ROLE OF M3 MUSCARINIC RECEPTOR IN REGULATION OF IMMUNITY TO INFECTIOUS PATHOGENS

Alykhan Vira

This thesis is submitted to the University of Cape Town in fulfillment of requirements for a Doctor of Philosophy (PhD)

Division of Immunology

Department of Clinical Laboratory Sciences

Faculty of Health Sciences

University of Cape Town

January 2013

Plagiarism Declaration

I, Alykhan Vira hereby:

(a) grant the University of Cape Town free licence to reproduce the above thesis in whole or in part, for the purpose of research;

(b) declare that:

(i) the above thesis is my own unaided work, both in concept and execution, and that apart from the normal guidance from my supervisor, I have received no assistance except as stated below:

where assistance was received from collaborators in generating the data this has been duly acknowledged

(ii) neither the substance nor any part of the above thesis has been submitted in the past, or is being, or is to be submitted for a degree at this University or at any other university, except as stated below:

_____

I am now presenting the thesis for examination for the degree of PhD.

Candidate Signature:……..

Publications

During the tenure of my PhD studies (commencing 1st August 2009), the following manuscripts for which I am first author or joint first author have been published or have been submitted for publication.

1. **Vira, A, ME. Selkirk, C. Schnoeller, F. Culley, M. Darby, J. Wess, F. Brombacher, WGC. Horsnell.** Acetylcholine signalling via the M3 muscarinic receptor is required for optimal primary and recall immunity to *Nippostrongylus brasiliensis*. (Submitted for Review December 2012)

2. **Horsnell, W. G.*, A. Vira*, F. Kirstein, H. Mearns, J. C. Hoving, A. J. Cutler, B. Dewals, E. Myburgh, M. Kimberg, B. Arendse, N. White, A. Lopata, P. E. Burger, and F. Brombacher.** IL-4Ralpha-responsive smooth muscle cells contribute to initiation of T_H2 immunity and pulmonary pathology in *Nippostrongylus brasiliensis* infections. Mucosal Immunol (2010) **4:**83-92.

*** - These authors have contributed equally.**

ACKNOWLEDGEMENTS:

A great many people have made it possible for me to reach this stage of my PhD. My co-supervisors Prof. Frank Brombacher and Dr. William Horsnell have been exceptional mentors, providing intellectual support at every step. Our collaborators on this project, especially Prof. Murray Selkirk and Dr. Corinna Schnoeller have been great source of critical comments and study design. My gratitude goes out to the International Center for Genetic Engineering and Biotechnology (ICGEB) for a 4 year fellowship which provided me with the financial support during my PhD tenure.

I was also fortunate to have support and technical assistance from my fellow lab members. Special thanks to Umeshree, Matthew, Sumaiyya, Dunja, Claire, Natalie, Anita, Reto, Suraj and Frank for help with various experiments, intellectual input and critical reading of my thesis. I would also like to thank Wendy, Rayaana, Lizette, Ronnie, Sandra, Chadrick and George for providing technical expertise.

Finally, I would like to thank my friends, who over the course of my stay in Cape Town, have become like family and without whose moral support I would not have the motivation to get through my academic pursuits. Nousheen, whose constant encouragement has helped keep me sane. My greatest gratitude goes out to my parents whose unwavering support and love, not only during the last 4 years, but throughout my life has been the source of my achievement.

List of Abbreviations:

ACh – Acetylcholine

AChE – Acetylcholinesterase

APC – Antigen Presenting cells

AT – Atropine

BCR – B cell receptor

BMDM – Bone marrow derived macrophages

BSA – Bovine Serum Albumin

CAP – Cholinergic anti-inflammatory pathway

CD – Cluster of differentiation

CFU – Colony forming units

ChAT – Choline acetyltransferase

CNS – Central Nervous system

DMEM – Dulbecco's Modified Eagle Medium

ELISA – Enzyme Linked Immunosorbent Assay

FACS – Fluorescence activated cell sorting

GPCR – G Protein coupled receptor

HRP – Horseradish peroxidase

IFN-γ – Interferon Gamma

IgG – Immunoglobulin

IL- Interleukin

IMDM – Iscove's Modified Dulbecco's Medium

LB – Luria Broth

LPS - Lipopolysaccharide

M3R – M3 muscarinic receptor

$M3R^{-/-}$ - M3R deficient mouse strain

mAChR – Muscarinic acetylcholine receptor

MHC – Major Histocompatibility Complex

mRNA – Messenger RNA

N.brasiliensis – *Nippostronglyus brasiliensis*

nAChR – Nicotinic acetylcholine receptor

NK cell – Natural Killer cells

NTS – Non-tyhpoidal Salmonella

Oxo-M – Oxotermorine M

PAS – Periodic Acid Schiff

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

S.typhimurium – *Salmonella enterica* sp. Typhimurium

SEM – Standard error of Mean

Tbet - T-box expressed in T cells

TCR – T cell receptor

T_H1 – T helper type 1 immune response

T_H2 – T helper type 2 immune response

TLR – Toll-like Receptor

TNF-α – Tumour Necrosis Factor alpha

Contents

List of Figures and Tables

Literature Review

Materials and Methods

Results

Conclusion

ABSTRACT

During the last decade, cholinergic signaling via acetylcholine and its receptors has emerged as an important regulator of immunity. Acetylcholine binds to and signals through two types of receptors; nicotinic and muscarinic receptors. Studies have shown that signaling through nicotinic receptors, particularly the α7 subtype on macrophages has potent anti-inflammatory effects. However, the role for muscarinic receptor has not yet been conclusively characterized. In this study, we demonstrate that M3 muscarinic receptor subtype is required for optimal protective immunity to two pathogens; the nematode *Nippostrongylus brasiliensis* and the bacterium *Salmonella enterica* sp. Tyhpimurium. M3R deficient mice ($M3R^{-/-}$) were susceptible to infection with *N.brasiliensis* with decreased production of the protective cytokine IL-13. Furthermore, stimulation of lymphocytes with muscarinic agonists enhanced T_H2 cytokine production in an M3R dependent manner. We identified CD4 T cells as the main immune cell type affected in $M3R^{-/-}$ mice with protection elicited by adoptive transfer of antigen experienced CD4 T cells completely abrogated in the absence of M3R. We also found that M3R was required for regulating a T_H1 biased immune response induced by *S.typhimurium* and $M3R^{-/-}$ mice were more susceptible having up to 20 fold higher bacterial burden in the spleen at day 27. Similarly, CD4 T cells from $M3R^{-/-}$ mice had reduced production of IFN-γ and expression of its upstream transcription factor Tbet. Following infection with both pathogens, a lower proportion of CD4 T cells were activated in $M3R^{-/-}$ mice and also had reduced expression of the T cell costimulatory receptor CD28, hence providing a potential mechanism for M3R mediated immune regulation. Our data provides the first conclusive evidence of a role for the muscarinic receptor subtype M3 in enhancing T helper type 1 and 2 immune responses following infection. Unlike, nicotinic receptors which dampen immune responses, we find that muscarinic receptors are pro-inflammatory and function to enhance immune responses, influencing the activity of CD4 T cells possibly via the expression of the T cell co-stimulatory molecule CD28.

LITERATURE REVIEW

Literature Review

1. Immunity:

The immune system is an important physiological response mechanism that protects against entry of foreign microorganisms or particles that may harm the body. At the same time, the immune system also has to recognize and maintain tolerance to self-antigens and commensal microorganisms as an inappropriate response towards these can be destructive to the host (Janeway 1992; Van Parijs and Abbas 1998; Lathrop, Bloom et al. 2011). The immune system has thus developed two distinct arms, the innate and adaptive systems, to recognize and control pathogenic insult as well as to develop long term memory immunity which can protect against future infections by the same pathogen (Murphy, Travers et al. 2012).

1.1. Innate and Adaptive Immunity:

The innate immune system is the rapid response arm of the immune system and is activated within hours of a pathogenic infection (Fearon and Locksley 1996). A number of cell types such as macrophages, neutrophils, dendritic cells, natural killer cells, eosinophils and mast cells are associated with innate immunity (Janeway and Medzhitov 2002). A common feature of these cells is their ability to detect a broad spectrum of pathogens using pattern recognition receptors on their cell surface such as the Toll-like receptors (TLR) (Medzhitov and Janeway 1997). Effector mechanisms for innate immune cells include phagocytosis of pathogens into specialized structures called phagosomes where they can be destroyed and in the case of Antigen Presenting Cells (APCs) display the pathogenic antigens for activating the adaptive immune system. Innate immune cells also contain stores of toxic molecules and enzymes such as perforin, granzymes, and cathelicidin that can directly

destroy extracellular pathogens and infected cells. Innate immunity includes non-cellular components such as the complement system, a number of plasma proteins that can bind to pathogens and assist in their uptake and destruction (Beutler 2004). An important feature of the innate immune system is its ability to activate the adaptive immune system which then ensures complete elimination of the pathogen as well as development of immune memory for the specific pathogen in case of a re-infection (Medzhitov and Janeway 1997).

The adaptive immune response comprises mainly of T and B lymphocytes that develop later in an infection but are highly specific for the pathogen. T and B lymphocytes express a broad repertoire of T cell (TCR) and B cell (BCR) receptors respectively which theoretically span the entire antigen spectrum of foreign organisms. The specificity of the adaptive immune response arises from activation and proliferation of only those clones of T and B lymphocytes that express receptors complementary to pathogen antigens displayed by APCs. T lymphocytes are further classified into CD4 Helper T cells and CD8 Cytotoxic T cells (Murphy, Travers et al. 2012). CD4 T cells produce immunomodulatory proteins called cytokines that can assist B cell development into antibody-producing cells and to activate phagocytes such as macrophages to enhance intracellular pathogen killing (Adams and Hamilton 1984; Parker 1993). CD8 T cells can directly kill other host cells infected by viruses or intracellular pathogens using a variety of toxic enzymes such as perforins (Sallusto, Geginat et al. 2004). B lymphocytes upon activation develop into plasma cells which secrete antibodies that bind specifically to pathogens and other foreign antigens. Antibody bound foreign particles undergo enhanced uptake by macrophages and are thus more readily eliminated (Murphy, Travers et al. 2012).

The immune system is an exquisitely controlled system, with the innate and adaptive immune system working together to limit spread of pathogens and to develop memory immunity that responds more rapidly when the pathogen is encountered a second time. However, uncontrolled inflammation targeted at the pathogen can also damage the host's own tissue and therefore must be tightly regulated. The body also has to control aberrant immune responses to self-antigens, benign environmental antigens and beneficial commensal micro-organisms. The immune system has thus developed a mechanism of regulating the immune system comprising of regulatory T cells (Josefowicz, Lu et al. 2012). Regulatory T cells are specialized CD4 T cells that express the transcription factor *Forkhead box P3* (FoxP3) and can limit inflammatory responses from other immune cells (Fontenot, Gavin et al. 2003; Khattri, Cox et al. 2003; Fontenot, Rasmussen et al. 2005). Regulatory CD4 T cells employ cell surface receptors such as the T cell inhibitory receptor CTLA-4 (Read, Malmstrom et al. 2000; Read, Greenwald et al. 2006) and secrete cytokines such as IL-10 and TGF-β in order to achieve immune suppression (Li, Wan et al. 2007; Rubtsov, Rasmussen et al. 2008). Other mechanisms also exist for controlling the immune system including apoptosis of activated immune cells and secretion of steroids such as glucocorticoids (Krammer 2000; De Bosscher and Haegeman 2009)

1.2. Classes of Immune response : T_H1 Versus T_H2

CD4 T lymphocytes are central regulators of the adaptive immune system and ensure that an appropriate immune response is generated that facilitates clearance of pathogens but at the same time limits tissue damage. The first attempt at classifying CD4 Helper T cells into distinct subsets was proposed by Mosmann and Coffman as the T Helper type 1 (T_H1) and T Helper type 2 (T_H2) based on their cytokine profile (Mosmann and Coffman 1989). The T_H1 response is pro-inflammatory and considered to be protective against intracellular pathogens mainly bacteria,

protozoan and viral infections. Cytokines that are characteristic of a T_H1 response include IFN-γ, TNF-α and IL-12 can activate macrophages to promote intracellular killing as well as to act in a positive feedback loop to increase pro-innflammatory cytokine production (Szabo, Sullivan et al. 2003).

T_H2 responses on the other hand are characterized by the cytokines IL-4, IL-5 and IL-13 and is associated with resolving helminth infections and conversely causing allergic reactions (Mosmann and Sad 1996). T_H2 unlike T_H1 responses are not inflammatory and may have evolved to prevent tissue destruction during chronic helminth infections. Effector mechanisms induced by T_H2 cytokines are also geared towards expulsion of helminth parasites and include increased smooth muscle contractility, mucus production from goblet cells, epithelial cell turnover and recruitment of eosinophils (Anthony, Rutitzky et al. 2007). This T_H2 induced immunopathology is beneficial during helminth infection however can also be detrimental in the case of allergic reactions (Yazdanbakhsh, van den Biggelaar et al. 2001). The T_H1/T_H2 paradigm has more recently been extended to include other distinct responses such as T_H17, another pro-inflammatory response that develops during fungal infections and regulatory T cells (T_{reg}), a subset that actively downregulates inflammation and CD4 T cell activity (Mosmann and Sad 1996; Korn, Bettelli et al. 2009).

Other classes of immune responses such as the T helper 17 (T_H17) and Regulatory T cell have since been discovered to expand our understanding of the distinct classes of immune response.

2. Acetylcholine and the immune system:

Acetylcholine is the first neurotransmitter discovered in humans and is responsible for nerve impulse transmission across synapses in the central nervous system. Acetylcholine synthesis, however, has also been identified in a number of non-neuronal cellular sources indicating that acetylcholine may have alternative functions outside of the nervous system (Wessler, Kirkpatrick et

al. 1998; Kawashima and Fujii 2004). It is also produced by a variety of lower organisms without a nervous system such as plants, bacteria and protists, suggesting that this molecule is an ancient signaling molecule which has been retained by evolution in higher organisms (Wess, Eglen et al. 2007).

Acetylcholine in humans has diverse roles depending on its cellular source as well as its target cell. Neuronal sources of acetylcholine are predominantly responsible for maintaining nerve impulse transmission across synaptic junctions where acetylcholine produced by the pre-synaptic neuron binds to an appropriate receptor on the post-synaptic neuron (Caulfield 1993; Dajas-Bailador and Wonnacott 2004). In addition, acetylcholine acts as an important signaling molecule in neuromuscular junctions where it stimulates muscular contraction upon binding specific receptors on smooth muscles (Caulfield 1993). Non-neuronal cellular sources of acetylcholine also possess the enzyme necessary to synthesize acetylcholine as well as the enzymes for its transport, storage and degradation. These sources include skin keratinocytes, bone forming osteoclasts and importantly components of the immune system such as CD4 T cells, B cells, macrophages and dendritic cells (Fujii, Takada-Takatori et al. 2008; Kawashima and Fujii 2008).

Over the past decade, a number of studies have shown an emerging role for acetylcholine in immunity. Tracey *et al.* have, in a series of papers, shown that acetylcholine secretion following vagus nerve stimulation, particularly from a subset of activated CD4 T cells in the spleen, can suppress pro-inflammatory immune responses in a murine model of endotoxaemia (Borovikova, Ivanova et al. 2000; Wang, Yu et al. 2003; Rosas-Ballina, Olofsson et al. 2011). However, very little is known about the role of acetylcholine and its associated molecular machinery in regulating host immune cell responses especially in response to infectious pathogens.

2.1. Acetylcholine receptors:

Acetylcholine acts as a signaling molecule by binding specifically to receptors on the surface of target cells. In mammalian systems, acetylcholine binds to and signals via two distinct families of receptors known as the nicotinic and muscarinic receptors (Felder 1995).

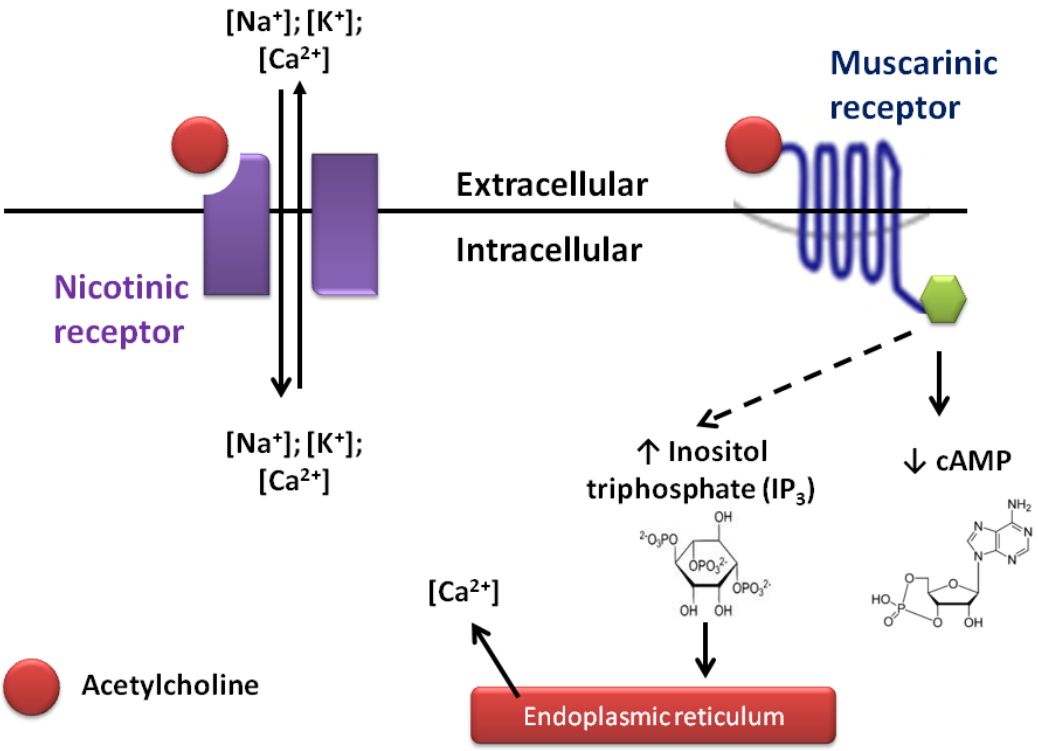


Fig 1. Different mechanisms of action of two distinct acetylcholine receptors; nicotinic receptor, a ligand gate ion channel and muscarinic receptor, a GPCR.

2.1.1. Nicotinic receptor:

Nicotinic receptors are pentameric cell surface receptors, comprising of one (homo-meric) or more (hetero-meric) types of subunits, which upon binding acetylcholine undergo a rapid conformational change to allow influx of Na^+ ions into and efflux of K^+ out of target cells. This mainly occurs at the synapse between two neurons and is the classical method of propagating nerve impulses. Some subunit configurations also allow entry of Ca^{2+} ions which results in activation of calcium sensitive

genes and enzymes. The prototypical agonist for nicotinic receptors is nicotine from which the receptor derives its name (Brejc, van Dijk et al. 2001).Nicotinic receptors are mainly found in the synapses of neurons or at the neuromuscular junction. Immune cells such as macrophages and CD4 T cells also express functional nicotinic receptors (Toyabe, Iiai et al. 1997; Wang, Yu et al. 2003).

2.1.2. Muscarinic receptor:

Muscarinic receptors are classical G-protein coupled receptors (GPCRs) that also bind acetylcholine to initiate a downstream signaling cascade resulting in influx of ions into the cell, which unlike with the nicotinic receptor, is a slower process and involves a number of intermediate secondary messengers. The initiation of signaling proceeds via a G protein that is bound to a GPCR, and can further activate a number of important signaling molecules such as kinases (Caulfield 1993; Felder 1995).

Muscarinic receptors are divided into 5 subtypes namely the M1-M5 and can be placed in two distinct groups depending on the associated G protein and secondary messengers utilized. These receptors have also been highly conserved in mammalian species as analysis of amino acid sequences shows a high degree of similarity (over 90% in most cases). The M1, M3 and M5 receptors are activating in nature and bound to the G protein G_q protein which upon stimulation causes an increase in the secondary messenger inositol triphosphate (IP3). In contrast, the M2 and M4 receptor are inhibitory, associate with the G_i protein and upon stimulation results in a decrease in cyclic AMP (cAMP), another secondary messenger. (Caulfield and Birdsall 1998; Abrams, Andersson et al. 2006).

Muscarinic receptors are widely expressed both in the central nervous system and periphery where they regulate a number of physiological processes. In the central nervous system, muscarinic

receptors are involved in motor control, temperature and cardiovascular regulation. In the periphery, muscarinic receptors regulate processes such as cardiac rate, smooth muscle contraction and glandular secretions (saliva and insulin) (Wess 1993; Wess, Eglen et al. 2007).

2.2. Acetylcholine synthesis and transport enzymes

Acetylcholine is a 146 KDa molecule synthesized from the abundant cellular precursors acetyl-CoA and choline in a reaction that is catalyzed by the enzyme Choline acetyltransferase (ChAT) (Oda 1999). This reaction occurs primarily in the pre-synaptic neuron where the newly synthesized acetylcholine is packaged into vesicles and transported by the Vesicular acetylcholine transporter (VAChT) (Arvidsson, Riedl et al. 1997). However, neuronal cells are not the only source of acetylcholine in the body. A number of non-neuronal cell types have also been identified which are capable of synthesizing and secreting acetylcholine on the basis of expression of the ChAT enzyme which acts as a useful marker for non-neuronal sources as acetylcholine is labile and prone to degradation (Wessler, Kilbinger et al. 2001). Therefore a number of molecular techniques such as RT-PCR, western blotting and GFP tagged ChAT have been used so far to identify cell types capable of producing acetylcholine (Rathenberg, Gartner et al. 2002). Some studies have even been able to detect measurable amounts of acetylcholine using High Pressure Liquid Chromatography (HPLC) from non-neuronal cells as a further proof of its synthesis by non-neuronal cells (Klapproth, Reinheimer et al. 1997; Fujii, Tajima et al. 1999). Cells such as bone osteoclasts, skin keratinocytes, and immune cells such as CD4 T cells, dendritic cells have all been shown to express ChAT (Wessler, Kilbinger et al. 2001). Interestingly, dendritic cells up-regulate ChAT mRNA expression levels after stimulation implying that changes in acetylcholine production may be important in regulating immune responses (Kawashima, Yoshikawa et al. 2007).

2.3. Degradation of acetylcholine (Acetylcholinesterase)

Acetylcholine is a potent signaling molecule and needs to be removed rapidly after its release to prevent continuous stimulation of nerves and muscles. The hydrolysis of ACh is catalyzed by Acetylcholinesterase (AChE), an enzyme that is found mainly in post-synaptic neurons (Massoulie and Bon 1982) and red blood cells (Steck 1974) but can also be produced by immune cells such as macrophages and T cells (Szelenyi, Bartha et al. 1982; Klegeris, Budd et al. 1994). In neurons, AChE breaks down acetylcholine into acetate and choline, which is recycled back to the pre-synaptic neuron to be re-synthesized. In immune cells, it has been postulated that acetylcholinesterase is required to lower acetylcholine in the surrounding milieu hence reduce its anti-inflammatory effects (Nizri, Hamra-Amitay et al. 2006). This principle was conclusively demonstrated when a microRNA (miRNA) that targets AChE for degradation was identified (Shaked, Meerson et al. 2009). In macrophages, AChE levels are tightly regulated by the microRNA miR-132 which targets AChE transcripts for degradation with subsequent increase in the acetylcholine levels. In activated macrophages, there is a significant increase in miR-132 as a regulatory measure in order to limit pro-inflammatory cytokine production via acetylcholine. Conversely, when AChE transcripts are modified to render them insensitive to miR-132, there is an increase in pro-inflammatory cytokines IL-1β and IL-6 as a result of decreased acetylcholine availability. This further demonstrates that acetylcholine is an important regulator of the immune system and acts as an anti-inflammatory signal at least for macrophages (Shaked, Meerson et al. 2009). The body has thus developed various mechanisms to regulate the availability of acetylcholine by regulating enzymes involved in its synthesis and degradation so as to achieve the desired level of inflammation. Since the nervous system is the major producer of acetylcholine, it also raises the possibility that it can control the immune system through this ancient signaling molecule (O'Neill 2009).

Table 1: Functions of different components of the cholinergic system in regulating immunity

Cholinergic component	Cell type	Immune Function	Reference
Choline acetyltransferase (marker of acetylcholine production)	CD4 T cell	- Inhibit TNF-α production from macrophages during endotoxemia.	(Rosas-Ballina, Olofsson et al. 2011)
	B cell	- Control neutrophil recruitment during inflammation	(Reardon, Duncan et al. 2013)
Acetylcholinesterase	Macrophage	- Decreased AChE level during inflammation (miRNA-132 mediated) enhances ACh mediated anti-inflammatory effects.	(Shaked, Meerson et al. 2009)
	Central Nervous System (CNS)	- AChE inhibitors reduce IL-1β in brain	(Pollak, Gilboa et al. 2005)
α7 nicotinic receptor	Macrophage	- Anti-inflammatory. Binds acetylcholine to reduce TNF-α production	(Wang, Yu et al. 2003)
M1 muscarinic	CD8 T cell	- Development of cytolytic T cell responses *in vitro*. No defect in $M1R^{-/-}$ CD8 T cells *in vivo*.	(Zimring, Kapp et al. 2005; Vezys, Masopust et al. 2007)
M3 muscarinic	CD4 T and B lymphocytes (human cell lines)	- Stimulation induces Ca^{2+} influx and c-fos gene expression	(Fujii and Kawashima 2000)
Muscarinic receptor (subtype not identified)	Human T cell line (Jurkat)	- Enhance IL-2 production blocked by atropine and M3 receptor specific antagonist 4-DAMP	(Nomura, Hosoi et al. 2003)

2.4. The Cholinergic Anti-Inflammatory Pathway (CAP)

Historically, the immune system was viewed as an autonomous physiological process that operated outside the influence of the central nervous system. However, this dogma was challenged when a study by Tracey *et al* published 10 years ago showed that electrical stimulation of the vagus nerve can significantly inhibit pro-inflammatory cytokine production and improve survival during LPS induced endotoxemia in a murine model (Borovikova, Ivanova et al. 2000). This led to a paradigm shift in which the immune system was highly influenced by the nervous system was termed Cholinergic Anti-inflammatory Pathway (CAP). Further studies by the Tracey lab demonstrated that vagus nerve stimulation affected macrophages in the spleen which produced lower amounts of TNF-α, the cytokine that drives pathophysiology of LPS induced toxic shock. The receptor that regulates this effect is the alpha7 subunit of the nicotinic receptor (α7nAChR) on macrophages as vagal stimulation in α7nAChR deficient mice did not result in TNF-α suppression (Wang, Yu et al. 2003).

The actual source of acetylcholine remained ambiguous as no acetylcholine-secreting cholinergic nerves have been found in the spleen which could serve as a link between the vagus nerve and macrophages (Rosas-Ballina, Ochani et al. 2008). However, this discrepancy was resolved by a study which showed that the source of acetylcholine is a certain population of activated ($CD44^{hi}CD62L^{lo}$) CD4 T cells in the spleen that expresses choline acetyltransferase (ChAT).The vagus nerve endings which innervate the T cell zones in the spleen are adrenergic, producing the neurotransmitter noradrenalin which can directly stimulate the ChAT positive CD4 T cell subpopulation to increase acetylcholine secretion. When this ChAT positive population was transferred into nude mice that lack functional T cells, it restored the anti-inflammatory effect of vagus nerve stimulation (Rosas-

Ballina, Olofsson et al. 2011). This was the first clear evidence of a non-neuronal source of acetylcholine modulating the immune system (Trakhtenberg and Goldberg 2011).

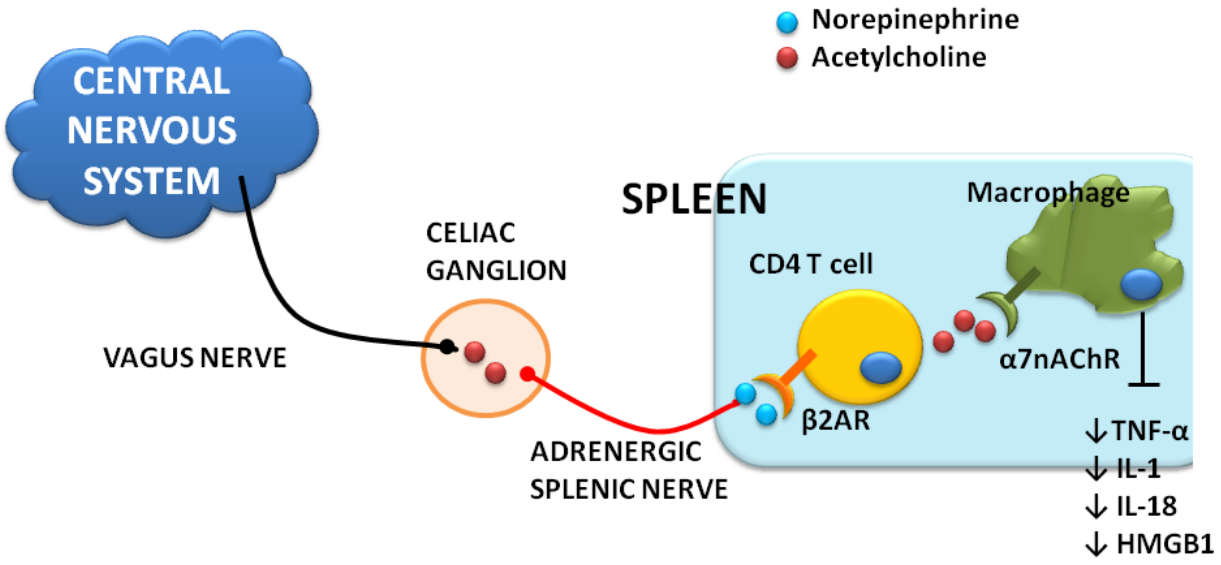


Fig 2: Regulation of cytokine production by the Cholinergic Anti-Inflammatory Pathway (CAP). Adapted from Andersson and Tracey, *Journal of Experimental Medicine* (Andersson and Tracey 2012)

Regulation of inflammation by the cholinergic anti-inflammatory pathway has now been shown for many other disease models such as collagen induced arthritis (Zhang, Han et al. 2008), sepsis (Huston, Gallowitsch-Puerta et al. 2007), ischemia-reperfusion injury (Bernik, Friedman et al. 2002) and DSS induced colitis (Ghia, Blennerhassett et al. 2006) indicating that CAP via the vagus nerve may be one of the main ways by which the body limits excessive inflammation. The existence of CAP also raises the possibility of pharmacological interventions targeting this pathway. Since the nicotinic receptor plays a central role in CAP, exogenous administration of nicotine may control the inflammation. This has been observed in human clinical trials where patients suffering from

ulcerative colitis, a painful condition caused by excessive inflammation in the gut, were treated with nicotine. A significantly higher proportion of patients had complete remission after nicotine treatment suggesting that CAP can be manipulated pharmacologically to reduce inflammation in certain diseases (Sandborn, Tremaine et al. 1997; Guslandi 1999).

3. M3 muscarinic receptor:

3.1. Structure and classical signal transduction pathway.

Muscarinic M3 receptor (M3R) is a G-protein coupled receptor with the characteristic 7-transmembrane region that is structurally similar to the classic GPCR rhodopsin. Human M3 receptor is made up of 590 aa and is highly similar to murine M3 receptor (589 aa) with a sequence similarity of 92% (pairwise sequence alignment using LALIGN) as well as across other species such as pig and rat (Caulfield and Birdsall 1998) implying that the M3R has been highly conserved and is likely to exhibit similar functions across species.

The N-terminal end of the M3 receptor is extracellular and is heavily glycosylated whereas the C-terminal is located intracellularly and associates with the $G_{q/11}$ family of G-proteins which are responsible for initiating the downstream signaling cascade (Caulfield 1993). The M3R, like all muscarinic receptors, is a cationic amine receptor which binds its ligand acetylcholine using an Aspartic residue in the third extracellular loop. This binding initiates a downstream signaling cascade that starts with phosphorylation of the α subunit of the $G_{q/11}$, which detaches from the C-terminal end of the M3R to activate Phospholipase C (PLC) through a series of kinases (Exton 1996). PLC then cleaves a phospholipid to generate diacyglycerol and inositol triphosphate, both of which can

act as secondary messengers. This results in the release of Ca^{2+} ions from intracellular sources and subsequent increase in Ca^{2+} ion concentration, which is responsible for mediating the functional effects of M3R such as smooth muscle contraction and glandular secretion (Lanzafame, Christopoulos et al. 2003).

A major problem with studying muscarinic receptors is the structural similarity particularly at the active site of the 5 subtypes. However, the crystal structure of the M3 receptor has recently been solved making it easier for rational design of subtype specific ligands (Kruse, Hu et al. 2012).

3.2. Characterization of M3 muscarinic receptor knockout mouse.

Transgenic mice lacking each of the 5 muscarinic receptor subtypes have been generated and are reproductively viable thus making them a valuable tool in dissecting the role of individual receptor subtypes (Hamilton, Loose et al. 1997; Gomeza, Shannon et al. 1999; Gomeza, Zhang et al. 1999; Yamada, Lamping et al. 2001; Yamada, Miyakawa et al. 2001). All 5 knockout mouse strains have been systematically tested for wide range of physiological defects providing subtype specific roles for the muscarinic receptor. These insights have allowed pharmaceutical companies to design more subtype selective and ultimately more clinically relevant drugs (Wess, Eglen et al. 2007). Some examples of physiological functions identified include inhibition of β-amyloid particle deposition (M1), regulation of heart rate (M2), smooth muscle contractility (M3) and decrease in cocaine addiction (M5).

The M3 receptor knockout mice ($M3R^{-/-}$) was first reported in a study that characterized functional defects in peripheral autonomic organs in this mutant mouse strain. The M3 receptor gene (*Chrm3* in mice) was disrupted by replacing part of the normal gene with a neomycin cassette and deletion was

confirmed using western blotting to show complete absence of M3 receptors in the brain of homozygous $M3R^{-/-}$ mice (Matsui, Motomura et al. 2000). This mouse strain has thus become a valuable tool in dissecting various functional roles of the M3R (Wess, Eglen et al. 2007).

3.3. Roles of non-neuronal M3 receptor.

The muscarinic M3 receptor is highly expressed on non-neuronal cells in the periphery (outside of the nervous system) particularly on smooth muscle cells (Eglen, Reddy et al. 1994; Eglen, Hegde et al. 1996); exocrine glands such as pancreas (Gautam, Han et al. 2006), salivary glands (Gautam, Heard et al. 2004); keratinocytes (Ndoye, Buchli et al. 1998); tumour cells (Oppitz, Busch et al. 2008; Raufman, Samimi et al. 2008) and immune cells such as CD4 T cells, macrophages, and dendritic cells (Fujii, Watanabe et al. 2003; Kawashima, Yoshikawa et al. 2007). Due to this widespread expression, the M3R has been associated with a wide range of physiological roles, some of which have been experimentally verified and other hypothetical roles with some experimental evidence.

3.3.1. Smooth muscle contraction:

The parasympathetic nervous system controls smooth muscle contraction by using acetylcholine to stimulate muscarinic receptors resulting in a contractile response (Nadel and Barnes 1984). M3 muscarinic receptor has been identified as the major regulator of this process using M3R specific antagonists as well as M3R deficient mouse strains (Loenders, Rampart et al. 1992; Matsui, Motomura et al. 2000). This discovery was important pharmacologically, as a number of specific M3 inhibitors were developed to target this contractile response which is an important pathological feature of asthma (broncho-constriction), COPD (Gosens, Zaagsma et al. 2006) and urinary bladder

disorders (Hegde and Eglen 1999) resulting in approved drugs such as Spiriva® (Tiotropium bromide) and Darifenacin®.

Smooth muscle contraction is initiated by acetylcholine binding to a M3 muscarinic receptor which activates a signaling cascade which results in release of Ca^{2+} ions from intracellular stores and extracellular entry. This increase in Ca^{2+} in the cytosol facilitates contraction through major structural proteins such as actin and myosin (Ebashi and Ebashi 1964; Bremel 1974).

3.3.2. Secretion from exocrine and endocrine glands:

M3 receptor expression has been observed in a number of peripheral organs, particularly on secretory cells of certain exocrine and endocrine glands where its function has been well characterized. Exocrine glands such as the salivary gland are responsive to acetylcholine produced by parasympathetic neurons resulting in secretion of saliva by acinar cells (Baum 1993). Exocytosis of saliva from acinar cells is initiated by an increase in intracellular Ca^{2+}, a process dependent on muscarinic receptor stimulation similar to that observed in smooth muscle cells (Turner and Sugiya 2002). *In vivo*, M1 and M3 muscarinic receptors were identified as the main subtypes regulating this process using muscarinic receptor deficient mice (Gautam, Heard et al. 2004).

Control of endocrine secretion, most notably insulin secretion, by the M3 muscarinic receptor has been extensively studied due to the potential therapeutic application in obesity and diabetes (Gilon and Henquin 2001). Insulin is produced and secreted by β-pancreatic cells found in the islets of Langerhans in response to increased blood glucose levels after feeding. Acetylcholine acts in synergy with glucose to increase intracellular Ca^{2+} which drives the exocytosis of insulin (Garcia, Hermans et al. 1988). M3 muscarinic receptor on pancreatic β-cells is the major receptor regulating this process

as demonstrated using β-cell specific M3 receptor knockout mice (Gautam, Han et al. 2006). Interestingly, in models of obesity and obesity-induced insulin tolerance, M3 receptor deficient mice have a more favorable outcome suggesting that, in these models at least, antagonizing M3 receptor function may be beneficial (Gautam, Gavrilova et al. 2006). Another interesting observation is that in mice, unlike humans, the pancreatic islets are well innervated with parasympathetic neurons which act as a source of acetylcholine. However in humans, alpha cells which also reside in the islets of Langerhans, are the main source of acetylcholine that prime nearby β-cells to secrete insulin (Rodriguez-Diaz, Dando et al. 2011).

3.3.3. Proliferation of tumor cells:

Expression of the M3 muscarinic receptor has been identified in a number of carcinoma cell lines such colon cancer, prostate carcinoma, gastric cancer as well as primary cancer cells (Frucht, Jensen et al. 1999; Kodaira, Kajimura et al. 1999). A characteristic feature of tumor cells is their ability to proliferate rapidly and muscarinic receptors particularly the subtypes M1 and M3 were associated with modulating growth of cells *in vitro* (Brown, Sah et al. 1997). Colon cancer cell lines exhibit different levels of M3 expression, with high expressing cell lines showing Ca^{2+} mobilization and increased proliferation when stimulated with muscarinic agonists which could be blocked by specific antagonists of M3 receptors (Frucht, Jensen et al. 1999). Acetylcholine acts as the autocrine growth signal via M3R at least for one of the colon cancer cell line (H508) which can secrete acetylcholine and this increased proliferation can be inhibited by atropine and a specific M3R antagonist p-sila-difenidol (Cheng, Samimi et al. 2008). Interestingly, in a murine model of colon neoplasia induced by azoxymethane, mice lacking M3R ($M3R^{-/-}$) had a significantly reduced number and size of tumors suggesting that M3 receptors are also important for regulating cellular proliferation *in vivo* (Raufman, Samimi et al. 2008). This is not only limited to colon cancer, but was also observed in a small cell

lung carcinoma model both *in vivo* and *in vitro* where administration of Darifenacin, a M3R specific antagonist, inhibited tumor growth (Song, Sekhon et al. 2007). The growth stimulating effect of M3R is likely due to its effect on pro-proliferative signaling proteins Mitogen Activated Protein Kinase (MAPK) and Epidermal Growth Factor (EGF) (Cheng and Raufman 2005; Song, Sekhon et al. 2007).

3.3.4. Regulation of Apoptosis:

Apoptosis is a term used to describe programmed cell death, a series of controlled processes by which a target cell undergoes cell death and is consequently removed without eliciting inflammatory responses (Elmore 2007). This process is commonly observed during removal of infected cells, auto-reactive thymocytes in the thymus, and cells with damaged DNA caused by UV radiation (Zimmermann and Green 2001). Muscarinic receptors have been shown to be protective against chemical induced apoptosis in a number of cell lines *in vitro* by using non-specific muscarinic agonists such as oxotremorine-M (Lindenboim, Pinkas-Kramarski et al. 1995; De Sarno, Shestopal et al. 2003). Muscarinic receptor subtypes M1, M3 and M5 that couple to the $G_{q/11}$ pathway significantly attenuated apoptosis when transfected into a Chinese Hamster Ovary (CHO) cell line where as M2 and M4 which couple to a different G protein do not. The C-terminal tail of M3 muscarinic receptor was determined as the main regulator of the anti-apoptotic effect, and this effect is independent of classical muscarinic activated pathways such as Phospholipase C, Janus Kinase (JNK) and extracellular regulated kinase (ERK) activation (Budd, McDonald et al. 2003). Stabilization of the anti-apoptotic protein Bcl-2 was identified as the potential anti-apoptosis mechanism as M3 receptor activation inhibits Bcl-2 degradation (Budd, Spragg et al. 2004). However, how M3 muscarinic receptor regulates Bcl-2 protein levels remains unknown.

3.4. M3 receptor on immune cells.

The M3 receptor as highlighted in the previous section plays an important role in diverse cell types, in most cases by initiating a signaling cascade which results in influx of intracellular calcium, a secondary messenger. However, its role on immune cells remains elusive. Before the discovery of the cholinergic anti-inflammatory pathway (CAP), Kawashima and colleagues published a number of studies characterizing the expression of cholinergic components particularly muscarinic and nicotinic receptors on immune cell types (Kawashima and Fujii 2004). After the prominent immunological role demonstrated for nicotinic receptor in CAP (Wang, Yu et al. 2003), it was hypothesized that a potential role exists for muscarinic receptors which also feature prominently on immune cells.

M3R, together with other muscarinic subtypes, is expressed by a number of human leukemic T cell lines such as Jurkat, CEM and MOLT-3 as well as in peripheral blood mononuclear cells (PBMCs) and alveolar macrophages (Kaneda, Kitamura et al. 1993; Hellstrom-Lindahl and Nordberg 1996; Sato, Fujii et al. 1999; Koarai, Traves et al. 2012). Stimulation of CEM T cells with oxotremorine-M induced calcium influx which could be inhibited by 4-DAMP, an M3 specific inhibitor indicating that the M3 receptor is functional on these cells (Fujii and Kawashima 2000; Fujii and Kawashima 2000). The M3 receptor has also been identified in murine mononuclear leukocytes, dendritic cells and macrophages (Kawashima, Yoshikawa et al. 2007). Therefore, there is enough evidence to show conclusively that M3 receptors exist on immune cells. However, few studies have tried to identify an immune related function for this receptor. *In vitro*, polarization of naïve CD4 T cells to T_H1, T_H2 or T_H17 using an appropriate cocktail of cytokines results in changes in expression levels of muscarinic and nicotinic receptors. Interestingly, M3R is significantly down-regulated at the mRNA and protein level in cytokine-induced T_H1, T_H2 and T_H17 CD4 T cells (Qian, Galitovskiy et al. 2011).

M3 receptors have not been investigated for potential immune cell function *in vivo* but muscarinic receptors in general and certain subtypes have been studied. Continuous administration of low-dose atropine, a muscarinic antagonist, by osmotic pumps results in a significant attenuation of inflammation induced by turpentine. It also reduced antigen specific T cell proliferation and number of antibody producing cells after immunization with sheep red blood cell (SRBC), a commonly used antigen. The opposite effect was observed when oxotremorine-M was administered, with increased T cell proliferation SRBC antibody production indicating that muscarinic receptors, unlike nicotinic receptors, may be pro-inflammatory (Razani-Boroujerdi, Behl et al. 2008). M1 but not M3 receptors were also found to be important for generation of functional cytotoxic T lymphocytes (CTL) from CD8 T cells *in vitro (Z*imring, Kapp et al. 2005). *In vivo*, however, M1R is not required for optimal CD8 responses as M1R specific knockout mice are not susceptible to viral infection (Vezys, Masopust et al. 2007). Antibody responses are also affected by certain muscarinic receptor subtypes. M1R/M5R double knockout mice produce significantly lower total and antigen specific IgG1 but not IgM after immunization with ovalbumin. OVA restimulated splenocytes from these cells also secrete less IFN-γ and IL-6 indicating a role for M1 and/or M5 receptors in cytokine production and antibody class switching (Fujii, Tashiro et al. 2007).

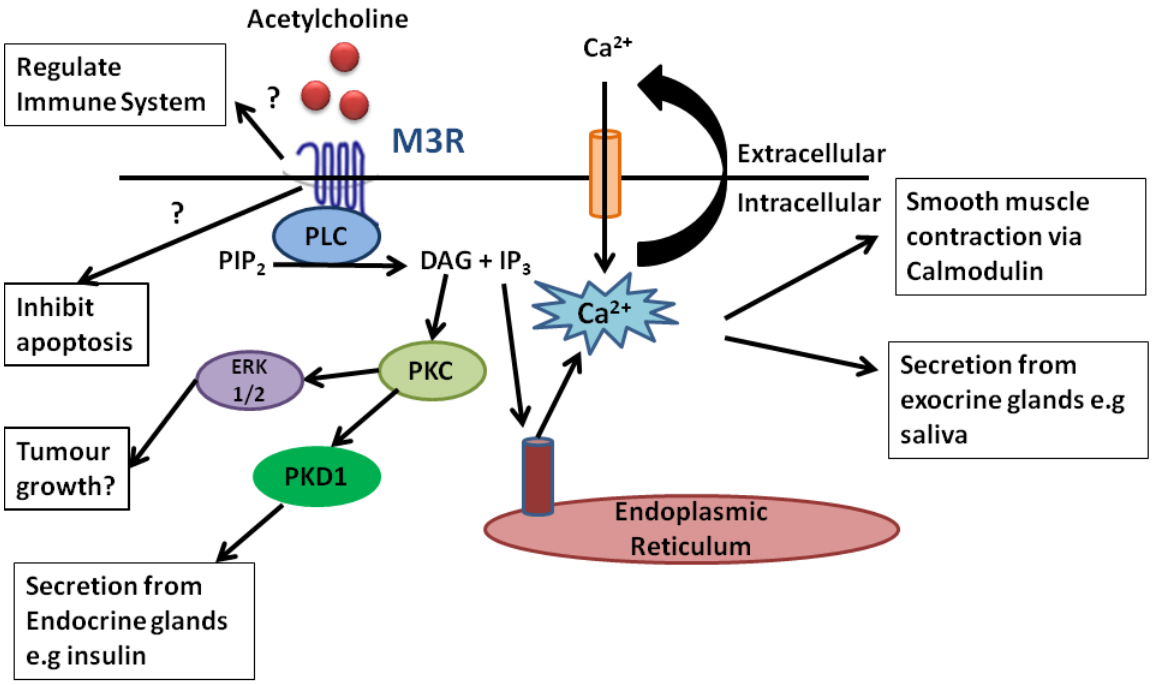


Fig 3. Known and hypothesized non-neuronal roles of M3 Muscarinic receptor signaling.

Abbreviations: PLC – Phospholipase C; PIP_2 – Phosphotidylinositol 4,5-bisphosphate; DAG – Diacylglycerol; IP_3 – Inositol triphosphate; PKC – Protein Kinase C; ERK 1/2 – Extracellular signal-regulated Kinase 1/2 ; PKD1- Protein Kinase D1.

4. Murine infectious disease models:

Murine models of infectious diseases are an important tool for studying cellular mechanism of immune responses to various human (and related) pathogens and to facilitate translation of these findings to human studies. Studying immune responses are particularly useful during early stage vaccine development as well as to identify correlates of protection such as cytokines and other cytotoxic molecules. Murine models for all major classes of pathogens such as bacteria, viruses, protists, fungi, and helminths have been developed and together with genetically modified mouse strains provide a powerful platform to study a broad range of host-pathogen interactions. Below, the two pathogens used in this study will be reviewed.

4.1 Hookworm: *Nippostrongylus brasiliensis*:

Hookworms are a major human parasite infecting an estimated 740 million people worldwide, causing significant morbidity primarily as a result of iron-deficiency anemia (Hotez, Brooker et al. 2004). The two major species that commonly infect humans are *Necator americanus* and *Ancylostoma duodenale* however a murine model for these does not exist. Instead, the nematode parasite *Nippostrongylus brasiliensis*, which naturally infects rats but has also been adapted to infect mice, is commonly used in the laboratory to simulate human hookworm infections as it shares similarity in terms of life cycle and class of immune response (Loukas and Prociv 2001; Gause, Urban et al. 2003).

4.1.1 Life cycle of *N.brasiliensis*.

N.brasiliensis infection begins when the L3 infective stage larvae penetrate the skin of the host and after 24-48 hours migrate to the lungs via the circulatory system. In the lungs, the L3 larvae moult into L4 stage larvae which are coughed up and swallowed thereby entering the jejunum where they develop into egg-laying adults. Eggs are passed out in faeces, hatch in the soil and develop into L3 stage larvae which are capable of infecting the next host so as to propagate the life cycle. Adult worms are naturally expelled, damaged but alive, from immunocompetent mice within 2 weeks of infection (Ogilvie and Hockley 1968; Finkelman, Shea-Donohue et al. 1997).

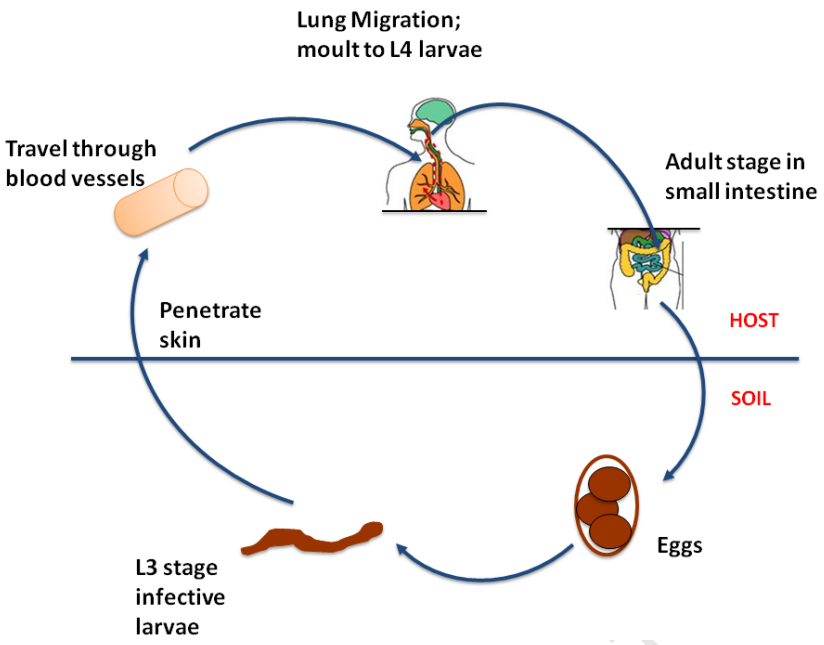


Fig 4. Life cycle of typical gastrointestinal nematode parasite such as *Nippostrongylus brasiliensis* in mammalian hosts.

4.1.2 Role of immune and non-immune cells during T Helper type 2 response to *N.brasiliensis*.

N.brasiliensis infection elicits a highly polarized T Helper type 2 immune response (T_H2 characterized by the production of cytokines IL-4, IL-5 and IL-13 together with increased eosinophilia and IgE production (Urban, Madden et al. 1992). The IL-4 receptor-alpha subunit (IL-4Rα) which forms the receptor that binds IL-4 and IL-13, as well as the T_H2 transcription factor STAT-6 are essential for expulsion of *N.brasiliensis* (Urban, Noben-Trauth et al. 1998). Interestingly, IL-13 but not IL-4 is the major cytokine that drives parasite expulsion as IL-$4^{-/-}$ mice but not IL-$13^{-/-}$ mice are able to effectively resolve the infection (McKenzie, Bancroft et al. 1998).

The importance of IL-13 in parasite expulsion is widely accepted however there was much debate regarding the cellular source of this IL-13 as well as mechanisms involved in initiating a T_H2 response. The use of genetically engineered reporter mice in which IL-13 producing cells also

synthesize green fluorescent protein (GFP) have allowed researchers to identify new populations of IL-13 producing cells (Price, Liang et al. 2010).

CD4 T cells are essential for expulsion of *N.brasiliensis* as depleting CD4 T cells by anti-CD4 antibodies delays parasite expulsion as long as the antibodies are administered (Katona, Urban et al. 1988). Nude athymic mice that lack functional CD4 T cells are also unable to clear parasites providing further evidence that CD4 T cells are important (Jacobson and Reed 1974). Recently, a new innate immune cell population has been discovered that produces high levels of IL-13 rapidly after *N.brasiliensis* infection and has been hypothesized as the main cellular sources of this cytokine early after infection. These new cell types termed nuocytes (Neill, Wong et al. 2010) lack expression of classical T and B cell markers but proliferate rapidly in response to IL-25 and IL-33 to secrete IL-13 and are essential for expulsion of *N.brasiliensis*. This was shown by using *Il17br*$^{-/-}$ *Il1rl1*$^{-/-}$ mice that lack nuocytes and are unable to clear the parasite but when adoptively transferred with *in vitro* expanded IL-13 sufficient nuocytes achieve expulsion similar to wildtype controls (Neill, Wong et al. 2010). Together nuocytes and CD4 T cells represent the essential component of the innate and adaptive immune system required for *N.brasiliensis* expulsion.

Non-immune populations such as smooth muscle cells and mucus-secreting goblet cells are involved in mediating effector responses that ensures parasite expulsion from the gut (Anthony, Rutitzky et al. 2007). An increase in intestinal smooth muscle contraction is observed in response to *N.brasiliensis* infection, IL-4 and IL-13 in a STAT-6 dependent manner and is hypothesized to be required for physical expulsion of parasite (Zhao, McDermott et al. 2003). Apart from direct physiological responses, IL-4Ra signaling on smooth muscle cells also mediates T_H2 immune response resulting in a delay in both parasite expulsion and T_H2 dependent development of pulmonary pathology after *N.brasiliensis* infection (Horsnell, Cutler et al. 2007; Horsnell, Vira et al.

2011). *N.brasiliensis* infection also results in an increase in mucus secreting goblet cells in the intestine and lungs which provides protection against infection by toxic molecules in mucus such as Muc5-AC, RELM-β (Herbert, Yang et al. 2009; Hasnain, Evans et al. 2011) and/or prevent adherence of the parasite in the gut (McKenzie, Bancroft et al. 1998; Finkelman, Shea-Donohue et al. 2004).

4.1.3 M3 muscarinic receptor and *N.brasiliensis*:

Increase in smooth muscle contractility is a characteristic feature of *N.brasiliensis* infection and has been identified as a potential contributing factor for parasite expulsion however a direct link has not been established (Finkelman, Shea-Donohue et al. 2004). These contractions were measured in jejunum sections *ex vivo* in response to increasing doses of acetylcholine (Zhao, McDermott et al. 2003). Interestingly, acetylcholine stimulated intestinal smooth muscle contractions are highly dependent on M3 receptors as $M3R^{-/-}$ mice have severely reduced cholinergic contractions but still remain viable (Matsui, Motomura et al. 2002). However, whether this affects *N.brasiliensis* infection *in vivo* has not been studied. A further study in our laboratory showed that mice lacking IL-4Rα on smooth muscle cells (SM-MHC^{Cre}IL-$4R\alpha^{-/lox}$) have a delayed expulsion of *N.brasiliensis* which is correlated with a reduced expression of M3 receptor (at the mRNA level) in the intestine. This implies a potential causal effect between delayed clearance and M3 receptor expression in the small intestine (Horsnell, Cutler et al. 2007). We have also shown that SM-MHC^{Cre}IL-$4R\alpha^{-/lox}$ mice have a delay in onset of T_H2 dependent pulmonary pathology after *N.brasiliensis* infection

4.2 Bacterial pathogen*: Salmonella enterica* sp. Typhimurium

Salmonella enterica serovar Typhimurium (here referred to as *S.typhimurium*) is a Gram negative bacterium that causes non-typhoidal salmonellosis (NTS); a disease characterized by diarrheal gastroenteritis in immunocompetent adult humans. However, in young children and

immunocompromised individuals such as those with HIV, it can be fatal (Hohmann 2001; Morpeth, Ramadhani et al. 2009) causing invasive NTS characterized by febrile (fever) systemic illness and a variety of clinical symptoms such as diarrhea, splenomegaly and anemia present in the majority of the cases (Feasey, Dougan et al. 2012). Invasive NTS has a high incidence of mortality causing deaths in 22-47% of the cases even with appropriate treatment (Gordon, Banda et al. 2002; Gordon, Graham et al. 2008). Apart from its lethal effects on humans, *S.typhimurium* is also responsible for disease burden in common livestock such as cattle, chickens and pigs finally making its way into humans through the food chain. Of particular concern is the increase in antibiotic resistance in *S.typhimurium* with certain isolates being resistant to upto four commonly used antibiotics (Threlfall 2000).

In the laboratory setting, *S.typhimurium* has been used extensively in murine models to study systemic salmonellosis (Collins, Mackaness et al. 1966; Simon, Tennant et al. 2011). The advantage of using this bacterium is its fast growth rate and is the same species that also infects humans making translational studies easier (Mittrucker and Kaufmann 2000; Mastroeni and Sheppard 2004).

4.2.1 Generation of the *Salmonella typhimurium* ΔaroA (SL3261) mutant strain.

S.typhimurium multiplies rapidly *in vivo* in mice, infecting various organs such as the liver and spleen and has 100% mortality within 10 days (Mittrucker, Raupach et al. 2000). To allow for longer observation time, a number of genetically altered strains of *S.typhimurium* with specific deletion in key pathways were generated resulting in attenuated strains which are not fatal (O'Callaghan, Maskell et al. 1988).

S.typhimurium requires certain metabolites for growth and virulence that cannot be obtained directly from its vertebrate host and must be synthesized *de novo*. Two such compounds are p-amino-benzoic acid (pABA) and 2,3-dihydroxybenzoate (DHB) required for synthesis of folates and an iron-acquiring compound called enterochelin respectively (Bacon, Burrows et al. 1951; Yancey, Breeding et al. 1979). pABA and DHB are synthesized from the important intermediate compound chorismate, a product of the aromatic synthesis (*aro*) pathway. Selectively deleting genes in this *aro* pathway generates auxotrophic strains of the bacteria that are unable to synthesize important virulence factors and thus highly attenuated. This was demonstrated in a study by Hoiseth and Stocker who generated a non-reverting *S.typhimurium* ΔaroA strain by inserting a tetracycline-resistance transposon Tn*10* into the aroA cluster of genes and selecting for tetracycline-sensitive strains which arise as a result of the natural DNA modification property of Tn*10* (Hoiseth and Stocker 1981). One of the strains obtained, SL3261 is highly attenuated *in vivo* with no fatality at an infection dose of 3 X 10^6 bacteria i.p. compared to the parent strain which has an LD_{50} of less than 20 bacteria. SL3261 is also an effective vaccine in mice providing 100% protection against oral or intraperitoneal challenge with lethal strains of *S.typhimurium* (Hoiseth and Stocker 1981).

4.2.2 Role of immune cells during *Salmonella typhimurium* infection.

The natural route of infection with *S.typhimurium* is orally with the bacteria rapidly spreading from the gut via Peyer's patches and mesenteric lymph nodes to infect organs such as spleen and liver (Carter and Collins 1974). *S.typhimurium* primarily replicates extracellularly in target organs however protective immunity arises from engulfment and control of bacterial replication within macrophages (Hsu 1989). Various murine models have been used to dissect the role for both innate and adaptive immune responses during *S.typhimurium* infection.

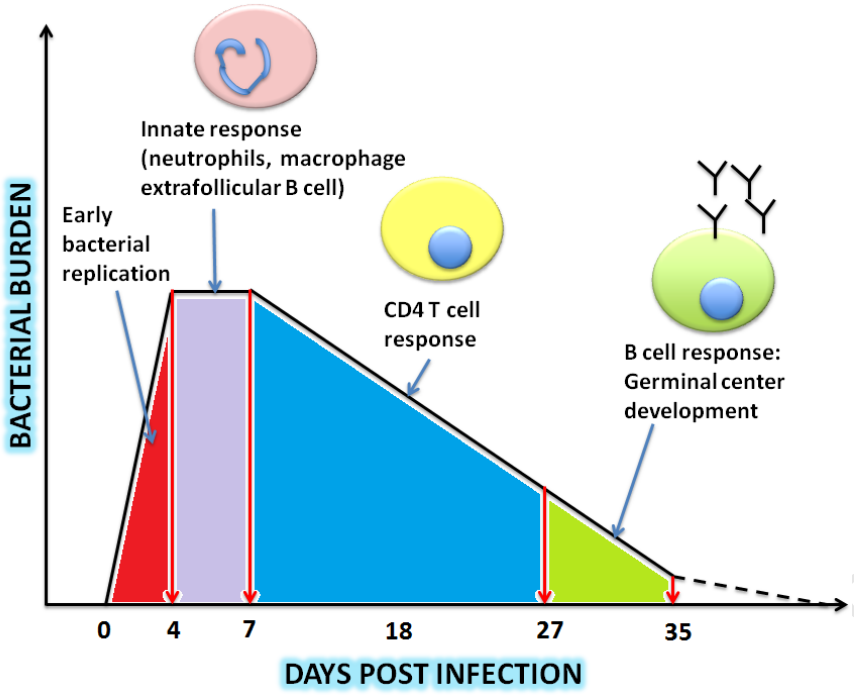


Fig 5. Progression of *S.typhimurium* ΔaroA infection in mice as measured by bacterial burden in the spleen at different time points. Key immune cells and responses at each stage are indicated.

Role of Innate immunity:

Phagocytes such as macrophages and neutrophils respond early after infection with *S.typhimurium* and play an important role in control of bacterial growth. Neutrophils are recruited early to the site of infection and can readily be detected in the peritoneum 4 hours after i.p. infection and undergo a 10-fold increase in the spleen by day 5 post-infection (Kirby, Yrlid et al. 2002). However, neutrophils only play a supportive function in controlling *S.typhimurium* infection and are not essential for protection (Cheminay, Chakravortty et al. 2004).

Macrophages are also recruited early into the spleen and increase 3 fold in number by day 5 post-infection (Kirby, Yrlid et al. 2002). Early bacterial growth is controlled by *Nramp1* (Natural resistance associated macrophage protein 1), a cation transporter located on phagosomes in macrophages and is essential for protection. Inbred mice strains that have nonfunctional *Nramp1* are highly susceptible to *S.typhimurium* (Malo, Vogan et al. 1994) and Nramp1$^{-/-}$ mice also quickly

succumb to infection and die within 6 days (Vidal, Tremblay et al. 1995). Macrophages also act as a source of T_H1 polarizing cytokines such as IL-12 and IL-18 that enhance production of IFN-γ required for macrophage activation and effective bacterial killing (Mastroeni, Harrison et al. 1996; Mastroeni, Clare et al. 1999)

Role of Adaptive Immunity

Innate control of bacterial replication protects mice from bacteremia, septic shock and death however the innate immune system on its own is not capable of eradicating *S.typhimurium*. The adaptive immune system is thus required for achieving sterile immunity as well as development of a memory immune response to subsequent re-infections.$RAG1^{-/-}$ mice which lack functional B and T cells but possess innate immune cells are capable of controlling attenuated *S.typhimurium* in the early stages of infection but succumb after weeks where as immune competent mice survive.

The major cell type that mediates adaptive immunity to *S.typhimurium* is the CD4 T cells and this is evident from the fact that antibody mediated CD4 T cell depletion and athymic nude mice (which lack T cells) are characterized by higher bacterial burdens (Nauciel 1990; Sinha, Mastroeni et al. 1997). Furthermore, TCR-$\alpha\beta^{-/-}$ mice which lack the classical MHCII interacting CD4 T cells are highly susceptible to attentuated *S.typhimurium aroA* but a difference in bacterial burden is only visible after day 21 highlighting the importance of these cells in the adaptive phase of the immune response. Mice deficient in other T cell types such as TCR-$\delta^{-/-}$ (lack γδ T cells) or beta-2m-/- (lack CD8 T cell) mice respond similarly to wildtype mice further emphasizing the central role played by CD4 T cells (Hess, Ladel et al. 1996). These essential T_H1 primed CD4 T cells develop in the spleen and other secondary lymphoid organs after interaction with monocyte-derived dendritic cells

(Flores-Langarica, Marshall et al. 2011) with conventional dendritic cells required for subsequent maintenance of the T_H1 cells (John, Rajagopal et al. 2002).

S.typhimurium induces a highly polarized T_H1 response and the cytokine IFN-γ is essential for control of bacterial replication. IFN-γ receptor deficient mice (IFN-$\gamma R^{-/-}$) whose cells cannot respond to IFN-γ die early within 21 days after infection with *Salmonella aroA* whereas wildtype mice all survive and clear the infection (Hess, Ladel et al. 1996). This demonstrates the critical and central role for IFN-γ during *S.typhimurium* infection. The development of T_H1 responses and IFN-g producing CD4 T cells is regulated by T-bet, a T_H1 specific T-box transcription factor (Szabo, Kim et al. 2000). Protection against some intracellular bacteria such as *Listeria monocytogenes* is not dependent on T-bet as T-$bet^{-/-}$ mice are capable of clearing the infection (Way and Wilson 2004). However, development of an effective T_H1 response to *S.typhimurium* is highly dependent on T-bet expression, with T-$bet^{-/-}$ mice succumbing to infection with attenuated strains (Ravindran, Foley et al. 2005).

Activation of T cells requires engagement of the TCR as well as co-stimulatory markers. An important co-stimulatory marker for CD4 T cells is CD28 which receives signals from professional antigen presenting cells via CD80 and CD86 (Lenschow, Walunas et al. 1996). Expression and engagement of CD28 is critical during *S.typhimurium* infection and $CD28^{-/-}$ mice die rapidly within the first 10 days after infection with a lethal strain. $CD28^{-/-}$ mice also remain chronically infected with an attenuated *S.typhimurium* strain whereas wildtype mice clear the infection within 5 weeks. CD28 engagement is also required to mediate T and B cell interactions for antibody class switching and $CD28^{-/-}$ mice do not produce *Salmonella* specific IgG1 and IgG2a antibodies (Mittrucker, Kohler et al. 1999).

B lymphocytes play a more variable role depending on the strain of *S.typhimurium* used and the route of infection. Igμ$^{-/-}$ mice which are completely devoid of B cells and antibodies are capable of clearing *S.typhimurium aroA* infection similarly to wildtype mice. Igμ$^{-/-}$ mice are more susceptible to an oral infection with lethal *S.typhimurium* but die as rapidly as wildtype mice after an intravenous infection with the same strain suggesting that B cells and antibodies may be more effective in preventing certain routes of infection over others. Early during infection, *S.typhimurium* induces a rapid and massive extrafollicular plasma cell response, producing low-affinity antibodies with development of germinal centers and high-affinity antibody production following later during infection. Therefore, even though B cells and high affinity antibodies are not essential for clearance of a primary infection with *S.typhimuirum aroA*, antibodies play an important role in limiting the spread of bacteria and colonization of splenic macrophages (Cunningham, Gaspal et al. 2007). *Salmonella* outer membrane proteins (Omp) particularly OmpD induces a strong T cell independent antibody response from a subset of B cells in the peritoneum known as the B1b. These antibodies are then capable of providing protection against re-infection making OmpD a potential vaccine candidate (Gil-Cruz, Bobat et al. 2009). B lymphocytes are also important for development of memory immune responses to lethal *S.typhimurium* after vaccination with the *aroA* strain (Mittrucker, Raupach et al. 2000). However, B cells can provide this post-vaccination protection independent of antibody production by directing an effecting T cell mediated immune response (Nanton, Way et al. 2012).

The pro-inflammatory cytokine TNF-α is also involved in controlling *S.typhimurium* although it is not essential for survival. TNF receptor (TNFαp55R$^{-/-}$) deficient mice are more susceptible to *Salmonella aroA* infection having a higher bacterial burden in the spleen and liver but can eventually clear the infection (Everest, Roberts et al. 1998). TNF-α is however essential for memory immune responses

after vaccination with attenuated Salmonella as all TNFαp55R$^{-/-}$ mice succumb to lethal infection compared to 100% survival in wildtype vaccinated mice (Everest, Roberts et al. 1998).

4.2.3 Cholinergic control of *Salmonella* and other bacterial infections.

Several studies have examined broadly the contribution of cholinergic system to immunity in various bacterial infection models. Acetylcholine is generally considered to be anti-inflammatory based on its action on nicotinic receptors (Tracey 2007). However raising acetylcholine levels *in vivo* by administering paraoxon, an acetylcholinesterase inhibitor, significantly improves survival after infection with a lethal strain of *S.typhimurium* (20% survival versus 80% survival after paraoxon). Paraoxon-administered mice also have a 10-fold lower bacterial burden in the spleen and higher serum levels of the T_H1 polarising cytokine IL-12 (Fernandez-Cabezudo, Lorke et al. 2010). This effect was specific for the acetylcholinesterase inhibiting property of paraoxon as co-administration of K-27 which reverses the inhibition restored the susceptibility of the mice to *S.typhimurium*. This study suggests that the role of acetylcholine may vary depending on the circumstance and can also act as a pro-inflammatory agent (Fernandez-Cabezudo, Lorke et al. 2010).

The anti-inflammatory property of nicotinic receptors inhibits immunity to bacterial infection. Mice deficient in the essential nicotinic subunit α7 (α7nAChR$^{-/-}$) have enhanced bacterial clearance as shown by a lower bacterial burden in the spleen, kidney and blood after an intra-peritoneal *Escherichia coli* infection (Giebelen, Le Moine et al. 2008). Also, activation of nicotinic receptors by topical application of nicotine or genetically deleting catestatin (Chga$^{-/-}$), a nicotinic antagonist, increased susceptibility to skin infections by *Staphylococcus aureus* and *Streptococcus* Group A. This

susceptibility resulted from a direct suppression of antimicrobial peptide production in the skin (Radek, Elias et al. 2010). These studies provide further evidence that nicotinic receptors are anti-inflammatory and thus prevent not only excessive inflammation during LPS induced endotoxic shock (Wang, Yu et al. 2003) but may also hamper anti-bacterial immunity.

Materials and Methods

Materials and Methods:

Mice. M3R$^{-/-}$ mice were obtained from Dr. Jurgen Wess (NIH; Bethesda, MD) in the C57BL/6 background and prior to use in this study were backcrossed to BALB/c background for 10 generation. Mice were bred under specific pathogen-free conditions at the University of Cape Town Animal Unit and used when they were between ages 6-10 weeks. Protocols for all experiments were reviewed and approved by the UCT Animal Ethics committees (UCT AEC Protocols number: 010/032 (*Salmonella typhimurium*) 011/008 (*Nippostrongylus brasiliensis*) and 011/021 (*Nippostrongylus brasiliensis* life cycle maintanence). M3R$^{-/-}$ mice were genotyped using the following set of primers (refer Results Fig 1.1 for schematic on generation of M3R$^{-/-}$ and genotyping):

A1: 5'-AAGACCACAGTAGCAGTG-3'

A2: 5'-CTCTCTACATCCATAGTCCC-3'

A3: 5'-TGGATGTGGAATGTGTGCGAGG-3'

***N.brasiliensis* life cycle maintanence:** *N.brasiliensis* L3 stage infectious larvae were maintained by routine passage through female Wistar rats. Female Wistar rats (at age 7 weeks; at least 170g) were infected with 5000L3 larvae subcutaneously and faecal pellets collected on days 6, 7 and 8 post-infection. Faecal pellets were soaked in water containing amphotericin-B (Fungizone, Sigma) for 1 hour prior to processing. Pellets were meshed into a paste and spread onto a filter paper placed on top of a gauze-pad soaked in amphotericin-B containing water. The faecal paste was then incubated at room temperature for one week to allow development of L3 stage infectious larvae of *N.brasiliensis*

and migration of these larvae to the edge of the filter paper. Filter paper edges containing L3 larvae were collected and transferred to a fresh petri dish, ready for infection.

***Nippostrongylus brasiliensis* infection in mice.** For primary infection, mice were infected subcutaneously with 500 *N. brasiliensis* L3 stage larvae. To enumerate adult worms, mice were killed at various times post-infection (p.i.), intestines opened longitudinally, incubated in 10 ml saline for 3 hrs at 37°C to allow parasite migration out of the intestines which were then counted under a dissecting microscope. For secondary infections, mice were infected as in primary infections with 500L3 larvae but treated at day 9 with ivermectin via drinking water to eliminate parasites, rested for 28 days and re-infected with 500 L3. Larvae were recovered from lungs at day 2 post-infection by finely slicing the tissue placing it in 5 ml saline for 3 hours, and parasites enumerated under a dissection microscope.

Flow cytometry. Single cell suspensions were prepared and $1x10^6$ cells incubated in PBS + 0.1% BSA, 1% normal rat serum, 1% FcεRII/III (Blocking antibody) and appropriate antibody cocktails in a total of 50ul in 96 well V-bottomed plates for 30 mins on ice. After incubation, cells were washed once with 200ul ice cold FACS Buffer (PBS + 0.1% BSA) and resuspended in 200ul FACS Buffer for acquisition. Cell populations were determined and acquired on a BD FACS Fortessa (Becton Dickinson). Prior to each acquisition run of samples, compensation beads (BD CompBeads; BD Biosciences) stained with appropriate fluorochrome antibodies were used to obtain correct compensation parameters. All analyses were performed using the FlowJo™ software (version 7.6.5).

The following antibodies were used in this study:

Antibody target	Fluorochrome	Clone Number	Company
B220/CD45R	Biotin	RA3-6B2	Homemade
CD3	AlexaFluor-700	17A2	eBioscience
CD4	PerCP	L3T4	BD Bioscience
CD8	V500	53-6.7	BD Bioscience
CD28	PE	37.51	BD Bioscience
CD44	FITC	IM7	BD Bioscience
CD62L	APC	MEL-14	BD Bioscience
CD19	APC	1D3	BD Bioscience
CD11c	Biotin	HL3	BD Bioscience
CD11b	FITC	M1/70	BD Bioscience
Gr1	PE	RB6-8C5	BD Bioscience
F4/80	PE-Cy7	BM8	eBioscience
TCRαβ	Biotin	H57-597	BD Bioscience
IFN-γ	PE	XMG1.2	BD Bioscience
TNF-α	APC	MP6-XT22	BD Bioscience

Immune cell populations were gated as follows:

CD4 T cells: $CD3^+CD4^+$; CD8 T cells: $CD3^+CD8^+$; B cells: $CD19^+B220^+$; Macrophages: $CD11b^+F4/80^+$; Dendritic cells: $CD11c^+$

CD4 T cells were further gated into naïve ($CD44^{lo}CD62L^{hi}$) and activated ($CD44^{hi}CD62L^{lo}$) T cell populations.

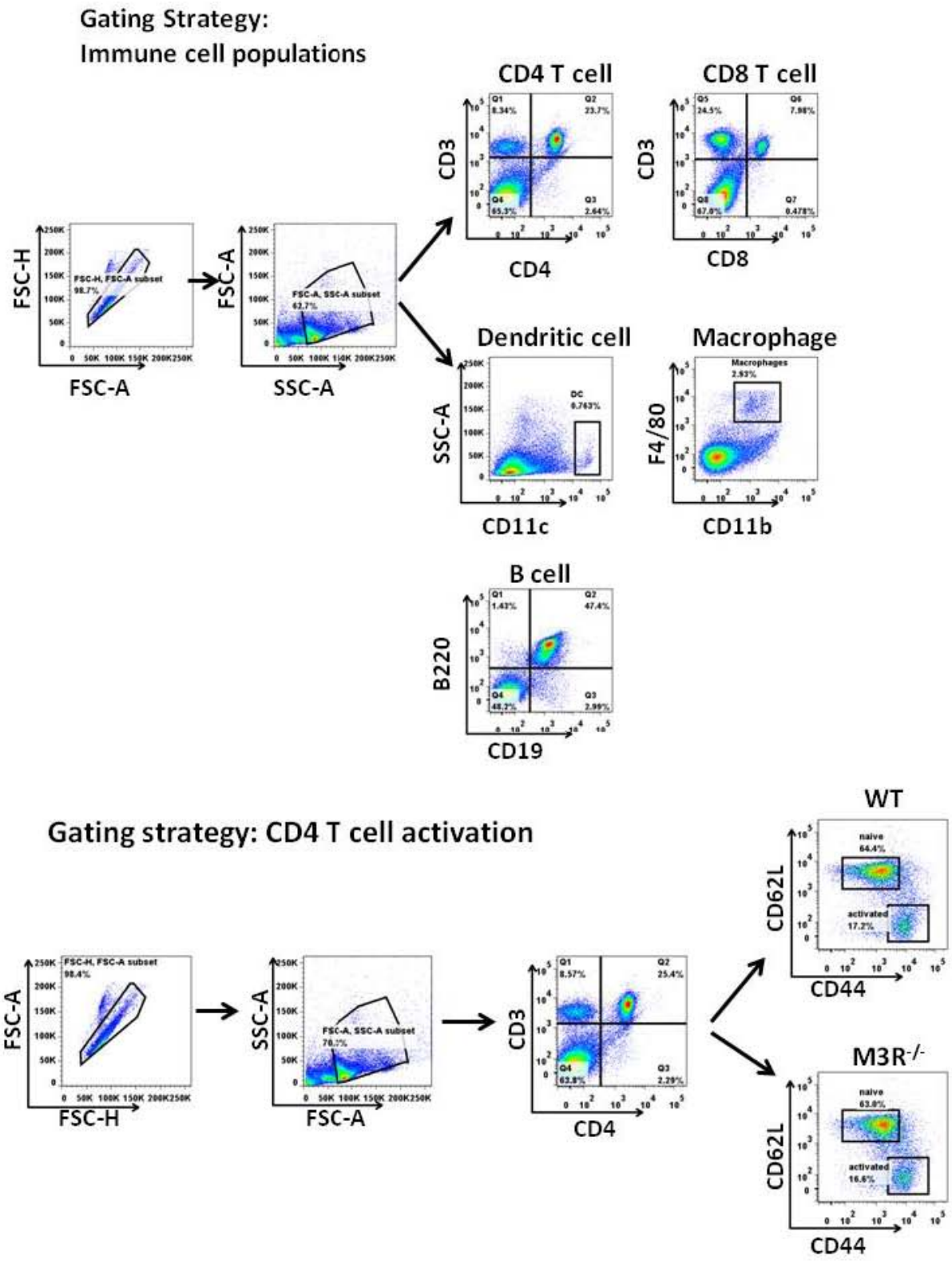


Fig 1: Gating strategy for immune cells in the spleen and mesenteric lymph node using flow cytometry. Splenocytes and lymphocytes were stained with a cocktail of antibodies including CD3, CD4, CD8, B220, CD19, CD11b, F4/80, CD11c to delineate important immune cell populations such as CD4 and CD8 T cells, B cells, macrophages and dendritic cells. CD4 T cells were also further gated to identify naïve and activated T cell populations.

Measurement of antibody and cytokine levels using Enzyme Linked ImmunoSorbent Assay (ELISA).

96-well flat-bottom ELISA plates (Nunc Maxisorp; Thermo Fisher Scientific) were coated with 50ul of monoclonal coating antibodies specific for IL-13 (BD Bioscience) and IFN-γ (homemade clone: ANI8KL6) or total antibody isotype overnight at 4°C. For *N.brasiliensis* specific antibody ELISAs, plates were coated with 10ug/ml somatic antigen (NES) and for *Salmonella* specific antibody ELISAs plates were coated with 1 X 10^7 heat-killed *S.typhimurium aroA* per well. After overnight incubation, plates were blocked using 2% casein milk powder in PBS for 3 hours at 37°C. Samples were added at 50ul per well and in three concentrations (neat, 1 in 3 and 1 in 9 dilutions) overnight at 4°C. Each ELISA plate also contained a cytokine or antibody standard diluted out across one row. Next, a biotin conjugated polyclonal antibody specific to the cytokine IL-13, IFN-γ or antibody isotype of interest (BD Biosceince) was added in 50ul of FACS buffer and incubated for 3 hours at 37°C. Streptavidin-conjugated Horseradish Peroxidase (HRP) (KPL Inc.; MD, USA) was used for detection and added at a concentration of 0.02 μg/ml for 1 hour at 37°C followed by a thorough wash with PBS + 0.05% Triton X-100 and visualized using a commercially available substrate solution (KPL Inc.; MD, USA). All cytokine and antibody ELISAs had been previously optimized in our lab with respect to coating and secondary antibody concentrations by titration.

For cytokine re-stimulation, spleen, mesenteric LN and lung cells were plated at 1 X 10^6 cells per well in 48 well plates pre-coated with 20 ug ml^{-1} anti-CD3 (homemade – clone: 17A2) and restimulated for 72 hrs. Homogenates of lung and intestinal sections were prepared using a Polytron homogenizer and all samples standardized to 5 mg ml^{-1} protein prior to ELISA.

Blood was collected in BD Microtainer™ tubes (BD Bioscience) containing clot-activator gel and centrifuged at 8000 rpm for 5 mins to separate blood plasma from cellular material. Plasma was removed and used for determination of total and antigen specific antibody ELISA as outlined above.

Cholinergic stimulation. Lymph node cells were plated at 1 x 10^6 cells per well in a 96 well plate and stimulated with 0.1 μg ml^{-1} (sub-optimal) anti-CD3 (clone: 145-2C11), 10 μM acetylcholine (Sigma) + 10 μM BW284C51 (Sigma), 10 μM Oxotremorine M (Oxo M) (Sigma), 10 μM muscarine (Sigma) or buffer control for 24 hours. Supernatants were analysed for cytokines by ELISA as described.

Measurement of intestinal contraction. Ileal sections (1 cm) were obtained from mice by cutting the intestine 10 cm from the junction with the stomach. This section was hooked onto a force transducer, placed in PBS maintained at 37^0C in an organ bath, and stimulated with ACh from 10^{-9}M to 10^{-3}M. In between stimulations, the intestinal segment was allowed to return to baseline contraction (at least 5 min). All measurements were recorded using the Powerlab acquisition unit (ADInstruments) and analysed using the Chart5 program and the amplitude measured as the difference between the peak and trough of the contraction reported in millinewtons (mN).

Adoptive transfer of CD4 T cells. CD4 T cells were purified from MLNs by positive selection using CD4 MACS beads (L3T4, MACS Miltenyi, Holland) according to the manufacturer's protocol. Cells were further purified by flow cytometry to obtain purities above 95%, and 5x10^5 purified CD4

T cells from infected animals or $1x10^6$ purified CD4 T cells from naive animals transferred into naive WT or $M3R^{-/-}$ mice intravenously. Recipient mice were infected 24 hrs later with 500 L3 and killed 5 days post infection.

cDNA synthesis and RT-PCR. RNA was extracted using the Qiagen RNeasy Mini kit (Qiagen, Germany) as per manufacturer's protocols. RNA was converted to cDNA either using random primers and Superscript Reverse Transcriptase (for Imperial College generated data) (Invitrogen) or using the Roche Transcriptor First Strand cDNA synthesis kit (Roche Applied Science) for quantitative PCR, which was performed on a Roche Lightcycler 480 using FastStart SybrGreen (Roche Applied Science) as detection dye. The following primer pairs were used:

Gene	Forward Primer	Reverse Primer
M1R	5'-GGACAACAACACCAGAGGAGA-3'	5'-GAGGTCACTTTAGGGTAGGG-3'
M2R	5'-TGAAAACACGGTTTCCACTTC-3'	5'-GATGGAGGAGGCTTCTTTTTG-3'
M3R	5'-TTTACATGCCTGTCACCATCA-3'	5'-ACAGCCACCATACTTCCTCCT-3'
M4R	5'-TGCCTCTGTCATGAACCTTCT-3'	5'-TGGTTATCAGGCACTGTCCTC-3'
M5R	5'-CTCTGCTGGCAGTACTTGGTC-3'	5'-GTGAGCCGGTTTTCTCTTCTT-3'
β-actin	5'-TGGAATCCTGTGGCATCCATGAAAC-3'	5'-TAAAACGCAGCTCAGTAACAGTCCG-3'
IL-13	5'-CTCCCTCTGACCCTTAAGGAG-3'	5'-GAAGGGGCCGTGGCGAAACAG-3'
IFN-γ	5'-GCTCTGAGACAATGAACGCT-3'	5'-AAAGAGATAATCTGGCTCTGC-3'
Tbet	5,-AACTTTGAGTCCATGTACGC-3'	5'-TATCCTTGGGCTGGCCT-3'
TNF-α	5'-TCTCATCAGTTCTATGGCCC-3'	5'-GGGAGTAGACAAGGTACAAC-3'

Histology. Lung and intestinal sections were fixed with 4% formalin in PBS solution immediately after dissection. After overnight fixation, sections were embedded in wax and cut into thin 5uM sections, then stained with Periodic Acid Schiff (PAS) stain to distinguish mucus-producing goblet cells. Cells containing mucus were distinguished by the dark purple colour formation following PAS staining. A Nikon Eclipse 90i microscope was used to capture micrographs using supplied software (NIS Elements; Nikon).

***Salmonella typhimurium aroA* infection in mice:**

Salmonella enterica sp Typhimuirum *aroA* (SL3261) was kindly donated by Prof. Adam Cunningham (University of Birmingham). *S.typhimurium* for infection was prepared from frozen stocks and used during the log phase of growth. Briefly, 10mls of LB agar (10g tryptic soy broth, 5g Yeast extract and 10g NaCl in 1 liter distilled water autoclaved.) were inoculated with a loop of frozen *S.typhimurium aroA* and incubated at 37 °C on a shaker set at 180rpm. The absorbance (OD) of the bacterial culture at 600nm was monitored periodically until a value of 0.9 was reached (previously established as log phase growth). 1ml of the bacterial culture was removed and centrifuged at 10,000rpm for 5 min, washed twice with sterile PBS and finally resuspended in 1ml sterile PBS. This bacterial stock was diluted 1 in 200 prior to infecting each mouse intrapertioneally with 200ul. The infectious dose was determined by plating the inoculum on LB agar plates and incubating overnight at 37°C. We consistently obtained an infectious dose of between 5 X 10^5 to 8 X 10^5 CFU per mouse using this method.

Determination of *S.typhimurium aroA* spleen bacterial burden:

Mice infected with *S.typhimurium aroA* were killed at various time points of days 7, 18, 27 and 35 post infection and the spleens removed and weighed before further processing. Spleens were then cut into three pieces, each piece was weighed individually, and one piece used for determining bacterial burden. Spleen sections were processed through a 100 μm filter using 1ml of antibiotic-free media and transferred to a 48-well plate. Serial dilutions (1 in 10) of the spleen homogenate were performed upto a final concentration of 10^{-3}. 100ul of appropriate dilution (based on day post-infection and expected bacterial burden) was plated out on LB agar (LB tryptic soy broth + 12% bacterial-grade agar) and incubated overnight at 37°C. Individual bacterial colony forming units (CFU) were enumerated for each spleen section and back-calculated to obtain total CFU per spleen.

Generation of bone marrow chimera mice with B cell specific deletion of M3R.

We used mixed bone marrow chimeras to generate mice that lacked M3R expression only on B cells. Recipient B cell deficient (μMT) mice were irradiated with two doses of 500 rads (5 Grays) over 5 mins using a ^{60}Co source, with doses separated by 3 hours. All recipient mice were given acidified drinking water (100ul concentrated hydrochloric acid in 500mls drinking water) one week prior to and upto 10 days after irradiation. Naive wildtype, M3R$^{-/-}$ and μMT mice were used as donors for bone marrow cells which was extracted from the femur and tibia by flushing out with cold DMEM media containing 10% FCS. Bone marrow (BM) cells from each strain were enumerated and mixed in the ratio of 80% μMT BM cells and 20% WT or M3R$^{-/-}$ BM cells. Recipient mice were rested overnight prior to transfer of 5 X 10^6 mixed BM cells intravenously and rested for a further 6 weeks to allow for effective repopulation of the immune system. After 6 weeks, blood was collected from

the tail vein of chimera mice and the percentage of B cells was determined using flow cytometry to ensure all mice were effectively repopulated.

Generation of bone marrow derived macrophages (BMDM)

Wildtype and M3R$^{-/-}$ mice were killed using halothane inhalation and the femur and tibia of both limbs were removed. Muscle tissue attached to the bone was carefully scraped off using a scalpel, and the femur and tibia separated at the knee joint. The top and bottom of both bones were sliced open, and a syringe with 20 ml of cold DMEM/10%FCS attached to a 23G needle was used to flush out the bone marrow into a 50 ml tube, bone marrow from all mice of the same strain were collected together and centrifuged at 1200 rpm for 5 min. Cells were resuspended in 10 ml of Pluznik media (recipe in Appendix A) which contained L929 cell line conditioned media, a source of M-CSF (macrophage growth factor) and counted. A total of 6 X 10^6 BM cells were added to 60 ml of Pluznik media in special Teflon coated bags and incubated at 37^{o}C for 10 days to allow for proliferation and differentiation of bone marrow cells into macrophages. For harvesting, media in Teflon coated bags was decanted, and the bag washed with a further 90 ml of media to maximize the number of macrophages being harvested. All the decanted media was pooled, centrifuged at 1200 rpm for 5 min and cell pellet resuspended in a final volume of 10 ml of antibiotic free DMEM media/10% FCS.

In vitro BMDM kill assay with *S.typhimurium aroA*

Bone marrow derived macrophages (BMDM) generated as described above were plated at 5 X 10^5 cells per well in a 24 well plate and incubated overnight at 37°C to allow for macrophage adherence. During overnight incubation, half the BMDMs were treated with 100U/ml IFN-γ for activation and the other half were left untreated. The following day, cells were washed with 500 ul pre-warmed media and *S.typhimurium aroA,* from a log phase culture, was added at multiplicity of infection (MOI) of either 2 or 10 in a total volume of 500 ul. BMDMs and *S.typhimurium* were incubated for 4 hours at 37 °C to allow for bacterial uptake, after which the cells were washed with 100 ug/ml gentamycin for 2 hours to get rid of all extracellular bacteria. BMDMs were washed with media to remove the gentamycin and a further 500 ul of media was added. This was considered time 0 and after which cells were lysed at different time points by addition of 1 ml PBS with 0.05% Triton-X and the lysates diluted 10-fold before plating on LB agar plates. CFU was enumerated after overnight incubation at 37°C to obtain bacterial burden at different time points.

In vitro CD4 T cell differentiation into T_H1 or T_H2

CD4 T cells were isolated from mesenteric lymph nodes of naïve mice using flow cytometry to atleast 99% purity. FACS sorted CD4 T cells from the lymph node were plated at 1 X 10^5 cells/well on 96 well plates previously coated with 10 μg/ml anti-CD3 (BD Bioscience) and 5 μg/ml anti-CD28 (BD Bioscience). For T_H1 polarization, cells were incubated with 5 ng/ml rIL-12 (BD Bioscience) and 50 μg/ml anti-IL-4 (homemade Clone: 11B11). T_H2 polarization was induced by adding 50 ng/ml mouse IL-4 (BD Bioscience) and 50 μg/ml anti-IFN-γ. Medium alone was used as the neutral condition. The cells were cultured in a final volume of 100 μl for 72 hours. The cells

were then transferred to a fresh round bottomed 96 well plate and washed with medium after which they were resuspended in appropriate antibody cocktails with the addition of 20 U/ml IL-2 (BD Bioscience) and cultured for another 48 hours. The cells were then washed with fresh medium, replated at 2 X 10^5 cells/well and incubated on 96 well plate coated with 20 μg/ml anti-CD3 (BD Bioscience). Supernatants were harvested after 48 hours of restimulation and used for ELISA.

Statistical Analysis

All data obtained were analysed using GraphPad Prism software Ver. 5.0 (GraphPad Software Inc. La Jolla, CA)and included statistical tests such as Mann-Whitney two-tailed t test and Two-way ANOVA. Flow cytometry data was anaylsed using FlowJo software Ver 7.6.5 (Treestar Inc. Ashland, OR) kindly provided for free under the FlowJo for Africa initiative.

RESULTS

Chapter 1: Characterization of physiology and immune cell populations of M3R deficient mice in the naïve state:

The muscarinic receptor family comprises of a group of five subtypes, called M1-M5 (Caulfield and Birdsall 1998), that play important roles in regulating a diverse set of physiological functions including smooth muscle contractility, control of heart rate and promoting glandular secretions (Wess, Eglen et al. 2007). Historically, the muscarinic receptor was studied mainly done on neurons and its role in regulating nerve impulse transmission across synapses. However, more recently, studies have focused increasingly on the expression and function of muscarinic receptors on non-neuronal cells (Kawashima and Fujii 2008). One of the subtypes, M3 muscarinic receptor (M3R), has been widely studied due to its presence on a number of important cells and the nature of the responses it regulates. The M3R plays a major role in regulating smooth muscle contraction and has been the target for drugs to alleviate the symptoms of asthma and overactive bladder disorder. M3R also plays a significant role in promoting secretions such as saliva from salivary glands and insulin from the pancreas (Eglen 1996; Nakamura, Matsui et al. 2004; Gautam, Han et al. 2007).

The M3R deficient mouse ($M3R^{-/-}$) strain was generated by a number of groups and has been instrumental in confirming known roles as well as delineating further roles for the M3R and to study in detail it's mechanism of action. The $M3R^{-/-}$ mice used in this study were generated by our collaborator Dr. Jurgen Wess at the National Institute of Diabetes and Digestive and Kidney Disease, Bethseda, Maryland and its physiological properties have been characterized while still on a hybrid background of 129SvEv and C57Bl/6J (Matsui, Motomura et al. 2000; Yamada, Miyakawa et al. 2001). Initial studies with $M3R^{-/-}$ mice revealed that this mice are hypophagic (reduced feeding) and lean, with both male and female mice weighing about 22% less than their wildtype counterparts at week 12 of observation (Yamada, Miyakawa et al. 2001). $M3R^{-/-}$ mice were obtained by us in the

C57Bl/6J background but in order to perform infection experiments standardized in our laboratory, mice were backcrossed into the BALB/c background for at least 10 generations.

It has been known for a number of years that the M3 muscarinic receptor is expressed by immune cells such as CD4 T cells, CD8 T cells, dendritic cells and macrophages at the protein and mRNA level (Sato, Fujii et al. 1999; Kawashima, Yoshikawa et al. 2007). Subsequent work has also shown that *in vitro* activation of naïve CD4 T cells into T helper 1 (T_H1), T helper 2 (T_H2) or T helper 17 (T_H17) phenotype alters expression of M3R implying a possible functional role in T cell activation (Qian, Galitovskiy et al. 2011). However, whether these M3 receptors are required for immune cell development or function has not yet been conclusively determined.

In the first chapter of this study, we aim to determine whether genotypic background of the mice influence some observed phenotypic changes in $M3R^{-/-}$ mice from previous studies. We will also address the effect of M3 receptor deletion on development and function of major immune cell populations *in vivo*.

2.1 Backcrossing $M3R^{-/-}$ from C57BL/6 to BALB/c background maintains hypophagic phenotype and decreased intestinal smooth muscle contraction:

The gene for the M3 muscarinic receptor was deleted using a targeting vector to replace a 1.6kb XbaI/Sse83371 fragment of the M3R gene, which contained the translation start site, and introduced into a TC1 (129SvEv) embryonic stem cell by electroporation (Fig 1a). The targeting vector also contained two copies of the herpes simplex virus thymidine kinase gene and a neomycin resistance gene (*neo*) to allow for selection of successfully transformed embryonic stem cells (Fig 1a). The M3 muscarinic receptor deficient mice (referred to from now as $M3R^{-/-}$) had normal fertility and reproduced in the normal Mendelian frequency.

To determine whether backcrossing had maintained the deleted M3R allele we designed a multiplex PCR genotyping protocol using the primer set A1, A2 and A3 (sequence in Material&Methods) which yielded a 226 bp product for WT mice and a 170 bp product for $M3R^{-/-}$ mice Fig. 1B). This was confirmed by both melt curve analysis (real-timePCR) and running the PCR products on an agarose gel (Fig. 1C).

This study was done on C57BL/6 mice and therefore to determine whether genetic background switching to BALB/c influenced this phenotype, we weighed male and female $M3R^{-/-}$ mice (after 10 generations backcrossing into the BALB/c background) for 13 weeks post birth. We found that both male and female $M3R^{-/-}$ had a significantly lower body weight compared to age matched wildtype BALB/c mice (Fig 1D) and this decrease (22% in males and 18% in females) is similar to values reported in other studies (Yamada, Miyakawa et al. 2001) implying that BALB/c $M3R^{-/-}$ mice have a similar lean phenotype as that of their C57BL/6 counterparts.

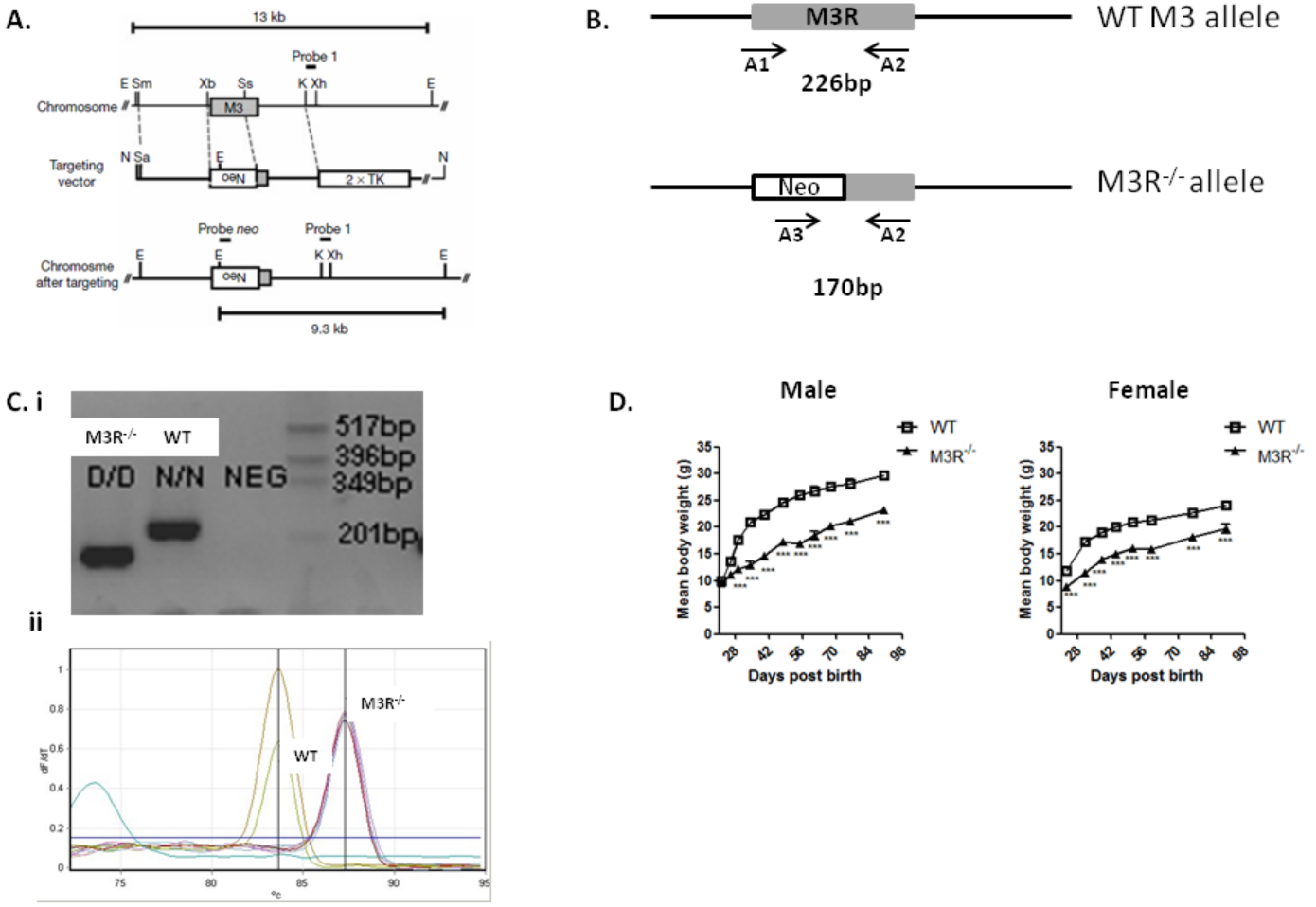


Fig 1.1 Generation, genotyping and characterization of M3R deficient mice (M3R$^{-/-}$) after backcrossing into BALB/c background (A) A schematic showing the generation of M3R$^{-/-}$ mice where the M3R gene was disrupted by replacing part of the coding sequence with a targeting vector containing neomycin resistance cassette. (Yamada, Miyakawa et al. 2001) (B) Design of the primer set for genotyping used to differentiate between normal M3R gene (WT) and disrupted M3R gene (M3R$^{-/-}$). (C) Mice were genotyped based on a difference in PCR product size (i) using agarose gel electrophoresis or melting point (ii) determined by melt curve analysis (D) WT and M3R$^{-/-}$ mice, born on the same day were weighed regularly from 3 weeks (post weaning) up to 13 weeks of age while being fed *ad-libitum* and the body weights plotted as shown. Data are shown as mean ± SEM and is representative of two independent experiments with 4 mice in each group. Statistical significance was calculated using the Mann-Whitney two-tailed t test and denoted by * $p<0.05$, ** $p<0.01$.

2.2 M3R deletion does not affect development of immune cell populations in naïve mice:

M3 receptors are expressed on a number of non-neuronal cells including importantly immune cells. At the transcription and protein level, M3 receptors have been shown on T cells, macrophages and dendritic cells (Kawashima, Yoshikawa et al. 2007; Qian, Galitovskiy et al. 2011). However, a functional role for M3R on immune cells has yet to be identified. Other muscarinic receptors notably M1R have been implicated in differentiation into cytolytic CD8 T cell *in vitro* (Zimring, Kapp et al. 2005) but this function was not replicated *in vivo* using an M1R/M5R double knockout mice (Vezys, Masopust et al. 2007). To determine whether M3R plays a role in development of major immune cell populations we examined proportions and numbers of the major immune cell types such as CD4 T cells, CD8 T cells, B cells, dendritic cells and macrophages in two important immune organs, spleen and lymph nodes. These different cell types were identified on a flow cytometer using the following markers: CD3+CD4+ (CD4 T cells); CD3+CD8+ (CD8 T cells); CD19+B220+ (B cells); CD11c+ (Dendritic cells) and F4/80+CD11b+ (macrophages) (Fig 1.2D).

Spleens of $M3R^{-/-}$ mice (0.096g±0.01g) were significantly smaller, weighing 30% less than WT spleens (0.143g±0.01g) (Fig 1.2Ai) however there was no significant difference in cellularity (Fig1.2Aii). The smaller spleen weight of $M3R^{-/-}$ mice can be explained by a similar difference in body size compared to WT mice. When the absolute numbers of immune cell types were examined in the spleens of naïve WT and $M3R^{-/-}$, similar numbers were obtained for CD4 T cells, CD8 T cells, B cells, macrophages and Dendritic cells (Fig 1.2B.i.). Similarly, in the mesenteric LN (MLN) there were equivalent proportions of CD4 T cells, CD8 T cells and B cells (Fig 1.2B.ii.). Therefore, despite studies showing expression of M3R on these immune cell types, there was no developmental defect in the absence of M3R. We also examined the proportion of activated ($CD44^{hi}CD62L^{lo}$) and naïve ($CD44^{lo}CD62L^{hi}$) CD4 T cells in spleen and MLN as an indicator of basal CD4 T cell activation in

naïve mice. No difference was found between WT and $M3R^{-/-}$ CD4 T cells and thus M3R deletion does not affect basal CD4 T cell activation (Fig 1.2C. i and ii). Finally, we examined whether M3R deletion affects differentiation of CD4 T cells into either the T helper 1 or T helper 2 phenotype *in vitro*. Isolated MLN WT and $M3R^{-/-}$ CD4 T cells were incubated with IFN-γ and anti-IL-4 antibody (T_H1 condition) or IL-4 and anti-IFN-γ antibody (T_H2 condition) for 5 days after which cells were transferred to a fresh plate and stimulated with anti-CD3 and anti-CD28 for 3 days. Degree of differentiation was determined by measuring IFN-γ or IL-4 production after stimulation and similar levels were produced by WT and $M3R^{-/-}$ CD4 T cells under T_H1 (IFN-γ) and T_H2 (IL-4) conditions (Fig 1.2D). Thus, there is no defect in T_H1 or T_H2 differentiation by CD4 T cells in the absence of M3R under optimal *in vitro* conditions.

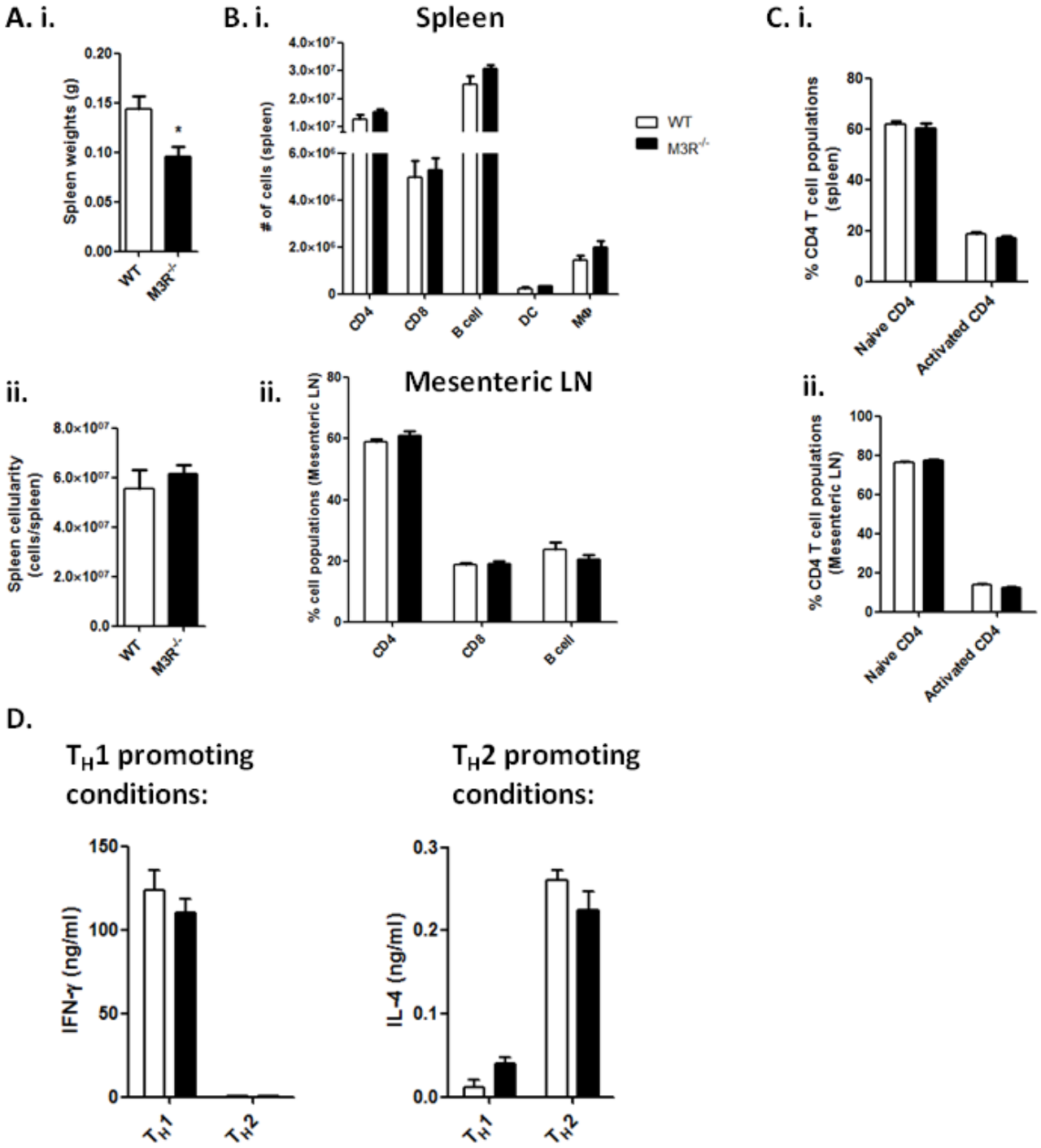


Fig 1.2. M3R does not affect development of immune cell populations in spleen and mesenteric lymph nodes (MLN) in naïve mice (A.) Spleens from WT and M3R$^{-/-}$ mice were weighed and then processed to single cell suspension to determine (ii) spleen cellularity. (B.) (i) The numbers of major immune cell populations namely CD4 T cell, CD8 T cell, B cell, macrophages and dendritic cells in the spleen were determined in WT and M3R$^{-/-}$ naïve mice using specific cell surface markers on a flow cytometer. (ii) Proportion of B cells, CD4 and CD8 T cells in the MLN were determined by flow cytomtery. (C) Proportion of activated and naïve CD4 T cells were determined for (i) spleen (ii) MLN by first gating on CD3+CD4+ T cells followed by gating on activated (CD44^{hi}CD62L^{lo}) and naïve (CD44^{lo}CD62L^{hi}). (D.) Isolated WT and M3R$^{-/-}$ CD4 T cells from MLN were subjected to TH1 or TH2 differentiation *in vitro* using a cocktail of cytokines and antibodies as outlined in Materials & Methods section. Data are shown as mean ± SEM and is representative of two independent experiments with 6 mice in each group. Statistical significance was calculated using the Mann-Whitney two-tailed t test and denoted by * $p<0.05$, ** $p<0.01$.

2.3 M3R deficiency affects expression of co-receptors CD28 and TCRαβ on CD4 T cells in naïve mice.

We have shown that M3R deletion is not required for development of major immune cell populations including activated and naïve subsets of CD4 T cells in the spleen and mesenteric lymph node despite published studies indicating expression of M3R by these cells (Qian, Galitovskiy et al. 2011). Despite no apparent differences in basal activation of CD4 T cells, it is possible that M3R exerts its effects at the level of expression of immune receptors on the surface of CD4 T cells such as the co-receptors TCRαβ and CD28 thereby impacting the quality of induced T cell activation. TCRαβ and CD28 are essential co-receptors for antigen recognition and activation of CD4 T cells by Antigen Presenting cells (APC) (Ward 1996; Jenkins, Khoruts et al. 2001). Splenocytes and MLN cells from naïve mice were stained with CD3, CD4, CD44 and CD62L antibodies to delineate populations of activated and naïve CD4 T cells using flow cytometry. Cells were also stained with antibodies for co-receptors CD28 and TCRαβ with relative expression levels determined by geometric mean (Geo Mean). Interestingly, $M3R^{-/-}$ CD4 T cells from both the spleen and MLN displayed significantly reduced expression of CD28, and furthermore this reduction was most prominent in the activated ($CD44^{hi}CD62L^{lo}$) subset of CD4 T cells (Fig 1.3A). Meanwhile, TCRαβ expression was more similar between WT and $M3R^{-/-}$ CD4 T cells, except for activated CD4 T cells in the spleen where it was marginally, but significantly, higher on $M3R^{-/-}$ CD4 T cells (Fig 1.3A). Since CD28 ligation by the co-stimulatory molecules CD80/CD86 on APCs is an essential step for CD4 T cell activation (Lenschow, Walunas et al. 1996), reduced expression may result in defective T cell activation following interaction with APCs.

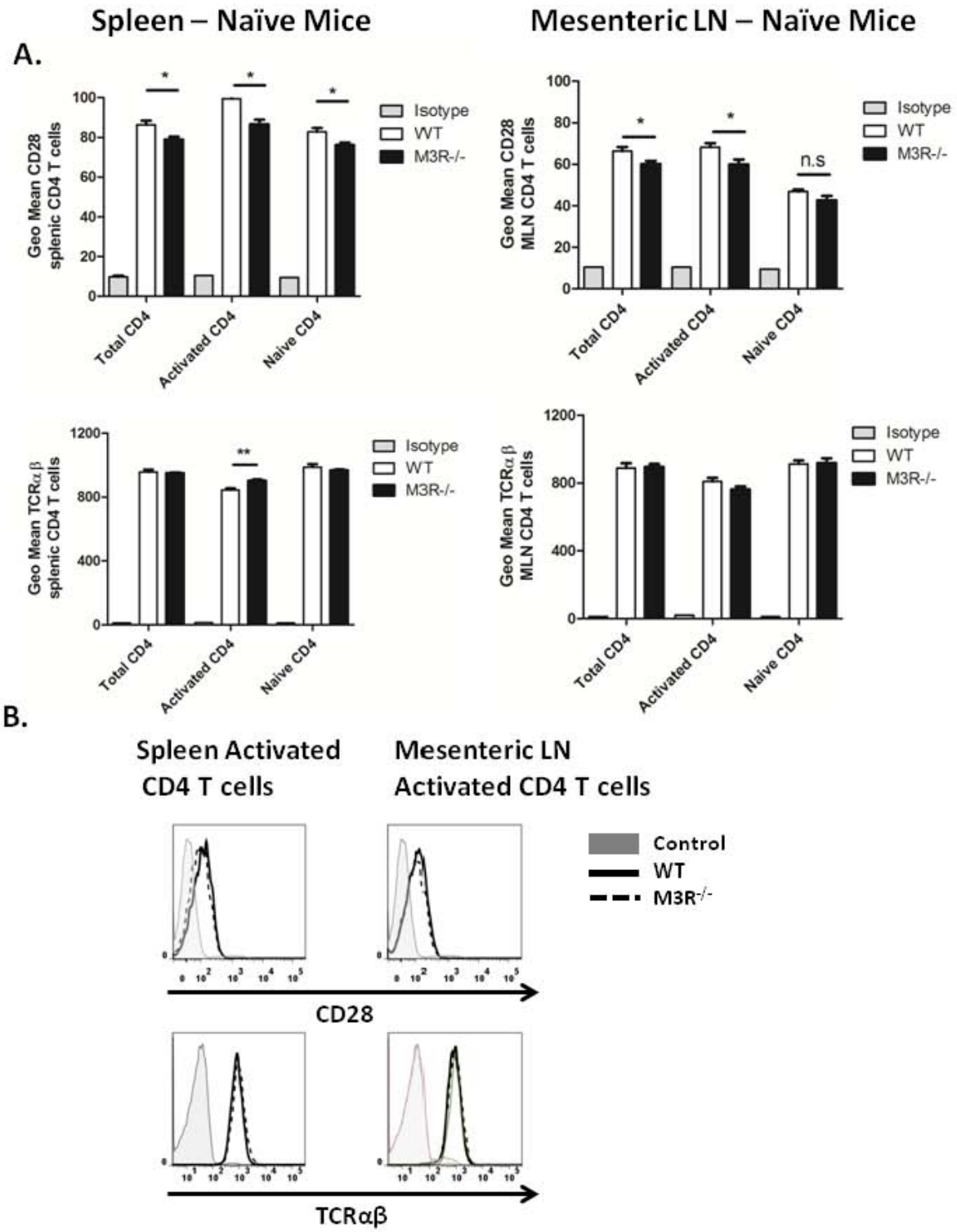


Fig 1.3 M3R deficiency affects expression of co-receptors CD28 and TCRαβ on CD4 T cells in naïve mice (A) Geometric means of CD28 and TCRab expression on total CD4 T cells, activated ($CD44^{hi}CD62L^{lo}$) and naïve ($CD44^{lo}CD62L^{hi}$) CD4 Tcells derived from spleen and mesenteric lymph nodes of naïve uninfected mice. (B) Representative histograms showing CD28 and TCRab expression on activated CD4 T cells from the spleen and MLN of WT (solid line) and $M3R^{-/-}$ mice (dashed line). Cells were stained with an antibody panel consisting of CD3, CD4, CD44, CD62L, CD28 and TCRαβ, acquired on a BD Fortessa and analysed using FlowJo software. Data is shown as mean ± SEM and is from one experiment with 6 mice in each group. Statistical significance was calculated using the Mann-Whitney two-tailed t test and denoted by * $p<0.05$, ** $p<0.01$.

3. Discussion:

Muscarinic receptors and particularly the M3 receptor subtype exist on a number of non-neuronal cells such as smooth muscle cells, insulin-secreting β cells in the pancreas and immune cells. The function of M3R has been well characterized for smooth muscle contraction and glandular secretions however its role on immune cells is not known. Therefore in our study, we set out to elucidate a role for the M3R in controlling immune cell functioning using targeted gene deficient mice lacking the M3R ($M3R^{-/-}$) and a number of murine models of infectious diseases. The $M3R^{-/-}$ mouse strain was generated and kindly given to us by Dr Jurgen Wess in the C57BL/6 background.

In order to perform standardized experiments using model pathogens in our laboratory, the $M3R^{-/-}$ mice first had to be backcrossed to the BALB/c background for at least 10 generations prior to use. In the first chapter, we present data to determine whether the $M3R^{-/-}$ mice still retain the major phenotypes reported for this mouse strain as well as to characterize the development of major immune cell populations in the naïve state (i.e prior to infection with model pathogens). We found that $M3R^{-/-}$ mice even in the BALB/c background maintained their distinct hypophagic and lean phenotype with both male and female mice weighing approximately 20% less than their wildtype counterparts as reported previously (Yamada, Miyakawa et al. 2001). We also obtained the expected product sizes using the genotyping primers designed to detect the wildtype and modified allele confirming that backcrossing into BALB/c has maintained the M3R deletion genetically as well as phenotypically.

The existence of both subtypes of acetylcholine receptors, nicotinic and muscarinic, has been reported on immune cells (Kawashima, Yoshikawa et al. 2007). An important anti-inflammatory role has since been described for nicotinic receptor in downregulating TNF-α production from

macrophages. Muscarinic receptors have also been found on numerous immune cells, both from murine and human primary and immortalized cell lines. A muscarinic subtype, the M1R, is required for development of CD8 T cells *in vitro*, however *in vivo* mice lacking the M1R were able to generate fully functional CD8 T cells. M3R in particular have been found in CD4 T cells, CD8 T cells (Qian, Galitovskiy et al. 2011), B cells (Fujii and Kawashima 2000), dendritic cells and macrophages (Kawashima, Yoshikawa et al. 2007). Using $M3R^{-/-}$ mice we showed that M3R was not required for development of major immune cells such as CD4 T cell, CD8 T cell, B cells, macrophages and dendritic cells in the spleen or lymph nodes as both wildtype and M3R had similar proportions and number of immune cells in the lymphoid organs examined. We also focused on subsets of CD4 T cells and found that under specific pathogen free condition (SPF) the M3R mice were able to maintain basal levels of T cell activation as measured by $CD44^{hi}$ and $CD62L^{lo}$ expression. We further tested whether functionally M3R were required for CD4 T cells to develop into either T_H1 or T_H2 phenotype in vitro. Using a cocktail of antibodies and cytokines, we found that naïve $M3R^{-/-}$ CD4 T cells are fully capable of generating a T_H1 or a T_H2 equivalent to wildtype CD4 T cell. Therefore, it appeared that even though a number of immune cells express the M3R, it was not required for their development and in the case of CD4 T cells did not prevent development of a T helper 1 or 2 type of response *in vitro*. Muscarinic receptors, like other G protein coupled receptors (Ritter and Hall 2009) interact with a number of other proteins such as ion channel proteins, scaffolding proteins and other membrane receptors (Borroto-Escuela, Agnati et al. 2012). M3R has also been shown to interact with and modulate mammalian odorant receptor signaling (Li and Matsunami 2011). We therefore investigated whether M3R deficiency affects expression of two key co-receptors, TCRαβ and CD28, on the surface of CD4 T cells. In naïve mice, $M3R^{-/-}$ CD4 T cells and mainly the activated CD4 T cell subset express significantly lower levels of CD28 but not TCRαβ on both splenic and MLN derived CD4 T cells. CD28 is an essential co-receptor on T cells which binds the

co-stimulatory molecules CD80/CD86 on APCs to initiate an immune response (Lenschow, Walunas et al. 1996). Lower levels of CD28 expression may be indicative of a reduced potential for CD4 T cell activation and subsequent dampening or delay of immune responses. In order to investigate this further, we employed the use of murine infectious pathogens to study the role of M3R on protective immunity to pathogens.

Chapter 2: Role of M3R in regulating T_H2 immunity after infection with *Nippostrongylus brasiliensis*.

1. Introduction:

In the previous chapter, we determined that naive $M3R^{-/-}$ mice did not have any obvious defect in basal development of immune cell populations. However it still remains to be determined whether M3R deletion influences the development of an immune response following a pathogenic infection.

In this chapter, we aimed to determine the contribution of M3R signalling to immunity induced by *Nippostrongylus brasiliensis* by utilising M3R gene deficient mice ($M3R^{-/-}$ mice). *Nippostrongylus brasiliensis* is a common laboratory pathogen used to study T helper 2 immune response mediated disease resolution and closely resembles the important human hookworms *Ancylostoma duodenale* and *Necator americanus* (Gause, Urban et al. 2003), which affects up to two billion people in the world and is responsible for severe economic losses in livestock (Hotez, Brindley et al. 2008). The nematode parasite *N.brasiliensis* induces a T_H2 response in murine hosts, with IL-13 signalling via IL4Rα essential for clearance of infection (Urban, Noben-Trauth et al. 1998). This pathway is also necessary for enhanced smooth muscle contractility, thought to be an important factor in expelling parasites from the intestine (Zhao, McDermott et al. 2003; Akiho, Lovato et al. 2005). Previous studies in our laboratory using mice with smooth muscle cells deficient in IL-4Rα showed delayed parasite expulsion. Associated with this deficit was reduced T_H2 cytokine production in mesenteric lymph nodes, delayed goblet cell hyperplasia, and reduced intestinal M3 muscarinic receptor (M3R) expression (Horsnell, Cutler et al. 2007). This was an interesting finding, as the M3R is the major mAChR expressed on smooth muscle, and has been shown to drive most of the contractile response in the ileum (Matsui, Motomura et al. 2002). In addition to the expected defect in smooth muscle

function, the study revealed a surprising effect of M3R deficiency on adaptive immune responses elicited *in vivo* during infection with *N. brasiliensis* particularly related to CD4 T cell function.

2. Results:

2.1. Absence of M3R results in delayed parasite expulsion and reduced T_H2 cytokine production after a primary *N.brasiliensis* infection.

To determine whether expression of M3 receptor is important for *in vivo* immunity to *N. brasiliensis*, we infected $M3R^{-/-}$ with 500L3 larvae and examined parasite burden at day 7, 9 and 12 post infection. At 7 days post infection $M3R^{-/-}$ mice had a 2-fold higher adult worm burden in the small intestine compared to WT mice. In normal wildtype (WT) BALB/c mice, the infection is self-resolving and the parasites are cleared from the small intestine by day 9. However, expulsion of parasites was delayed in the M3R knockout mice ($M3R^{-/-}$) with adult worms recovered at day 9 where as none were recovered from WT mice but by day 12 $M3R^{-/-}$ mice had also cleared the infection (Fig. 2.1 A).

Increased intestinal hypercontraction in response to carbachol, a cholinergic agonist, has been observed during *N. brasiliensis* infection and is hypothesised to be a potential effector mechanism for parasite expulsion (Rodriguez-Palmero, Hara et al. 1999). $M3R^{-/-}$ mice have previously been shown to be refractory to ACh-induced hypercontractility (Matsui, Motomura et al. 2002). We observed increased ACh-driven hypercontraction in infected wildtype mice but this was completely abrogated in infected $M3R^{-/-}$ mice (Fig. 2.1 B), suggesting that M3R-mediated hypercontractility of smooth muscle may indeed contribute to parasite expulsion but is not essential as $M3R^{-/-}$ mice are totally devoid of cholinergic induced contractions yet are capable of expelling *N.brasliensis* from the gut.

Next we investigated whether M3R influences the development of T_H2 immune responses which may contribute to the observed delay in expulsion of *N.brasiliensis*. Analysis of whole tissue cytokine levels in the intestine revealed a trend for reduced IL-13 levels in $M3R^{-/-}$ compared to WT mice at day 7, although this did not reach statistical significance, with no difference observed at day 9 post-infection (Fig. 2.1 Ci). IL-13 was also significantly reduced 3-fold at the transcript level in isolated MLN CD4 T cells from $M3R^{-/-}$ mice at day 7 (Fig. 2.1 Cii) suggesting that the defect in parasite expulsion may be immune driven. To further characterize the CD4 T cell phenotype in the absence of M3R, we used flow cytometry to determine CD4 T cell activation and costimulatory marker expression. There was a higher percentage of CD4 T cells in the MLN of $M3R^{-/-}$ mice but a lower proportion of these cells were activated as defined by markers $CD44^{hi}CD62L^{lo}$ indicating a possible defect in activation but not development in the absence of M3R (Fig. 2.1 D). The proportion of naïve CD4 T cells, defined by $CD44^{lo}CD62l^{hi}$, in contrast were significantly higher in $M3R^{-/-}$ mice further supporting the claim that in the absence of M3R, CD4 T cell activation is attenuated (Fig 2.1D). CD4 T cells and in particular the activated $CD4^{+}CD62L^{lo}$ T cells had a lower expression of the co-stimulatory marker CD28 (Fig. 2.1E). This reduced CD28 expression may explain the defect in T_H2 cytokine production as ligation of this receptor is important for T_H2 differentiation and production of associated cytokines such as IL-4 and IL-5 by CD4 T cells *in vitro* and *in vivo* (Rulifson, Sperling et al. 1997; Rodriguez-Palmero, Hara et al. 1999). These data indicate that expulsion of *N.brasiliensis* and production of IL-13 is delayed in $M3R^{-/-}$ mice, suggesting that expression of M3R on $CD4^{+}$ T cells is required for optimal IL-13 production possibly via CD28 co-stimulation in response to *N. brasiliensis* infection.

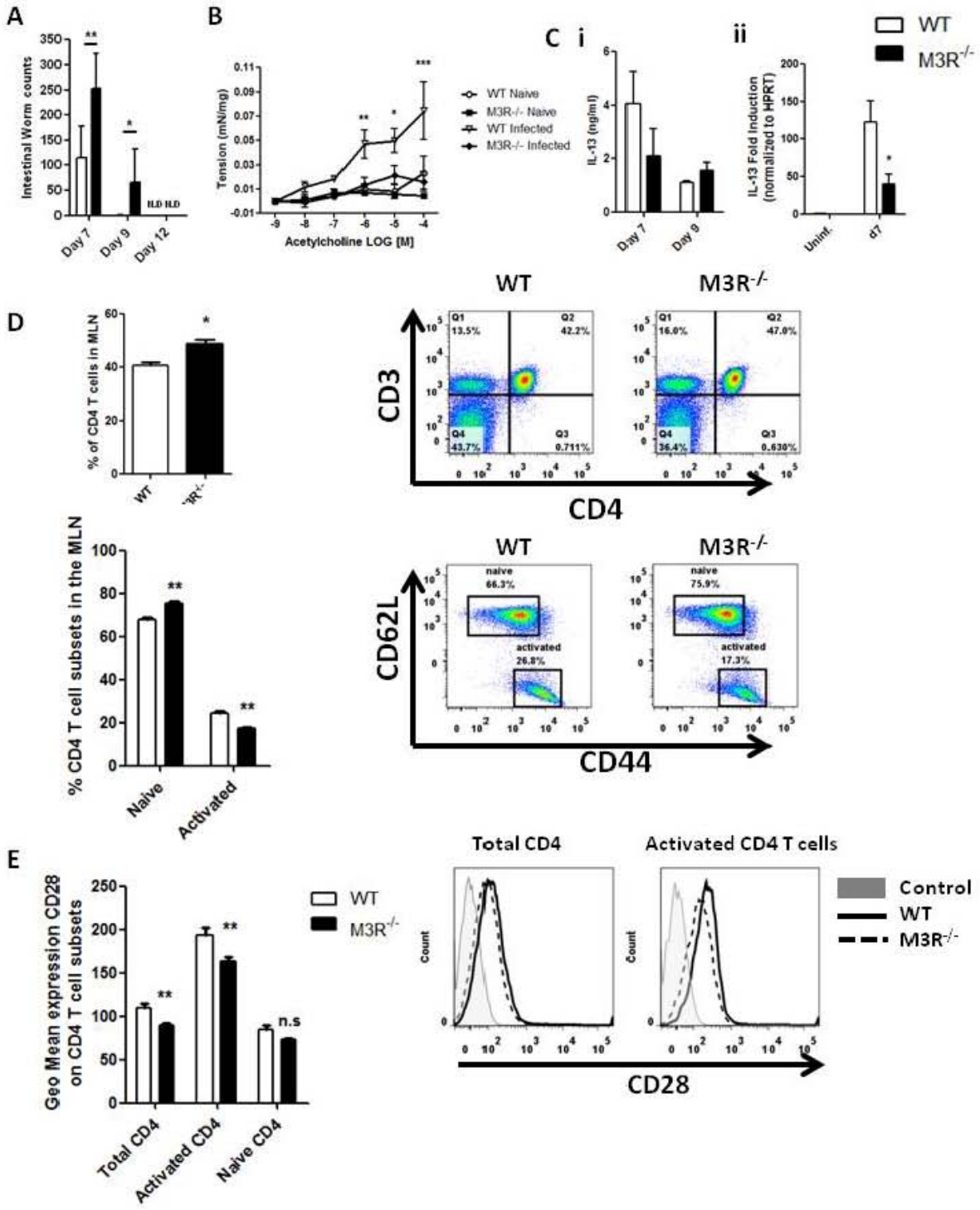


Fig 2.1. M3R deficient mice have delayed clearance of a primary *N.brasiliensis* infection. (A) Adult worms in the intestine were enumerated at days 7, 9 and 12 post-infection in WT (white bar) and M3R-/- (black bar) mice. (B) Contraction of jejunum sections in response to varying doses of acetylcholine measured using a force transducer at day 9 post-infection. (C) (i) IL-13 in intestinal homogentaes (5mg/ml final protein conc at days 7 and 9 post-infection detected by ELISA. (ii) IL-13 mRNA in sorted CD4 T cells from MLN of WT and M3R-/- mice at day 7 post infection. (D) Percentage of CD4 T cells in MLN at d7 p.i and proportion of activated CD4 T cells ($CD3^+CD4^+CD44^{hi}CD62L^{lo}$) within total CD4 T cells. (E) Expression of the co-stimulatory marker CD28 on total CD4 T cells and activated CD4 T cells. Data are shown as mean ± SEM and is representative of two independent experiments with 6 mice in each group. Statistical significance was calculated using the Mann-Whitney two-tailed t test and denoted by * $p<0.05$, ** $p<0.01$.

2.2. M3R expression is increased on CD4 T cells after *N.brasiliensis* infection.

CD4 T cells express M3R and differentiation of CD4 T cells to T_H1, T_H2 or T_H17 phenotype *in vitro* using appropriate cytokine mixes results in downregulation of M3R both at the transcript and protein levels (Qian, Galitovskiy et al. 2011). However, regulation of M3R on CD4 T cells *in vivo* after infection is not known. Therefore we used flow cytometry and RT-PCR to further characterize the expression of M3R on CD4 T cells after *N.brasiliensis* infection. An antibody that detects cell surface M3R specifically by flow cytometry is not commercially available and therefore we used the pan-muscarinic receptor antibody M35 which detects all subtypes (Carsi-Gabrenas, Van der Zee et al. 1997). At day 7 post-infection with *N. brasiliensis*, there was a two-fold increase in the number of muscarinic receptor positive CD4+ T cells in the MLN compared to normal uninfected control mice (Fig. 2.2 Ai), and the vast majority of these cells produced IL-4 (Fig. 2.2 Aii). Analysis of muscarinic receptor expression by PCR showed that T (CD90+) cells isolated from MLN expressed M1, M3, M4 and M5 receptor subtypes (Fig. 2.2 Bi). Moreover, expression of the M3 receptor as measured by real-time PCR was significantly increased 3-fold on $CD3^+CD4^+$ T cells from MLN of infected mice (Fig. 2.2 Bii). This is in contrast with the reported *in vitro* situation where M3R expression was reduced after T_H2 differentiation of CD4 T cells using IL-4 and anti-IFN-γ antibodies (Qian, Galitovskiy et al. 2011).

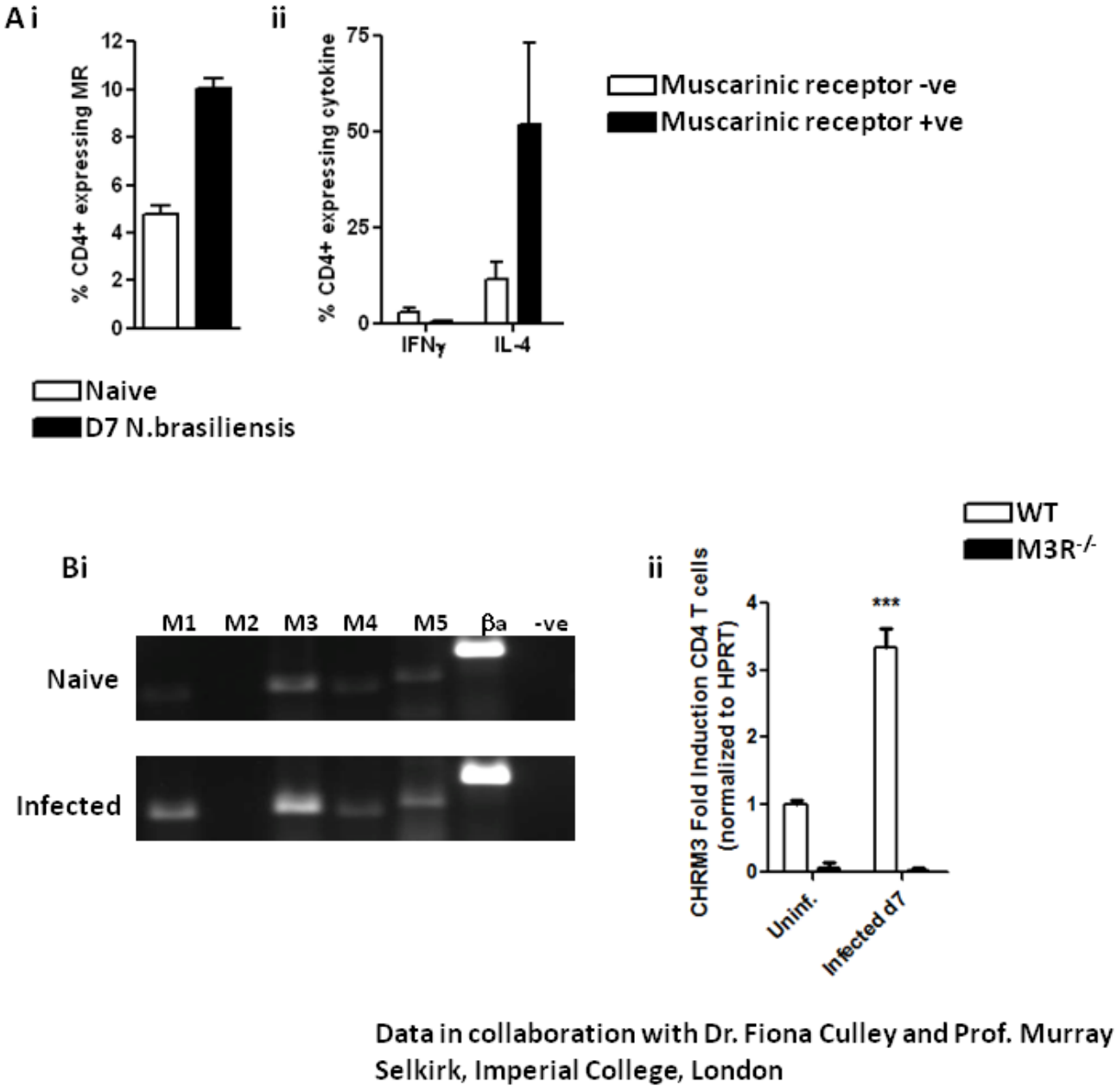
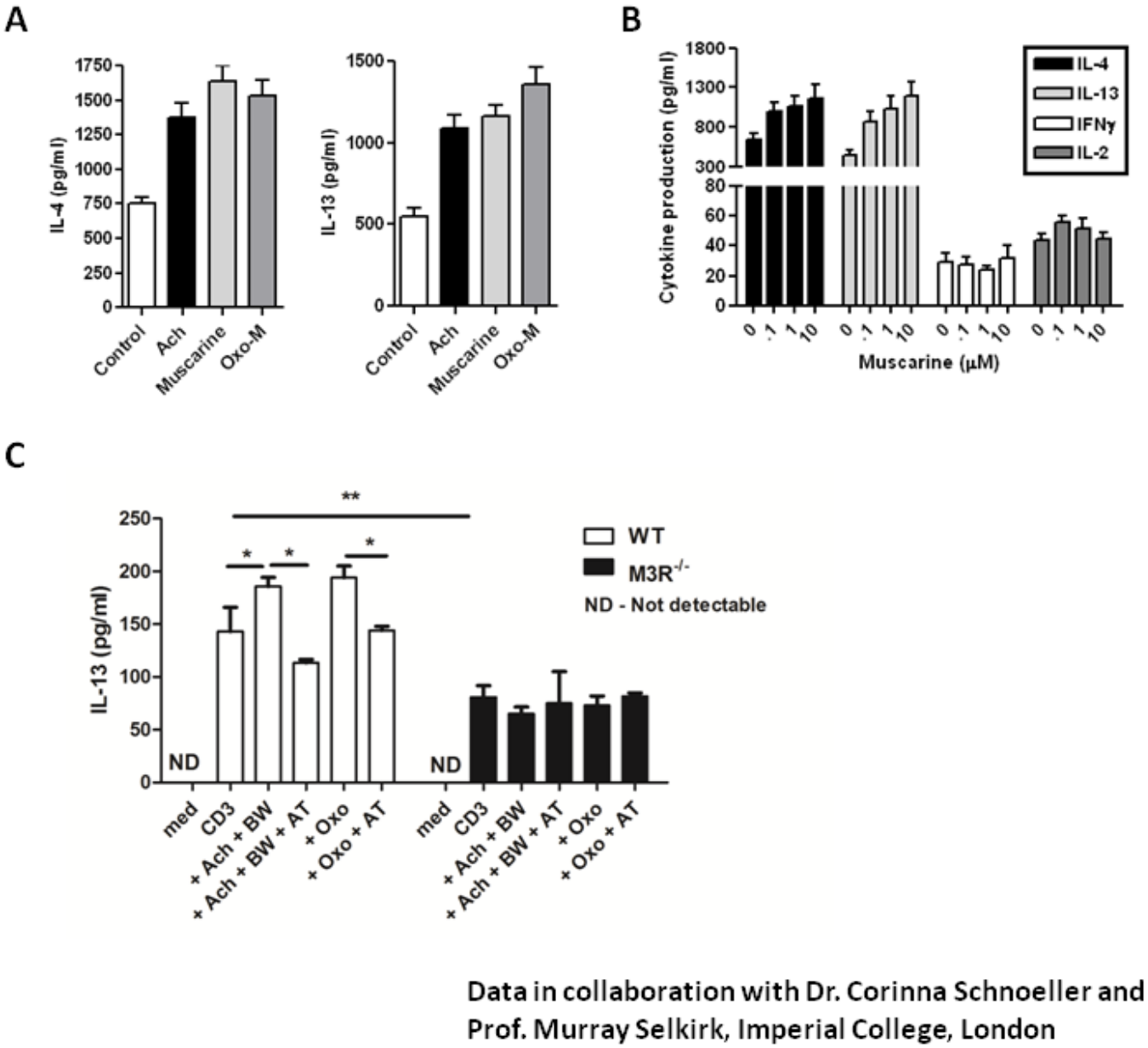


Fig 2.2: Muscarinic receptors are associated with T_H2 cytokine production after *N.brasiliensis* infection. **(**A) (i) Muscarinic receptor expression on CD4 T cells in the MLN after infection is determined by flow cytometry and staining with M35, a nonspecific muscarinic receptor antibody. (ii) Muscarinic receptor positive and negative CD4 T cells were examined for intracellular cytokine production. (B) (i) Semi- quantitative PCR of muscarinic receptor subtypes and (ii) Quantification of relative M3 receptor muscarinic expression in mRNA extracted from MLN CD4 T cells from naive mice and after infection with *N.brasiliensis*. Data are shown as mean ± SEM and is representative of two independent experiments with 5-6 mice in each group. Statistical significance was calculated using the Mann-Whitney two-tailed t test and denoted by * $p<0.05$, ** $p<0.01$.

2.3. Muscarinic receptor stimulation enhances T_H2 cytokine production.

CD4 T cells upregulate expression of muscarinic receptors particularly M3R after infection with *N.brasiliensis*. To determine whether these receptors are functional, we restimulated MLN cells of infected mice with suboptimal anti-CD3 (0.1μg/ml) in the presence of the muscarinic receptor agonists acetylcholine, muscarine and oxotremorine-M. Treatment with all three agonists at a concentration of 10μM enhanced production of the T_H2 cytokines IL-4 and IL-13 by 2-fold (Fig 2.3A). Furthermore for muscarine, the prototypical muscarinic receptor agonist, this increase was dose dependent and specific for T_H2 cytokines but not for IL-2 or the T_H1 cytokine IFN-γ (Fig 2.3B). Production of IL-13 is significantly reduced in $M3R^{-/-}$ MLN cells after sub-optimal CD3 stimulation providing a potential cause for reduced IL-13 in the intestine of $M3R^{-/-}$ mice. Interestingly, the stimulation of $M3R^{-/-}$ cells with the cholinergic agonist acetylcholine, together with the acetylcholinesterase inhibitor BW (to increase half-life of acetylcholine) (Bois, Hummel et al. 1980), does not result in IL-13 enhancement providing strong evidence that M3R is the key muscarinic subtype regulating this process. This was also observed for the muscarinic receptor specific agonist oxotremorine-M. Furthermore, treatment of wildtype mesenteric lymph node cells with atropine, a muscarinic antagonist, completely abrogated agonist mediated cytokine enhancement (Fig 2.3C). These data confirm that muscarinic receptors and M3R in particular are functional and enhance production of T_H2 cytokine IL-13 by lymphocytes after *N.brasiliensis* infection.



Data in collaboration with Dr. Corinna Schnoeller and Prof. Murray Selkirk, Imperial College, London

Fig 2.3: Muscarinic receptor stimulation with agonist enhances T_H2 cytokine production after *N.brasiliensis* infection dependent on M3R. (A) IL-4 and IL-13 produced by restimulated CD4 T cells isolated from MLN cells after *N.brasiliensis* infection and after treatment with various muscarinic receptor agonists acetylcholine (Ach), muscarine and oxotremorine-M (at 10uM each) and measured by specific ELISA. (B) Dose dependent effect of muscarine treatment on cytokine production by MLN cells. (C) Muscarinic agonist acetylcholine (with BW – Acetylcholinesterase inhibitor), oxotremorine-M and antagonist atropine (AT) treatment of mesenteric LN cells in the absence of M3R. IL-13 was measured in the supernatant after 24 hours Data are shown as mean ± SEM and is representative of two independent experiments. Statistical significance was calculated using the Mann-Whitney two-tailed t test and denoted by * $p<0.05$, ** $p<0.01$.

2.4. Absence of M3R significantly impairs memory immune response during a secondary *N.brasiliensis* infection.

CD4 T cells co-ordinate the adaptive phase of the immune response and are also important for immunological memory against *N.brasiliensis* and provide protection in mice during re-infection (Harvie, Camberis et al. 2010). We next investigated whether expression of the M3R influenced recall immune responses following secondary infection with *N. brasiliensis*. WT and $M3R^{-/-}$ mice were infected subcutaneously with 500 infective larvae (L3), treated with ivermectin 10 days post-infection, rested for 28 days and then re-infected with 500 L3. Much higher numbers of parasites were recovered from the lungs of $M3R^{-/-}$ mice at day 2 post-infection compared to WT mice (Fig. 2.4 A). This was accompanied by significantly reduced levels of IL-4 and IL-13 in lung and intestinal homogenates from $M3R^{-/-}$ mice after secondary infection (Fig. 2.4 B). As expected, infection resulted in a dramatic increase in the numbers of mucus-producing goblet cells in the airway epithelia of WT mice, but these were notably absent in the lungs of $M3R^{-/-}$ mice (Fig. 2.4 C). Mucus secretion by goblet cells has long been suggested as an effector mechanism against *N. brasiliensis* and other enteric nematodes (Miller, Huntley et al. 1981). Recent work has confirmed this, as mice deficient in expression of the mucins Muc2 and Muc5A exhibit delayed expulsion of a number of nematode species, and Muc5A was also demonstrated to have a direct detrimental effect on parasite viability (Hasnain, Wang et al. 2010; Hasnain, Evans et al. 2011).

It has recently been demonstrated that the lung is a critical site for priming CD4+ T cell mediated immunity to *N. brasiliensis* (Harvie, Camberis et al. 2010), and that lung-resident CD4+ T cell populations are responsible for co-ordinating recall responses (G. Le Gros, International Immunology meeting Kobe Japan). However, total and activated CD4+ T cell populations in the lung, the latter identified by expression of CD62L, were similar in WT and $M3R^{-/-}$ mice (Fig. 2.4 D). It therefore appears that M3 receptor expression is required for the generation of an effective

memory response in the lung after a secondary *N. brasiliensis* challenge, and that this involves timely production of IL-13 and hyperplasia of mucus-secreting goblet cells.

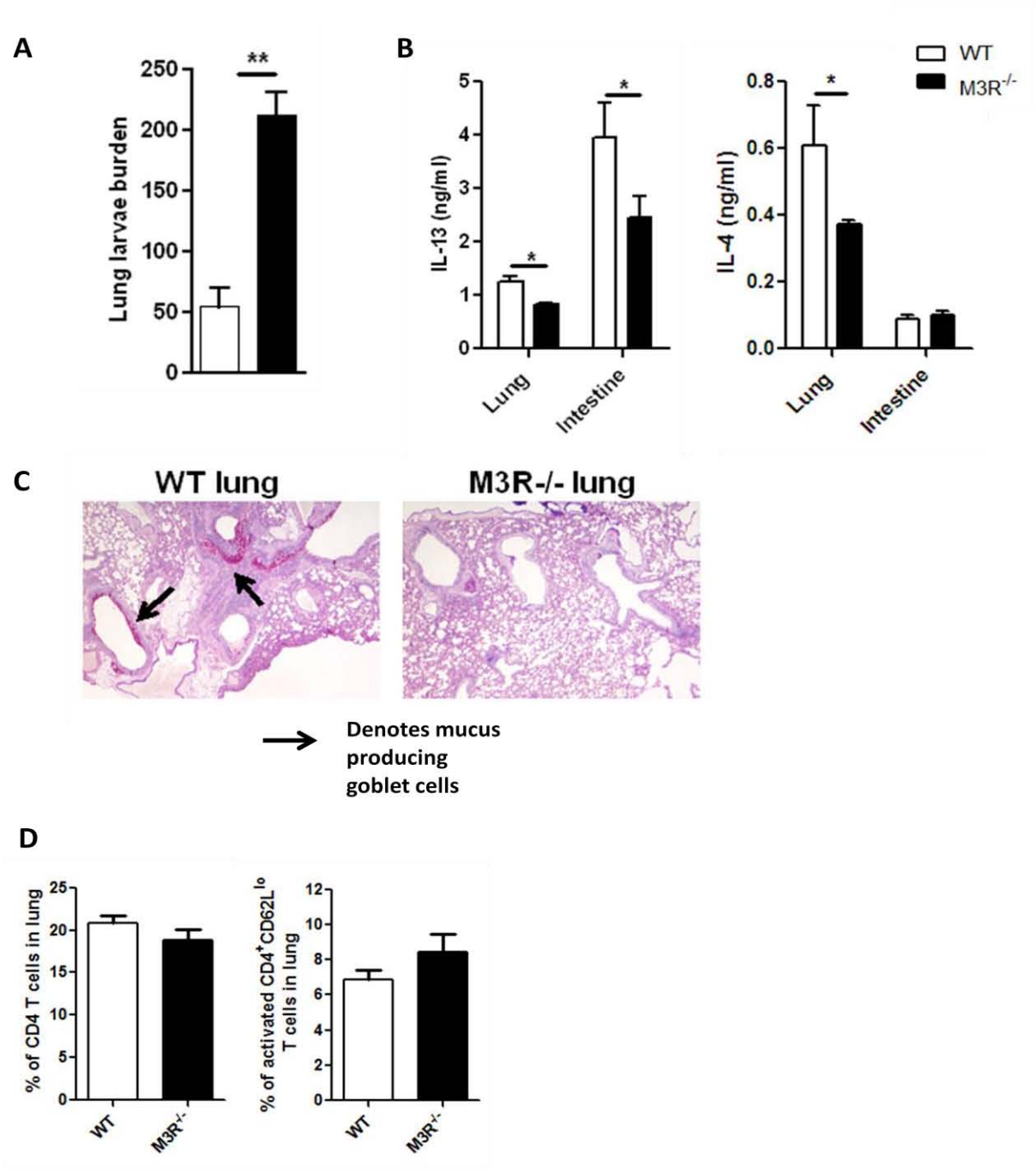


Fig 2.4 M3R deficient mice have impaired memory immune responses during a secondary infection with *N.brasiliensis*. (A) Number of *N.brasiliensis* larvae in the lung at day 2 post-secondary infection. (B) Levels of cytokine IL-4 and IL-13 in total lung and intestinal homogenates at day 2 pi. (C) Lung histological sections stained with Periodic Acid Schiff (PAS) stain at day 2 pi. Arrows indicate mucus producing-goblet cells. (E). Analysis of total and activated CD4 T cells expressed as a percentage of total cells in the lung at day 2 post-secondary infection. Data are shown as mean ± SEM and is representative of two independent experiments with 6 mice in each group. Statistical significance was calculated using the Mann-Whitney two-tailed t test and denoted by * $p<0.05$, ** $p<0.01$.

2.5. Expression of M3R is essential for producing functional CD4 T cells *in vivo*.

$CD4^+$ T cells are required for primary resolution of *N. brasiliensis*, as $CD4^+$ T cell depleted mice are unable to expel parasites (Hamilton, Loose et al. 1997) and may also be important in facilitating memory immune response. Since M3 receptors are significantly upregulated on CD4 T cells after *N. brasiliensis* infection and CD4 T cells are important in mediating adaptive immune response, we investigated whether M3R expression is required on CD4 T cells to confer protection against *N. brasiliensis in vivo*. CD4 T cells were sorted from the MLN of infected WT and $M3R^{-/-}$ mice at day 7 p.i. and 5 X 10^5 CD4 T cells were then adoptively transferred into naive BALB/c recipient mice which were then infected with 750L3 larvae. On day 5 p.i, adult worm burdens in mice receiving WT CD4 T cells were significantly lower compared to control BALB/c having received no cells (Fig 2.5A). However recipient mice receiving $M3R^{-/-}$ CD4 T cells had similar worm burdens to control Balb/c mice indicating that no protection was provided by the transfer of $M3R^{-/-}$ CD4 T cell (Fig 2.5A). Furthermore, restimulated splenocytes from recipient mice with WT CD4 T cells produce more IL-13 as compared to $M3R^{-/-}$ CD4 T cell recipients which had levels similar to naive mice that had not received any cells (Fig 2.5B). WT CD4 T cell recipients also had fewer total and activated CD4 T cells in the spleen (Fig 2.5C) and lungs (Fig 2.5D) compared to $M3R^{-/-}$ CD4 T cell recipient mice and naive BALB/c mice. This implies that $M3R^{-/-}$ CD4 recipients had to mount a much stronger cellular response in response to *N.brasiliensis* infection as a result of higher parasite burden compared to WT CD4 T cell recipients further supporting their increased susceptibility. Hence we can conclude that M3R deletion results in defective CD4 T cells that are unable to confer protection to *N. brasiliensis* when adoptively transferred to naive mice.

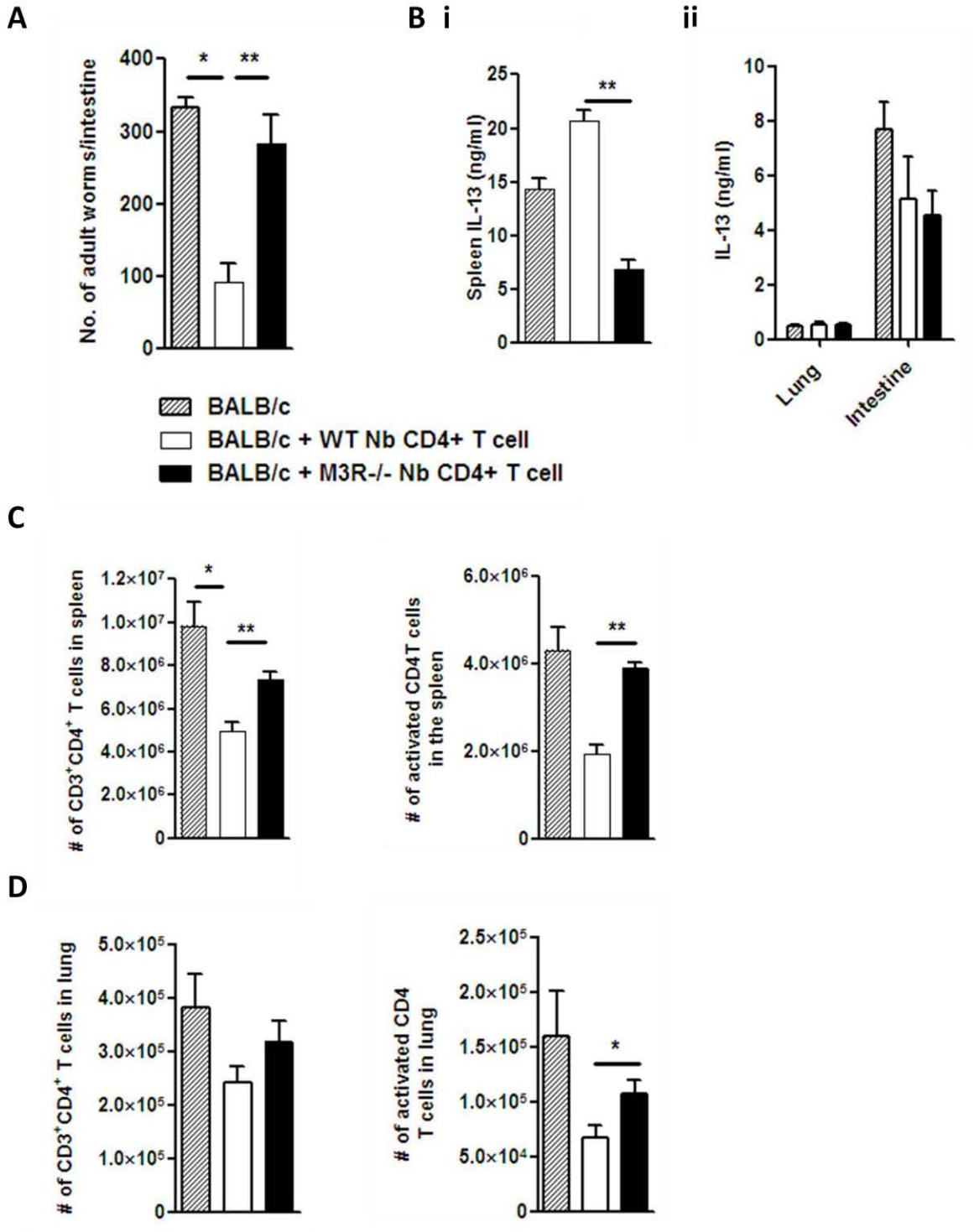


Fig 2.5. Adoptively transferred CD4 T cells from infected M3R$^{-/-}$ mice do not confer protection against *N.brasiliensis in vivo*. (A) Number of adult worms in the intestine at day 5 post infection of naive Balb/c mice were determined after transfer of 5 X 10^5 CD4 T cells from either infected WT or M3R$^{-/-}$ mice (B) (i) IL-13 levels in supernatant of MLN cells after anti-CD3 restimulation of splenocytes were determined by ELISA. (ii) IL-13 levels in whole lung and intestinal homogenates (5 mg/ml) were determined by ELISA. Numbers of total and activated CD4 T cells were determined in the (C) spleen and (D) lung of recipient mice at day 5 post-infection with *N.brasiliensis*. Data are shown as mean ± SEM and is representative of two independent experiments with 5-6 mice in each group. Statistical significance was calculated using the Mann-Whitney two-tailed t test and denoted by * $p<0.05$, ** $p<0.01$.

3. Discussion:

In this chapter we provide comprehensive characterization of the role of M3 muscarinic receptor following a pathogenic infection using a nematode parasite *Nippostrongylus brasiliensis*, as a model pathogen. *N.brasiliensis* induces a strong T_H2 response with the cytokine IL-13 being essential for its expulsion and in a previous study from our lab we have demonstrated that gene deficient mice that have a delayed clearance of *N.brasiliensis* also express lower levels of M3R mRNA in the small intestine. Our current data extends the previous study by showing that $M3R^{-/-}$ mice are more susceptible to N.*brasiliensis* during both primary and secondary infections and muscarinic receptors, particularly the M3 muscarinic receptor, enhances the T_H2 immune response during infection. This highlights an opposing effect of the two acetylcholine receptor subtypes, as nicotinic receptors have a well-documented role for inhibiting immune responses. This study therefore expands the paradigm of cholinergic signalling on development of immunity to cover not only anti-inflammatory but also a pro-inflammatory role for the cholinergic-immune system interaction. In a previous study, administration of muscarinic antagonist atropine significantly attenuated inflammation *in vivo* in response to turpentine suggesting a potential pro-inflammatory role for muscarinic receptors (Razani-Boroujerdi, Behl et al. 2008). However, we demonstrate this conclusively using a transgenic mouse strain lacking M3R and in the context of a pathogenic infection. We show that i*n vivo*, the M3R is important for optimal expulsion of parasites from the intestine, associated with increased IL-13 production. Importantly, the absence of M3R had a profound effect on activation of CD4 T cells from the mesenteric lymph node. This was also correlated to a lower expression of the co-stimulatory receptor CD28 on the CD4 T cells however whether this is a cause or effect of the absence of M3R has yet to be determined. CD28 is essential for T_H2 differentiation (Rulifson, Sperling et al. 1997; Rodriguez-Palmero, Hara et al. 1999) and blocking of CD28 *in vivo* delayed

N.brasiliensis expulsion and IL-13 production from CD4 T cell (data not shown, part of H.Ndlovu PhD Thesis in submission). M3Rs are important in primary resolution of *N.brasiliensis* however, the role of M3Rs is more apparent during secondary infection, where recall responses in the lung are important (Harvie, Camberis et al. 2010). M3R deficient mice are highly susceptible to secondary infection with a four-fold higher parasite burden by the second day of infection. The amount of T_H2 cytokines, both IL-4 and IL-13, were also reduced in the lung of $M3R^{-/-}$ mice resulting in a severely reduced goblet cell hyperplasia and mucus production in the airways. The attenuated activation of CD4 T cells in $M3R^{-/-}$ mice prompted us to examine the dynamics of M3R expression on this cell type in more detail. We show that mRNA for M3R is upregulated on CD4 T cells after infection with *N. brasiliensis*, and that muscarinic agonists act as a co-stimulatory signal for type 2 cytokine production by lymphocytes. Acetylcholine stimulates increased production of IL-13 from mesenteric lymph node cells of infected mice. Since acetylcholine is the major natural ligand for cholinergic receptors, this effect could result from stimulation of nicotinic or muscarinic receptors. However, the enhancement in cytokine production was completely abrogated in the presence of the muscarinic receptor specific antagonist atropine or in $M3R^{-/-}$ lymphocytes. Therefore, this is conclusive evidence that M3 muscarinic receptor is functional on lymphocytes and responds to stimulation by enhancing the production of T_H2 cytokines such as IL-13.

Finally, we show that M3Rs are required for effective CD4 T cell functioning in recall immunity. CD4 T cells with functional M3R were able to confer significant three-fold protection when transferred into *N.brasiliensis* infected naive mice, a protective effect that was lost when M3R deficient CD4 T cells were transferred. Transfer of M3R sufficient CD4 T cells was also correlated to an increase in IL-13 production from restimulated splenocytes.

Functionally selective M3R antagonists are widely used clinically as bronchodilators to treat asthma and chronic obstructive pulmonary disease (Moulton and Fryer 2011). The development of M3R-selective agonists is currently the goal of many academic and industrial laboratories (Wess, Eglen et al. 2007). Our findings suggest that such compounds may improve adaptive immunity, providing a rational basis for the development of novel classes of drugs aimed at enhancing immune function in various pathophysiological conditions.

Our findings demonstrate a striking effect on T_H2 immunity and also on fundamental immune cell function, namely CD4 T cell actvation and co-stimulation as well as cytokine production from lymphocytes. These findings may suggest that the influence exerted by M3R signalling is not confined to T_H2 immunity and may a have broader fundamental influence on immunity. Therefore, in order to determine whether the effect of M3R is localized to T_H2 responses or extends more broadly to T_H1 pro-inflammatory immune response and other classes of pathogens, we used a model bacterial pathogen model, *Salmonella typhimurium*.

Chapter 3: Role of M3R in regulating T_H1 immunity after infection with a bacterial pathogen *Salmonella enterica* sp. Typhimurium ΔaroA.

1. Introduction

In the previous chapter, we have shown that M3 receptor is required for optimal immunity against *N.brasiliensis* and acts as an important regulator of CD4 T cell function. However it still remains unclear whether a role for M3R is restricted to T_H2 responses or is applicable to other classes of immune responses particularly the T_H1 immune responses. To dissect the involvement of M3R in T_H1 responses we used a bacterial pathogen *Salmonella enterica* sp. Typhimurium (referred to from now as *S.typhimurium*). *S.typhimurium* is a Gram negative bacterium that causes non-typhoidal salmonellosis (NTS); a disease characterized by diarrheal gastroenteritis in immunocompetent adult humans but in small children and HIV infected individuals results in system infection (invasive) NTS that has high mortality rates of between 22% and 47% (Gordon, Banda et al. 2002; Morpeth, Ramadhani et al. 2009; MacLennan, Gilchrist et al. 2010).

S.typhimurium is a well established model in mice to study the progression of NTS and the associated immune response (Santos, Zhang et al. 2001; Mastroeni and Sheppard 2004). However in order to mimic disease progression in mice to that seen in humans as well as to study the adaptive immune response we use attenuated strains of *Salmonella* as wildtype strains are lethal, causing severe infection and death in mice within 10 days (O'Callaghan, Maskell et al. 1988). Therefore for this study we used the *aroA* SL3261 strain (Hoiseth and Stocker 1981) that has a well-defined kinetics of progression and immunocompetent mice are able to clear the infection within 35 days (O'Callaghan, Maskell et al. 1988).

S.typhimurium induces a robust T_H1 immune response that is essential for bacterial clearance (Mittrucker and Kaufmann 2000) with CD4 T cells and IFN-γ being critical in protection and bacterial clearance (Hess, Ladel et al. 1996). As muscarinic receptor regulation of T_H1 immune response has not been well studied, *S.typhimurium* is a good model to employ in order to address this question. Previous studies have shown that administration of the non-subtype selective muscarinic receptor antagonist, atropine *in vivo* inhibits turpentine induced inflammation suggesting a pro-inflammatory role for muscarinic receptors (Razani-Boroujerdi, Behl et al. 2008). *In vitro*, artificially skewing naïve CD4 T cells to the T_H1 phenotype reduces expression of M3R at the mRNA and protein level providing further evidence that this receptor may be involved in regulating T_H1 immune responses (Qian, Galitovskiy et al. 2011). However, there is no conclusive evidence of immune system regulation by muscarinic receptor in general and M3R in particular

In this chapter, we will examine the effect of M3R on development of T_H1 immune response to a bacterial pathogen *S.typhimurium* by comparing kinetics of disease progression in wildtype and $M3R^{-/-}$ mice particularly bacterial clearance and immune cell functioning.

2. Results:

2.1. M3 receptor is required for effective *in vivo* clearance of *S.typhimurium*.

To determine the role of M3R during a primary bacterial infection, WT and $M3R^{-/-}$ mice were infected with 5 X 10^5 *S.typhimurium* bacteria i.p and killed at day 7, 18, 27 and 35 post-infection. *S.typhimurium* infection is lethal in mice but a genetic modification introduced into the *aroA* gene significantly attenuates its virulence and normal wildtype mice are capable of resolving the infection (Hoiseth and Stocker 1981). In our model, by day 35 WT mice had significantly reduced bacterial burden with most mice having less than 500 bacteria in the spleen (Fig 3.1A). The greatest reduction in bacterial burden (100 fold) occurred between day 18 and day 27 where the adaptive immune system and particularly CD4 T cells are known to play an important role in clearance. $M3R^{-/-}$ mice were more susceptible to *S.typhiumurium* infection with a higher bacterial burden at day 18 (3-fold), 27 (20-fold) and 35 (5-fold). This increased susceptibility is also evident from weight loss where WT mice suffer 5% drop in body weight at day 1 post-infection but quickly recover by day 4 however $M3R^{-/-}$ have a peak weight loss of 16% of body weight and only recover 25 days post-infection (Fig 3.1B) Interestingly, bacterial burdens in WT and $M3R^{-/-}$ were similar during the early stages of the infection when the innate immune system is dominant (Kirby, Yrlid et al. 2002), however during the latter adaptive immune phase beginning from week 2 (McSorley, Cookson et al. 2000), a striking difference in bacterial burden is evident. A characteristic feature of *S.typhimurium* infection in murine hosts is marked splenomegaly (Jackson, Nanton et al. 2010) which is also observed in humans with nontyphoidal salmonella (Morpeth, Ramadhani et al. 2009). Spleens of $M3R^{-/-}$ mice were significantly smaller compared to WT mice post-infection (Fig 3.1C) however when we determined relative splenomegaly (=Spleen weight post-infection/Average pre-infection spleen weight), both WT and $M3R^{-/-}$ mice had a similar degree of splenomegaly with an 8-fold increase in spleen weight

at day 18 post-infection (Fig 3.1D). Therefore, in the absence of M3R, mice are more susceptible to infection with an attenuated strain of *S.typhimurium* as evidenced by increased splenic bacterial burden and weight loss.

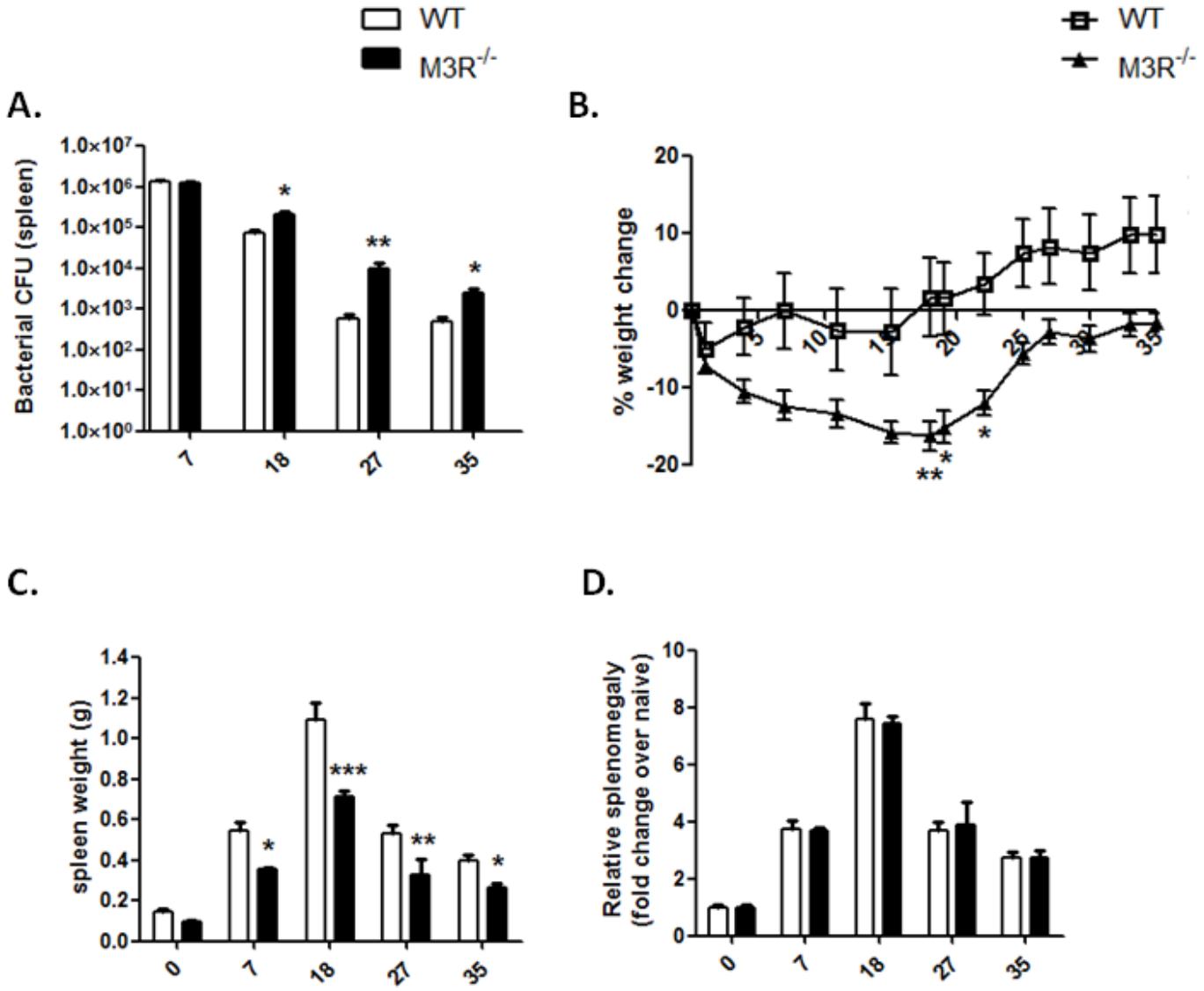


Fig 3.1. M3R$^{-/-}$ mice are more susceptible to *Salmonella typhimurium* AroA infection compared to WT Balb/c mice. (A) Mice were infected with 8X10^5 CFU of an attenuated strain of *S.typhimurium* aroA i.p and monitored for 35 days post-infection. (B) Mice were weighed to determine changes in body weight after infection with *S.typhimurium* upto day 35 post-infection. (C) Spleens were weighed individually (D) Splenomegaly was determined as fold change over average spleen weight of naïve mice. Data are shown as mean ± SEM and is representative of 2-3 independent experiments with 6 mice in each group. Statistical significance was calculated using Two-way ANOVA and Mann-Whitney two-tailed t test and denoted by * $p<0.05$, ** $p<0.01$.

2.2. M3R is required for optimal immune response and IFN-γ production during primary infection with *S.typhimurium*.

In the absence of M3R, mice are more susceptible to infection with *S.typhimurium aroA*. To determine whether this increase in susceptibility had an underlying immunological basis, we examined the kinetics of key immune cell populations and production of the pro-inflammatory cytokine IFN-γ. Overall, as the infection progressed there was a steady increase in total splenic cellularity upto day 18, after which it decreased to levels similar to that of naïve mice by day 35 and this kinetic was not disrupted in $M3R^{-/-}$ mice (Fig 3.2A). This increase in cellularity has also been observed in C57BL/6 mice infected with another attenuated strain of *S.typhimurium* (Jackson, Nanton et al. 2010). Total CD4 T cell numbers did not increase dramatically after infection, with peak numbers observed at day 27 where CD4 T cells were two-fold higher compared to naïve mice. There was a trend towards higher CD4 T cell numbers in $M3R^{-/-}$ mice however this did not reach statistical significance (Fig 3.2B). CD19+B220+ B cells remain constant upto day 18 post-infection in both WT and $M3R^{-/-}$ mice. However at day 27, there is a greater than 2 fold increase in the total number of B cells in WT mice but this was not observed for $M3R^{-/-}$ mice (Fig 3.2C). Germinal center (GC) formation is delayed in S.typhimurium infection, only appearing when bacterial burdens are reduced (Cunningham, Gaspal et al. 2007). Therefore, the increase in B cells in WT but not $M3R^{-/-}$ mice is possibly a result of the failure of $M3R^{-/-}$ mice to clear bacteria effectively and hence a further delay in GC formation.

Macrophages are recruited rapidly to the spleen following infection and by day 7 are significantly higher (6 fold) compared to naïve mice. The number of macrophages in the spleen correlated to bacterial burden and returned to base line levels by day 35 when infection was resolved. $M3R^{-/-}$ mice had a significantly greater number of macrophages in the spleen compared to WT mice at day 18, resulting from the increased bacterial burden during this time point (Fig 3.2D). Macrophages play an

important role both as a host cell (Ibarra and Steele-Mortimer 2009) for *S.typhimurium* as well as effector cells when activated by CD4 T cell derived IFN-γ (VanCott, Chatfield et al. 1998). Since IFN-γ is critical for protection and clearance of *S.typhimurium* (Hess, Ladel et al. 1996), we measured this cytokine in supernatants from anti-CD3 restimulated splenocytes. Highest amounts of IFN-γ are produced early after infection at day 7 by innate immune cells such as Natural Killer (NK) cells, neutrophils and macrophages (Ramarathinam, Niesel et al. 1993; Kirby, Yrlid et al. 2002). However, by day 18 and 27 IFN-γ production by $M3R^{-/-}$ splenocytes is markedly reduced (Fig 3.2E). This decrease in IFN-γ synthesis may be the key mechanism driving enhanced susceptibility of $M3R^{-/-}$ mice as it is strongly correlated with increased bacterial burdens.

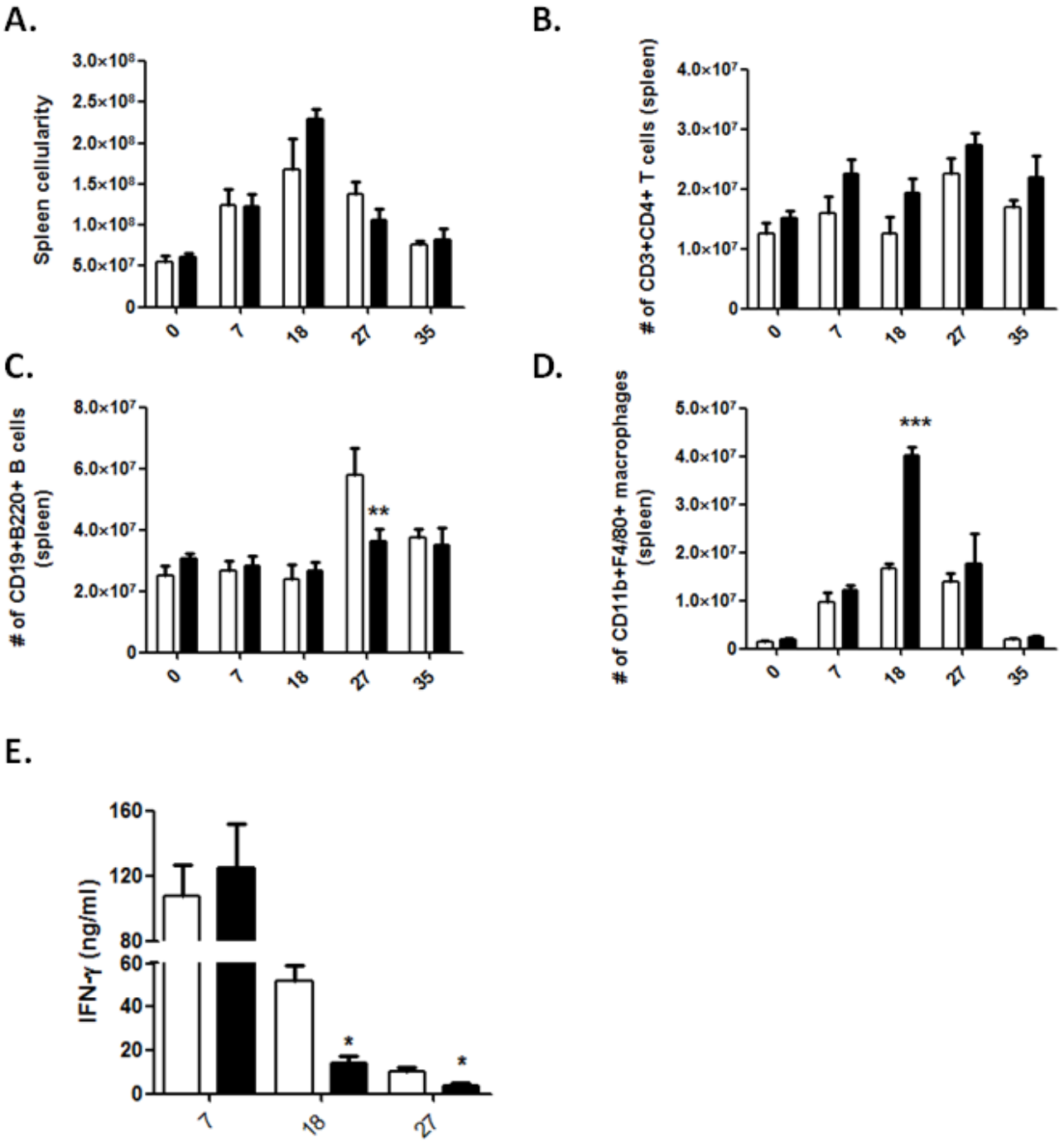


Fig 3.2. M3R is required for optimal immune response and IFN-γ production during primary infection with *S.typhimurium*. (A) Total spleen cellularity was determined at different time points. Splenocytes were then analysed by flow cytometry using surface markers for different immune cells and number of cells were calculated for (B) $CD3^+CD4^+$ T cells (C) $CD19^+B220^+$ B cells (D) $CD11b^+F4/80^+$ splenic macrophages. (E) 1 $X10^6$ splenocytes per well from WT and $M3R^{-/-}$ mice were stimulated with heat-killed *S.typhimurium* for 24 hours and IFN-γ was measured in the supernatants by ELISA. . Data are shown as mean ± SEM and is representative of 2-3 independent experiments with 6 mice in each group. Statistical significance was calculated using Two-way ANOVA and Mann-Whitney two-tailed t test and denoted by * $p<0.05$, ** $p<0.01$.

2.3. M3R does not affect memory immune response to *S.typhimurium* after vaccination with heat-killed bacteria.

We have shown previously that in the absence of M3R both primary and memory immune responses to *N.brasiliensis* are diminished and M3R$^{-/-}$ mice also have delayed clearance of a primary *S.typhimurium* infection. We therefore wanted to investigate the effect of M3R deletion on memory immune response to *S.typhimurium*. WT and M3R$^{-/-}$ mice were vaccinated with 1 X10^{7} CFU of heat-killed S.*typhimurium aroA*, rested for 4 weeks after which the mice were re-infected with 1 x 106 live *aroA* bacteria and killed on day 7 post infection. Heat-killed *Salmonella* has been shown to elicit protective responses in mice when used as a vaccination against a lethal *S.typhimurium* strain. Vaccination of wildtype mice with heat-killed bacteria results in protection with bacterial burdens reduced 500 fold compared to unvaccinated mice (Fig3.3A). Interestingly, M3R-/- mice also have a similar level of protection after vaccination (WT – 6350 bacteria/spleen; M3R$^{-/-}$ - 6324 bacteria/spleen – Fig 3.3A) indicating that memory immune response at least against an attenuated strain of *S.typhimurium* is not dependent on M3R.Splenocytes from vaccinated mice secrete significantly lower amounts of IFN-γ which correlated with the lower bacterial burden at day 7 post-infection however there was no difference between WT and M3R$^{-/-}$ vaccinated mice (Fig 3.3B). Vaccinated M3R$^{-/-}$ mice had a higher proportion of B cells (WT- 9.35% M3R$^{-/-}$-11.8%) and a similar proportion of total CD4 T cells. However M3R$^{-/-}$ mice had a significantly lower proportion of activated CD4 T cells (WT – 4.7% M3R$^{-/-}$- 2.5%) as measured by the expression of CD44^{hi} and CD62L^{lo}(Fig3.3C). Therefore we can conclude that deletion of M3R does not affect vaccination induced protection against *S.typhimurium*. Vaccinated WT mice also have a higher proportion of activated CD4 T cells (2-fold) compared to M3R$^{-/-}$ mice however this does not translate to IFN-γ secretion which is similar in both strains.

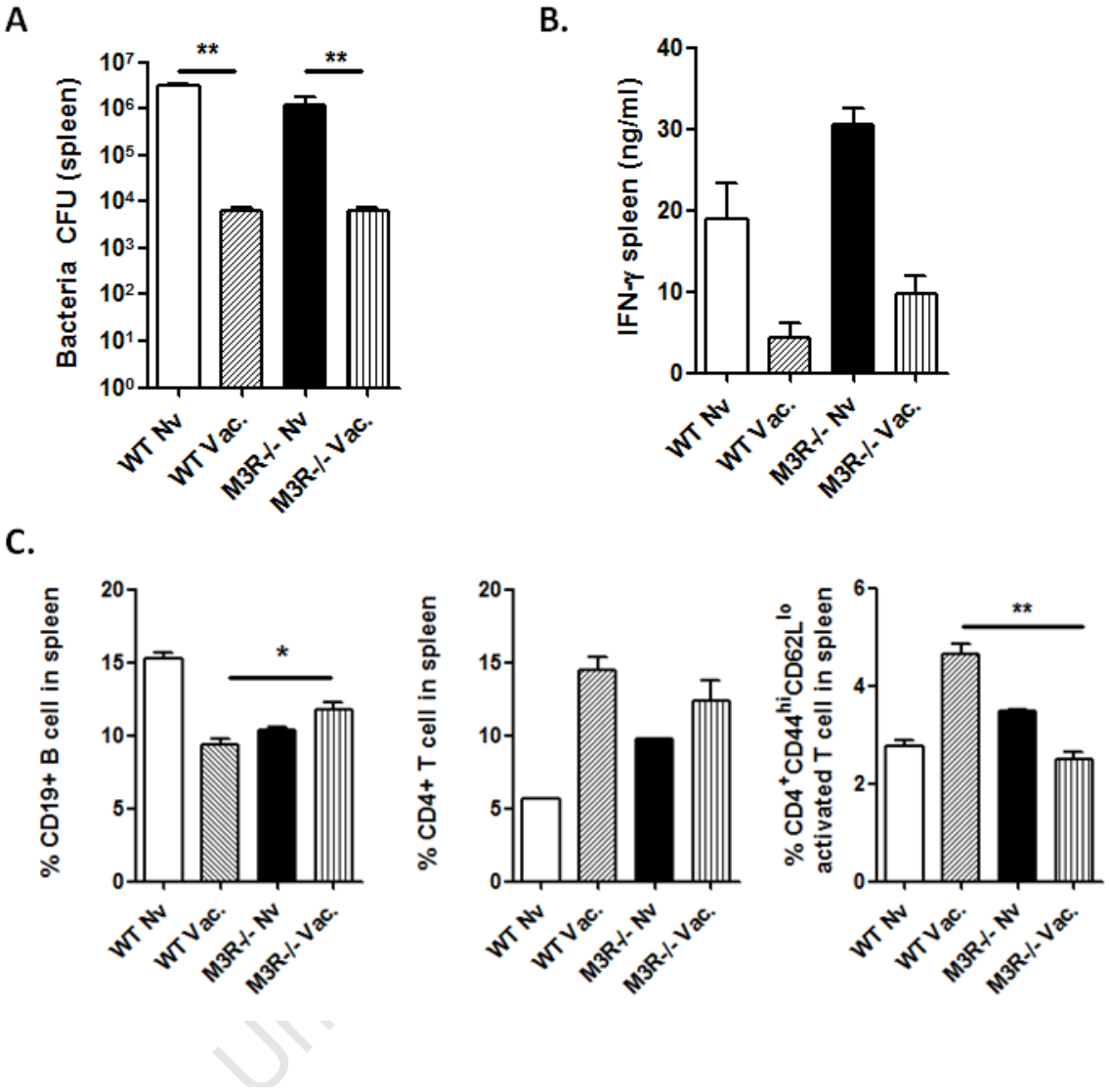


Fig 3.3 M3R is not required for memory immune response to *S.typhimurium* after vaccination with heat-killed bacteria. Mice were vaccinated with 1 X10^7 heat-killed *S.typhimurium* aroA, rested for 4 weeks and re-infected with 1 x 10^6 live aroA bacteria. (A) Spleen bacterial burden was determined at day 7 post-secondary infection (B) 1 x 10^6 splenocytes were restimulated with heat-killed bacteria for 72 hours and IFN-γ levels were measured by ELISA. (C) Flow cytometry was used to determine the proportion of B cells (CD19+), CD4 T cells (CD3+CD4+) and activated CD4 T cells (CD3+CD4+CD44^{hi}CD62L^{lo}). Data are shown as mean ± SEM and is representative of two independent experiments with 5 mice in each group. Statistical significance was calculated using the Mann-Whitney two-tailed t test and denoted by * $p<0.05$, ** $p<0.01$.

2.4. M3R deficient macrophages are effective at bacterial killing *in vitro.*

Protective immunity against *S.typhimurium* is highly dependent on phagocytosis and intracellular killing by macrophages (Lindgren, Stojiljkovic et al. 1996). To determine whether the susceptibility of $M3R^{-/-}$ mice is due to a defect in the bactericidal capacity of macrophages, an *in vitro* pathogen kill assay using bone marrow derived macrophages (BMDM) was setup. BMDMs, generated from WT and $M3R^{-/-}$ mice, were infected with *S.typhimurium aroA* at a multiplicity of infection (MOI) of 2 and 10 either in the presence or absence of 100ng/ml IFN-γ for activation. Bactericidal activity was determined by counting the number of viable colony forming bacteria remaining at various time points upto 24 hours post-infection. WT and $M3R^{-/-}$ macrophages were both capable of killing *S.typhimurium aroA in vitro* when added at an MOI of 2 or 10 and there was no difference in bactericidal capacity in the absence of M3R (Fig 3.4). Furthermore, overnight treatment with 100ng/ml IFN-γ did not improve the bactericidal capacity of macrophages against *S.typhimurium*. We can conclude that M3R deficiency on macrophages does not affect its bactericidal capacity and thus may not be responsible for the observed *in vivo* susceptibility of $M3R^{-/-}$ mice.

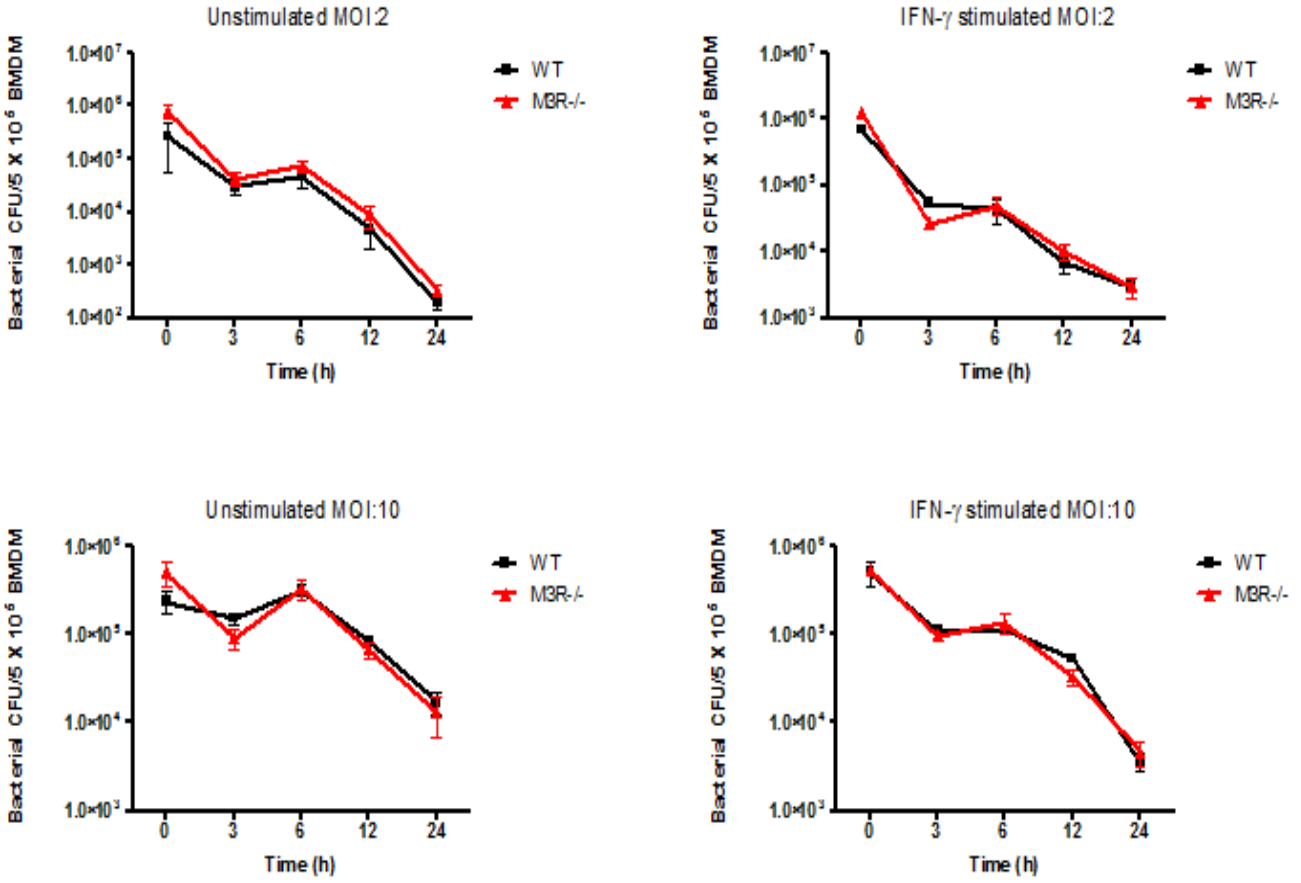


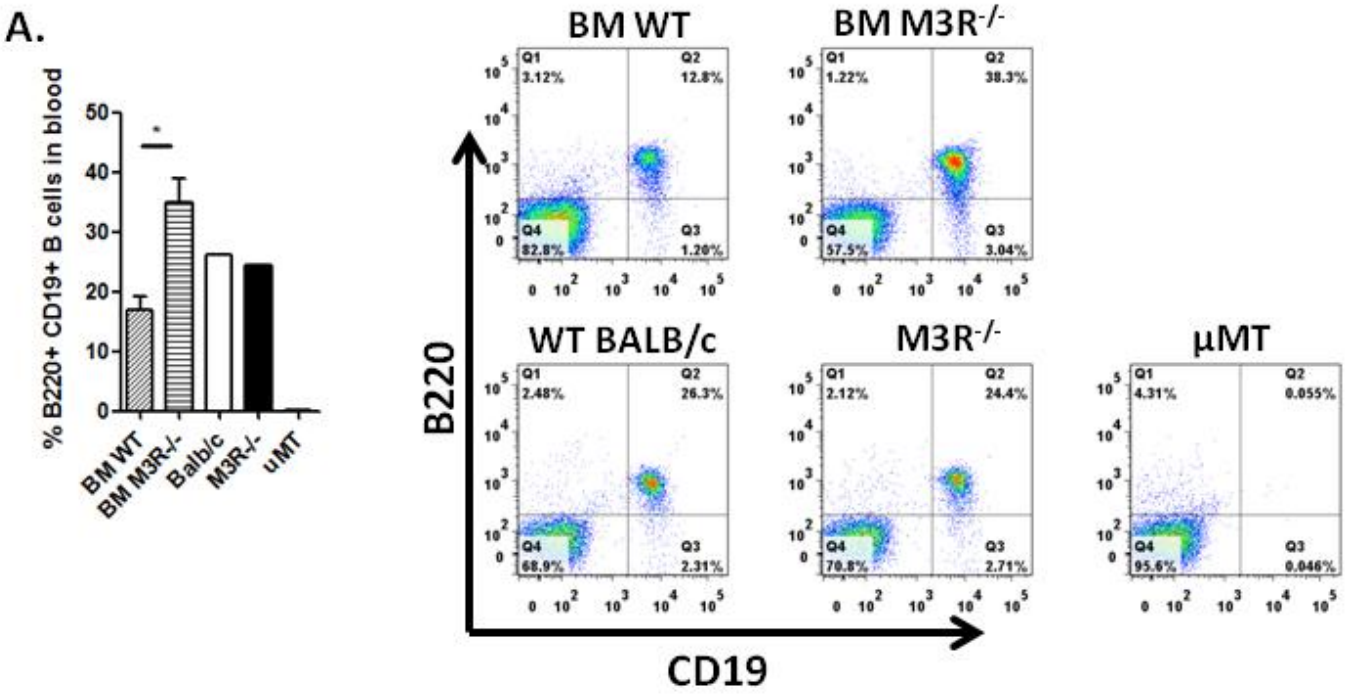
Fig 3.4: WT and M3R$^{-/-}$ bone marrow derived macrophages have similar bactericidal capacity *in vitro*. BMDMs were generated from WT and M3R$^{-/-}$, plated at 5X10^5 cells/well in 24 well plates and either left unstimulated or stimulated with 100ng/ml IFN-g overnight to activate them. *S.typhimurium AroA* was added at MOI of 2 or 10 and bacterial burden was followed over a period of 24 hours by plating colony plating on LB agar plates. Each treatment was plated out in triplicate.

2.5. M3R expression on B lymphocytes is not required for immunity against *S.typhimurium.*

Igμ$^{-/-}$ mice (μMT) are totally devoid of B cells but are still able to clear an infection with attenuated *S.typhimurium* (*aroA*) similarly to wildtype mice thus implying that B cells might not be important atleast for a primary infection with an attenuated strain (Mittrucker, Raupach et al. 2000). However, B cells are important in vaccination induced protective immunity to lethal *S.typhimurium* infection by enhancing T cell mediated immunity independent of its ability to produce antibody (Nanton, Way et al. 2012). Furthermore, specific antibodies also reduce the extracellular spread of *S.typhimurium* and its colonization of splenic macrophages (Cunningham, Gaspal et al. 2007) and can also provide protection aginast lethal strains (McSorley and Jenkins 2000). We have shown that M3R$^{-/-}$ mice have lower B cell numbers at day 27 in the spleen following *S.typhimurium* infection. To determine whether B cell M3R expression plays a role in bacterial clearance, we generated a bone marrow chimera where only B cells did not express M3R (refer Materials and Method). B cell repopulation of recipient mice was checked 6 weeks after bone marrow transfer in the blood. Irradiated μMT mice receiving WT and M3R$^{-/-}$ bone marrow had detectable levels of B cells in the blood compared to normal μMT thus confirming the chimeras were successful (Fig 3.5A). Mice were then infected with 1X 10^6 bacteria per mouse and killed at day 27 post-infection. As expected from previous results, normal M3R$^{-/-}$ had a greater than 10 fold higher bacterial burden to normal WT mice. However, no significant difference in bacterial burden was observed between WT and B cell-M3R$^{-/-}$ chimeric mice and therefore concluding that M3R on B cells does not contribute to *S.typhimurium aroA* clearance (Fig 3.5B). Both chimeric mice had a higher bacterial burden than un-irradiated μMT mice which may be due to toxic effects of radiation on other immune cells. M3R expression on B cells also does not affect antigen specific antibody production as both WT and B cell-M3R$^{-/-}$ chimeras had similar levels of *Salmonella* specific Ig1 and Ig2a (Fig 3.5D). Serum antibodies from both strains of chimeric

mice were also able to confer equivalent protection to naïve mice *in vivo* after opsonization of *S.typhimurium aroA* (Fig 3.5E) indicating that both the quantity and quality of *Salmonella* specific antibodies are not affected after M3R deletion on B cells.

An interesting observation was made regarding number of B cells in the two chimeras. B cell-M3R$^{-/-}$ chimera mice had significantly more B cells (2-fold) in the blood prior to infection compared to WT chimeras. This was also observed in splenic B cell populations at day 27 post infection where B cell numbers were 4-fold higher in B cell-M3R$^{-/-}$ chimeras (Fig 3.5B). This data suggests that in the absence of M3R, there is increased proliferation relative to M3R sufficient cells as evidenced by the significantly higher numbers of B cells when M3R was selectively deleted in this cell type.



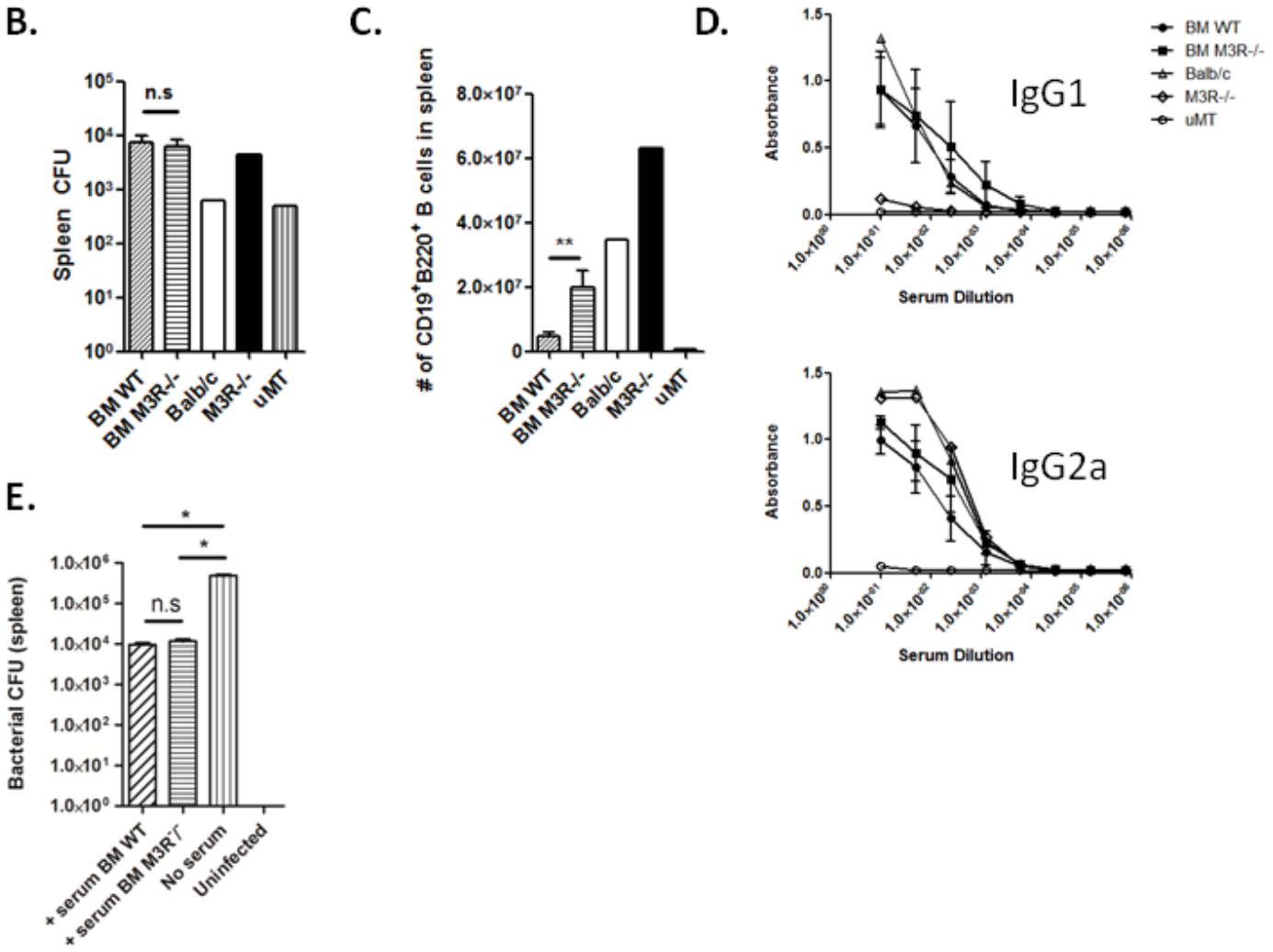


Fig 3.5 Deletion of M3R on B cells only does not contribute to increased susceptibility to *S.typhimurium*. B cell specific deletion of M3R was obtained by mixed bone marrow chimeras where B cell deficient uMT mice were irradiated and reconstituted with bone marrow (BM) made up of 80% uMT BM and 20% of either WT (BM WT) or M3R$^{-/-}$ bone marrow (BM M3R$^{-/-}$). (A) After resting for 6 weeks, B cell reconstitution of BM chimeras was checked in blood (B) Mice were infected with 1 X 10^6 aroA bacteria and spleen bacterial burden determined on day 27 post-infection. (C) B cell numbers were determined in the spleen at day 27 using the markers CD19 and B220. (D) Production of *Salmonella* specific IgG1 and IgG2a antibodies was determined in BM chimeras at day 27 using ELISA. (E) Effectiveness of antibodies produced by WT or M3R-/- B cells was tested in vivo by opsonization. Serum from WT and M3R-/- B cell specific bone marrow chimeras was heated at 65^{o}C for 30 mins to inactivate complement and incubated with 2.5 X 10^6 bacteria/ml at 10% serum concentration for 30 mins at room temperature. Naïve BALB/c mice were infected with opsonized S.typhimurium aroA and killed after 5 days and spleen bacterial burden enumerated. Data are shown as mean ± SEM and is representative of one independent experiments with 6 mice in each group. Statistical significance was calculated using the Mann-Whitney two-tailed t test and denoted by * $p<0.05$, ** $p<0.01$.

2.6. CD4 T cells from M3R$^{-/-}$ mice have reduced activation and CD28 expression *in vivo* after *S.typhimurium* infection.

CD4 T cells are essential for mounting an effective immune response to *S.typhimurium* and in nude athymic mice which lack CD4 T cells, attenuated strains of *S.typhimurium* including *aroA* are lethal (Coynault and Norel 1999). We have shown that in a T_H2 biased immune response to *N.brasiliensis*, M3R expression on CD4 T cells enhances expulsion of the nematode parasite in a primary infection. Therefore we set out to determine whether CD4 T cell function is also influenced by M3R during a T_H1-dominant immune response induced by *S.typhimurium*.

At the transcript level, M3R expression was reduced by more than 2-fold at day 18 and 27 following *S.typhimurium* infection (Fig 3.6A), which differs from the 3-fold increase observed after infection with *N.brasiliensis*. Activation of splenic CD4 T cells, as determined by expression of the marker CD62L (CD3+CD4+CD62L^{lo}), was severely impaired in M3R$^{-/-}$ mice at day 18 where 21% of its CD4 T cells were activated compared to 36% for WT mice (Fig 3.6B). Despite bacterial burdens being similar in WT and M3R$^{-/-}$ mice at day 18, this difference in activation of T cells may account for the large (100 fold) difference in bacterial burdens observed at later time points (day 27). By day 27, the percentage of activated CD4 T cells in the M3R$^{-/-}$ mice increases to the same level as in WT mice (35.8% - WT and 33.2% - M3R$^{-/-}$). Interestingly, reduced CD4 T cell activation in M3R$^{-/-}$ mice at day 18 was correlated with a significant decrease in expression of the co-stimulatory marker CD28 on the CD4 T cells (Fig 3.6C). Reduced CD28 expression was also observed after *N.brasiliensis* infection indicating that M3R may have a similar mechanism of action in both T_H1 and T_H2 biased immune responses

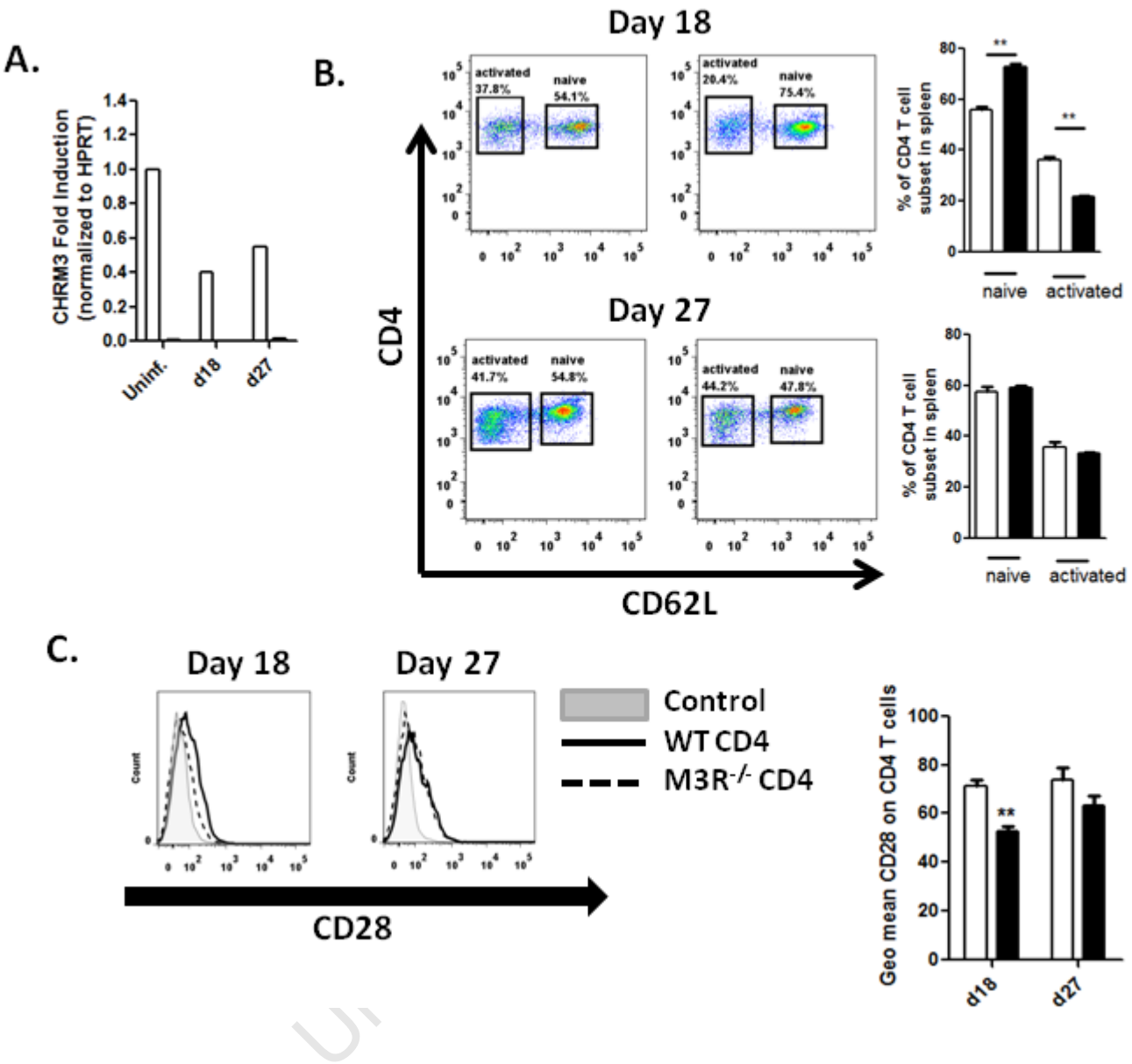


Fig 3.6: M3R transcription is regulated in CD4 T cells after *S.typhimurium* infection and CD4 T cells from M3R$^{-/-}$ mice have a reduced CD4 T cell activation status at day 18 post-infection. (A) Real time quantification of the M3 receptor transcript (CHRM3) in CD4 T cells sorted by flow cytomtery from the spleen of infected WT and M3R$^{-/-}$ mice at day 18 and 27 p.i. (B)Splenocytes at day 18 and day 27 were stained with the following cell surface markers CD3, CD4, CD62L and CD28 to determine proportion of activated CD4 T cells denoted by CD3^{+}CD4^{+}CD62L^{lo} and (C) expression of the costimulatory marker CD28. Data are shown as mean ± SEM and is representative of two independent experiments with 6 mice in each group. Statistical significance was calculated using the Mann-Whitney two-tailed t test and denoted by * $p<0.05$, ** $p<0.01$.

2.7. CD4 T cells from M3R$^{-/-}$ mice have reduced expression of the T_H1 transcription factor T-bet correlated with reduced pro-inflammatory cytokine production.

To further probe the effect of M3R deletion on CD4 T cell function after infection with *S.typhimurium*, we determined expression levels of two pro-inflammatory cytokines Interferon-gamma (IFN-γ) and Tumour Necrosis Factor alpha (TNF-α) as well as the T_H1 inducing transcription factor *T-box expressed in T cells* (T-bet) (Szabo, Kim et al. 2000) at the mRNA level. IFN-γ production by CD4 T cells at the mRNA level was greatly increased (110-fold) at day 18 post-infection suggesting that this is one of the main features of CD4 T cell activation after *S.typhimurium* infection. M3R$^{-/-}$ CD4 T cells in contrast had a significantly lower IFN-γ production (30-fold compared to WT uninfected) (Fig 3.7A) which correlates with its lower activation status. By day 27 IFN-g levels returned to almost base-line levels suggesting that CD4 T cell-mediated production of IFN-γ occurred mainly between d18 and d27. This period also correlates with a significant decrease (100-fold) in spleen bacterial burden (Fig 3.1A). IFN-γ is produced by a number of cell types after infection, with natural killer (NK) cells being an important early source (Schafer and Eisenstein 1992) while during the later stages of infection this role is taken up by *Salmonella* specific T_H1 CD4 T cells. IFN-γ is essential for control of *S.typhimurium* infection, and infection with an attenuated strain is lethal in IFN-γR$^{-/-}$ mice highlighting the importance of this cytokine (Hess, Ladel et al. 1996). Another pro-inflammatory cytokine, TNF-α was also reduced in M3R$^{-/-}$ CD4 T cells but to a lesser extent compared to IFN-γ (Fig 3.7A). We also observed a significantly lower infection-induced enhancement in T-bet mRNA synthesis in the absence of M3R (44-fold in WT versus 15-fold in M3R$^{-/-}$) (Fig 3.7B). Since differentiation of naïve T cells into the T_H1 lineage and synthesis of IFN-γ is controlled by the transcription factor T-bet (Szabo, Kim et al. 2000), the reduced IFN-γ production in the absence of M3R likely results from a defect at step prior to increase in transcription factor levels. T-bet is also essential for controlling *S.typhimurium* infection and T-bet$^{-/-}$

mice do not generate IFN-γ producing CD4 T cells and succumb to infection (Ravindran, Foley et al. 2005). Decrease IFN-g at the mRNA level also translated into decreased IFN-γ production at the protein level. $M3R^{-/-}$ mice had a 2 fold lower proportion and number of IFN-γ^{+} CD4 T cells in the spleen at day 18 (Fig 3.7C). A very small percentage of CD4 T cells were positive for TNF-α, and therefore it was difficult to determine whether the secretion of this cytokine was also reduced. The decrease in IFN-γ was specific for CD4 T cells, as the CD4 negative population had similar levels of IFN-γ producing cells in the WT and $M3R^{-/-}$ mice (Fig 3.7D). The non-CD4 T cell population had a higher proportion of TNF-α^{+} cells, possibly as a result of higher bacterial burden at day 18 post-infection.

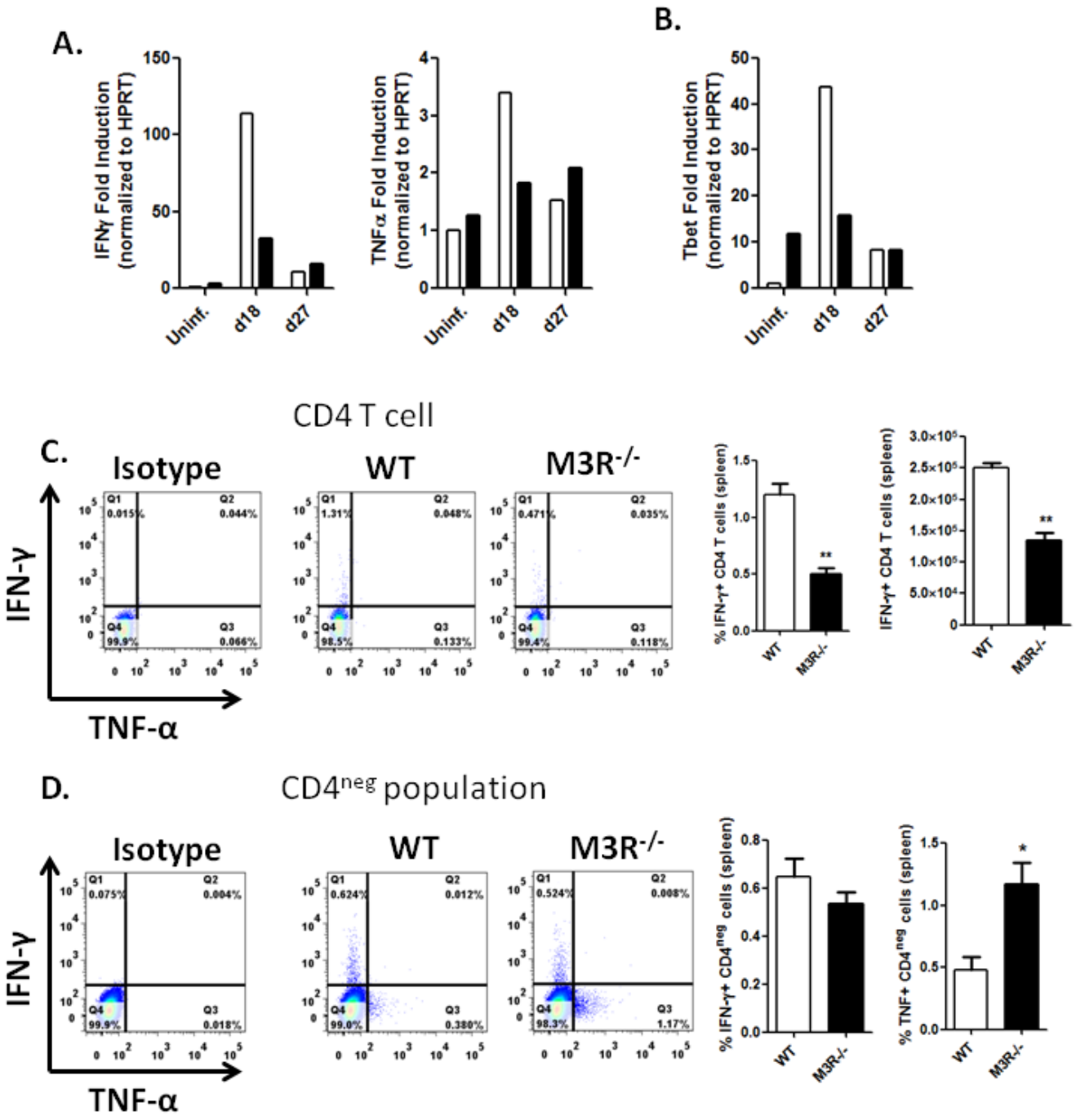


Fig 3.7: M3R$^{-/-}$ CD4 T cells have reduced levels of the T_H1 transcription factor Tbet which correlates with decreased CD4 T cell IFN-γ production at day 18 post-infection. Splenic CD4 T cells were purified using flow cytometry to greater than 99% purity and RNA was extracted using the Qiagen Mini RNA kit. RNA was reverse transcribed to cDNA and real time PCR ($\Delta\Delta C_T$ method) used to determine levels of (A) Pro-inflammatory cytokines IFN-γ and TNF-α as well as (B) the TH1 specific transcription factor Tbet. All genes were normalized to the housekeeping gene HPRT and the fold change was calculated relative to WT CD4 T cells from uninfected mice. Splenocytes from mice at day 18 post infection were stimulated with heat-killed *S.typhimurium* for 24 hours with monensin added in the last 4 hours to block cytokine secretion. Cells were first stained with CD3 and CD4, after which the cells were fixed, permeabilized and stained with IFN-γ and TNF-α to detect cytokine secretion in (C) CD4+ T cells and (D) CD4^{neg} splenocyte populations. Data are shown as mean ± SEM and is from one independent experiment with 5-6 mice in each group. Statistical significance was calculated using the Mann-Whitney two-tailed t test and denoted by * $p<0.05$, ** $p<0.01$.

3. Discussion:

In this chapter we investigate whether M3R regulates the T helper type 1 response, launched as a protective response to a bacterial infection. This added a new dimension to our previous data which demonstrated that M3R enhance T_H2 responses and thus attribute a broader role for the M3R in regulating two fundamentally different classes of immune response launched against a bacterial and helminth pathogen.

We used an attenuated strain of the bacterial pathogen *Salmonella typhimurium aroA*, which can naturally be cleared by wildtype mice after a robust CD4 T cell mediated T_H1 response. As expected wildtype mice were able to clear *S.typhimurium aroA* gradually over 5 weeks. In contrast, $M3R^{-/-}$ mice are more susceptible to infection, with significantly higher bacterial burden beginning on day 18 post-infection up to the end of the infection period. This increased susceptibility was also evident from the significantly higher weight loss in the $M3R^{-/-}$ mice which had lost almost 20% of their body weight while WT mice had recovered. A number of studies exist that highlight involvement of cholinergic signaling either via acetylcholine or one of its receptors in bacterial infection. *In vivo* inhibition of acetylcholinesterase using a chemical inhibitor which raises acetylcholine levels significantly improved survival after lethal *S.typhimurium* infection and increased serum IL-12p40 implying a possible pro-inflammatory role for increased acetylcholine (Fernandez-Cabezudo, Lorke et al. 2010). With respect to acetylcholine receptor involvement in bacterial infection, nicotinic receptor deficient mice ($n\alpha7AChR^{-/-}$) have improved control of *Escherichia coli* burden after an intraperitoneal infection indicating that nicotinic receptors, via their anti-inflammatory property, inhibit bacterial clearance (Giebelen, Le Moine et al. 2008). However, our study is the first demonstration of muscarinic receptor involvement in optimal control of bacterial infection possibly via enhancing the T helper type 1 immune response.

We further investigated the effect of M3R deletion on the kinetics of immune cell recruitment and/or development in the spleen after infection with *S.typhimurium*. While CD4 T cell numbers were similar in WT and M3R$^{-/-}$ mice, the numbers of B cells and macrophages differed significantly at various times after infection. B cells were increased after germinal center formation in WT mice (Cunningham, Gaspal et al. 2007) but this was not observed in M3R$^{-/-}$ mice. There were also significantly more macrophages in the spleens of M3R$^{-/-}$ mice at day 18 possibly as a result of increased bacterial burden. Macrophages are activated by IFN-γ from a variety of sources such as NK cells and neutrophils early in the infection and CD4 T cells during the adaptive phase (Ramarathinam, Niesel et al. 1993; Ravindran, Foley et al. 2005) in order to effectively kill intracellular S.typhimurium. Splenocytes from M3R$^{-/-}$ mice produced less IFN-γ compared to wildtype mice particularly in the adaptive phase of the immune system. Due to the central role played by IFN-γ, this defect could account for the increased susceptibility in the absence of M3R.

We then probed the functional role of M3R in each of these immune cell populations using various techniques such as bone marrow chimeras, in vitro bactericidal activities and isolation of target cells. Macrophages readily phagocytose *S.tyhpimurium* , playing an important role in bacterial clearance *in vivo* (Mastroeni 2002) and are also capable of killing various strains including the *aroA* strain in vitro (Fields, Swanson et al. 1986). We found that M3R$^{-/-}$ deficient bone marrow derived macrophages were able to kill *S.typhimurium aroA* similarly to wildtype macrophages and thus a defect in macrophage bactericidal activity does not account for increased susceptibility of the M3R$^{-/-}$ mice. However, even though we demonstrate that *ex vivo* generated M3R$^{-/-}$ macrophages may be efficient in killing *Salmonella*, we cannot rule out that splenic macrophages in M3R$^{-/-}$ mice may have a reduced bactericidal capacity as a result of interaction with its environment consisting of other immune cells and cytokine mediators.

We also generated a mouse strain with a B cell specific deletion of M3R using bone marrow chimeras to determine the M3R dependent contribution of this immune cell type. B cells are required for optimal control of a primary lethal *S.typhimurium* infection as well as memory induced protection after vaccination (Mittrucker, Raupach et al. 2000). B cell deficient mice are capable of clearing attenuated *aroA- S.typhimurium* strain effectively (Mittrucker, Raupach et al. 2000); however B cells have been shown to impair protective immune response by producing IL-10 in a Myd88 dependent manner even after infection with attenuated *S.typhimurium* strains (Neves, Lampropoulou et al. 2010). We showed that mice which selectively lacked M3R only on B cells were capable of capable of clearing *S.typhimurium* similarly to BM chimeras with normal B cells suggesting this immune cell type is not involved in driving susceptibility in $M3R^{-/-}$ mice. We did however find that B cell specific $M3R^{-/-}$ mice had increased numbers of B cells in the blood (2-fold) prior to infection as well as in the spleen (4 fold) post-infection. This highlights a potential role for M3R in regulating cell proliferation or cell death, an area for future investigation. Indeed M3R has been previously implicated in regulating proliferation in a number of cancer cells particularly colon cancer lines (Raufman, Samimi et al. 2008) as well as apoptosis (Budd, Spragg et al. 2004).

Finally, we examined CD4 T cell function in $M3R^{-/-}$ mice following *S.typhimurium aroA* infection as CD4 T cells are critical for protection and bacterial clearance and attenuated S.*typhimurium* is lethal in mice lacking CD4 T cells (Hess, Ladel et al. 1996). During peak of T cell response in this mice (day 18) (Srinivasan, Foley et al. 2004), M3R expression is significantly reduced in CD4 T cells by 60% compared to pre-infection levels which agrees with previously published data on in vitro generated T_H1 CD4 T cells (Qian, Galitovskiy et al. 2011). CD4 T cell activation at day 18 is markedly reduced in $M3R^{-/-}$ mice correlated to the increased bacterial burden observed at this time point and although the higher bacterial burden persists at day 27, CD4 T cell activation in $M3R^{-/-}$ mice reached a similar

level to wildtype mice. Similarly CD28 expression on $M3R^{-/-}$ CD4 T cells is significantly lower at day 18 but not at day 27 post-infection. This pattern is remarkably similar to that observed during *Nippostrongylus brasiliensis* infection indicating that M3R regulates CD4 T cell activation and CD28 expression in both T_H1 and T_H2 skewed disease models. CD28 is essential for immunity against *S.typhimurium*, and $CD28^{-/-}$ mice are highly susceptible even to the attenuated aroA- strain (Mittrucker, Kohler et al. 1999). CD28 is important for development of a T_H1 response particularly IFN-g production (Lenschow, Walunas et al. 1996) and this was postulated as the main mechanism for the observed increase in susceptibility of $CD28^{-/-}$ mice to *S.typhimurium* (Mittrucker, Kohler et al. 1999), further supported by the fact that IFN-$\gamma R^{-/-}$ mice are similarly highly susceptible to *S.typhimurium* (Hess, Ladel et al. 1996). In the $M3R^{-/-}$ mice, we also observed a decrease in IFN-γ mRNA levels in CD4 T cells as well as the number of IFN-γ secreting CD4 T cells in the spleen after an *S.typhimurium* infection, suggesting that a similar mechanism of CD28 costimulation driven IFN-γ production by CD4 T cells is responsible for the increased susceptibility. Interestingly, IFN-γ secretion by non-CD4 T cells was not affected, providing further evidence that M3R mainly influences CD4 T cell function. Mice lacking the muscarinic receptors M1 and M5 ($M1R^{-/-}M5R^{-/-}$) also have reduced production of pro-inflammatory cytokines IFN-γ and TNF-α after ovalbumin immunization (Kawashima, Fujii et al. 2012), demonstrating that other muscarinic subtypes may also be involved in modulating pro-inflammatory immune responses. Furthermore a key question still remains whether M3R directly regulates CD28 expression and function or acts indirectly via other receptors and/or proteins and if other muscarinic receptor subtypes such as M1 and M5 may act in synergy to enhance immune responses. The latter question can be answered by generating triple knockout mice lacking all 3 muscarinic subtypes (M1, M3 and M5) that have a similar mechanism of action of increasing intracellular Ca^{2+} concentration.

This chapter provides the first comprehensive characterization of a bacterial infection in the $M3R^{-/-}$ mice and identifies an important role for the M3 muscarinic receptor in generation of an effective T_H1 immune response. We also determined that $M3R^{-/-}$ CD4 T cells, but not B cells or macrophages, have markedly reduced T-bet mediated IFN-γ production and CD28 expression which strongly correlated with higher bacterial burdens and increased susceptibility to infection. This study also supports previously published literature that muscarinic receptors are pro-inflammatory (Razani-Boroujerdi, Behl et al. 2008; Oenema, Kolahian et al. 2010), and further also provides more conclusive data using receptor deficient mice, muscarinic agonists and two fundamentally different infectious disease models as well as identifying the major muscarinic receptor subtype responsible.

CONCLUSION

CONCLUSION

Cholinergic signaling is an important physiological pathway regulated by the neurotransmitter acetylcholine via its two receptor subtypes, nicotinic and muscarinic receptors (Albuquerque, Pereira et al. 2009). Classically known for its role in nerve impulse transmission (Picciotto, Higley et al. 2012), the cholinergic system has now been described in numerous non-neuronal cells such as smooth muscle cells (Roffel, Elzinga et al. 1990), skin keratinocytes (Ndoye, Buchli et al. 1998), bone osteoclasts (En-Nosse, Hartmann et al. 2009) and immune cells (Kawashima, Fujii et al. 2012). Over the last decade, an increasing number of studies have described a significant role for acetylcholine in immunity, particularly the dampening of pro-inflammatory immune responses of macrophages by signaling via the α7 subunit of nicotinic receptors (Wang, Yu et al. 2003). This pathway, termed the Cholinergic Anti-inflammatory Pathway (CAP) (Tracey 2007), is regulated by acetylcholine secreted from a subset of activated CD4 T cells in the spleen (Rosas-Ballina, Olofsson et al. 2011). Other studies have characterized different aspects of the cholinergic system in immune cells such as expression of nicotinic and muscarinic receptors upon activation (Qian, Galitovskiy et al. 2011), the role for acetylcholinesterase in controlling inflammation (Shaked, Meerson et al. 2009) and nicotinic receptors in bacterial infections (Giebelen, Le Moine et al. 2008; Radek, Elias et al. 2010). However, despite conclusive evidence of cholinergic regulation of immunity and the key role played by nicotinic receptors, the role for muscarinic receptors which also exist on many of the same immune cells remains unknown. In this study, we showed that M3 muscarinic receptor also plays an important in regulating immunity, however unlike the nicotinic receptor which is anti-inflammatory (Caulfield and Birdsall 1998; Wang, Yu et al. 2003); the M3 muscarinic receptor enhances immune responses. This finding is supported by published studies which ascribe a potential pro-inflammatory function for muscarinic receptors. Administration of a non-subtype selective muscarinic antagonist atropine *in vivo*, which reduces muscarinic activity, also reduced sterile

inflammation induced by turpentine as well as antigen specific T and B cell responses to an inert antigen (Razani-Boroujerdi, Behl et al. 2008).

Muscarinic receptor is a family of five subtypes (M1-M5) (Caulfield 1993) and in our study we identified the M3 subtype as the major receptor responsible for regulating immunity to pathogens using genetically engineered mouse strain lacking the M3 receptor ($M3R^{-/-}$). In the absence of M3R, mice were more susceptible to two pathogens, the nematode parasite *Nippostrongylus brasiliensis* and the bacterium *Salmonella enterica* sp. Typhimurium. This increase in susceptibility was marked by increased pathogen burden and strongly correlated with a decrease in key immune responses such as production of cytokines IL-13 and IFN-γ which are essential for protection during *N.brasiliensis* and *S.typhimurium* infections respectively. Furthermore, stimulation of lymphocytes from *N.brasiliensis* infected mice with muscarinic agonists significantly enhanced production of IL-13, an effect that was not observed in $M3R^{-/-}$ lymphocytes. This provides a potential mechanism by which stimulation of M3R enhances immune responses. Other muscarinic receptor subtypes such as M1 and M5 have previously been suggested to regulate immunity by being required for development of CD8 T cells *in vitro*, however this effect was not observed *in vivo* (Vezys, Masopust et al. 2007). Our study is thus the first *in vivo* demonstration of a muscarinic receptor subtype involved in the regulation of immunity.

M3 receptor is expressed on a number of immune cells such as macrophages, dendritic cells, B cells, CD4 and CD8 T cells in mice but has also been shown on human immune cells (Fujii and Kawashima 2000; Kawashima, Yoshikawa et al. 2007; Qian, Galitovskiy et al. 2011). We identified CD4 T cells as the key immune cell population affected in the absence of M3R. After *N.brasiliensis* and *S.typhimurium* infection, M3R was required for the optimal activation of CD4 T cells as the activation (as determined by $CD44^{hi}$ and $CD62L^{lo}$ expression) of $M3R^{-/-}$ CD4 T cells was

significantly lower. Production of important cytokines IL-13 and IFN-γ by CD4 T cells was also significantly reduced at the mRNA and protein level. Interestingly, basal CD4 T cell activation in naïve mice prior to infection with the two pathogens was similar in the presence or absence of M3R. In the case of *N.brasiliensis*, protective immunity obtained by adoptive transfer of antigen experienced CD4 T cell was completely abrogated in the absence of M3R. This is a clear indication that M3R is required for an effective pathogen induced activation of CD4 T cells which have optimal cytokine production and capability for pathogen clearance.

Our study indicates the M3R is required for optimal development of both T_H1 and T_H2 immunity and therefore may be acting at a stage prior to differentiation of the immune response. However, some differences do exist between T_H1 and T_H2 response particularly in relation to the expression of M3R at the messenger RNA level on CD4 T cells. After *N.brasiliensis,* infection when the immune environment is skewed towards a T helper 2 immune response, expression of M3R is increased CD4 T cells whereas after *S.typhimurium* infection which induces a T helper 1 response M3R expression is decreased. Since T_H1 responses are pro-inflammatory and cause significant tissue pathology if left uncontrolled (Rennick, Fort et al. 1997), decreased M3R expression on CD4 T cells may be a self-regulatory mechanism to reduce production of cytokines such as IFN-γ. T_H2 responses on the other hand help limit pro-inflammatory immune pathology during helminth infections (Hunter and McKay 2004; Dewals, Hoving et al. 2010) and therefore enhanced M3R expression which may result in cholinergic mediated increase in T_H2 cytokine production will be beneficial to the hosts.

The mechanism of action by which M3R controls the activation of CD4 T cells has not been conclusively identified. The classical route of T cell activation involved interaction between MHCII molecules on antigen presenting cells (APC), carrying peptides and a complementary T cell receptor

(TCR) on CD4 T cells. A secondary costimulatory interaction between CD80/86 on APCs and CD28 on CD4 T cells is required to complete the activation signal (Lenschow, Walunas et al. 1996). We found that expression of CD28 on $M3R^{-/-}$ CD4 T cells was significantly lower compared to wildtype CD4 T cells in naïve mice as well as after *N.brasiliensis* and *S.typhimurium* infection. M3R control of CD28 expression either directly or indirectly may explain the reduced activation of CD4 T cells in the absence of this receptor. The central role of CD28 in controlling pathogen infection is also highlighted by increased susceptibility of $CD28^{-/-}$ mice to *N.brasiliensis* and *S.typhimurium* infection (Mittrucker, Kohler et al. 1999). Therefore, M3R mediated decrease in CD28 expression may account for the increased susceptibility of the $M3R^{-/-}$ mice. Further work is required to verify the link between CD28 and M3R and to determine whether this link is causal or a correlation.

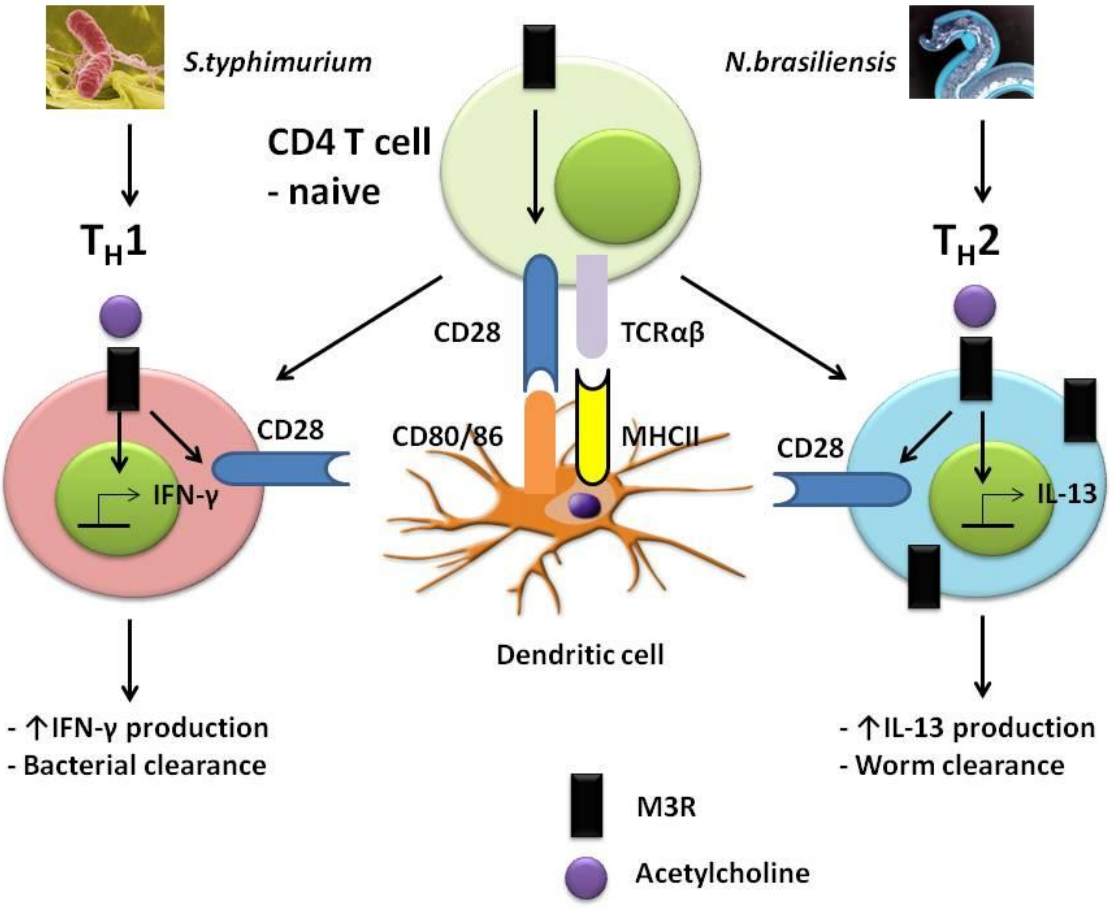


Fig 1. Proposed model of action of M3 muscarinic receptor in regulation of CD4 T cell function after pathogen infection.

Our study is the first conclusive demonstration of a role for muscarinic receptors in immunity. Using genetically engineered mouse strains and pathogen models, we have identified M3 muscarinic subtype as the main subtype required for enhancing protective immune response and clearance of two distinct pathogens, a nematode parasite *N.brasiliensis* and bacterial pathogen *S.typhimurium*. Cholinergic regulation of immunity is an important emerging paradigm and our study identifies a role for muscarinic receptors that will have potential benefits in improving immune responses to pathogens.

Future work

We have identified a key role for muscarinic receptor in regulating immunity, raising interesting questions that will be the focus of future research. Firstly, we need to determine whether immune regulatory function of M3R is also observed in human immune cells. Given the highly conserved nature of the M3R (92% similarity between human and murine M3R), it is highly likely that this function is also conserved. To confirm this we will measure cytokine production after restimulation of peripheral blood mononuclear cells (PBMCs) in the presence of muscarinic agonists and antagonists to determine whether muscarinic stimulation enhances cytokine production similarly to the murine model. Modest reductions in IL--5 and IL-13 production after tiotropium treatment of phytohemagluttinin (PHA) stimulated PBMCs has been reported supporting the theory that M3 receptors behave similarly in human cells (Ohta, Oda et al. 2010). We will examine antigen specific responses particularly after vaccination and in bronchoaveolar lavage (BAL) cells of people that regularly inhale tiotropium for relieving asthma.

Secondly, we found that M3R regulate CD4 T cell function possibly via regulating expression of the co-stimulatory receptor CD28. It still remains to be determined whether there is a direct correlation between M3R and CD28. A second potential mechanism by which M3R can affect CD4 T cell function is by regulating intracellular calcium (Ca^{2+}) levels. M3R binding of its agonists such as acetylcholine results in influx of Ca^{2+} ions into the cytosol (Caulfield 1993). This influx is also a characteristic feature of CD4 T cell activation (Lewis 2001) and therefore it remains to be investigated whether Ca^{2+} influx resulting from T cell activation is dysregulated in the absence of M3R.

Of the muscarinic subtypes, the M3R is targeted by a number of antagonistic drugs for relieving symptoms of aberrant smooth muscle contraction in overactive bladder disorder such as darifenacin and asthma and chronic obstructive pulmonary disease (COPD) such as tiotropium bromide (Wess, Eglen et al. 2007). Findings from our study raise important concerns about unintended consequences of the potential immune modulating properties of M3R antagonists. On the one hand, this may be beneficial as treatment of asthma related bronchoconstriction using tiotropium (Casaburi, Mahler et al. 2002; Kerstjens, Engel et al. 2012) may have the additional benefit of inhibiting T_H2 responses that cause other pathological features of asthma such as IL-13 dependent IgE production and mucus hypersecretion in the lungs (Barnes 2008). This has already been shown in mice, where tiotropium inhalation treatment of mice with ovalbumin induced allergy resulted in a significant reduction in T_H2 immune responses (Ohta, Oda et al. 2010). However on the other hand, we show that M3R is also required for an effective pro-inflammatory T_H1 response. Therefore, blocking M3R using tiotropium in the lung may enhance susceptibility of the users to common viral and bacterial infections of the lung such as influenza, TB and pneumonia as these require T_H1 responses for control (Kadioglu and Andrew 2004; Doherty and Andersen 2005; Forrest, Pride et al. 2008). Hence in light of our findings, future studies of M3R antagonists used as therapeutic drugs must also consider the immunomodulatory side effects of muscarinic receptor blocking. Additionally, M3R antagonists may also be used to exploit the immune enhancing property of the M3R, and therefore we need to determine whether administration of approved M3R antagonist drugs *in vivo* can help suppress cytokine production in conditions where excessive pro-inflammation is detrimental. Conversely, it will be interesting to determine whether immune responses during a natural infection or after vaccination can be enhanced by administering muscarinic agonists. The crystal structure of M3R has recently been solved (Kruse, Hu et al. 2012) and will further aid the discovery of M3R specific agonists and antagonists thus providing a further degree of selectivity

during *in vivo* immune modulation. Regulation of immunity by muscarinic receptors represents an exciting new avenue in the search for better, more effective ways in controlling our immune system.

REFERENCES

Abrams, P., K. E. Andersson, et al. (2006). "Muscarinic receptors: their distribution and function in body systems, and the implications for treating overactive bladder." Br J Pharmacol **148**(5): 565-578.

Adams, D. O. and T. A. Hamilton (1984). "The cell biology of macrophage activation." Annu Rev Immunol **2**: 283-318.

Akiho, H., P. Lovato, et al. (2005). "Interleukin-4- and -13-induced hypercontractility of human intestinal muscle cells-implication for motility changes in Crohn's disease." Am J Physiol Gastrointest Liver Physiol **288**(4): G609-615.

Albuquerque, E. X., E. F. Pereira, et al. (2009). "Mammalian nicotinic acetylcholine receptors: from structure to function." Physiol Rev **89**(1): 73-120.

Anthony, R. M., L. I. Rutitzky, et al. (2007). "Protective immune mechanisms in helminth infection." Nat Rev Immunol **7**(12): 975-987.

Arvidsson, U., M. Riedl, et al. (1997). "Vesicular acetylcholine transporter (VAChT) protein: a novel and unique marker for cholinergic neurons in the central and peripheral nervous systems." J Comp Neurol **378**(4): 454-467.

Bacon, G. A., T. W. Burrows, et al. (1951). "The effects of biochemical mutation on the virulence of Bacterium typhosum; the loss of virulence of certain mutants." Br J Exp Pathol **32**(2): 85-96.

Barnes, P. J. (2008). "Immunology of asthma and chronic obstructive pulmonary disease." Nat Rev Immunol **8**(3): 183-192.

Baum, B. J. (1993). "Principles of saliva secretion." Ann N Y Acad Sci **694**: 17-23.

Bernik, T. R., S. G. Friedman, et al. (2002). "Cholinergic antiinflammatory pathway inhibition of tumor necrosis factor during ischemia reperfusion." J Vasc Surg **36**(6): 1231-1236.

Beutler, B. (2004). "Innate immunity: an overview." Mol Immunol **40**(12): 845-859.

Bois, R. T., R. G. Hummel, et al. (1980). "Presynaptic and postsynaptic neuromuscular effects of a specific inhibitor of acetylcholinesterase." J Pharmacol Exp Ther **215**(1): 53-59.

Borovikova, L. V., S. Ivanova, et al. (2000). "Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin." Nature **405**(6785): 458-462.

Borroto-Escuela, D. O., L. F. Agnati, et al. (2012). "Muscarinic acetylcholine receptor-interacting proteins (mAChRIPs): targeting the receptorsome." Curr Drug Targets **13**(1): 53-71.

Brejc, K., W. J. van Dijk, et al. (2001). "Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors." Nature **411**(6835): 269-276.

Bremel, R. D. (1974). "Myosin linked calcium regulation in vertebrate smooth muscle." Nature **252**(5482): 405-407.

Brown, J. H., V. Sah, et al. (1997). "Pathways and roadblocks in muscarinic receptor-mediated growth regulation." Life Sci **60**(13-14): 1077-1084.

Budd, D. C., J. McDonald, et al. (2003). "The C-terminal tail of the M3-muscarinic receptor possesses anti-apoptotic properties." J Biol Chem **278**(21): 19565-19573.

Budd, D. C., E. J. Spragg, et al. (2004). "Signalling of the M3-muscarinic receptor to the anti-apoptotic pathway." Biochem J **381**(Pt 1): 43-49.

Carsi-Gabrenas, J. M., E. A. Van der Zee, et al. (1997). "Non-selectivity of the monoclonal antibody M35 for subtypes of muscarinic acetylcholine receptors." Brain Res Bull **44**(1): 25-31.

Carter, P. B. and F. M. Collins (1974). "The route of enteric infection in normal mice." J Exp Med **139**(5): 1189-1203.

Casaburi, R., D. A. Mahler, et al. (2002). "A long-term evaluation of once-daily inhaled tiotropium in chronic obstructive pulmonary disease." Eur Respir J **19**(2): 217-224.

Caulfield, M. P. (1993). "Muscarinic receptors--characterization, coupling and function." Pharmacol Ther **58**(3): 319-379.

Caulfield, M. P. and N. J. Birdsall (1998). "International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors." Pharmacol Rev **50**(2): 279-290.

Cheminay, C., D. Chakravortty, et al. (2004). "Role of neutrophils in murine salmonellosis." Infect Immun **72**(1): 468-477.

Cheng, K. and J. P. Raufman (2005). "Bile acid-induced proliferation of a human colon cancer cell line is mediated by transactivation of epidermal growth factor receptors." Biochem Pharmacol **70**(7): 1035-1047.

Cheng, K., R. Samimi, et al. (2008). "Acetylcholine release by human colon cancer cells mediates autocrine stimulation of cell proliferation." Am J Physiol Gastrointest Liver Physiol **295**(3): G591-597.

Collins, F. M., G. B. Mackaness, et al. (1966). "Infection-immunity in experimental salmonellosis." J Exp Med **124**(4): 601-619.

Coynault, C. and F. Norel (1999). "Comparison of the abilities of Salmonella typhimurium rpoS, aroA and rpoS aroA strains to elicit humoral immune responses in BALB/c mice and to cause lethal infection in athymic BALB/c mice." Microb Pathog **26**(6): 299-305.

Cunningham, A. F., F. Gaspal, et al. (2007). "Salmonella induces a switched antibody response without germinal centers that impedes the extracellular spread of infection." J Immunol **178**(10): 6200-6207.

Dajas-Bailador, F. and S. Wonnacott (2004). "Nicotinic acetylcholine receptors and the regulation of neuronal signalling." Trends Pharmacol Sci **25**(6): 317-324.

De Bosscher, K. and G. Haegeman (2009). "Minireview: latest perspectives on antiinflammatory actions of glucocorticoids." Mol Endocrinol **23**(3): 281-291.

De Sarno, P., S. A. Shestopal, et al. (2003). "Muscarinic receptor activation protects cells from apoptotic effects of DNA damage, oxidative stress, and mitochondrial inhibition." J Biol Chem **278**(13): 11086-11093.

Dewals, B., J. C. Hoving, et al. (2010). "Control of Schistosoma mansoni egg-induced inflammation by IL-4-responsive CD4(+)CD25(-)CD103(+)Foxp3(-) cells is IL-10-dependent." Eur J Immunol **40**(10): 2837-2847.

Doherty, T. M. and P. Andersen (2005). "Vaccines for tuberculosis: novel concepts and recent progress." Clin Microbiol Rev **18**(4): 687-702.

Ebashi, S. and F. Ebashi (1964). "A New Protein Factor Promoting Contraction of Actomyosin." Nature **203**: 645-646.

Eglen, R. M. (1996). "Muscarinic M2 and M3 receptor function in smooth muscle." Proc West Pharmacol Soc **39**: 57-60.

Eglen, R. M., S. S. Hegde, et al. (1996). "Muscarinic receptor subtypes and smooth muscle function." Pharmacol Rev **48**(4): 531-565.

Eglen, R. M., H. Reddy, et al. (1994). "Muscarinic acetylcholine receptor subtypes in smooth muscle." Trends Pharmacol Sci **15**(4): 114-119.

Elmore, S. (2007). "Apoptosis: a review of programmed cell death." Toxicol Pathol **35**(4): 495-516.

En-Nosse, M., S. Hartmann, et al. (2009). "Expression of non-neuronal cholinergic system in osteoblast-like cells and its involvement in osteogenesis." Cell Tissue Res **338**(2): 203-215.

Everest, P., M. Roberts, et al. (1998). "Susceptibility to Salmonella typhimurium infection and effectiveness of vaccination in mice deficient in the tumor necrosis factor alpha p55 receptor." Infect Immun **66**(7): 3355-3364.

Exton, J. H. (1996). "Regulation of phosphoinositide phospholipases by hormones, neurotransmitters, and other agonists linked to G proteins." Annu Rev Pharmacol Toxicol **36**: 481-509.

Fearon, D. T. and R. M. Locksley (1996). "The instructive role of innate immunity in the acquired immune response." Science **272**(5258): 50-53.

Feasey, N. A., G. Dougan, et al. (2012). "Invasive non-typhoidal salmonella disease: an emerging and neglected tropical disease in Africa." Lancet **379**(9835): 2489-2499.

Felder, C. C. (1995). "Muscarinic acetylcholine receptors: signal transduction through multiple effectors." FASEB J **9**(8): 619-625.

Fernandez-Cabezudo, M. J., D. E. Lorke, et al. (2010). "Cholinergic stimulation of the immune system protects against lethal infection by Salmonella enterica serovar Typhimurium." Immunology **130**(3): 388-398.

Fields, P. I., R. V. Swanson, et al. (1986). "Mutants of Salmonella typhimurium that cannot survive within the macrophage are avirulent." Proc Natl Acad Sci U S A **83**(14): 5189-5193.

Finkelman, F. D., T. Shea-Donohue, et al. (1997). "Cytokine regulation of host defense against parasitic gastrointestinal nematodes: lessons from studies with rodent models." Annu Rev Immunol **15**: 505-533.

Finkelman, F. D., T. Shea-Donohue, et al. (2004). "Interleukin-4- and interleukin-13-mediated host protection against intestinal nematode parasites." Immunol Rev **201**: 139-155.

Flores-Langarica, A., J. L. Marshall, et al. (2011). "T-zone localized monocyte-derived dendritic cells promote Th1 priming to Salmonella." Eur J Immunol **41**(9): 2654-2665.

Fontenot, J. D., M. A. Gavin, et al. (2003). "Foxp3 programs the development and function of CD4+CD25+ regulatory T cells." Nat Immunol **4**(4): 330-336.

Fontenot, J. D., J. P. Rasmussen, et al. (2005). "Regulatory T cell lineage specification by the forkhead transcription factor foxp3." Immunity **22**(3): 329-341.

Forrest, B. D., M. W. Pride, et al. (2008). "Correlation of cellular immune responses with protection against culture-confirmed influenza virus in young children." Clin Vaccine Immunol **15**(7): 1042-1053.

Frucht, H., R. T. Jensen, et al. (1999). "Human colon cancer cell proliferation mediated by the M3 muscarinic cholinergic receptor." Clin Cancer Res **5**(9): 2532-2539.

Fujii, T. and K. Kawashima (2000). "Calcium signaling and c-Fos gene expression via M3 muscarinic acetylcholine receptors in human T- and B-cells." Jpn J Pharmacol **84**(2): 124-132.

Fujii, T. and K. Kawashima (2000). "YM905, a novel M3 antagonist, inhibits Ca2+ signaling and c-fos gene expression mediated via muscarinic receptors in human T cells." Gen Pharmacol **35**(2): 71-75.

Fujii, T., S. Tajima, et al. (1999). "Constitutive expression of mRNA for the same choline acetyltransferase as that in the nervous system, an acetylcholine-synthesizing enzyme, in human leukemic T-cell lines." Neurosci Lett **259**(2): 71-74.

Fujii, T., Y. Takada-Takatori, et al. (2008). "Basic and clinical aspects of non-neuronal acetylcholine: expression of an independent, non-neuronal cholinergic system in lymphocytes and its clinical significance in immunotherapy." J Pharmacol Sci **106**(2): 186-192.

Fujii, T., Y. Watanabe, et al. (2003). "Upregulation of mRNA encoding the M5 muscarinic acetylcholine receptor in human T- and B-lymphocytes during immunological responses." Neurochem Res **28**(3-4): 423-429.

Fujii, Y. X., A. Tashiro, et al. (2007). "Diminished antigen-specific IgG1 and interleukin-6 production and acetylcholinesterase expression in combined M1 and M5 muscarinic acetylcholine receptor knockout mice." J Neuroimmunol **188**(1-2): 80-85.

Garcia, M. C., M. P. Hermans, et al. (1988). "Glucose-, calcium- and concentration-dependence of acetylcholine stimulation of insulin release and ionic fluxes in mouse islets." Biochem J **254**(1): 211-218.

Gause, W. C., J. F. Urban, Jr., et al. (2003). "The immune response to parasitic helminths: insights from murine models." Trends Immunol **24**(5): 269-277.

Gautam, D., O. Gavrilova, et al. (2006). "Beneficial metabolic effects of M3 muscarinic acetylcholine receptor deficiency." Cell Metab **4**(5): 363-375.

Gautam, D., S. J. Han, et al. (2007). "Role of the M3 muscarinic acetylcholine receptor in beta-cell function and glucose homeostasis." Diabetes Obes Metab **9 Suppl 2**: 158-169.

Gautam, D., S. J. Han, et al. (2006). "A critical role for beta cell M3 muscarinic acetylcholine receptors in regulating insulin release and blood glucose homeostasis in vivo." Cell Metab **3**(6): 449-461.

Gautam, D., T. S. Heard, et al. (2004). "Cholinergic stimulation of salivary secretion studied with M1 and M3 muscarinic receptor single- and double-knockout mice." Mol Pharmacol **66**(2): 260-267.

Ghia, J. E., P. Blennerhassett, et al. (2006). "The vagus nerve: a tonic inhibitory influence associated with inflammatory bowel disease in a murine model." Gastroenterology **131**(4): 1122-1130.

Giebelen, I. A., A. Le Moine, et al. (2008). "Deficiency of alpha7 cholinergic receptors facilitates bacterial clearance in Escherichia coli peritonitis." J Infect Dis **198**(5): 750-757.

Gil-Cruz, C., S. Bobat, et al. (2009). "The porin OmpD from nontyphoidal Salmonella is a key target for a protective B1b cell antibody response." Proc Natl Acad Sci U S A **106**(24): 9803-9808.

Gilon, P. and J. C. Henquin (2001). "Mechanisms and physiological significance of the cholinergic control of pancreatic beta-cell function." Endocr Rev **22**(5): 565-604.

Gomeza, J., H. Shannon, et al. (1999). "Pronounced pharmacologic deficits in M2 muscarinic acetylcholine receptor knockout mice." Proc Natl Acad Sci U S A **96**(4): 1692-1697.

Gomeza, J., L. Zhang, et al. (1999). "Enhancement of D1 dopamine receptor-mediated locomotor stimulation in M(4) muscarinic acetylcholine receptor knockout mice." Proc Natl Acad Sci U S A **96**(18): 10483-10488.

Gordon, M. A., H. T. Banda, et al. (2002). "Non-typhoidal salmonella bacteraemia among HIV-infected Malawian adults: high mortality and frequent recrudescence." AIDS **16**(12): 1633-1641.

Gordon, M. A., S. M. Graham, et al. (2008). "Epidemics of invasive Salmonella enterica serovar enteritidis and S. enterica Serovar typhimurium infection associated with multidrug resistance among adults and children in Malawi." Clin Infect Dis **46**(7): 963-969.

Gosens, R., J. Zaagsma, et al. (2006). "Muscarinic receptor signaling in the pathophysiology of asthma and COPD." Respir Res **7**: 73.

Guslandi, M. (1999). "Long-term effects of a single course of nicotine treatment in acute ulcerative colitis: remission maintenance in a 12-month follow-up study." Int J Colorectal Dis **14**(4-5): 261-262.

Hamilton, S. E., M. D. Loose, et al. (1997). "Disruption of the m1 receptor gene ablates muscarinic receptor-dependent M current regulation and seizure activity in mice." Proc Natl Acad Sci U S A **94**(24): 13311-13316.

Harvie, M., M. Camberis, et al. (2010). "The lung is an important site for priming CD4 T-cell-mediated protective immunity against gastrointestinal helminth parasites." Infect Immun **78**(9): 3753-3762.

Hasnain, S. Z., C. M. Evans, et al. (2011). "Muc5ac: a critical component mediating the rejection of enteric nematodes." J Exp Med **208**(5): 893-900.

Hasnain, S. Z., H. Wang, et al. (2010). "Mucin gene deficiency in mice impairs host resistance to an enteric parasitic infection." Gastroenterology **138**(5): 1763-1771.
Hegde, S. S. and R. M. Eglen (1999). "Muscarinic receptor subtypes modulating smooth muscle contractility in the urinary bladder." Life Sci **64**(6-7): 419-428.
Hellstrom-Lindahl, E. and A. Nordberg (1996). "Muscarinic receptor subtypes in subpopulations of human blood mononuclear cells as analyzed by RT-PCR technique." J Neuroimmunol **68**(1-2): 139-144.
Herbert, D. R., J. Q. Yang, et al. (2009). "Intestinal epithelial cell secretion of RELM-beta protects against gastrointestinal worm infection." J Exp Med **206**(13): 2947-2957.
Hess, J., C. Ladel, et al. (1996). "Salmonella typhimurium aroA- infection in gene-targeted immunodeficient mice: major role of CD4+ TCR-alpha beta cells and IFN-gamma in bacterial clearance independent of intracellular location." J Immunol **156**(9): 3321-3326.
Hohmann, E. L. (2001). "Nontyphoidal salmonellosis." Clin Infect Dis **32**(2): 263-269.
Hoiseth, S. K. and B. A. Stocker (1981). "Aromatic-dependent Salmonella typhimurium are non-virulent and effective as live vaccines." Nature **291**(5812): 238-239.
Horsnell, W. G., A. J. Cutler, et al. (2007). "Delayed goblet cell hyperplasia, acetylcholine receptor expression, and worm expulsion in SMC-specific IL-4Ralpha-deficient mice." PLoS Pathog **3**(1): e1.
Horsnell, W. G., A. Vira, et al. (2011). "IL-4Ralpha-responsive smooth muscle cells contribute to initiation of TH2 immunity and pulmonary pathology in Nippostrongylus brasiliensis infections." Mucosal Immunol **4**(1): 83-92.
Hotez, P. J., P. J. Brindley, et al. (2008). "Helminth infections: the great neglected tropical diseases." J Clin Invest **118**(4): 1311-1321.
Hotez, P. J., S. Brooker, et al. (2004). "Hookworm infection." N Engl J Med **351**(8): 799-807.
Hsu, H. S. (1989). "Pathogenesis and immunity in murine salmonellosis." Microbiol Rev **53**(4): 390-409.
Hunter, M. M. and D. M. McKay (2004). "Review article: helminths as therapeutic agents for inflammatory bowel disease." Aliment Pharmacol Ther **19**(2): 167-177.
Huston, J. M., M. Gallowitsch-Puerta, et al. (2007). "Transcutaneous vagus nerve stimulation reduces serum high mobility group box 1 levels and improves survival in murine sepsis." Crit Care Med **35**(12): 2762-2768.
Ibarra, J. A. and O. Steele-Mortimer (2009). "Salmonella--the ultimate insider. Salmonella virulence factors that modulate intracellular survival." Cell Microbiol **11**(11): 1579-1586.
Jackson, A., M. R. Nanton, et al. (2010). "Innate immune activation during Salmonella infection initiates extramedullary erythropoiesis and splenomegaly." J Immunol **185**(10): 6198-6204.
Jacobson, R. H. and N. D. Reed (1974). "The immune response of congenitally athymic (nude) mice to the intestinal nematode Nippostrongylus brasiliensis." Proc Soc Exp Biol Med **147**(3): 667-670.
Janeway, C. A., Jr. (1992). "The immune system evolved to discriminate infectious nonself from noninfectious self." Immunol Today **13**(1): 11-16.
Janeway, C. A., Jr. and R. Medzhitov (2002). "Innate immune recognition." Annu Rev Immunol **20**: 197-216.
Jenkins, M. K., A. Khoruts, et al. (2001). "In vivo activation of antigen-specific CD4 T cells." Annu Rev Immunol **19**: 23-45.
John, B., D. Rajagopal, et al. (2002). "Role of IL-12-independent and IL-12-dependent pathways in regulating generation of the IFN-gamma component of T cell responses to Salmonella typhimurium." J Immunol **169**(5): 2545-2552.

Josefowicz, S. Z., L. F. Lu, et al. (2012). "Regulatory T cells: mechanisms of differentiation and function." Annu Rev Immunol **30**: 531-564.
Kadioglu, A. and P. W. Andrew (2004). "The innate immune response to pneumococcal lung infection: the untold story." Trends Immunol **25**(3): 143-149.
Kaneda, T., Y. Kitamura, et al. (1993). "Presence of m3 subtype muscarinic acetylcholine receptors and receptor-mediated increases in the cytoplasmic concentration of Ca2+ in Jurkat, a human leukemic helper T lymphocyte line." Mol Pharmacol **43**(3): 356-364.
Katona, I. M., J. F. Urban, Jr., et al. (1988). "The role of L3T4+ and Lyt-2+ T cells in the IgE response and immunity to Nippostrongylus brasiliensis." J Immunol **140**(9): 3206-3211.
Kawashima, K. and T. Fujii (2004). "Expression of non-neuronal acetylcholine in lymphocytes and its contribution to the regulation of immune function." Front Biosci **9**: 2063-2085.
Kawashima, K. and T. Fujii (2008). "Basic and clinical aspects of non-neuronal acetylcholine: overview of non-neuronal cholinergic systems and their biological significance." J Pharmacol Sci **106**(2): 167-173.
Kawashima, K., T. Fujii, et al. (2012). "Critical roles of acetylcholine and the muscarinic and nicotinic acetylcholine receptors in the regulation of immune function." Life Sci **91**(21-22): 1027-1032.
Kawashima, K., K. Yoshikawa, et al. (2007). "Expression and function of genes encoding cholinergic components in murine immune cells." Life Sci **80**(24-25): 2314-2319.
Kerstjens, H. A., M. Engel, et al. (2012). "Tiotropium in asthma poorly controlled with standard combination therapy." N Engl J Med **367**(13): 1198-1207.
Khattri, R., T. Cox, et al. (2003). "An essential role for Scurfin in CD4+CD25+ T regulatory cells." Nat Immunol **4**(4): 337-342.
Kirby, A. C., U. Yrlid, et al. (2002). "The innate immune response differs in primary and secondary Salmonella infection." J Immunol **169**(8): 4450-4459.
Klapproth, H., T. Reinheimer, et al. (1997). "Non-neuronal acetylcholine, a signalling molecule synthezised by surface cells of rat and man." Naunyn Schmiedebergs Arch Pharmacol **355**(4): 515-523.
Klegeris, A., T. C. Budd, et al. (1994). "Acetylcholinesterase activation of peritoneal macrophages is independent of catalytic activity." Cell Mol Neurobiol **14**(1): 89-98.
Koarai, A., S. L. Traves, et al. (2012). "Expression of muscarinic receptors by human macrophages." Eur Respir J **39**(3): 698-704.
Kodaira, M., M. Kajimura, et al. (1999). "Functional muscarinic m3 receptor expressed in gastric cancer cells stimulates tyrosine phosphorylation and MAP kinase." J Gastroenterol **34**(2): 163-171.
Korn, T., E. Bettelli, et al. (2009). "IL-17 and Th17 Cells." Annu Rev Immunol **27**: 485-517.
Krammer, P. H. (2000). "CD95's deadly mission in the immune system." Nature **407**(6805): 789-795.
Kruse, A. C., J. Hu, et al. (2012). "Structure and dynamics of the M3 muscarinic acetylcholine receptor." Nature **482**(7386): 552-556.
Lanzafame, A. A., A. Christopoulos, et al. (2003). "Cellular signaling mechanisms for muscarinic acetylcholine receptors." Receptors Channels **9**(4): 241-260.
Lathrop, S. K., S. M. Bloom, et al. (2011). "Peripheral education of the immune system by colonic commensal microbiota." Nature **478**(7368): 250-254.
Lenschow, D. J., T. L. Walunas, et al. (1996). "CD28/B7 system of T cell costimulation." Annu Rev Immunol **14**: 233-258.
Lewis, R. S. (2001). "Calcium signaling mechanisms in T lymphocytes." Annu Rev Immunol **19**: 497-521.

Li, M. O., Y. Y. Wan, et al. (2007). "T cell-produced transforming growth factor-beta1 controls T cell tolerance and regulates Th1- and Th17-cell differentiation." Immunity **26**(5): 579-591.
Li, Y. R. and H. Matsunami (2011). "Activation state of the M3 muscarinic acetylcholine receptor modulates mammalian odorant receptor signaling." Sci Signal **4**(155): ra1.
Lindenboim, L., R. Pinkas-Kramarski, et al. (1995). "Activation of muscarinic receptors inhibits apoptosis in PC12M1 cells." J Neurochem **64**(6): 2491-2499.
Lindgren, S. W., I. Stojiljkovic, et al. (1996). "Macrophage killing is an essential virulence mechanism of Salmonella typhimurium." Proc Natl Acad Sci U S A **93**(9): 4197-4201.
Loenders, B., M. Rampart, et al. (1992). "Selective M3 muscarinic receptor antagonists inhibit smooth muscle contraction in rabbit trachea without increasing the release of acetylcholine." J Pharmacol Exp Ther **263**(2): 773-779.
Loukas, A. and P. Prociv (2001). "Immune responses in hookworm infections." Clin Microbiol Rev **14**(4): 689-703, table of contents.
MacLennan, C. A., J. J. Gilchrist, et al. (2010). "Dysregulated humoral immunity to nontyphoidal Salmonella in HIV-infected African adults." Science **328**(5977): 508-512.
Malo, D., K. Vogan, et al. (1994). "Haplotype mapping and sequence analysis of the mouse Nramp gene predict susceptibility to infection with intracellular parasites." Genomics **23**(1): 51-61.
Massoulie, J. and S. Bon (1982). "The molecular forms of cholinesterase and acetylcholinesterase in vertebrates." Annu Rev Neurosci **5**: 57-106.
Mastroeni, P. (2002). "Immunity to systemic Salmonella infections." Curr Mol Med **2**(4): 393-406.
Mastroeni, P., S. Clare, et al. (1999). "Interleukin 18 contributes to host resistance and gamma interferon production in mice infected with virulent Salmonella typhimurium." Infect Immun **67**(2): 478-483.
Mastroeni, P., J. A. Harrison, et al. (1996). "Effect of interleukin 12 neutralization on host resistance and gamma interferon production in mouse typhoid." Infect Immun **64**(1): 189-196.
Mastroeni, P. and M. Sheppard (2004). "Salmonella infections in the mouse model: host resistance factors and in vivo dynamics of bacterial spread and distribution in the tissues." Microbes Infect **6**(4): 398-405.
Matsui, M., D. Motomura, et al. (2002). "Mice lacking M2 and M3 muscarinic acetylcholine receptors are devoid of cholinergic smooth muscle contractions but still viable." J Neurosci **22**(24): 10627-10632.
Matsui, M., D. Motomura, et al. (2000). "Multiple functional defects in peripheral autonomic organs in mice lacking muscarinic acetylcholine receptor gene for the M3 subtype." Proc Natl Acad Sci U S A **97**(17): 9579-9584.
McKenzie, G. J., A. Bancroft, et al. (1998). "A distinct role for interleukin-13 in Th2-cell-mediated immune responses." Curr Biol **8**(6): 339-342.
McSorley, S. J., B. T. Cookson, et al. (2000). "Characterization of CD4+ T cell responses during natural infection with Salmonella typhimurium." J Immunol **164**(2): 986-993.
McSorley, S. J. and M. K. Jenkins (2000). "Antibody is required for protection against virulent but not attenuated Salmonella enterica serovar typhimurium." Infect Immun **68**(6): 3344-3348.
Medzhitov, R. and C. A. Janeway, Jr. (1997). "Innate immunity: impact on the adaptive immune response." Curr Opin Immunol **9**(1): 4-9.
Medzhitov, R. and C. A. Janeway, Jr. (1997). "Innate immunity: the virtues of a nonclonal system of recognition." Cell **91**(3): 295-298.
Miller, H. R., J. F. Huntley, et al. (1981). "Immune exclusion and mucus trapping during the rapid expulsion of Nippostrongylus brasiliensis from primed rats." Immunology **44**(2): 419-429.

Mittrucker, H. W. and S. H. Kaufmann (2000). "Immune response to infection with Salmonella typhimurium in mice." J Leukoc Biol **67**(4): 457-463.
Mittrucker, H. W., A. Kohler, et al. (1999). "Critical role of CD28 in protective immunity against Salmonella typhimurium." J Immunol **163**(12): 6769-6776.
Mittrucker, H. W., B. Raupach, et al. (2000). "Cutting edge: role of B lymphocytes in protective immunity against Salmonella typhimurium infection." J Immunol **164**(4): 1648-1652.
Morpeth, S. C., H. O. Ramadhani, et al. (2009). "Invasive non-Typhi Salmonella disease in Africa." Clin Infect Dis **49**(4): 606-611.
Mosmann, T. R. and R. L. Coffman (1989). "TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties." Annu Rev Immunol **7**: 145-173.
Mosmann, T. R. and S. Sad (1996). "The expanding universe of T-cell subsets: Th1, Th2 and more." Immunol Today **17**(3): 138-146.
Moulton, B. C. and A. D. Fryer (2011). "Muscarinic receptor antagonists, from folklore to pharmacology; finding drugs that actually work in asthma and COPD." Br J Pharmacol **163**(1): 44-52.
Murphy, K., P. Travers, et al. (2012). Janeway's immunobiology. New York, Garland Science.
Nadel, J. A. and P. J. Barnes (1984). "Autonomic regulation of the airways." Annu Rev Med **35**: 451-467.
Nakamura, T., M. Matsui, et al. (2004). "M(3) muscarinic acetylcholine receptor plays a critical role in parasympathetic control of salivation in mice." J Physiol **558**(Pt 2): 561-575.
Nanton, M. R., S. S. Way, et al. (2012). "Cutting Edge: B Cells Are Essential for Protective Immunity against Salmonella Independent of Antibody Secretion." J Immunol **189**(12): 5503-5507.
Nauciel, C. (1990). "Role of CD4+ T cells and T-independent mechanisms in acquired resistance to Salmonella typhimurium infection." J Immunol **145**(4): 1265-1269.
Ndoye, A., R. Buchli, et al. (1998). "Identification and mapping of keratinocyte muscarinic acetylcholine receptor subtypes in human epidermis." J Invest Dermatol **111**(3): 410-416.
Neill, D. R., S. H. Wong, et al. (2010). "Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity." Nature **464**(7293): 1367-1370.
Neves, P., V. Lampropoulou, et al. (2010). "Signaling via the MyD88 adaptor protein in B cells suppresses protective immunity during Salmonella typhimurium infection." Immunity **33**(5): 777-790.
Nizri, E., Y. Hamra-Amitay, et al. (2006). "Anti-inflammatory properties of cholinergic up-regulation: A new role for acetylcholinesterase inhibitors." Neuropharmacology **50**(5): 540-547.
Nomura, J., T. Hosoi, et al. (2003). "The presence and functions of muscarinic receptors in human T cells: the involvement in IL-2 and IL-2 receptor system." Life Sci **72**(18-19): 2121-2126.
O'Callaghan, D., D. Maskell, et al. (1988). "Characterization of aromatic- and purine-dependent Salmonella typhimurium: attention, persistence, and ability to induce protective immunity in BALB/c mice." Infect Immun **56**(2): 419-423.
O'Neill, L. A. (2009). "Boosting the brain's ability to block inflammation via microRNA-132." Immunity **31**(6): 854-855.
Oda, Y. (1999). "Choline acetyltransferase: the structure, distribution and pathologic changes in the central nervous system." Pathol Int **49**(11): 921-937.
Oenema, T. A., S. Kolahian, et al. (2010). "Pro-inflammatory mechanisms of muscarinic receptor stimulation in airway smooth muscle." Respir Res **11**: 130.

Ogilvie, B. M. and D. J. Hockley (1968). "Effects of immunity of Nippostrongylus brasiliensis adult worms: reversible and irreversible changes in infectivity, reproduction, and morphology." J Parasitol **54**(6): 1073-1084.

Ohta, S., N. Oda, et al. (2010). "Effect of tiotropium bromide on airway inflammation and remodelling in a mouse model of asthma." Clin Exp Allergy **40**(8): 1266-1275.

Oppitz, M., C. Busch, et al. (2008). "Distribution of muscarinic receptor subtype M3 in melanomas and their metastases." J Cutan Pathol **35**(9): 809-815.

Parker, D. C. (1993). "T cell-dependent B cell activation." Annu Rev Immunol **11**: 331-360.

Picciotto, M. R., M. J. Higley, et al. (2012). "Acetylcholine as a neuromodulator: cholinergic signaling shapes nervous system function and behavior." Neuron **76**(1): 116-129.

Pollak, Y., A. Gilboa, et al. (2005). "Acetylcholinesterase inhibitors reduce brain and blood interleukin-1beta production." Ann Neurol **57**(5): 741-745.

Price, A. E., H. E. Liang, et al. (2010). "Systemically dispersed innate IL-13-expressing cells in type 2 immunity." Proc Natl Acad Sci U S A **107**(25): 11489-11494.

Qian, J., V. Galitovskiy, et al. (2011). "Plasticity of the murine spleen T-cell cholinergic receptors and their role in in vitro differentiation of naive CD4 T cells toward the Th1, Th2 and Th17 lineages." Genes Immun **12**(3): 222-230.

Radek, K. A., P. M. Elias, et al. (2010). "Neuroendocrine nicotinic receptor activation increases susceptibility to bacterial infections by suppressing antimicrobial peptide production." Cell Host Microbe **7**(4): 277-289.

Ramarathinam, L., D. W. Niesel, et al. (1993). "Salmonella typhimurium induces IFN-gamma production in murine splenocytes. Role of natural killer cells and macrophages." J Immunol **150**(9): 3973-3981.

Rathenberg, J., A. Gartner, et al. (2002). "Modification of choline acetyltransferase by integration of green fluorescent protein does not affect enzyme activity and subcellular distribution." Cell Tissue Res **308**(1): 1-6.

Raufman, J. P., R. Samimi, et al. (2008). "Genetic ablation of M3 muscarinic receptors attenuates murine colon epithelial cell proliferation and neoplasia." Cancer Res **68**(10): 3573-3578.

Ravindran, R., J. Foley, et al. (2005). "Expression of T-bet by CD4 T cells is essential for resistance to Salmonella infection." J Immunol **175**(7): 4603-4610.

Razani-Boroujerdi, S., M. Behl, et al. (2008). "Role of muscarinic receptors in the regulation of immune and inflammatory responses." J Neuroimmunol **194**(1-2): 83-88.

Read, S., R. Greenwald, et al. (2006). "Blockade of CTLA-4 on CD4+CD25+ regulatory T cells abrogates their function in vivo." J Immunol **177**(7): 4376-4383.

Read, S., V. Malmstrom, et al. (2000). "Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation." J Exp Med **192**(2): 295-302.

Reardon, C., G. S. Duncan, et al. (2013). "Lymphocyte-derived ACh regulates local innate but not adaptive immunity." Proc Natl Acad Sci U S A.

Rennick, D. M., M. M. Fort, et al. (1997). "Studies with IL-10-/- mice: an overview." J Leukoc Biol **61**(4): 389-396.

Ritter, S. L. and R. A. Hall (2009). "Fine-tuning of GPCR activity by receptor-interacting proteins." Nat Rev Mol Cell Biol **10**(12): 819-830.

Rodriguez-Diaz, R., R. Dando, et al. (2011). "Alpha cells secrete acetylcholine as a non-neuronal paracrine signal priming beta cell function in humans." Nat Med **17**(7): 888-892.

Rodriguez-Palmero, M., T. Hara, et al. (1999). "Triggering of T cell proliferation through CD28 induces GATA-3 and promotes T helper type 2 differentiation in vitro and in vivo." Eur J Immunol **29**(12): 3914-3924.

Roffel, A. F., C. R. Elzinga, et al. (1990). "Muscarinic M3 receptors mediate contraction of human central and peripheral airway smooth muscle." Pulm Pharmacol **3**(1): 47-51.

Rosas-Ballina, M., M. Ochani, et al. (2008). "Splenic nerve is required for cholinergic antiinflammatory pathway control of TNF in endotoxemia." Proc Natl Acad Sci U S A **105**(31): 11008-11013.

Rosas-Ballina, M., P. S. Olofsson, et al. (2011). "Acetylcholine-synthesizing T cells relay neural signals in a vagus nerve circuit." Science **334**(6052): 98-101.

Rubtsov, Y. P., J. P. Rasmussen, et al. (2008). "Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces." Immunity **28**(4): 546-558.

Rulifson, I. C., A. I. Sperling, et al. (1997). "CD28 costimulation promotes the production of Th2 cytokines." J Immunol **158**(2): 658-665.

Sallusto, F., J. Geginat, et al. (2004). "Central memory and effector memory T cell subsets: function, generation, and maintenance." Annu Rev Immunol **22**: 745-763.

Sandborn, W. J., W. J. Tremaine, et al. (1997). "Transdermal nicotine for mildly to moderately active ulcerative colitis. A randomized, double-blind, placebo-controlled trial." Ann Intern Med **126**(5): 364-371.

Santos, R. L., S. Zhang, et al. (2001). "Animal models of Salmonella infections: enteritis versus typhoid fever." Microbes Infect **3**(14-15): 1335-1344.

Sato, K. Z., T. Fujii, et al. (1999). "Diversity of mRNA expression for muscarinic acetylcholine receptor subtypes and neuronal nicotinic acetylcholine receptor subunits in human mononuclear leukocytes and leukemic cell lines." Neurosci Lett **266**(1): 17-20.

Schafer, R. and T. K. Eisenstein (1992). "Natural killer cells mediate protection induced by a Salmonella aroA mutant." Infect Immun **60**(3): 791-797.

Shaked, I., A. Meerson, et al. (2009). "MicroRNA-132 potentiates cholinergic anti-inflammatory signaling by targeting acetylcholinesterase." Immunity **31**(6): 965-973.

Simon, R., S. M. Tennant, et al. (2011). "Mouse models to assess the efficacy of non-typhoidal Salmonella vaccines: revisiting the role of host innate susceptibility and routes of challenge." Vaccine **29**(32): 5094-5106.

Sinha, K., P. Mastroeni, et al. (1997). "Salmonella typhimurium aroA, htrA, and aroD htrA mutants cause progressive infections in athymic (nu/nu) BALB/c mice." Infect Immun **65**(4): 1566-1569.

Song, P., H. S. Sekhon, et al. (2007). "M3 muscarinic receptor antagonists inhibit small cell lung carcinoma growth and mitogen-activated protein kinase phosphorylation induced by acetylcholine secretion." Cancer Res **67**(8): 3936-3944.

Srinivasan, A., J. Foley, et al. (2004). "Massive number of antigen-specific CD4 T cells during vaccination with live attenuated Salmonella causes interclonal competition." J Immunol **172**(11): 6884-6893.

Steck, T. L. (1974). "The organization of proteins in the human red blood cell membrane. A review." J Cell Biol **62**(1): 1-19.

Szabo, S. J., S. T. Kim, et al. (2000). "A novel transcription factor, T-bet, directs Th1 lineage commitment." Cell **100**(6): 655-669.

Szabo, S. J., B. M. Sullivan, et al. (2003). "Molecular mechanisms regulating Th1 immune responses." Annu Rev Immunol **21**: 713-758.

Szelenyi, J. G., E. Bartha, et al. (1982). "Acetylcholinesterase activity of lymphocytes: an enzyme characteristic of T-cells." Br J Haematol **50**(2): 241-245.
Threlfall, E. J. (2000). "Epidemic salmonella typhimurium DT 104--a truly international multiresistant clone." J Antimicrob Chemother **46**(1): 7-10.
Toyabe, S., T. Iiai, et al. (1997). "Identification of nicotinic acetylcholine receptors on lymphocytes in the periphery as well as thymus in mice." Immunology **92**(2): 201-205.
Tracey, K. J. (2007). "Physiology and immunology of the cholinergic antiinflammatory pathway." J Clin Invest **117**(2): 289-296.
Trakhtenberg, E. F. and J. L. Goldberg (2011). "Immunology. Neuroimmune communication." Science **334**(6052): 47-48.
Turner, R. J. and H. Sugiya (2002). "Understanding salivary fluid and protein secretion." Oral Dis **8**(1): 3-11.
Urban, J. F., Jr., K. B. Madden, et al. (1992). "The importance of Th2 cytokines in protective immunity to nematodes." Immunol Rev **127**: 205-220.
Urban, J. F., Jr., N. Noben-Trauth, et al. (1998). "IL-13, IL-4Ralpha, and Stat6 are required for the expulsion of the gastrointestinal nematode parasite Nippostrongylus brasiliensis." Immunity **8**(2): 255-264.
Van Parijs, L. and A. K. Abbas (1998). "Homeostasis and self-tolerance in the immune system: turning lymphocytes off." Science **280**(5361): 243-248.
VanCott, J. L., S. N. Chatfield, et al. (1998). "Regulation of host immune responses by modification of Salmonella virulence genes." Nat Med **4**(11): 1247-1252.
Vezys, V., D. Masopust, et al. (2007). "Analysis of CD8+ T cell-mediated anti-viral responses in mice with targeted deletions of the M1 or M5 muscarinic cholinergic receptors." Life Sci **80**(24-25): 2330-2333.
Vidal, S., M. L. Tremblay, et al. (1995). "The Ity/Lsh/Bcg locus: natural resistance to infection with intracellular parasites is abrogated by disruption of the Nramp1 gene." J Exp Med **182**(3): 655-666.
Wang, H., M. Yu, et al. (2003). "Nicotinic acetylcholine receptor alpha7 subunit is an essential regulator of inflammation." Nature **421**(6921): 384-388.
Ward, S. G. (1996). "CD28: a signalling perspective." Biochem J **318 (Pt 2)**: 361-377.
Way, S. S. and C. B. Wilson (2004). "Cutting edge: immunity and IFN-gamma production during Listeria monocytogenes infection in the absence of T-bet." J Immunol **173**(10): 5918-5922.
Wess, J. (1993). "Molecular basis of muscarinic acetylcholine receptor function." Trends Pharmacol Sci **14**(8): 308-313.
Wess, J., R. M. Eglen, et al. (2007). "Muscarinic acetylcholine receptors: mutant mice provide new insights for drug development." Nat Rev Drug Discov **6**(9): 721-733.
Wessler, I., H. Kilbinger, et al. (2001). "The biological role of non-neuronal acetylcholine in plants and humans." Jpn J Pharmacol **85**(1): 2-10.
Wessler, I., C. J. Kirkpatrick, et al. (1998). "Non-neuronal acetylcholine, a locally acting molecule, widely distributed in biological systems: expression and function in humans." Pharmacol Ther **77**(1): 59-79.
Yamada, M., K. G. Lamping, et al. (2001). "Cholinergic dilation of cerebral blood vessels is abolished in M(5) muscarinic acetylcholine receptor knockout mice." Proc Natl Acad Sci U S A **98**(24): 14096-14101.
Yamada, M., T. Miyakawa, et al. (2001). "Mice lacking the M3 muscarinic acetylcholine receptor are hypophagic and lean." Nature **410**(6825): 207-212.

Yancey, R. J., S. A. Breeding, et al. (1979). "Enterochelin (enterobactin): virulence factor for Salmonella typhimurium." Infect Immun **24**(1): 174-180.

Yazdanbakhsh, M., A. van den Biggelaar, et al. (2001). "Th2 responses without atopy: immunoregulation in chronic helminth infections and reduced allergic disease." Trends Immunol **22**(7): 372-377.

Zhang, P., D. Han, et al. (2008). "Inhibition of the development of collagen-induced arthritis in Wistar rats through vagus nerve suspension: a 3-month observation." Inflamm Res **57**(7): 322-328.

Zhao, A., J. McDermott, et al. (2003). "Dependence of IL-4, IL-13, and nematode-induced alterations in murine small intestinal smooth muscle contractility on Stat6 and enteric nerves." J Immunol **171**(2): 948-954.

Zimmermann, K. C. and D. R. Green (2001). "How cells die: apoptosis pathways." J Allergy Clin Immunol **108**(4 Suppl): S99-103.

Zimring, J. C., L. M. Kapp, et al. (2005). "Regulation of CD8+ cytolytic T lymphocyte differentiation by a cholinergic pathway." J Neuroimmunol **164**(1-2): 66-75.

APPENDIX

Reagent Recipes:

Anaesthetic:

1.2 ml Anaket V (Ketamine)

0.8 ml 2% Rompun (Xylazine)

8 ml 1X PBS

ELISA Blocking Buffer:

20 g Milk Powder

1000 ml 1X PBS

ELISA Dilution Buffer:

10g Bovine Serum Albumin (BSA)

1X PBS

ELISA Substrate Buffer (For Alkaline Phosphatase assay):

0.2 g NaN_3

97 ml di-ethanolamine

0.8 g $MgCl_2.6H_2O$

700 ml ddH2O

Adjust pH to 9.8 and make up to final volume of 1L using ddH_2O

Add 10mg of to 10ml of Substrate buffer (1mg/ml final concentration) prior to use.

ELISA Washing Buffer

20 g KCl 800 g NaCl

50 ml Tween-20 144 g $Na_2HPO_4.H_2O$

20 g KH_2PO_4

Make up to 5 L with ddH_2O

Flow Cytometry Buffer:

1 g BSA (0.1% BSA)

Make up to 1L with 1X PBS and filter sterilize. Store at 4^0C

Iscove's Modified Dulbecco's Medium (IMDM) (Gibco):

Full composition available from Gibco – Invitrogen Product number: 12440-053

Contains Phenol Red (0.04mM), D-Glucose (25mM), HEPES (25mM)

Luria Broth medium for *S.typhimurium* Δ*aroA*

10 g Tryptic Soy powder

5 g Yeast extract

10 g NaCl

Make up to 1L with ddH2O, autoclave at 121^0C for 10 mins and store at 4^0C

For agar, add 12 g of bacterial grade agar to 1L of LB medium before autoclaving.

1X Phosphate Buffered Saline (PBS):

8 g NaCl 1.44g NaH_2PO_4

0.2 g KCl 0.24 g KH_2PO_4

Make upto 1L with ddH_2O and adjust pH to 7.4. Filter sterilize prior to use.

PLUZNIK media

10% Inactivated fetal calf serum

5% Inactivated horse serum

30% L929 conditioned medium (source of Macrophage Colony stimulating factor – M-CSF)

50U/ml Penicillin/Streptomycin

2 mM L-glutamine

1 mM Na-Pyruvate

50 μM β-Mercaptoethanol

Make upto a final volume of 500mls using DMEM medium. Filter sterilize and store at 4^0C.

Red Cell Lysis Buffer

8.26 g NH_4Cl

1 g $KHCO_3$

0.037 g EDTA

Make up to 1L with ddH_2O, adjust pH to 7.4 and autoclave at 121^0C for 10 mins to sterilize.

Tissue Lysis Buffer:

5mM EDTA

10% Glycerol

0.1% Sodium dodecylsulphate (SDS)

0.5% Nonidet P-40

5mM PMSF

150mM NaCl

25mM Tris-Cl pH 7.5

1% Triton-X 100

0.5% deoxycholate

Make up to 1L prior to use.