestinal response to infection, and effective MMC generation and mastocytosis cannot occur in the absence of MCs. Other models of MC deficiency include *Mcpt5-Cre, iDTR*, *Mcpt5-Cre* and *R-DTA*, and these models have advantages over traditional W/W-v and Wsh/Wsh models, as mast-cell deficiency in these models is unrelated to c-Kit functioning, which has been confirmed to contribute to MC depletion (Dudeck *et al.*, 2011a). These models could be used to confirm the results presented herein if Wsh/Wsh and Mas-TRECK mice are not depleted of MMCs.

Overall, these studies have highlighted the differences in the biology of mucosal mast cells and connective tissue mast cells, and by studying two types of MC-deficient mice (Wsh/Wsh and Mas-TRECK), this study confirmed the importance of evaluating different models of MC-deficiency in the study of both the pathology and parasite expulsion of *T. spiralis* and concludes that MCs are crucial for protection against and expulsion of *T. spiralis*. However, it is evident that Wsh/Wsh and Mas-TRECK MC-deficient mice are not entirely deficient in mucosal MC.

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APPENDIXES

Carnoy's fixative solution

- EtOH 90 ml
- Chloroform 45 ml
- Acetic Acid 15 ml

Clark's fixative solution

- EtOH 112.5 ml
- Acetic Acid 37.5 ml

Coating Buffer A (0.1 M Na_2CO_3)
Added to 500 ml distilled H_2O, pH adjusted to 8.2:

- Na_2CO_3 5.29 g

Coating Buffer for IgE ELISA (0.05 M Na_2CO_3/$NaHCO_3$)
Added to 500 ml d.H_2O, pH adjusted to 9.6:

- Na_2CO_3 2.65 g
- $NaHCO_3$ 2.1 g

Digest Buffer
In 500 ml of d. H_2O:

- Pepsin 2.5 g
- NaCl 4.5 g
- HCl 2.5 ml

Blocking Buffer

- 10% Fetal Calf Serum
- PBS
- 2 x 50 ml FCS added to 500ml d. H_2O containing 5 PBS tablets

Hank's Solution
To 1 L d. H_2O:

- Hank's Balanced Salts 9.75 g

PBS (20x)
Dissolved into 500 ml of d. H_2O (d. H_2O) and pH adjusted to 7.2:

- HNa_20_2P *$12H_20$ 58.4 g
- NaCl 160 g
- KH_2PO_4 4.8 g
- KCl 4 g

RPMI

500 ml bottle of RPMI contains:

- HEPES solution (pH 7.2) 7.5 ml
- 3.5 M NaHCO3 2.75 ml

Added to bottle:

- Amphotericin B 11 ml
- Pen/Strep 5.5ml

Complete RPMI (added before use):

- FCS 50 ml
- L-Glutamine solution 5.5ml

Wash Buffer (0.05% PBS-Tween)

- PBS (20x) 250 ml
- d. H_2O 4.75 L
- Tween 20 2.5 ml

Diphtheria Toxin (D.T)

- PBS 8 ml
- Diphtheria toxin 8µl

2% 3-aminopropyltriethoxysilane (APES) (500ml)

- APES 10 ml
- Acetone 490 ml

Safranin (O) 0.5% in 0.125 M HCL

- d.H_2O 500ml
- HCL 5.3625 ml
- Safranin (O) 2.5 g

Toluidine Blue 0.5% in 0.5 M HCL

- d.H_2O 500 ml
- Con. HCL 21.45 ml
- Toluidine Blue 2.5 g

0.0.5% Tween PBS

- 20xPBS 250ml
- d. H_2O 475ml
- 0.05% Tween 25ml