Significance of cross-reactive antibody responses and isotype bias in malaria- helminth co-infection

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Declaration

I, Karen Fairlie-Clarke, confirm that the composition of this thesis and the research it documents are my own work. However scientific research is collaborative and I would like to acknowledge the role of my colleagues in each of the studies.

Chapter 2: Dr. Andrea Graham assisted with experimental sampling.

Chapter 3: Dr Andrea Graham provided assistance with experimental sampling, as did Dr. Simmi Mahajan. A subset of the experiments described in this chapter also contributed to a collaborative study investigating cytokine responses and lung pathology, which was overseen in its entirety by Dr Andrea Graham who also performed the statistical analysis for publication.

Chapter 4: David Gray contributed to the experimental design. Kathryn Watt and Sheelagh Duncan provided technical assistance.

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Table of Contents

Abstract

The socio-economic and geographical distribution of malaria overlaps with that of many parasitic helminths and in these areas co-infections are common. Co-infection with helminths can influence disease outcome causing either exacerbation or amelioration of malaria. Understanding the complex host-parasite interactions that lead to these different disease outcomes is important for the success of control programmes aimed at these parasites.

The immune system has evolved diverse types of response (e.g. T-helper 1 (Th1) and T-helper 2 (Th2)) to efficiently combat infection with 'microparasites' and helminths respectively. When faced with co-infection however, the need for the host to multi-task means it must manage these counter-regulatory responses. In this study a murine model of malaria-hookworm (*Plasmodium chabaudi- Nippostrongylus brasiliensis*) co-infection was utilised to investigate how changes in T-helper bias affect malaria disease outcome. Antibody isotypes were used as indicators of Th1/Th2 bias and revealed that helminth co-infection reduced the malaria-specific Th1 response. Counter-intuitively this resulted in 'protection' from malaria with co-infected mice having reduced peak *P. chabaudi* parasitaemia and suffering less severe anaemia.

In addition to providing a measure of Th1/Th2 bias, analysis of antibody responses revealed the occurrence of cross-reactive antibodies. The potential for these cross-reactive antibodies to influence disease outcome was investigated but in this murine model resource-mediated mechanisms of parasite regulation appear to be responsible for the 'protection' that co-infection affords.

The question of why cross-reactive antibodies are produced has important immunological and ecological implications. Cross-reactive responses may arise through some physiological constraint on the immune mechanisms that usually result in antibody-specificity. However experiments designed to investigate if the specificity of antibodies is constrained by availability of antigen suggest that this is not the case in the model system used here. There is also the possibility that production of cross-reactive antibodies represents an evolutionary optimal strategy for a host faced with unpredictable exposure to a variety of parasites. However a major finding of this study indicates these two taxonomically distinct parasite species share antigens, which in itself is crucial to understanding host-parasite interactions in a co-infection setting.

The main findings of this thesis are relevant to co-infection studies in general and the implications for both evolutionary and applied biology are discussed.

List of Abbreviations

ABTS – 2,2'-azino-bis(3-ethylbenzthiazoline)-6-sulphonic acid

BCR – B Cell Receptor

ELISA – Enzyme Linked Immunosorbent Assay

FCS – Foetal Calf Serum

HRP – Horse Radish Peroxidase

IFNγ- Interferon gamma

Ig – Immunoglobulin

IL – Interleukin

IVC – Individually Ventilated Cages

L3 – 3rd stage larvae (infective)

MSP-1$_{19}$ – Merozoite Surface Protein-1$_{19}$

Nb - *Nippostrongylus brasiliensis*

NbA – *Nippostrongylus brasiliensis* antigen

NK cell – Natural Killer cell

PBS – phosphate buffered saline

Pcc – Plasmodium chabaudi chabaudi

Pc lysate – *Plasmodium chabaudi* parasitised Red Blood Cell lysate

pNPP – p-nitrophenyl phosphate

pRBC – parasitised (*Pcc*) red blood cell antigen

RBC- red blood cell

RPMI – Roswell Park Memorial Institute (media)

TBS – Tris Buffered Saline

TBST – Tris Buffered Saline with 1%Tween

TCR – T Cell Receptor

TGFβ- Transforming Growth Factor beta

Th – T- helper cell

TNFα- Tumour Necrosis Factor alpha

T-reg – regulatory T-cell

Materials and Methods

MICE

Female BALB/c mice were used in all experiments and were aged 10-12 weeks at the start of each experiment. Mice were either bought from Harlan UK (Bicester, UK) or bred in house at the Anne Walker animal facility. Mice were always housed in individually ventilated cages (IVC) with a 12h: 12h light: dark cycle, food (41b diet) and water were provided ad libitum.

PARASITES

Plasmodium chabaudi chabaudi (*Pcc*) the rodent malaria parasite was originally isolated from *Thamnomys rutilans* (thicket rats) and cloned by serial dilution and passage (Beale, Carter et al. 1978), in all experiments the AS strain of this parasite was used. Parasites were stored as frozen blood stabilates in liquid nitrogen and recovered by passage through donor mice prior to inoculation of experimental animals. Frozen stabilates were resuspended in 0.4ml citric saline and 2 donor mice were inoculated i.p (0.2ml/mouse). $1x10^6$ infected red blood cells (RBC) in 100µl diluent (50% Foetal Calf Serum (FCS): 47.5% Ringer's solution: 0.025% heparin) from these mice were passaged into 2 more donor mice and infected RBC from one of these donors were used to inoculate the experimental mice.

Nippostrongylus brasiliensis (*Nb*) worms were maintained by serial passage through Sprague-Dawley rats. Third stage (L3) larvae were obtained by culturing the faeces of infected rats at 26^{o}C for a minimum of 5 days (Lawrence, Gray et al. 1996). Prior to injection into mice the L3s were washed 10 times in PBS and counted on a Wild Heerbrugg dissecting microscope at x60 magnification. The L3s were then resuspended at 200 L3/50µl in PBS.

INFECTION PROTOCOL

The details of infection regime (timing and parasite dose) for each set of experiments are reported in the relevant results chapter.

N. brasiliensis infection was initiated by subcutaneous (s.cut) injection of 200 3rd stage infective larvae (L3) suspended in PBS (50µl). Individual syringes were preloaded with the *Nb* L3s and held in an upright position to ensure accurate doses were administered. Control mice received a sham injection of PBS (50µl s.cut).

P. chabaudi was administered by intraperitoneal (i.p) injection of infected RBC in 100µl diluent (50% Foetal Calf Serum (FCS): 47.5% Ringer's solution: 0.025% heparin). Control mice received a sham injection of 1x10^5 naïve RBC in 100µl diluent (50% FCS: 47.5% Ringer's solution: 0.025% heparin) i.p.

MONITORING *Pcc* PARASITAEMIA, HOST ANAEMIA & BODY WEIGHT

Parasitaemia

Asexual *Pcc* parasitaemia was measured daily throughout malaria infection by microscopy of thin blood smears stained with Giemsa. Blood from the tail tip was spotted onto a microscope slide and spread into a smear, after air-drying the slide was fixed with 100% methanol and then immersed in 20% Giemsa for 20 minutes. Slides were then rinsed in water and allowed to air dry before being examined at x1000 magnification with oil immersion. The proportion of parasitised RBC was calculated by determining the total number of RBCs that had to be counted to discover 5 or 20 parasites (depending on stage of infection). If no parasites were observed in 50 fields the smear was classed as negative. Parasite density was calculated by multiplying the proportion of parasitised RBC by the RBC density of individual mice on the day of sampling. If throughout the course of infection the parasitised RBC density never exceeded 0.05 (10^{9}pRBC/ml) the infection was considered to have failed and the mouse was excluded from all further analyses.

Red Blood Cell Density (anaemia)

As a measure of anaemia in experimental animals red blood cell density was measured, 2µl of blood from the tail vein was collected by capillary and diluted in 80ml of ISOTON II diluent (Beckman Coulter). The number of RBCs was counted using the Z1 series Coulter counter (Beckman Coulter).

Body weight

As a measure of the pathology of disease body weight was measured daily using a platform scale with a setting specifically designed for live animals.

PARASITE ANTIGENS

***Nippostrongylus brasiliensis* antigen (NbA)** is a crude homogenate of *Nb* worms prepared from a stock of frozen adults, which were harvested from the intestines of infected Sprague-Dawley rats. The worms were defrosted and crushed in 500µl of sterile phosphate buffered saline (PBS) using a 1ml glass homogeniser, the preparation was kept on ice at all times. The preparation was then left for 1 hour before further homogenising. Finally the preparation was centrifuged at 13, 000 rpm (17 000g) for 10 mins and the supernatant removed. The supernatant was passed through a 0.22µm syringe driven filter (Millipore) and the protein concentration determined by Bradford assay.

Parasitised Red Blood Cell Antigen (pRBC) is a crude preparation of *Pcc* infected RBCs. Mice were infected with *Pcc* and the parasitaemia monitored daily. When the parasitaemia reached 20% or more the mice were bled under anaesthetic by cardiac puncture with a heparinised syringe. The RBC were washed in PBS and centrifuged at 1000g for 10 mins. The preparation was then lysed by repeated cycles of freeze (-80^{o}C) thaw. Finally the protein concentration was determined by Bradford assay.

***Plasmodium chabaudi* lysate (*Pc* lysate)** is also a crude preparation of malaria infected RBCs but undergoes further purification than pRBC (see above) to remove

host proteins. Mice were infected with *Pcc* and the parasitaemia monitored daily. When the parasitaemia reached 20% or more the mice were bled under anaesthetic by cardiac puncture with a heparinised syringe. This whole blood was diluted in 10ml RPMI media. 15ml of 67% Percoll was then underlayed by slowly pipetting it to the base of the tube. The sample was centrifuged at 3000rpm (6450g) for 10 min to allow separation of cells in the Percoll gradient and the interface containing parasitised RBC was harvested. The cells were washed twice with 50ml RPMI media by centrifugation at 2000rpm (6450g) for 5 mins. Antibodies were cleaved from the cells by incubation with trypsin at 37^{o}C for 10 mins. Cells were washed twice in RPMI as before and then resuspended in 10ml of 0.05% saponin to lyse the RBC membrane. Cells were then centrifuged at 2000rpm (6450g) for 10 min and washed with PBS until the supernatant was clear. The pellet was resuspended in 1ml sterile PBS and the parasites lysed by 5 cycles of freeze (-80^{o}C) thaw. Finally the protein concentration was determined by Bradford assay.

Merozoite Surface Protein-1$_{19}$ is a recombinant malaria protein, which was originally sequenced, cloned and expressed from *Pcc* AS clone, as described previously [39]. In brief the MSP-1$_{19}$ nucleotide sequence was inserted into *Pichia pastoris* vector pIC9K and protein expression carried out in *Pichia pastoris* strain SMD1169. An initial stock of MSP-1$_{19}$ protein was a kind gift from Jean Langhorne as was the plasmid, which enabled large-scale in house expression of the recombinant protein. The recombinant protein is HIS-tagged and was secreted into the yeast culture supernatant. This allowed purification of the protein from the supernatant via binding to a Nickel Sepharose column (HisTrap^{TM} HP column 17-5248-01 GE healthcare) using the AKTAPrime plus purification system. This one-step purification step yielded large quantities of MSP-1$_{19}$ protein that were dialysed against 1 x PBS before being stored at -20^{o}C or -80^{o}C until use. The purity of the recombinant protein was verified by SDS-page gel and revealed a single band (Figure 1). The potential for *Pichia* to artificially glycosylate the recombinant MSP-1$_{19}$ should be noted.

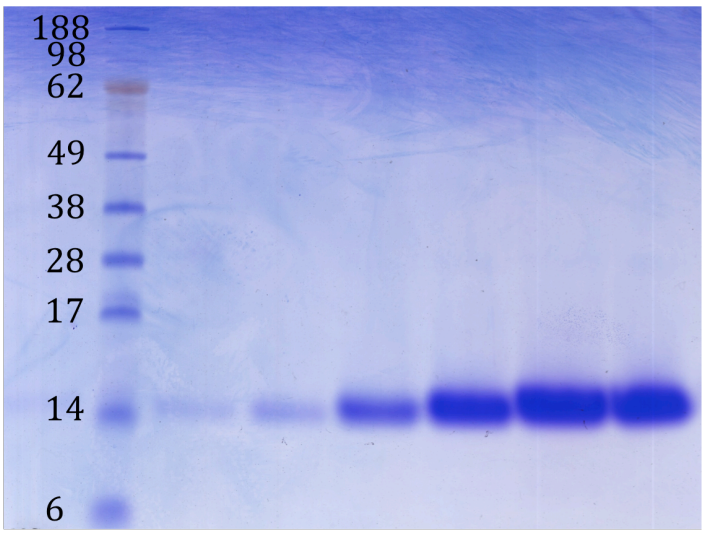


Figure 1: *SDS-page gel showing MSP-1$_{19}$ recombinant protein post-purification.* Each lane of the gel represents a fraction of the AktaPrime purification of MSP-1$_{19}$ from the culture supernatant, 4-12% BIS-TRIS gel, numbers represent the size of the protein markers (kDA) against which they are aligned.

ANTIBODY ANALYSIS

Blood sampling

On the final day of all experiments a terminal bleed from the brachial artery was performed under anaesthetic. Blood was either collected on Serasieve (Hughes & Hughes Ltd) or in heparin coated eppendorph tubes. The sample was then centrifuged at 13 000 rpm (17 000g) to separate the serum/ plasma fraction which was removed and stored at -80^{o}C.

In a subset of experiments blood samples were also collected on a daily basis for antibody analysis. 5µl of blood from the tail vein was collected by capillary and diluted in 5µl of heparin (1000U/ml)(SIGMA). The sample was centrifuged at 13 000 rpm (17 000g) to separate the plasma fraction which was removed and stored at -80^{o}C.

Enzyme Linked Immunosorbent Assay (ELISA)

Total IgE and IgG2a antibody responses were measured by sandwich ELISA. This method involved using immobilised 'capture' antibodies specific for a given isotype to bind the antibodies present in host plasma. A secondary, enzyme-conjugated antibody is added and the resulting sandwich complex is identified by a colorimetric change with the addition of substrate.

96 well maxisorp immunoplates (Nunc) were coated with capture antibody (see Table 1 for details) diluted in 0.06M carbonate buffer at a concentration of 2µg/ml in a final volume of 50µl/well. Plates were kept at 4^{o}C overnight to allow the antibodies to bind. Excess antibody was removed and non-specific binding sites were blocked with 5% Marvel diluted in Tris Buffered Saline (TBS) with 1%Tween (TBST), 100µl/well. Plates were incubated at 37 ^{o}C for 2 hours. At this stage and after each subsequent step the plates were washed five times with TBST. Plasma samples were added to the wells in a series of doubling dilutions from 1/100 – 1/800 using TBST as a diluent in a final volume of 50µl/well with the exception of IgE in plasma samples from terminal bleeds when a dilution of 1/20 was used. Purified antibodies of the relevant isotype were also added (50µl/well) to create a standard curve (see Table 1 for details) against which the sample concentrations were calibrated. Again TBST was used as a diluent. Plates containing the samples and standards were incubated at 37 ^{o}C for 2 hours. Secondary biotinylated 'detection' antibodies (see Table 1 for details) were diluted in TBST with 0.5% (FCS) (SIGMA) and added at a concentration of 2µg/ml in a final volume of 100µl/well. Plates were incubated at 37^{o}C for 1 hour before adding 100µl/ well of a 1/8000 dilution of Extravadin Peroxidase: TBST-5%FCS. Plates were incubated at 37^{o}C for 30 mins. Following the final three washes in TBST plates received an additional wash in distilled water before the TMB substrate was added (100µl/well). The development of the colorimetric change with the addition of substrate was allowed to develop at room temperature in the dark. The reaction was stopped with 100µl/well of 1M HCl that causes a final colour change from blue to yellow. The optical density was then

determined using an "Emax" ELISA microplate reader (Molecular Devices) with a 450nm filter.

Total IgG antibody responses were also measured by sandwich ELISA as described above. The details of the capture and detection antibodies used are given in Table 1. The detection antibody was HRP conjugated and so the addition of extravadin peroxidase was not required and rather than TMB, ABTS was used as the substrate.

Table 1: Details of the antibodies used in ELISA.

Antibody	Manufacturer and code	Concentration used in ELISA
IgG capture	AbD serotec 101001	1μg/ml
IgE capture	BD Pharmingen 553413	2μg/ml
IgG2a capture	BD Pharmingen 553446	2μg/ml
IgG detection	DAKO P0260	0.65μg/ml
IgE detection	BD Pharmingen 553419	2μg/ml
IgG2a detection	BD Pharmingen 553388	2μg/ml
IgE standard	BD Pharmingen 557079	Highest conc. 5μg/ml
IgG2a standard	BD Pharmingen 553459	Highest conc. 200μg/ml

Antigen Specific antibodies of the IgG, IgG1, IgG2a and IgG3 isotypes were measured by ELISA. 96 well maxisorp immunoplates (Nunc) were coated at 4^{o}C overnight with either recombinant or crude antigen preparations in 0.06M carbonate buffer in a final volume of 50μl per well. Recombinant MSP-1_{19} was used at a concentration of 1μg/ml, the crude antigens NbA and pRBC at 5μg/ml and Pc Lysate at 2μg/ml. Excess antigen was removed and non-specific binding sites were blocked with 5% FCS diluted in carbonate buffer (200μl/well) for 2 hours at 37oC. Wells were washed three times in TBST at this stage and following each subsequent step. Serum samples were added in serial dilutions 1/100- 1/204800 using TBST as a diluent, in a final volume of 50μl per well and incubated for 2 hours at 37^{o} C. Isotype specific detection antibodies were diluted in TBST in a final volume of 50μl per well. For IgG HRP conjugated rabbit anti-mouse IgG (DAKO P0260) was used at 1/2000. For IgG1, Horse Radish Peroxidase (HRP) conjugated goat anti-mouse IgG1

(Southern Biotech 1070-05) was used at 1/6000, HRP conjugated goat anti-mouse IgG2a (Southern Biotech 1080-05) at 1/4000 and HRP conjugated goat anti-mouse IgG3 (Southern Biotech 1100-05) was used at 1/1000. Plates were incubated for 1 hour at 37^{o}C. An additional wash in distilled water was carried out before the substrate 2,2'-azino-bis(3-ethylbenzthiazoline)-6-sulphonic acid (ABTS) (Insight Biotechnology) was added at 100µl per well. The reaction was allowed to develop for 20 minutes at room temperature. Optical density (OD) was determined using an "Emax" ELISA microplate reader (Molecular Devices) with a 405nm filter.

Antibody Titre Measures. For antigen-specific responses, in which a serial dilution of sera is measured by ELISA antibody titres can be calculated from the OD values. The mean plus 3 standard deviations of the optical density measured in control mice at 1/200 sera dilution was assigned as a 'cut-off' value. Titre was calculated in experimental mice as the reciprocal of the highest dilution at which the OD exceeded this cut-off. For example if at 1:800 sera dilution the OD value is greater than the cut-off this mouse has an antibody titre of 800. Any OD value that fell beneath the cut-off was deemed zero.

Protein-specific antibodies were measured by detection of antibodies that bound to antigen pre-treated with periodate. The ELISA was carried out as detailed for detection of antigen-specific antibodies (see above) with the following additional steps included after blocking with 5% FCS: carbonate buffer, prior to sample addition. TBST wash was followed by the addition of 10mM sodium (meta) periodate diluted in 50 mM sodium acetate in a final volume of 100 µl/well. Periodate oxidises carbohydrate to aldehydes and thus disrupts the carbohydrate epitope on the antigen. Plates were incubated at 37°C for 1 hour and then washed in 50 mM sodium acetate. To stop the activity of periodate, 100µl of 50 mM sodium borohydride solution was added to each well.

High avidity antibodies of the IgG1and IgG2a isotype were also measured by modifying the protocol used for ELISA of antigen specific responses. The modification involved an extra wash step with 6M Urea following incubation of the

serum samples. 6M urea is a hydrogen-bond dissociating agent and washing with this removed antibodies that were only weakly bound to the antigen coating the plate. This assay was always run in parallel to the standard antigen-specific ELISA protocol (detailed above) so that the 'avidity index' could be calculated by dividing the high avidity titre by the 'total' avidity titre.

WESTERN BLOT

Gel electrophoresis was carried out with the XCell *Sure*Lock mini-cell system (Invitrogen). *H. polygyrus*, *L. sigmodontis*, *N. brasiliensis* or *P. chabaudi* lysate antigens were prepared at a concentration of 25µg or 50µg in Nupage LDS Sample buffer (Invitrogen NP0007) with 20%NuPage sample reducing buffer (Invitrogen NP0009). Each antigen preparation was loaded into a separate well of a Nu-Page 4-12% Bis-Tris gel (Invitrogen NP0304). Gel electrophoresis was carried out at 200V for 35 mins in NuPage MES SDS Running buffer (Invitrogen NP0002) as per the manufacturer's instructions.

Transfer to Western blot membrane was carried out immediately after gel electrophoresis using the XCell II Blot module (Invitrogen). Each gel and nitrocellulose membrane (Amersham Hybond- ECL RPN303D) was sandwiched together between filter paper and blotting pads that had been presoaked in NuPage Transfer Buffer (Invitrogen NP0006-1) with 20% methanol. Transfer conditions of 35V for 75 mins were used.

Probing blot with *Pcc* anti-sera. Following transfer nitrocellulose membranes were blocked with 5% Marvel: TBST for 1 hour at room temperature (RT). Blots were incubated (with rocking) overnight at 4^{O}C with *Pcc* anti-sera (1:100 dilution in 5%Marvel: TBST). Blots were washed 5 times in TBST (20 min/ wash). Blots were then incubated for 1 hour at RT with rabbit anti-mouse IgG (DAKO P0260) (1:1000 dilution in 5%Marvel: TBST). Blots were washed again 5 times in TBST (20 min/ wash) and finally in TBS (2x 20 min wash). Blots were then incubated with ChemiGlow West (Alpha Innotech 60-12596-00) for 5 mins. The

chemiluminescence resulting from the reaction of the ‘ChemiGlow’ and bound antibodies was visualized on a gel scanner - FluorChem ™ SP (Alpha Innotech).

STATISTICAL ANALYSIS

All analyses were carried out in the statistical package JMP 8.0 (SAS) using generalised linear models. Details of the analyses are given in the results chapters.

BUFFER RECIPES

Phosphate Buffered Saline (PBS): As per manufacturers instructions, PBS tablets (SIGMA P4417) were dissolved in distilled water.

Tris Buffered Saline (TBS): Stock TBS (10x) – 437.5g NaCl, 121g Trisma base diluted in 5L of distilled water. TBS was used at 1x concentration (1:10 dilution of the 10x stock).

TBS-Tween (TBST): 1x TBS with addition of 1% Tween 20 Viscous Liquid (polyoxyethylenesorbitan monolaureate) (SIGMA P1379)

Carbonate Buffer: (0.06M Carbonate Buffer pH 9.6) is made by combining two carbonate solutions (A and B see below) in a 2:1 ratio i.e. 45.3ml of A and 18.2ml of B in 936.5ml dH_2O.

Solution A: 8.5g NaH_2CO_3 in 100ml dH_2O (1M)
Solution B: 10.6g Na_2CO_3 in 100ml dH_2O (1M)

Citrate Saline: 8.5g NaCl, 15g tri-sodium citrate (SIGMA S-4641) dissolved in 1L of distilled water.

Ringer's solution: 0.2g KCl, 9.0g NaCl, 0.2g $CaCl_2$ dissolved in 1L of distilled water.

Chapter 1: General Introduction

1.1 Background

The intimate relationship of parasites with their hosts is one of the features that make them so fascinating. As an undergraduate I was captivated by the ability of parasites to manipulate their host's behaviour to meet their own needs. *Taenia multiceps* parasites literally have their intermediate host (sheep) running round in circles as cysts develop in the brain. Circling has never been noted as the greatest strategy for evading predation by cunning canines. Perhaps the most striking example of parasites enhancing the predation of the host is the colourful, pulsating antennae of snails infected with *Leucochloridium paradoxum* that are simply irresistible to birds. These examples of parasite infestation provide sensational imagery for engaging undergraduates but it is the interactions between host and parasite that mediate disease outcome that has maintained my interest in the field of parasitology.

One of the fascinating things about parasites is the complex multi-stage life cycles that have evolved to promote invasion of, reproduction within and transmission between hosts. The vast diversity of parasite species means that is hard to think of an organ or cell that is not exploited as a niche for growth or reproduction. Exposure to an unpredictable array of parasite species each with its own complex life cycle and reproductive strategy presents a serious challenge to the host in terms of combating infection. For example a host must be equally capable of controlling the rapid replication of an intracellular parasite as expelling large multi-cellular parasitic helminths. The immune system has risen to the challenge and evolved diverse responses adapted to coping with these different parasites. However, in natural populations hosts are very rarely (if ever) infected by only one species of parasite, so the host must be able to multi-task. A core interest of mine is to understand how a host maximises its own fitness when simultaneously infected by multiple, potentially competing, parasite species.

Disease-causing parasites of humans rightly receive a great deal of attention and malaria probably warrants being the front-runner: in 2009 alone 225 million cases of malaria were reported including 781 000 deaths (W.H.O 2010). The 'neglected tropical diseases' of Leprosy, Trachoma, Onchocerciasis, Lymphatic filariasis, Schistosomiasis and soil-transmitted helminths are endemic in many of the same countries as malaria (Hotez and Molyneux 2008). In general these diseases are not fatal but cause chronic disability and can impact the severity of potentially fatal diseases such as malaria (Brooker, Akhwale et al. 2007). Targeting these diseases for control or eradication has been a focus of the World Health Organisation for several decades and the formulation of a Global plan for the eradication of these diseases (2008-2015) demonstrates the continued commitment to this cause (W.H.O 2010). It is pertinent to understand the effect of co-infection with these diseases on malaria disease outcome, as unfortunately although progress has been made neither the neglected diseases nor malaria is likely to be eradicated in the near future (W.H.O 2010; Zhang, MacArthur et al. 2010).

Several of the 'neglected tropical diseases' are caused by parasitic helminths (Kyelem, Fischer et al. 2011) and in areas where these parasites coincide with malaria, co-infection is common (Mazigo, Waihenya et al. 2010). Studies of co-infection in humans reveal contrasting affects of helminths on malaria disease outcome. In most cases exacerbation of disease is observed with increased severity of malarial attacks (Nacher, Singhasivanon et al. 2002; Spiegel, Tall et al. 2003; Sokhna, Le Hesran et al. 2004), or increased severity of malaria pathology such as anaemia and hepatosplenomegaly (Mwatha, Jones et al. 2003; Brooker, Akhwale et al. 2007). There are however reports of helminths reducing malaria parasite density (Briand, Watier et al. 2005) and protecting against cerebral malaria (Nacher, Gay et al. 2000). Understanding the intricacies of how these parasitic infections influence one another has important implications for the success of vaccination and drug administration programmes. If helminths do indeed increase the severity or prevalence of malaria then treating with anthelmintics may provide an affordable method of improving malaria control (Druilhe, Tall et al. 2005). In support of this idea, a study by Kirwan et al (Kirwan, Jackson et al. 2010) in which anthelmintics

were used to treat preschool children for *Ascaris lumbricoides* resulted in a significant reduction in the prevalence of malaria. On the flip side any protective effect of co-infection could easily be lost through drug clearance of helminths (Specht and Hoerauf 2007). This thesis tackles some of the complex within-host interactions that potentially mediate disease outcome in co-infection; focussing on the induction of the diverse immune responses that target malaria and helminths and the potential effect of competition between these parasites.

1.2 *Plasmodium* infection

Infection with *Plasmodium* species is initiated by the injection of sporozoites via the bite of an infected mosquito. These infectious forms migrate to the liver where they invade hepatocytes and undergo asexual replication resulting in the release of merozoites, which enter the circulatory system and invade red blood cells (RBC). Within the RBC a cycle of asexual replication occurs progressing through the trophozoite and schizont stages and culminating in the release of merozoites when the RBC ruptures. These free merozoites must then rapidly invade a new RBC to begin the cycle again (Figure 1).

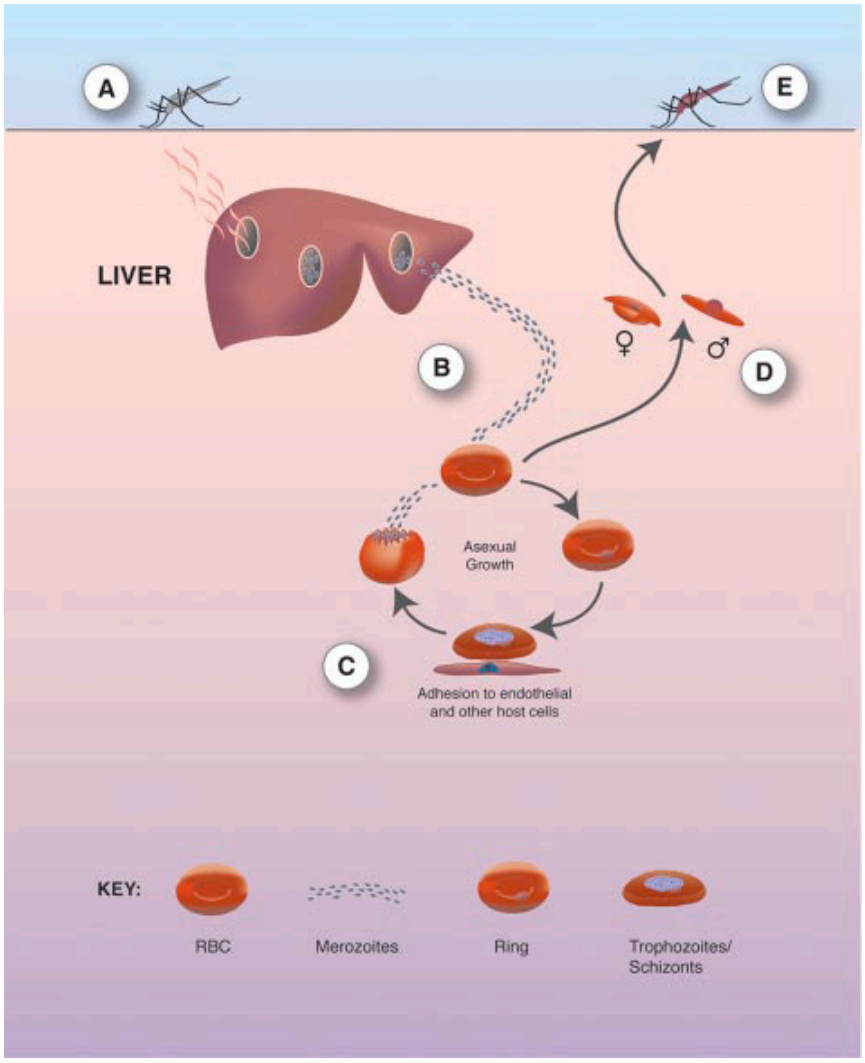


Figure 1: *Life cycle of Plasmodium species*.
Injection of sporozoites from infected mosquito (A) that migrate to the liver and invade hepatocytes (B). Replication within the liver produces merozoites that invade red blood cells and undergo asexual replication to produce more merozoites, which perpetuate the cycle (C). Some replication within red blood cells leads to the development of the sexual transmission forms (gametocytes) (D). These are ingested by the mosquito with a blood meal (E) and development within the vector results in sporozoites that can be injected to a new host. Figure reproduced with permission from Lamikanra et al (Lamikanra, Brown et al. 2007).

It is the cycle of invasion and rupturing of RBCs and the immune response to this stage of the parasite that causes most of the pathology associated with the disease (Tilley, Dixon et al. 2011). In particular the severe anaemia characteristic of malaria infection results partly from the mechanical destruction of RBC as they rupture on merozoite release. This anaemia is exacerbated by clearance of both infected and

uninfected RBC by phagocytosis and suppression of erythropoiesis (Totino, Magalhaes et al. 2010), as well as splenic retention of both infected and uninfected RBCs (Buffet, Safeukui et al. 2009). In addition to the uncomplicated symptoms of malarial disease (e.g. anaemia, fever) there are also complications such as cerebral malaria that can induce coma. Sequestration of infected RBC in the venules and capillaries of the brain combined with systemic inflammation (Good, Xu et al. 2005; Awandare, Goka et al. 2006) causes cerebral malaria in humans. The phenomenon of rosetting in which uninfected RBC adhere to infected RBC can also add to the blockage of blood vessels in the brain (White, Turner et al. 2010).

1.3 Soil-transmitted helminth infection

Whilst many helminth species are co-endemic with malaria, soil-transmitted helminths are the most prevalent and abundant in these areas (Brooker, Akhwale et al. 2007). The soil-transmitted helminths of humans include *Ascaris lumbricoides, Trichuris trichuria* and hookworms (*Ancylostoma duodenale* and *Necator americanus*). These infections are all contracted by ingestion of, or contact with, soil contaminated with eggs or larvae of these parasites. I will focus here on hookworm infection. Hookworm eggs hatch in soil and develop to produce infective 3rd stage larvae (L3), which are able to penetrate the skin of their host. Once they have entered the host the larvae migrate to the lungs and undergo further development to 4th stage larvae (L4). They then penetrate the airspace of the lungs and are coughed up and swallowed to the small intestine where they mature to adults. The eggs produced by the adults are passed out in the faeces and contaminate the environment (Figure 2).

The pathology of these helminths, which is proportional to parasite burden (Crompton and Nesheim 2002), relates to the anaemia and iron-deficiency caused by the adults directly consuming RBC and the tissue damage they cause by attaching to the gut wall (Gilman 1982; Crompton and Nesheim 2002). Migration of these worms through the lungs may also cause significant tissue damage and deterioration of lung function akin to emphysema and chronic obstructive pulmonary disease, although to

date this has only been explored in animal models of hookworm infection (Marsland, Kurrer et al. 2008).

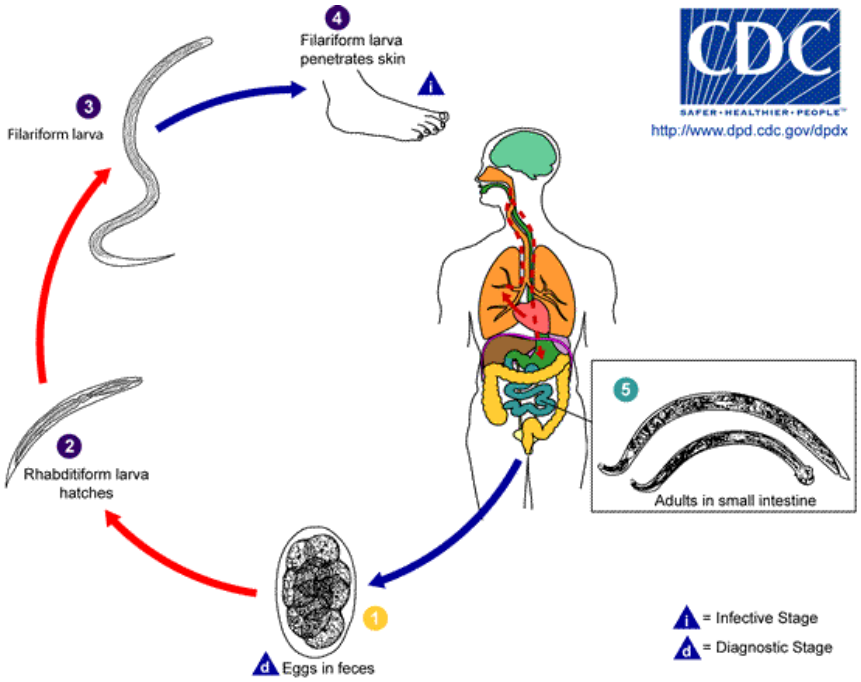


Figure 2*: Life cycle of hookworm species.*
Hookworm eggs in the soil (1) hatch to produce larvae (2) that develop and moult into the infective 3rd stage, which penetrate the skin of the host (3 and 4). On entering the host they migrate to the lungs from where they are coughed up and swallowed to the small intestine where they reside as adults (5) and release eggs back into the environment via faeces. Figure taken from Centres for Disease control and Prevention (CDC) website.

1.4 Malaria and helminths induce mutually inhibitory immune responses.

Asexual reproduction of *Plasmodium* parasites in RBCs results in the characteristic anaemia associated with malaria infection. In the murine model direct injection of merozoite-infected RBC is used as the mode of infection. It should be noted that sporozoite infection of mice via the bite of an infected mosquito results in lower peak asexual parasitaemia and a reduction in the magnitude of the immune response (Fonseca, Seixas et al. 2007). However, the quality of the immune response is similar

(Fonseca, Seixas et al. 2007) and using direct injection of merozoites means that the inoculating dose can be accurately controlled. Here I will concentrate on the immune response to the intra-erythrocytic stage.

Both innate and adaptive immune responses are required for the efficient control of malaria parasite growth. The initial control of parasite replication involves early production of the pro-inflammatory cytokines TNFα and IFNγ from macrophages and Natural Killer (NK) cells, which activate macrophages to produce anti-parasitic nitric oxide (Choudhury, Sheikh et al. 2000). The adaptive immune system promotes parasite-specific responses through T and B-cell receptor binding of parasite antigens and the early production of IFNγ by innate cells may play an important role in determining the type of adaptive immune response that is elicited by influencing T-cell differentiation (Su and Stevenson 2000). The dominant cytokines produced during malaria infection (TNFα, IFNγ and IL-12) drive the development of Th1 cells from naïve T-cells (Mosmann and Sad 1996). These Th1 cells perpetuate the production of IFNγ that results in isotype class switching in B-cells to produce IgG2a antibodies (Paul, Brown et al. 1987; Collins and Dunnick 1993; Else and Finkelman 1998). The production of these antibodies is crucial for the ultimate clearance of malaria infection (Weidanz, Batchelder et al. 2005) via opsonisation of parasitised RBC to facilitate phagocytosis by macrophages and by direct neutralisation of free merozoites before they can re-invade RBCs (Cavinato, Bastos et al. 2001; Mota, Brown et al. 2001; Bergmann-Leitner, Duncan et al. 2006; Bergmann-Leitner, Duncan et al. 2009).

Whilst the production of pro-inflammatory cytokines (TNFα, IFNγ) is beneficial in controlling initial parasite replication there is a fine line between benefit and cost as excessive production of these cytokines can exacerbate malaria pathology if they are not kept in check by the anti-inflammatory cytokines TGFβ and IL-10 (Omer and Riley 1998; Li, Sanni et al. 2003; Omer, de Souza et al. 2003). For example, the anaemia that is caused by the rupturing of RBC as merozoites burst out can be exacerbated by TNFα-mediated suppression of erythropoiesis (Li, Sanni et al. 2003). Other symptoms of pathology such as hypothermia and weight loss are also

associated with elevated levels of pro-inflammatory cytokines (Lamb, Brown et al. 2006). The main function of T-regulatory cells (T-regs) is to prevent excessive immune responses that could cause collateral damage (immunopathology) by suppressing T-cell proliferation via TGFβ and IL-10 (Pandiyan, Zheng et al. 2007). These cells occur naturally but can also be induced from naïve T-cells by TGFβ (Dardalhon, Awasthi et al. 2008). Of particular relevance to this study is the potential for the immune response induced by helminth infection to exert suppressive effects on malaria-specific Th1 response due to the counter-regulatory nature of Th1 and Th2 responses, discussed below.

Helminths induce potent Th2 immune responses characterised by the production of IL-4, IL-5 and IL-13 associated with infiltration of mast cells, eosinophils and basophils (Jackson, Friberg et al. 2009). Basophils in particular have recently been described as the innate cell crucial to the development of Th2 responses in *Nippostrongylus brasiliensis* infection (Nel, Hams et al. 2011). In contrast to Th1 cytokines that drive production of IgG2a antibodies, Th2 cytokines drive the production of IgE (Finkelman, Holmes et al. 1990) and IgG1 antibodies (Paul, Brown et al. 1987; Purkerson and Isakson 1992). The precise Th2 effector mechanisms that mediate protection against helminths are likely to vary amongst helminth species (Patel, Kreider et al. 2009). For example, alternative activation of macrophages driven by IL-4 is common to many helminth infections (Jenkins and Allen 2010) and has been described as having a protective effect in *Heligmosomoides polygyrus* infection by damaging migrating larvae (Anthony, Urban et al. 2006), whilst in other model systems neutrophils are associated with parasite killing – e.g. *Strongyloides stercoralis* and *Litomosoides sigmodontis* (Al-Qaoud, Pearlman et al. 2000; Padigel, Stein et al. 2007). More physiological processes are also involved with protection against gut-dwelling helminths such as *Nippostrongylus brasiliensis* and *Trichuris muris*; the so-called 'weep and sweep' mechanism whereby increased smooth muscle contractility combined with overproduction of mucous (both under the control of Th2-associated cytokines (Khan and Collins 2004)) lead to worm expulsion (de Veer, Kemp et al. 2007).

One of the key features of infection with helminths is the induction of T-regs, which combined with the predominant Th2 response provide an anti-inflammatory environment in which chronic helminth infection can persist (Maizels, Balic et al. 2004). Induction of suppressive T-regs, which as discussed previously, is essential for preventing excessive immune responses, has been hijacked by helminths to aid immune-evasion. The excretory/ secretory molecules of *H. polygyrus* have been shown to induce functional T-regs that suppress effector responses (Grainger, Smith et al. 2010). Helminth-induction of T-regs via the TGFβ pathway may well involve parasite homologues that mimic the action of mammalian TGFβ, such molecules have been identified in *H. polygyrus* and *N. brasiliensis* for example (Grainger, Smith et al. 2010; McSorley, Grainger et al. 2010). TGFβ in combination with IL-6 is also associated with the differentiation of Th17 cells, which are involved with the antimicrobial response to extracellular bacteria and inflammatory tissue responses (Ouyang, Kolls et al. 2008; Korn, Bettelli et al. 2009). IL-17 production by these cells is also associated with pathology in both helminth and malaria infection (Rutitzky, Lopes da Rosa et al. 2005; Wu, Chen et al. 2010). The cytokine driven differentiation of T-cells is summarised in Figure 3.

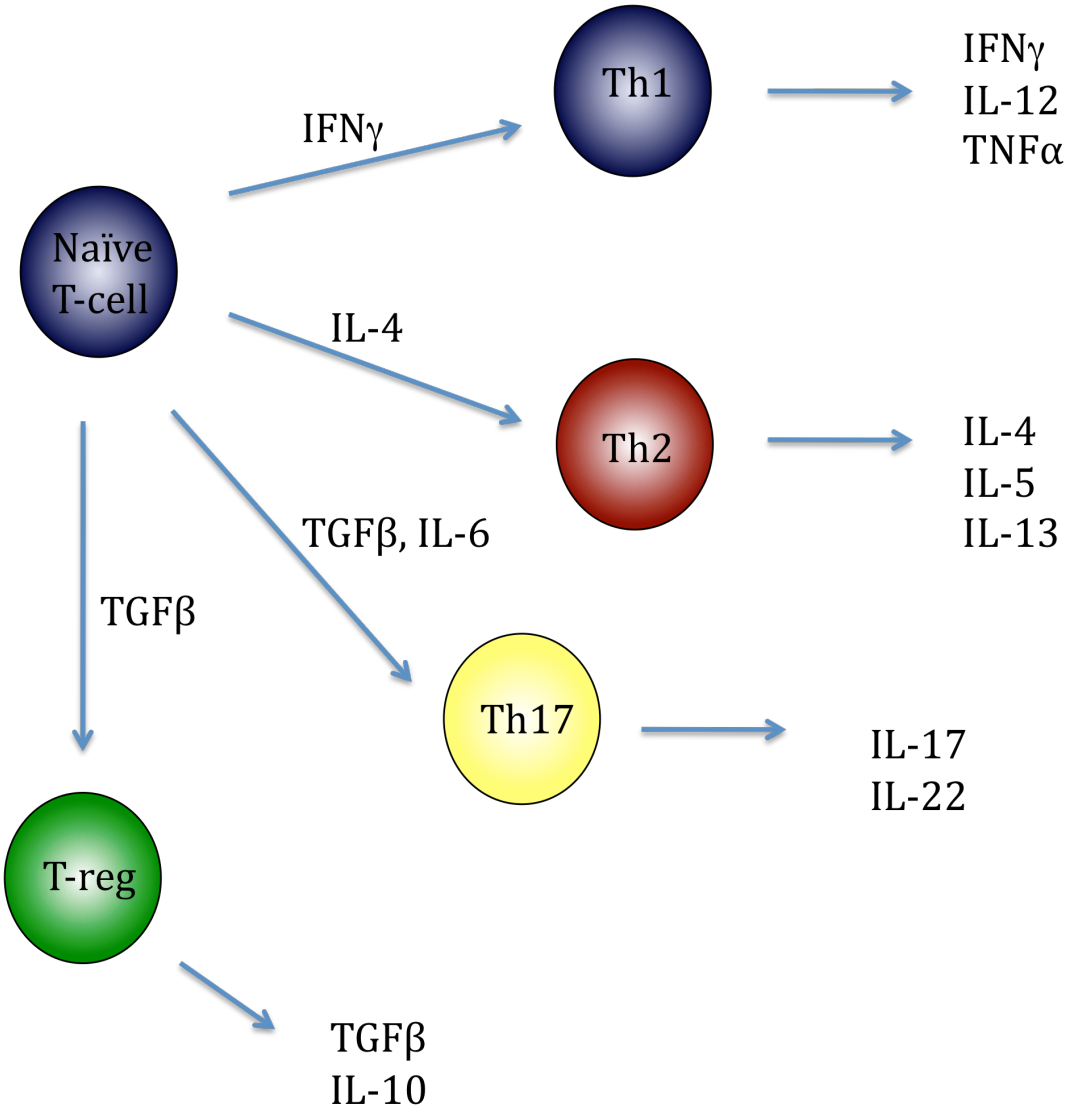


Figure 3: *Cytokine-driven differentiation of different T-cell subsets*. Naïve T-cells circulating in the periphery can be induced to differentiate to T-helper 1 (Th1) cells primarily via exposure to IFNγ, T-helper 2 (Th2) cells by IL-4, T-helper 17 (Th17) cells by TGFβ and IL-6 and T-regulatory cells (T-regs) can be induced by TGFβ. The main cytokines that these T-cell subsets secrete are shown.

Of particular importance to this study of co-infection is the fact that Th1 and Th2 responses induced by malaria and helminths respectively are counter-regulatory, such that mounting a strong response of one type suppresses the magnitude of the other (Mosmann and Sad 1996). In addition to Th1 and Th2 cytokines having inhibitory effects on one another helminth-driven induction of T-regs is also a mechanism through which responses to other infections such as malaria are

suppressed during co-infection (Tetsutani, Ishiwata et al. 2009; Metenou, Dembele et al. 2011). The modulation of Th1 responses in co-infection, apparent in both human (Hartgers, Obeng et al. 2009) and animal models (Su, Segura et al. 2005), has important implications for the severity of malaria disease. There is no consistent effect of helminth co-infection on malaria and it seems likely that the outcome is dependent on the pairings of different helminth and malaria species and host genotype (Helmby 2009). *Schistosoma mansoni*-malaria co-infection in particular has been well studied and can result in protection against cerebral malaria during *P. berghei* infection (Waknine-Grinberg, Gold et al. 2010). Similarly, schistosome infection reduced *P. chabaudi* associated mortality (Yoshida, Maruyama et al. 2000). However, *S. mansoni* in combination with these same malaria species has been reported to increase parasitaemia (Helmby, Kullberg et al. 1998; Legesse, Erko et al. 2004). Infection with the filarial nematode *L. sigmodontis* also elicits contrasting responses to malaria, exacerbating *P. chabaudi* parasitaemia (Graham, Lamb et al. 2005) yet protecting against challenge with *P. berghei* (Fernandez Ruiz, Dubben et al. 2009). The intestinal parasites *H. polygyrus* and *N. brasiliensis* seem to protect against the uncomplicated symptoms of malaria, for example anaemia and parasitaemia (Hoeve, Mylonas et al. 2009), hypothermia and hypoglycaemia (Segura, Matte et al. 2009).

Whilst understanding the influence of immune-mediated mechanisms of regulation on parasite growth is important, they are not the only means by which disease outcome may be affected. For a parasite the host is exploited as both a shelter and a food source and there is a strong likelihood that co-infecting parasites will compete for these resources. Consider malaria and helminths, *Plasmodium* absolutely requires host RBCs as a niche in which to replicate, whereas for many helminths these cells are a food source (Gilman 1982; Attout, Babayan et al. 2005). Hookworms in particular can cause severe anaemia (Hotez and Molyneux 2008) and this may impact on the availability of RBC for *Plasmodium* replication in a co-infection setting. Few studies consider both the resource- and immune-mediated mechanisms that may influence co-infection dynamics, although there are notable exceptions (Pedersen and Fenton 2007; Graham 2008).

1.5 Antibodies: technicality and serendipity of cross-reactivity.

The assays for measurement of antibodies are well established and I envisaged that using antibody isotypes as indicators of Th1/ Th2 bias in co-infection could provide a technically simple alternative to measuring other immune-molecules such as cytokines. The advantage of using antibodies is that they can be measured in small volumes of serum and are thus amenable to sampling in natural populations. One of the beauties of scientific research is that the findings can be unexpected, as is the case with the occurrence of cross-reactive antibodies that recognised antigens from a parasite they had never encountered. As I've mentioned something that fascinates me is how the host contends with the vast diversity of pathogens it is exposed to. The flexibility of these antibodies to bind different antigens (parasites) struck me as a potential way for the host to combat diverse parasites whilst conserving 'immune costs'. Of course this relies on the cross-reactive antibodies being functional *in vivo*. These cross-reactive antibodies also raise interesting immunological questions as to why they occur at all.

In theory, the diversity of B-cell receptors (BCR) and the process of somatic hypermutation enable the generation of an antibody that is perfectly matched to its cognate antigen (Pancer and Cooper 2006). The process of somatic hypermutation, which is initiated by antigen binding, is unique to B-cells and involves mutation of the antigen-binding site of the BCR. This 'new' receptor is selected for if it improves antigen binding and the culmination of this process is the secretion of antibodies of the same specificity as the BCR from plasma cells (Tarlinton and Smith 2000). If some constraint was imposed on somatic hypermutation, such as antigen availability or the numbers of rounds of B-cell division, it is feasible that cross-reactivity could occur. Imagine a baseball mitt that is moulded to the ball as the pitcher tosses the ball to and fro, repeated catches cement the impression of the ball in the leather so that it is unsuitable to catch other objects that it may have fit when brand new (e.g. a rubiks cube). The amount of catches determines the mitt's fit to the ball in the same way that binding to antigen determines the antibody's fit to antigen. With fewer catches

(exposure to antigen/ rounds of somatic hypermutation) the mitt may still fit other objects and an antibody may recognise other antigens (cross-react).

An alternative explanation for the occurrence of cross-reactive antibodies is that they are beneficial to the host. A beautiful example of when 'error' is beneficial is seen in the waggle-dance of the honeybee decoded by Karl von Frisch (Frisch 1967); foraging bees returning to the hive perform complicated dances, the angle and fervour of which communicate the direction and distance of food-sources. There is a certain amount of error in the dances repeated by an individual on returning to the hive (Okada, Ikeno et al. 2008) and this is thought to be an adaptation to encourage dance-followers to forage over a wider area than the dance directions convey (Weidenmuller and Seeley 1999), rather than an inability of bees to dance perfectly. This is analogous to antibody specificity/ cross-reactivity; if we suppose that the immune system is in fact capable of producing antibodies that are perfectly matched to their antigen then the maintenance of cross-reactivity ('error') is a strategy of the host to achieve a broader range of antigen recognition (binding) (Fairlie-Clarke, Shuker et al. 2009).

1.6 Are you a man or a mouse? : Animal models of malaria- helminth co-infection.

Dissecting the host-pathogen interactions that result in exacerbation or amelioration of disease in natural populations is complicated by variation in exposure to parasites, unknown infection histories and of course variation in host and parasite genotypes. Much of this variation can be controlled in animal models, which therefore provide a useful means of investigating the potential for immune- or resource-mediated mechanisms to influence disease outcome in co-infection. Although animal models are an invaluable tool care must nonetheless be taken in extrapolating the findings of these models to human disease, some of the reasons for this are discussed in more detail below.

It should thus be noted that no single species of murine malaria captures all the properties of *Plasmodium* infection in humans but by choosing particular combinations of laboratory mouse strain (e.g. BALB/c, C57BL/6, CBA) and parasite species (e.g. *Plasmodium berghei, Plasmodium yoelli, Plasmodium chabaudi*) many pathological aspects of the disease can accurately be reproduced. For example infection with *P. berghei* ANKA or *P. yoelli* 17XL results in cerebral malaria (Li, Seixas et al. 2001) reflecting one of the most severe complications of *P. falciparum* infection in humans. The characteristic symptoms of uncomplicated malaria, anaemia and fever associated with parasite replication, are reflected in *P. chabaudi* infection of C57BL/6 or BALB/c mice (Lamb, Brown et al. 2006), although in mice fever manifests as a drop in body-temperature (Li, Sanni et al. 2003). The diversity of helminth parasites in natural populations e.g. filarial, schistosome and hookworm infections are also represented in animal models (Yamada, Nakazawa et al. 1992; Hoffmann, Petit et al. 2000; Pearce and MacDonald 2002; Bungiro, Sun et al. 2008). Of greatest relevance to this thesis is the *N. brasiliensis* model that represents an acute hookworm infection and the *P. chabaudi* model of malaria in which asexual parasitaemia, anaemia and weight loss are all readily measured but cerebral malaria is not a symptom.

In combination these animal models provide systems in which to study the effects of co-infection on disease outcome. Importantly, animal models reflect the contrasting effects observed in human studies providing the opportunity to investigate the mechanistic causes of these different outcomes. Most murine studies of helminth co-infection report increases in malaria parasitaemia (Helmby, Kullberg et al. 1998; Su, Segura et al. 2005; Tetsutani, Ishiwata et al. 2009) and exacerbation of hepatosplenomegaly was also apparent in a murine model of Schistosome-malaria co-infection (Sangweme, Shiff et al. 2009). By contrast, a protective effect of *Schistosoma mansoni* on the cerebral malaria caused by *P. berghei* (Waknine-Grinberg, Gold et al. 2010) reflects the observation in humans co-infected with *Ascaris lumbricoides* and *P. falciparum* (Nacher, Gay et al. 2000). Two meta-analyses have attempted to make sense of some of the variation in these systems and draw general conclusions regarding the affect of helminth co-infection. The first

focussed on helminth-microparasite (viruses, fungi, bacteria or protozoa) co-infections and using a community ecology framework revealed that the control of microparasites was most severely impaired when the underlying helminth infection didn't impose resource limitation but strongly reduced the Th1 response (Graham 2008). The second concentrated on murine models of malaria-helminth co-infection and concluded that the response of hosts to malaria in single infection was important; if infection with malaria normally resolved then helminths increased malaria mortality as the host was unable to control malaria parasite replication. In contrast malaria-related pathology was reduced if in single infection the malaria was normally lethal (Knowles 2011).

Of course no animal model is perfect. There is dispute, for example, about the usefulness of *P. berghei* infection as a model for cerebral malaria as mice and humans exhibit different pathology that seems to be driven by different mechanisms; in mice there is an unequivocal role for pro-inflammatory cytokines in the development of cerebral malaria characterised by an accumulation of leukocytes and platelets in the brain whereas in humans blockage of the venules of the brain by sequestration of parasitized red blood cells is the main cause of cerebral malaria and the role of pro-inflammatory responses seems to be less important than in mice (White, Turner et al. 2010). Similarly, it should be noted that the parasitaemia associated with *P. chabaudi* is often much greater than that in human *Plasmodium* infection, where chronic low-level parasitaemia causes severe anaemia (Lamikanra, Brown et al. 2007). There are however similarities in the immune mediated mechanisms that influence anaemia and asexual parasitaemia in mice and humans. Increased levels of pro-inflammatory cytokines (e.g TNFα) are involved with suppression of erythropoiesis in mice (Li, Sanni et al. 2003) and an imbalance of the IL-10: TNFα ratio in Gambian children is associated with severe anaemia (Akanmori, Kurtzhals et al. 2000). Similarly the involvement of cytokines in Th1 cell-mediated control of parasitaemia is evident in mice and humans as is the protective function of cytophilic antibodies (Li, Seixas et al. 2001). Helminth infection models also have limitations; in some cases the mouse host does not support the full life cycle (Lawrence 1996) or if it does, the length of infection is

sometimes curtailed in comparison to the natural host (Maizels and Yazdanbakhsh 2003; Reece, Siracusa et al. 2008). However, the immune response to gastro-intestinal helminths in mice and humans is similar; a potent Th2 cytokine response is associated with enhanced eosinophilia and basophilia (Mearns, Horsnell et al. 2008; McSorley and Loukas 2010; Nel, Hams et al. 2011). Production of protective IgE antibody responses is also a common feature of helminth infection in these different hosts (Turner, Faulkner et al. 2005; Perona-Wright, Mohrs et al. 2008). Despite the caveats outlined here a wealth of research in animal models highlights their suitability for detailed investigation of immune responses throughout infection that would not be possible in humans. In this study I focus on a murine model of malaria-hookworm co-infection using the rodent malaria *Plasmodium chabaudi* and *Nippostrongylus brasiliensis*.

1.7 The *Plasmodium chabaudi- Nippostrongylus brasiliensis* co-infection model

The results presented in this thesis relate to the use of *Nippostrongylus brasiliensis* and *Plasmodium chabaudi* infection in BALB/c mice. I was particularly interested in investigating the potential for counter-regulation of Th1/ Th2 responses to be operating in this system. In order to minimise the induction of helminth associated T-reg responses I chose to use a parasite which in the mouse causes an acute infection i.e. *Nippostrongylus brasiliensis*. This helminth infection in mice captures the migratory phase of the infective larvae and their development to adults in the gut but rather than establishing a chronic infection, the adults are expelled within 7 days post infection ({Hoeve, 2009 #433} and Chapter 3). The blood stage of the rodent malaria parasite *Plasmodium chabaudi* (AS strain) was chosen as a model of asexual parasite replication within host RBC. The AS strain is considered to have moderate virulence {Mackinnon, 1999 #5} causing minimal mortality in BALB/c and thus allows the full course of infection to be monitored. Infection in this model is characterised by cycles of asexual replication that culminate in a peak of parasitaemia by day 10 post infection ({Hoeve, 2009 #433} and Chapter 3). This ‘acute’ phase of infection is largely controlled by pro-inflammatory cytokine responses (e.g., IFNγ and TNFα) and the initial peak of parasitaemia is resolved. It should be noted that cytophilic

antibodies are absolutely required for the ultimate resolution of infection without them mice fail to resolve the initial peak in asexual parasitaemia and suffer more severe recrudescence throughout infection {von der Weid, 1996 #82}.

1.8 Why this thesis?

Within the context of a larger project to investigate cytokine signalling and disease outcomes in the context of malaria- helminth co-infection I undertook the study reported in this thesis. I was motivated by an interest in understanding how hosts preserve their fitness when faced with infection by diverse parasites such as malaria and helminths. I envisaged that this would require management of the counter-regulatory Th1/ Th2 immune responses that had previously been reported in other co-infection systems. Using a murine model of malaria-hookworm co-infection and antibody isotypes as indicators of immune bias I discovered that the malaria-specific Th1 response was reduced during co-infection and interestingly that cross-reactive antibodies were induced by single parasite infection. Disease outcome was affected in a way that seemed counter-intuitive to the reduction in malaria-specific Th1 response, in that parasitaemia and anaemia were less severe. Intrigued by this and keen to acknowledge the importance of considering host-parasite interactions in a community ecology framework I investigated the potential for resource-mediated mechanisms to have a role in disease outcome. My findings indicated that in this model of co-infection the timing of helminth infection – and thus helminth-induced anaemia- may be particularly relevant in determining malaria disease outcome.

The serendipitous discovery of cross-reactive antibodies opened up several avenues of investigation; pertinent to disease ecology is the idea that these cross-reactive antibodies could have functional consequences in co-infection. Interesting immunological questions are also raised as to whether these cross-reactive antibodies arise because of a constraint on the immune processes that result in antigen specificity. It is also possible that these two distinct pathogens actually share antigens and western blots of antigen probed with *P. chabaudi* anti-sera provide evidence that this may indeed be the case. The final results chapter brings together

the themes and findings of the preceding chapters and addresses how increasing parasite dose may influence immune bias and antibody specificity/ cross-reactivity and is to my knowledge one of the few studies that considers this in the context of co-infection.

The order in which the results chapters of this thesis are presented reflects the development of my ideas and hypotheses and is summarised in Figure 4.

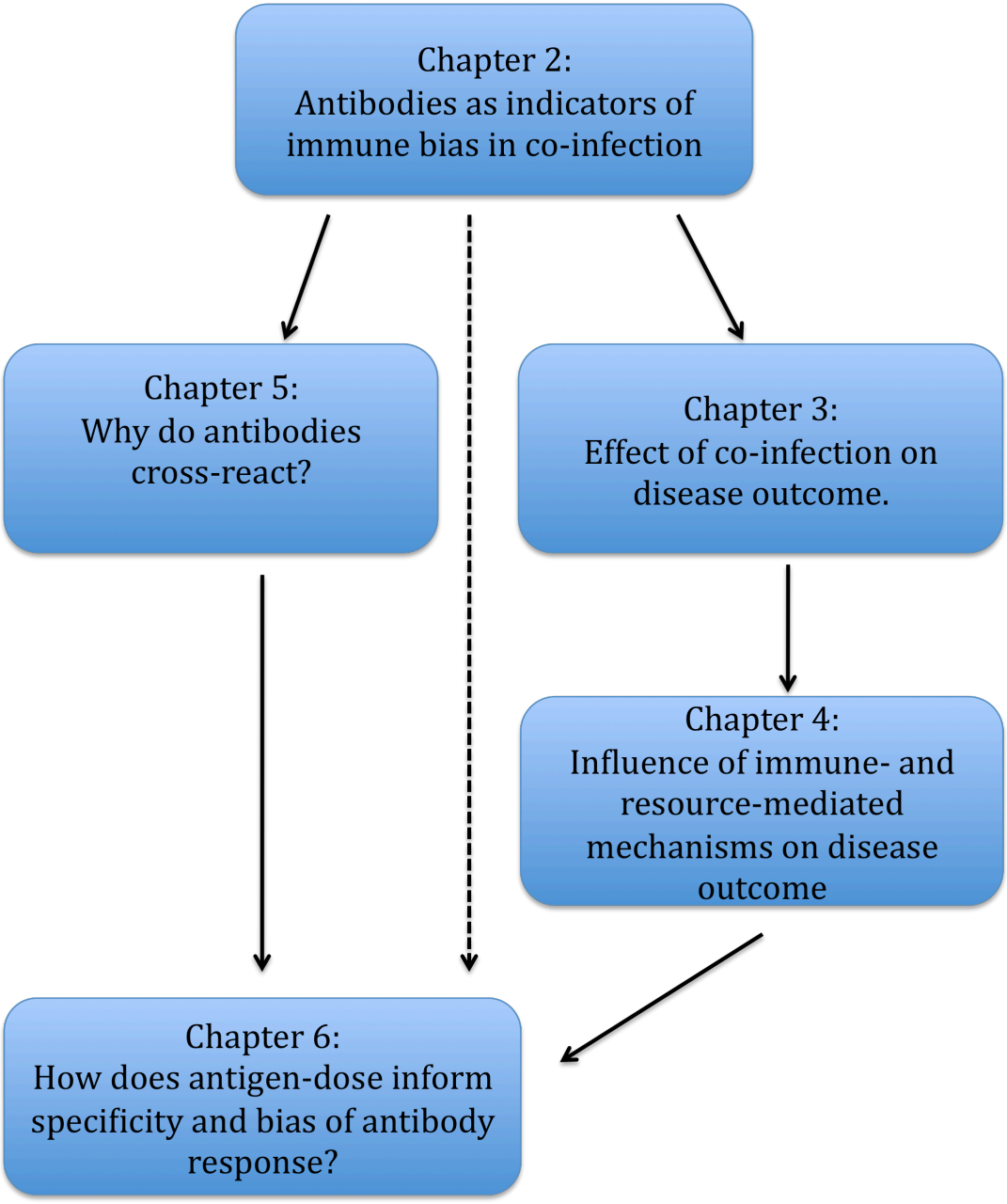


Figure 4: *Guide to the results chapters in this thesis.* Arrows indicate chapters that are linked by themes or findings of previous chapters.

Chapter 2: Antibody isotypes as indicators of immune bias.

2.1 Introduction

A wealth of research on infectious disease documents the need for different arms of the immune response to be deployed when combating bacteria, viruses and intracellular parasites (collectively 'microparasites') versus large extracellular organisms like parasitic helminths. Typically a T-helper 1 response (Th1) is induced by microparasites and a T-helper 2 (Th2) response during helminth infection (Abbas, Murphy et al. 1996; Miller, Smith et al. 2009; Kolbaum, Ritter et al. 2011). In addition Th17 cells have recently been described and are associated with the control of microbial infections (Liang, Tan et al. 2006). Importantly, T-regulatory cells (T-regs) prevent excessive inflammatory responses by suppressing the function of these effector T-cell subsets (Th1, Th2, Th17)(Pandiyan, Zheng et al. 2007). Th1 responses are characterised by the production of the cytokine IFNγ and the activation of cytotoxic and inflammatory responses (Mosmann and Sad 1996). For Th2 responses, the signature cytokines are IL-4, IL-5 and IL-13, which are associated with an increase in eosinophils, mast cells, basophils and alternatively activated macrophages (Jenkins and Allen 2010). Of fundamental importance is the fact that these Th1/Th2 responses are counter-regulatory which results in polarisation of the immune response toward Th1 or Th2 depending on the predominant cytokines in the milieu (Mosmann and Sad 1996). In individual infections polarisation of the immune response ensures the appropriate effector mechanisms are induced to combat infection. For a host co-infected with microparasites and helminths however both Th1 and Th2 responses are required and a skew in immune-bias toward Th1 or Th2 could have a significant impact on control of one of these infections. In addition helminths can stimulate expansion of T-regs (Finney, Taylor et al. 2007; Grainger, Smith et al. 2010), which could further influence immune bias through suppression of the Th1 response to microparasites (Metenou, Dembele et al. 2011). Parasite-induced polarisation of the immune response can also determine the host's response to other antigens, such as vaccines. Helminth-induced Th2 polarisation for example

impairs the protective Th1 response to tetanus and cholera vaccines and reduces their efficacy (Sabin, Araujo et al. 1996; Cooper, Chico et al. 2001; Nookala, Srinivasan et al. 2004). Similarly the challenge of creating a malaria vaccine is further complicated by an existing helminth-induced Th2 immune environment (Nacher 2001; Su, Segura et al. 2006). I am particularly interested in how helminth infection modulates the Th1 response necessary for the control of malaria (Li, Seixas et al. 2001) and use a murine model of malaria-hookworm co-infection (*P. chabaudi- N. brasiliensis*) to explore this.

In order to reach an understanding of how co-infection affects immune bias it is crucial to find a reliable indicator of both Th1 and Th2 responses. The distinct T-cell subsets that initiate the Th1 or Th2 response can be defined by their cytokine profile; an overproduction of IFNγ over IL-4 or IL-13 indicating Th1 for example. Whilst analysing cytokine production by antigen-specific T-cell recall assays (i.e. in-vitro stimulation of lymph node or spleen cell cultures with parasite antigens) can provide an accurate measure of Th1/ Th2 immune bias in murine models this requires sacrificing the individual. In malaria infection in particular it can also be difficult to assess T-cell responses due to immune-suppression associated with the peak of infection and apoptosis of splenocytes (Sanchez-Torres, Rodriguez-Ropon et al. 2001). Furthermore the production of cytokines by T-cells is often 'spiky' with a sharp peak being followed by a rapid waning of the response so their detection can be very sensitive to the time of sampling. It may be desirable to track the change in immune response throughout infection and for cytokines this requires sacrificing individuals at various timepoints. This greatly increases the number of animals required for an experiment and has the drawback that time-courses are not compiled from the same individual. Cytokine production can also be measured in serum but the sample volumes required for this can also prohibit repeated sampling. Ideally measuring immune bias should be achievable in a sample volume that facilitates repeated measures to be taken from the same individual, so that longitudinal data can be collected to further our understanding of changes throughout the course of infection.

Antibody isotypes are an attractive alternative to cytokines as indicators of Th1/Th2 bias as the cytokine environment drives their production by B-cells. In mice antibody isotype class switching in response to IFNγ results in B cells producing antibodies of the IgG2a isotype (Paul, Brown et al. 1987; Collins and Dunnick 1993; Else and Finkelman 1998), whereas the Th2 cytokine IL-4 switches B cells to produce IgG1 (Paul, Brown et al. 1987; Purkerson and Isakson 1992). Although the generation of IgG1 as a marker of Th2-type response is less definitive than IgG2a as a marker of a Th1-type response, the ratio of IgG1 to IgG2a provides a powerful indicator of immune bias (Da'Dara, Skelly et al. 2003; Arnold, Bumann et al. 2004; Li, Rush et al. 2004; Taylor, Ziman et al. 2007). IgE is also a useful marker of helminth-induced (Th2) responses (Perona-Wright, Mohrs et al. 2008; McCoy, Finkelman et al. 2010) and is driven by IL-4 production (Finkelman, Holmes et al. 1990). Measurement of antibody responses can be achieved with smaller sample volumes and poses fewer technical challenges than T-cell recall assays. Furthermore, antibody analysis captures more fully the history of infection as it reflects cumulative immunological activity, in contrast to the ex-vivo 'snapshot' of the immune environment provided by T-cell cytokine responses, which are readily altered by changes in the timing of sampling both *in vivo* and *in vitro*. Analysis of antibody responses of co-infected animals might therefore provide evidence of overall Th1-Th2 cell cross-regulation even when cytokine analysis may not.

This chapter focuses on the dynamics and interpretation of parasite antigen-specific antibody responses as indicators of immune bias in a host co-infected with *P. chabaudi* (*Pcc*) and *N. brasiliensis* (*Nb*). This type of data will provide the platform for investigating the overarching theme of this thesis, within-host ecology of immune responses during co-infection.

2.2 Experimental Design

Female BALB/c mice were infected with *Pcc* at a dose of $1x10^5$ parasitised RBC (i.p) with or without co-infection of 200 *Nb* L3 larvae (s.cut) on day 0. A group of mice infected with Nb only (200 L3 larvae s.cut) were also included in addition to a

control group, which received nRBC ($1x10^5$) and PBS as sham injections for *Pcc* and *Nb* respectively. There were 8 mice per infection group. Bodyweight, RBC density and asexual parasite density were measured daily throughout the course of infection. Serum was collected on day 20 p.i and ELISA was used to measure antigen-specific antibody responses to *Nb* crude antigen (NbA), crude malaria antigen (pRBC) and recombinant malaria antigen MSP-1_{19}. For details of how these antibody titres were calculated see Materials and Methods chapter.

To investigate the production of antibodies over the course of infection two further experiments were undertaken. The infection regime for singly or co-infected mice was as detailed above with the exception that one of these experiments continued until day 35 p.i. In addition to daily sampling of bodyweight, RBC density and asexual parasitaemia, 5µl of blood was taken from the tail daily and diluted in 5µl of heparin. This enabled a time-course of total IgG2a and IgE antibody production to be measured by ELISA, the concentration of each antibody isotype was calculated by comparison of optical density to a standard dilution series (see Materials & Methods chapter for details). Combining the data from these 2 time-course studies, excluding 11 mice with failed *Pcc* infection (peak parasite density <0.05 pRBC 10^9/ml) gave sample sizes of n=30 for *Nb*, n=39 for *Pcc*, n=29 for *Pcc*-*Nb* and n=18 for controls. See Box 1 for details of infections/ sample sizes from individual experiments.

BOX 1: Details of the number of infections from each of the 3 experiments that contribute to the data presented in this chapter. A note is also made of the contribution these experiments made to data presented in later chapters.

EXPERIMENT	Infection group	Sample size	Other chapters contributed to
1	Nb	8	Chapters 3 & 5
(Figure 1 & 2)	Pcc	8	
	Pcc-Nb	9	
	Control	4	
2	Nb	14	Chapter 3
(Figure 3 & 4)	Pcc	9	
	Pcc-Nb	7	
	Control	8	
3	Nb	16	Chapter 3
(Figure 3 & 4)	Pcc	30	
	Pcc-Nb	24	
	Control	10	

2.3 Statistical Analysis

The analyses of antigen-specific antibody responses at day 20 p.i were based on 24 mice (n=8 per infection group). All analyses were carried out in the statistical package JMP 8.0 (SAS) using generalised linear models (GLM). The generation of antibody titre creates ordinal data that were $\log_{10}$ transformed to satisfy the model assumptions of homogeneity-of-variance and normal distribution. Infection status (*Nb*, *Pcc* or *Pcc-Nb*) was included as a categorical factor and its ability to predict antibody response was formally evaluated. The maximal model was fitted first and minimal models were reached by sequentially removing non-significant terms (P-value > 0.05), beginning with interactions. Finally, whenever a factor was significant ($P < 0.05$), an All Pairs Tukey post-hoc test was carried out to identify

which groups of mice differed significantly in antibody induction, with respect to infection status.

To determine if differences in antibody isotype induction were apparent throughout infection I analysed the total antibody time-series data from two experiments combined. To analyse the time-series daily antibody concentrations (μg/ml) were averaged for each mouse and $\log_{10}$ transformed to satisfy the model assumptions of homogeneity-of-variance and normal distribution. Again GLMs were used for statistical analyses; main effects were worm presence or absence (*Nb*) and malaria presence or absence (*Pcc*) fitted as categorical variables, the main effects and the interaction between them (*Nb***Pcc*) test whether the response variable (antibody induction) is affected by *Pcc* infection, *Nb* infection or both. The effect of infection on immune response did not differ between experiments although the magnitude of responses did. Including "experiment" as a factor in all the analyses controlled for this difference between the two experiments. Maximal models were first fit to the data and the minimal models were reached by removing non-significant terms ($p > 0.05$) beginning with interactions. Significant interactions with "experiment" were removed if they were purely quantitative provided this did not alter the significance of the main effects remaining in the model. Finally, significant pairwise differences ($p < 0.05$) between groups were determined using the Tukey's All Pairs adjusted p-values for the most complex significant term in the minimal model (i.e. the interaction if significant).

2.4 Results

Antibody responses are effective indicators of immune bias in both single and co-infection

In the *Pcc-Nb* model of co-infection the nematode is cleared by day 7 and the peak of malaria parasitaemia is controlled by day 10 ((Hoeve, Mylonas et al. 2009) and Chapter 3 results). Antigen-specific serum antibody responses were measured by ELISA after 20 days of *Pcc-Nb* co-infection to investigate whether this could provide

a method to rapidly and quantitatively assess cumulated immune bias. In *Pcc*-only infected mice the typical Th1-cell response induced by malaria (Cavinato, Bastos et al. 2001; Su and Stevenson 2002) was reflected in the IgG2a response to the recombinant malaria antigen MSP-1_{19} (Figure 1a & Table 1) and a corresponding absence of the Th2 isotype (IgG1) to this antigen (Figure 1b). A significant up-regulation of IgG2a to the crude malaria antigen (pRBC) was not seen in *Pcc*-only mice when compared to *Nb* or *Pcc*-*Nb* mice (Figure 1c & Table 1). However a highly biased Th1-cell response to this antigen was indicated by the weak pRBC-specific IgG1 response (Figure 1d). In contrast, *Nb*-only infected mice had a characteristic Th2-biased antibody response (IgG1) to worm antigen (NbA) (Figure 1f & Table 1).

Having confirmed that the antibody response in single infections reflected the Th1 and Th2-cell biased response elicited by nematode and malaria infections respectively, I used antibody isotype analysis of IgG2a (Th1) and IgG1 (Th2) responses to both nematode and malaria specific antigens to determine if changes in bias due to co-infection could be detected in these responses. In other words would co-infection skew the Th1 cell response to *Pcc* to a more Th2 cell biased response and conversely, would the Th1 cell response induced by malaria have the capacity to alter the characteristic Th2-biased response to a nematode infection? The intermediate IgG1 response against NbA produced by co-infected mice (*Pcc*-*Nb*) (Figure 1f & Table 1) suggests that co-infection with *Pcc* down-regulated the anti-*Nb* specific Th2 response. It also appeared that responses in *Pcc*-*Nb* mice were further biased toward a Th1 cell response through the induction of IgG2a to NbA (Figure 1e & Table 1). However, the strong IgG2a response to the recombinant malaria antigen MSP-1_{19} detected in *Pcc*-only mice (Figure 1a) was also significantly reduced in *Pcc*-*Nb* mice (Figure 1a & Table1). These results strongly suggest that the isotype and hence cytokine bias of each single-species infection was significantly impacted by co-infection.

Levels of total IgE (Figure 2) support this observation where *Nb* mice have the highest level of IgE, *Pcc* mice are indistinguishable from control mice and the co-infected mice show intermediate levels. The immune bias that is apparent in serum

antibody isotype responses of *Pcc-Nb* infected mice is supported by cytokine responses in the lymph nodes of these mice, which were analysed as part of a different study. Again the bias of each single species infection was affected by co-infection; the *Pcc* associated IFNγ response (Th1) was significantly reduced in comparison to *Pcc*-only mice and the Th2 cytokines IL-13 and IL-5 were reduced in comparison to *Nb*-only mice (Hoeve, Mylonas et al. 2009).

TABLE 1: Results of GLM for antigen-specific and total IgE antibody responses at day 20 p.i. F statistics and P values of significant terms (P<0.05 denoted with *) come from the minimal model. Significant pairwise differences were determined from the Tukey's adjusted P-values of the most complex significant term in the model and are shown in Figures 1 and 2.

Response Variable	Factor	df	F ratio	P value
Anti-MSP-1_{19} IgG1	Infection	$F_{2,21}$	22.87	<0.0001*
Anti-MSP-1_{19} IgG2a	Infection	$F_{2,21}$	41.40	<0.0001*
Anti-pRBC IgG1	Infection	$F_{2,21}$	9.28	<0.0013*
Anti-pRBC IgG2a	Infection	$F_{2,21}$	2.55	0.1021
Anti-NbA IgG1	Infection	$F_{2,21}$	5.57	<0.0119*
Anti-NbA IgG2a	Infection	$F_{2,21}$	10.09	0.0009*
Total IgE	Infection	$F_{3,23}$	11.13	<0.0001*

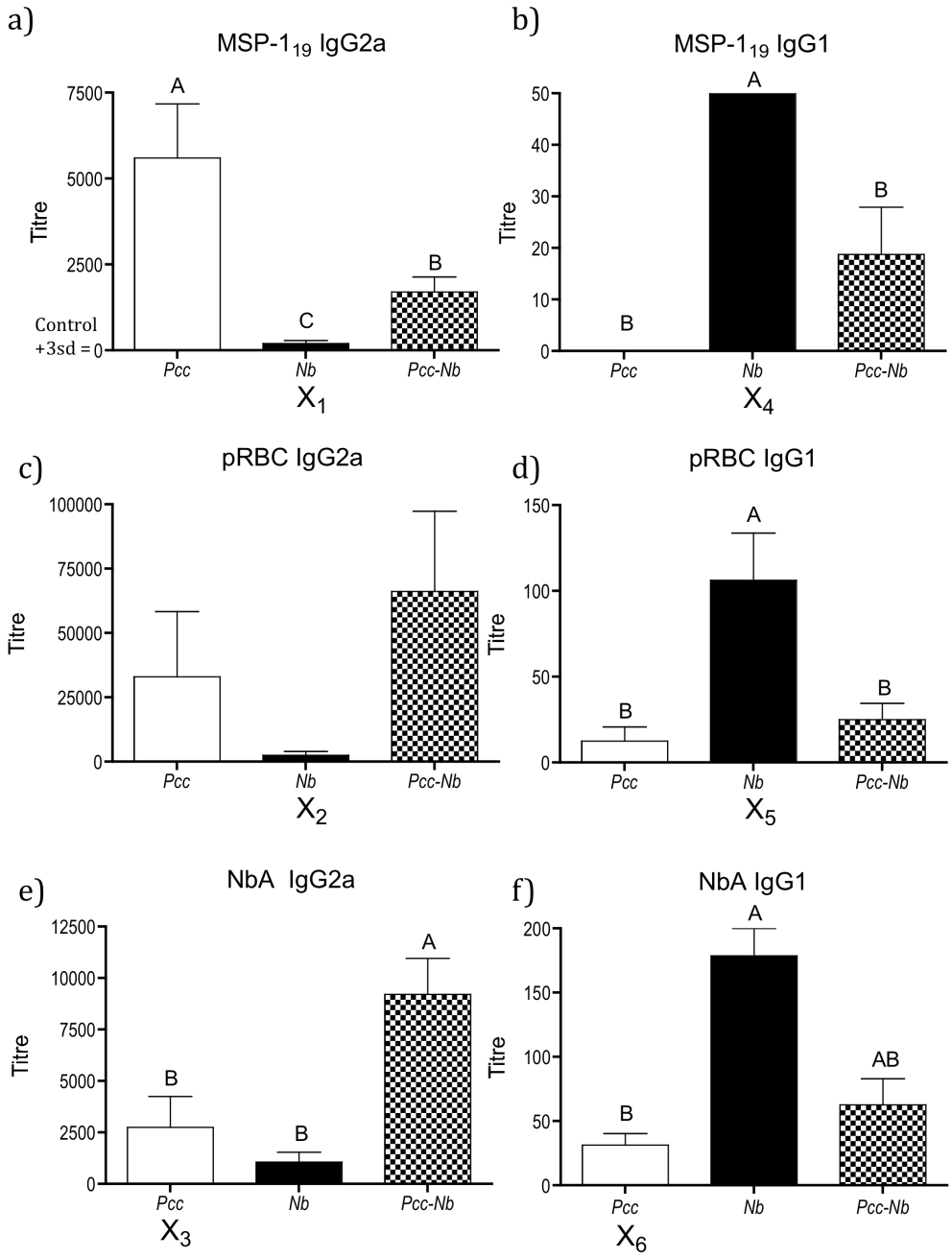


Figure 1: *Antibody isotype responses in infection and co-infection with* N. brasiliensis *and malaria.* Mice were infected with 200 *Nb* L3 larvae and/or 1x10^5 *Pcc*-infected RBCs on day 0. Serum antibody titres were measured at day 20 post-infection to worm antigen (NbA) and malaria antigens (MSP-1$_{19}$ and pRBC), Th1 responses indicated by IgG2a and Th2 responses by IgG1 are shown. All titres are above the mean +3 standard deviations of control mice at serum dilution of 1/200. This cutoff is represented as 0 on the y-axis. Single *Pcc* infections are shown in white bars, single *Nb* infections in black bars and co-infected mice (*Pcc-Nb*) are shown in the chequered bars. Graph shows mean and standard errors of 8 mice per group. Groups not connected by the same letter denote pairs that are significantly different ($p<0.05$) according to Tukey's Pairwise analysis. The letter X highlights those responses that are cross-reactive.

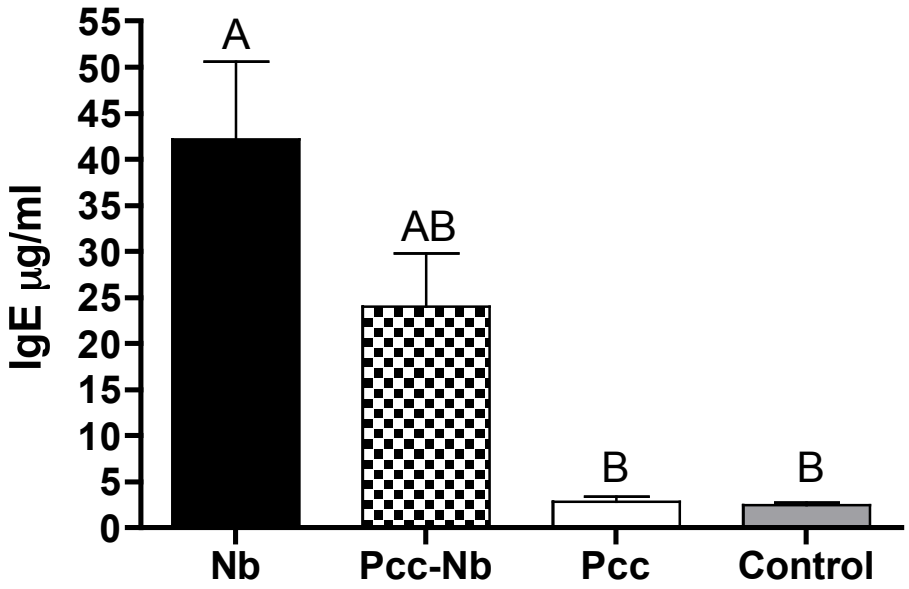


Figure 2: *Total IgE responses in infection and co-infection with* N. brasiliensis *and malaria.* Mice were infected with 200 Nb L3 larvae and/or 1x10^5 *Pcc*-infected RBCs on day 0. Serum antibody concentrations were measured at day 20 p.i. *Nb*-only mice are shown in black bars, single *Pcc* infection is shown in white bars and co-infected (*Pcc-Nb*) mice are shown in the chequered bars. Control mice are shown in grey. Graph shows mean and standard errors of 16 mice per group. Significant pairwise differences according to Tukey's adjusted P-values are indicated by letters. Groups that do not share letters are significantly different (P< 0.05 for all comparisons).

Cross-reactive antibody responses are observed in Pcc-Nb *co-infection*

Interestingly in the course of analyzing antibody responses I observed cross-reactivity, whereby *Nb* mice mounted detectable IgG2a and IgG1 responses to both recombinant and crude malaria antigens, which they were not exposed to during infection (indicated by X_1 & X_2 in Figures 1a & 1c and X_4 & X_5 in Figures 1b & d, respectively). The magnitude of these cross-reactive responses is particularly striking, with *Nb*-induced IgG2a titres against crude and recombinant malaria antigens reaching 2500 and 200 respectively. Also the cross-reactive *Nb*-induced IgG1 response to malaria antigens is comparable to the *Nb*-specific response (Figure

1f). Similarly, *Pcc* mice mounted cross-reactive responses to NbA (X_3 in Figure 1e and X_6 in Figure 1f). It is important to note that these titres although low are markedly greater than background responses (more than 3 standard deviations above mean concentration in control mice, which is represented as zero on the y-axis).

Determining the qualitative and quantitative aspects of the cross-reacting antibody responses are not only important for the practical analysis of immune bias but could be of real biological relevance during co-infection. As outlined above, antibody responses were biased, in terms of isotype, by infection status. The bias in isotype due to a particular infection (Th2 associated IgG1 induced during *Nb* infection, for example) was extended to non-specific antigens, as seen in the IgG1 response of *Nb* mice to both MSP-1_{19} and pRBC (X_4 and X_5 in Figures 1b & 1d). This raised the concern that cross-reactive antibodies may hinder the interpretation of immune bias in co-infection. However in the majority of cases, calculating titre from serial dilutions of sera and capitalising on the differences in strength of antigen-specific and cross-reactive responses clarified interpretation of immune bias in co-infected mice. For example, *Pcc*-specific IgG2a titres in *Pcc* mice were significantly higher than the cross-reactive response induced in *Nb* mice (Figure 1a). Thus, although *Nb* mice made cross-reactive IgG2a responses, these were no longer detectable with increasing dilution of sera (Figure 1a). Similarly, IgG1 responses to NbA were significantly higher in *Nb* mice than the cross-reactive response induced by *Pcc* mice (Figure 1f).

I concluded from this analysis that cross-reactive IgG1 responses to NbA were only detectable at dilutions less than 1:100 and thus higher dilutions could be used to avoid cross-reactivity when assessing antigen-specific antibody isotype profiles for the purpose of interpreting immune bias. However, increasing sera dilution did not always overcome the cross-reactivity observed, as IgG2a responses to NbA in *Pcc* mice were still observed at 1:2500 and did not differ significantly from the titre in *Nb* mice (X_3 in Figure 1e). This cross-reactivity warrants further investigation, as it is likely to be important biologically. Indeed even cross-reactive responses detectable only at high serum concentrations may still have functional relevance in vivo.

Antibody responses measured over time demonstrate dynamic changes in immune bias

One advantage of using antibody responses as indicators of Th1/ Th2-cell bias is that unlike cytokines they can be measured in small sample volumes e.g. 2-5μl – one tenth of that required for cytokine assays. This enabled repeated measures to be taken from the same animal so a profile of the antibody response throughout infection could be determined. To gain an overall picture of antibody responses in the different infection groups total (rather than antigen-specific) responses were measured by ELISA. IgM, IgG1, IgG2a and IgE responses were measured but as the IgM and IgG1 means were not different amongst the groups (data not shown) only the IgG2a and IgE data are discussed here. As expected from the analysis of antigen-specific data at day 20 p.i *Pcc* infection (*Pcc* or *Pcc*-*Nb*) resulted in production of IgG2a above the level seen in control or *Nb*-only mice from day 10 onwards (Figure 3a). Similarly, *Nb* (single or co-infection) showed production of IgE above the level measured in control or *Pcc*-mice from approximately day 8 p.i onwards (Figure 3b). Interestingly the down-regulation of IgE production evident in co-infected mice (Figure 2) is apparent from day 10 p.i but perhaps most striking at day 16 p.i. (Figure 3b) In an attempt to quantify these profiles of antibody production the average daily level of antibody was calculated for each mouse.

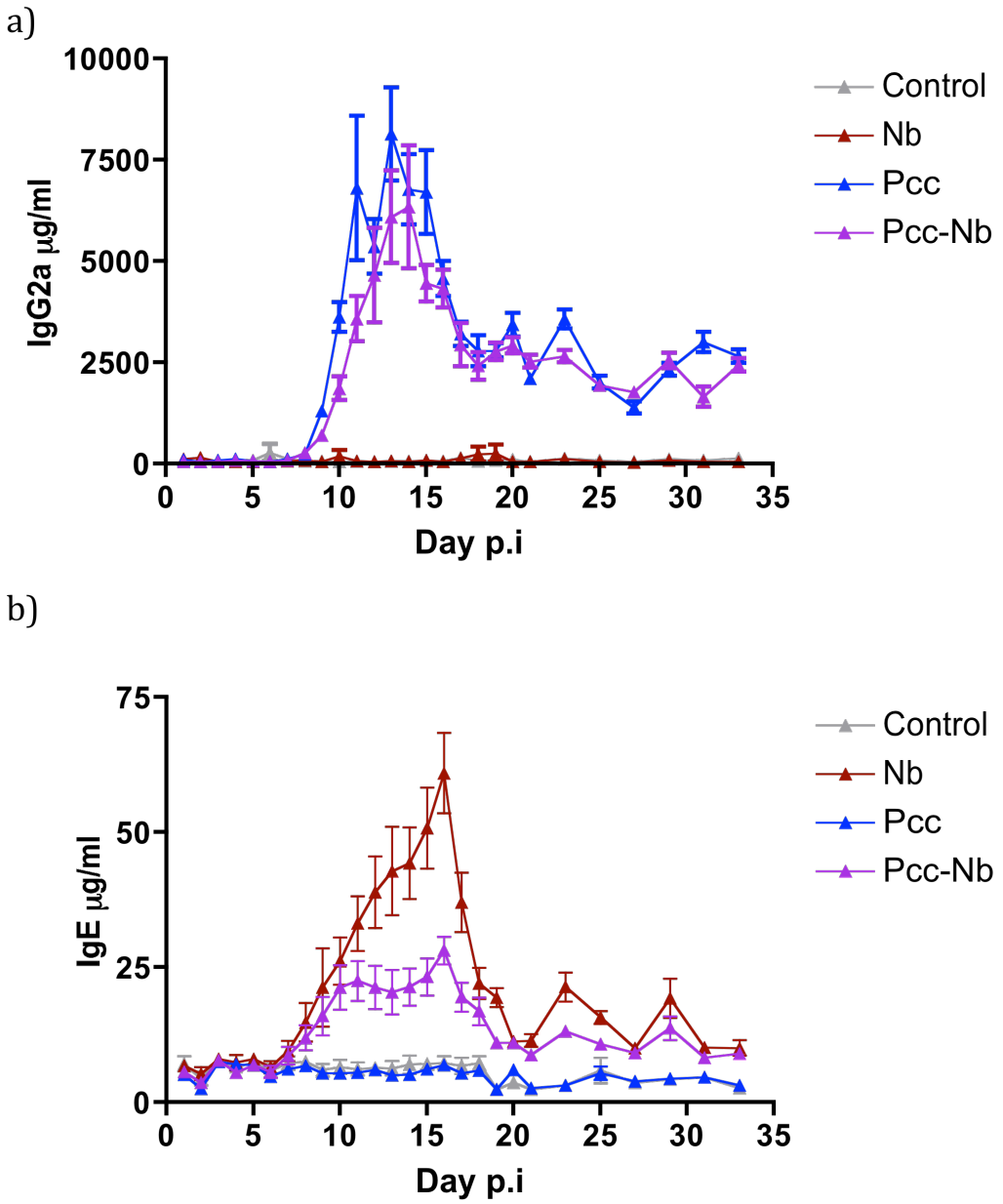


Figure 3: *Timecourse of total antibody responses throughout infection and co-infection with* N. brasiliensis *and malaria.* Mice were infected with 200 *Nb* L3 larvae and/or 1x10^5 *Pcc*-infected RBCs on day 0. Total antibody responses in serum were measured daily. IgG2a (Th1) responses a) and IgE (Th2) responses b) are shown. *Nb*-only mice are shown in red n=30, *Pcc*-only mice in blue n=39, co-infected (*Pcc-Nb*) mice in purple n=29 and control mice in grey n=18. Graph shows mean and standard errors of daily antibody concentrations (µg/ml).

For IgG2a the average daily antibody concentration in *Pcc* mice (singly or co-infected) was significantly greater than that of *Nb*-only and control mice (Figure 4a & Table 2). The lack of difference between *Pcc* and *Pcc-Nb* mice reflects the antigen-specific response to crude malaria antigen (pRBC) (Figure 1c). This summary of the antibody profile throughout the course of infection revealed significant differences in the level of IgE production between *Nb* and *Pcc-Nb* infected mice (Tukey's adjusted P-value 0.0302)(Figure 4b and Table2). Interestingly the down-regulation of IgE in co-infected mice seen from day 10 to day 16 p.i which was captured by analysis of average daily antibody concentrations (Figure 4b) was also evident in antibody responses measured in serum from the terminal bleed collected at the end of the infection, day 20 or day 35 (Figure 4c and Table 2). It should be noted that end point serum was collected through exsanguination via the brachial artery whereas daily samples were taken from the tail. The different source of sera may explain why day 33 timepoints (Figure 3b) do not reflect the differences evident at day 35 (Figure 4c). Examining immune responses throughout infection is extremely valuable for understanding the dynamics of changes in immune-bias. The fact that endpoint serum antibody concentrations accurately reflected changes occurring midway through infection highlights the use of antibodies as indicators of cumulated immune bias. Importantly measuring a time-series of antibody responses or analyzing endpoint responses are both compatible with measuring the course of parasitology and pathology to determine disease outcome.

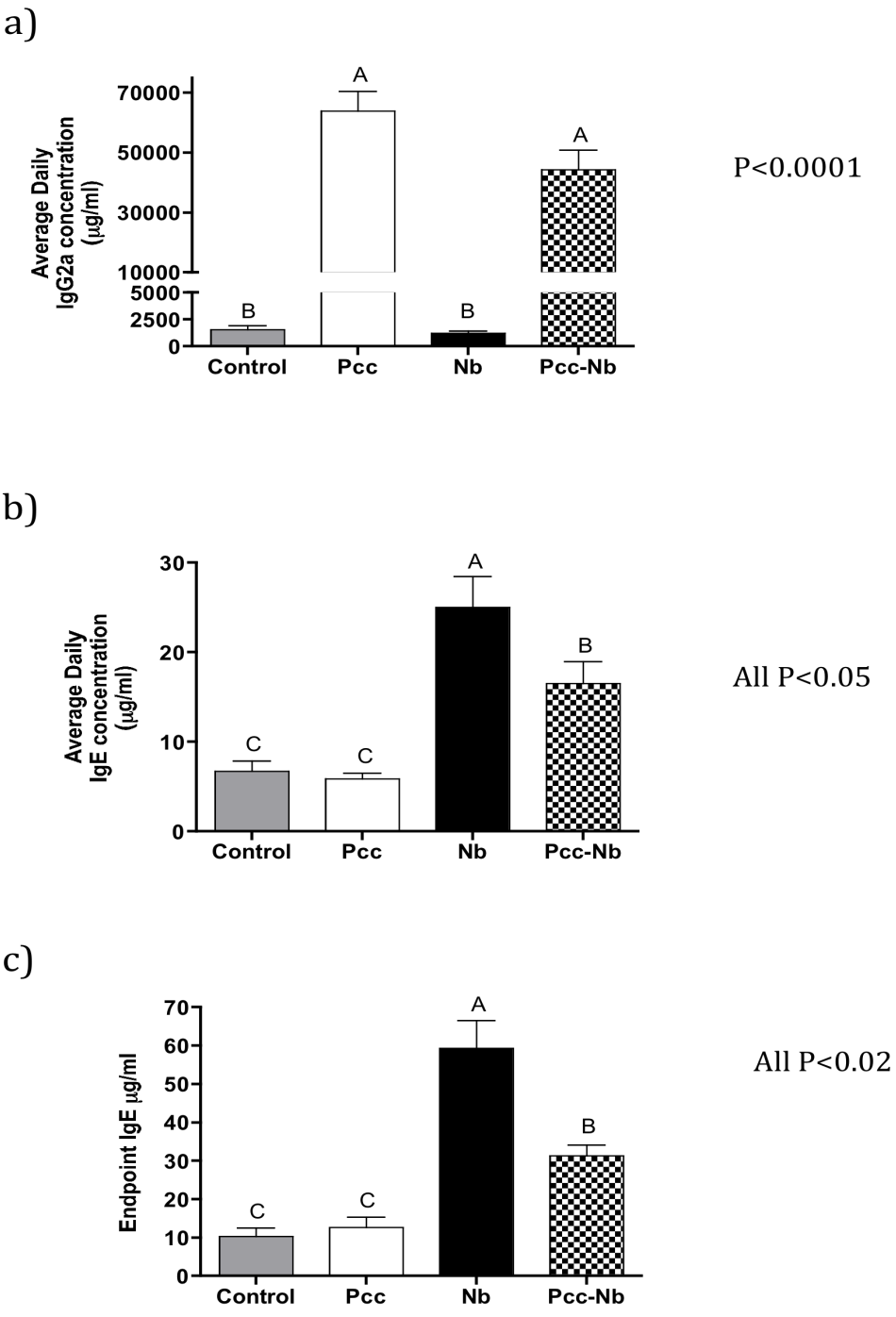


Figure 4: *Total antibody responses throughout infection and co-infection with* N. brasiliensis *and malaria*. Daily average concentrations for IgG2a a) and IgE b). Total IgE responses at the end of infection are shown c). *Nb*-only mice are shown in black bars n=30, *Pcc*-only mice in white bars n=39 and co-infected (*Pcc-Nb*) mice in chequered bars n=29. Control mice are shown in grey n=18. Graph shows mean and standard errors of antibody concentrations (µg/ml). Significant pairwise differences according to Tukey's adjusted P-values are indicated by letters. Groups that do not share letters are significantly different (P values for all comparisons are indicated).

TABLE 2: Results of GLM for the average daily production of total antibodies and the level of IgE antibody measured in serum harvested by terminal bleed (END DAY). Non-significant terms come from the last model before term was dropped and are shown in square brackets. F statistics and P values of significant terms (P<0.05 denoted with *) come from the minimal model. Significant pairwise differences were determined from the Tukey's adjusted P-values of the most significant complex term in the model and are shown in Figure 4.

Response Variable	df	Factor	F ratio	P value	Experiment effect size estimates (mean ± SE)
total IgG2a					
	$F_{1, 113}$	Experiment	887.48	<0.0001*	-0.87 ± 0.029
	[$F_{1, 112}$	Nb	0.90	0.3442]	
	$F_{1, 113}$	Pcc	628.00	<0.0001*	
	[$F_{1, 111}$	Nb*Pcc	0.23	0.6301]	
total IgE					
	$F_{1, 111}$	Experiment	81.97	<0.0001*	0.17 ± 0.019
	$F_{1, 111}$	Nb	160.07	<0.0001*	
	$F_{1, 111}$	Pcc	2.46	0.1196	
	$F_{1, 111}$	Nb*Pcc	4.86	0.0295*	
END DAY IgE					
	$F_{1, 111}$	Experiment	2.19	0.1417	
	$F_{1, 111}$	Nb	52.85	<0.0001*	
	$F_{1, 111}$	Pcc	6.31	0.0134*	
	$F_{1, 111}$	Nb*Pcc	11.21	0.0011*	

2.5 Discussion

In natural populations co-infection with multiple parasites is the norm and can result in either exacerbation or amelioration of disease (Petney and Andrews 1998; Cox 2001). Co-infection often exerts an influence on the host's immune response by altering the polarization of the Th1 and Th2 cell response; in various systems microparasites have been shown to down-regulate production of Th2 cytokines and helminths to suppress Th1 responses (Brady, O'Neill et al. 1999; Liesenfeld, Dunay et al. 2004; Kolbaum, Ritter et al. 2011). Whilst analysis of cytokine responses is definitive in describing Th1 and Th2-cell subsets it is not without its drawbacks; measurement of antigen-specific cytokine production by T-cells requires culture of spleen or lymph node cells and to obtain these in murine models involves sacrificing the host. Even measurement of cytokines in serum may necessitate sacrificing the animal due to the sample volumes required to perform these assays. In addition

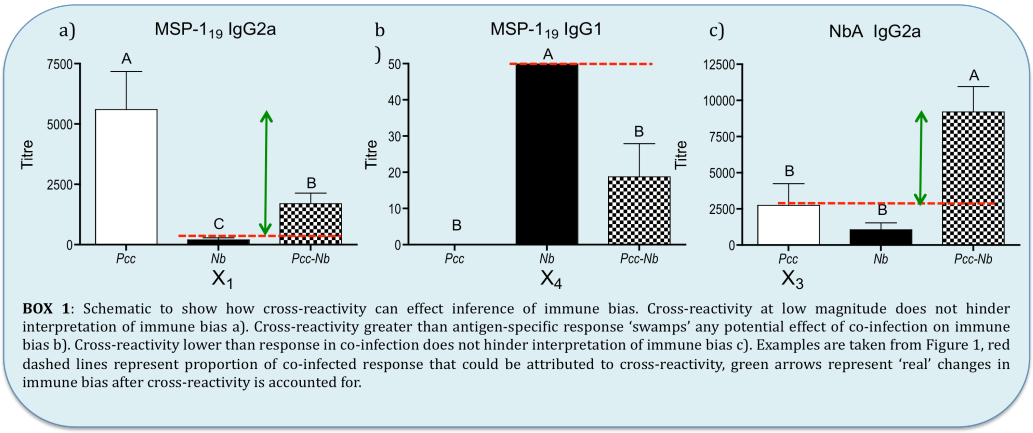
sudden spikes and rapid falls in cytokine production mean that the response must be sampled at its peak otherwise it could go undetected. The need to sacrifice mice to sample cytokine responses at their peak is incompatible with observing the full course of infection and thus the consequence of immune effects on parasite control or pathology. In human studies serum cytokine analysis is a viable option and avoids the technical challenges of harvesting and storing T-cells in the field. However cytokines can be sensitive to collection and storage methods and this may complicate their accurate measurement from field isolates (Flower, Ahuja et al. 2000; Kenis, Teunissen et al. 2002).

The cytokine environment directly influences antibody isotype production (Finkelman, Holmes et al. 1990) and as antibodies are more robust to freeze-thaw and can be measured in small volumes of serum they are a useful alternative to cytokines as indicators of immune bias. Analysis of antigen-specific antibody isotype reflected the Th1 or Th2 bias in mice singly infected with *Pcc* or *Nb* respectively; IgG2a (Th1) responses to *Pcc* antigens were enhanced in *Pcc*-only mice and conversely IgG1 (Th2) responses to worm antigen were significantly greater in *Nb*-only mice. The interpretation of Th1/Th2 antibody bias in co-infected animals was complicated by cross-reactivity and this is not a problem unique to the co-infection model used here (Naus, Jones et al. 2003; Lamb, Graham et al. 2005; Pierrot, Wilson et al. 2006; Fairlie-Clarke, Lamb et al. 2010). It is worth noting that cross-reactive responses were observed regardless of whether recombinant or crude antigens were used. Conclusions can still be drawn regarding the influence of a co-infecting parasite on immune bias using serum antibodies even when cross-reactivity is observed but this relies on the calculation of antibody titres to capitalise on the relative differences in antibody production amongst the different infection groups. In practical terms the change in bias due to a co-infecting parasite can be inferred by subtracting the cross-reactive response in single infection from that of co-infected mice. It is important to emphasise the need to calculate titre, as many models that aim to dissect the real effect of a co-infecting parasite on immune bias rely on antibody analysis at a fixed serum concentration, previously determined to fall within the linear range of the dilution curve (Rodriguez, Terrazas et al. 1999; Mutapi,

Ndhlovu et al. 2000; Mwatha, Jones et al. 2003; Naus, Jones et al. 2003; Lamb, Graham et al. 2005; Miller, Smith et al. 2009; Tetsutani, Ishiwata et al. 2009; Kolbaum, Ritter et al. 2011). This approach is usually taken in the interests of saving time and reagents as these kinds of studies use large numbers of animals to detect significant differences in antigen-specific responses between single and dual infection. This could prove to be a false economy as it may provide insufficient information to distinguish cross-reactive from bias-altering immune responses.

When antibody titres were calculated in this *Pcc*-*Nb* study, I was able to determine whether apparent alterations in antibody isotype profile on co-infection were due to actual changes in parasite-specific responses or reflected a cross-reactive response. For example, determining that cross-reactive IgG2a antibody titres in *Nb* mice were significantly lower than the antigen-specific response of *Pcc* mice meant that cross-reactivity contributed very little to the titre observed in *Pcc*-*Nb* mice (see Box 1 Figure a). This allowed me to conclude that the reduction in Th1 type antibody in *Pcc*-*Nb* mice was due to suppression of *Pcc*-specific Th1 responses by nematode infection. It is worth noting that had I not calculated titre and relied on optical density data derived from a single dilution of sera I may not have observed the difference between *Pcc* and *Pcc*-*Nb* mice and thus incorrectly concluded that there was no effect of co-infection on Th1 responses. Similarly, analysis of antibody titre enabled me to detect the reduction in anti-NbA IgG1 antibody in *Pcc*-*Nb* mice (Figure 1f), which suggests a *Pcc*-mediated bias toward a Th1 cell response. Antibody cross-reactivity poses more of a problem for the interpretation of changes in immune bias due to co-infection when it is of the isotype not typically associated with the parasite it recognises i.e. Th2 (IgG1) responses to malaria antigens or Th1 (IgG2a) responses to *Nb* antigens. For example, due to the magnitude of the *Nb*-induced IgG1 response to MSP-1_{19} it is impossible to tell if the response in *Pcc*-*Nb* mice is due entirely to this cross-reactivity or is a 'real' effect of Nb infection on cytokine and so antibody bias (see Box1 Figure b). The *Pcc*-induced IgG2a cross-reactive response to NbA is of a similar magnitude to the antigen-specific response of *Nb*-mice, yet the response in co-infected mice is significantly greater so even after

taking into consideration the proportion of this response that could be deemed cross-reactive there is still a significant effect of *Pcc* on immune bias (see Box 1 Figure c).



BOX 1: Schematic to show how cross-reactivity can effect inference of immune bias. Cross-reactivity at low magnitude does not hinder interpretation of immune bias a). Cross-reactivity greater than antigen-specific response 'swamps' any potential effect of co-infection on immune bias b). Cross-reactivity lower than response in co-infection does not hinder interpretation of immune bias c). Examples are taken from Figure 1, red dashed lines represent proportion of co-infected response that could be attributed to cross-reactivity, green arrows represent 'real' changes in immune bias after cross-reactivity is accounted for.

The induction of cross-reactive antibodies has important implications with regard to biological function. For example immune responses to malaria infection are typically characterised by a Th1 type (IgG2a) response, as we observed for *Pcc*-induced responses to the malaria antigens (pRBC and MSP-1$_{19}$). The propensity for *Nb* mice to induce atypical IgG1 antibody isotypes to malaria antigens is likely due to the helminth parasite promoting Th2 cytokines in the environment where the antibody response is established (e.g. lymph nodes)(Perona-Wright, Mohrs et al.). The biological consequences of the *Nb* driven IgG1 response to malaria antigens remains to be investigated, but as cytophilic IgG2a antibodies convey greater protection against malaria parasites than IgG1 antibodies (Su and Stevenson 2000; Cavinato, Bastos et al. 2001) there is the potential for cross-reactive *Nb*-induced IgG1 antibodies to be detrimental to parasite clearance and thus have real consequences in terms of disease outcome. The potential for cross-reactive responses of the appropriate isotype to have a functional role during infection raises the intriguing possibility that their production is a deliberate strategy of the host to combat diverse parasites (Fairlie-Clarke, Shuker et al. 2009).

In summary, the results presented in this chapter demonstrate that analysis of antibody isotype titres can be used to indicate Th1/Th2 immune bias in co-infection. Overall co-infected hosts exhibit intermediate antibody responses: *Pcc*-specific Th1

(IgG2a) responses were lower than *Pcc*-only mice and Th2 responses (*Nb*-specific IgG1 and total IgE) were reduced compared to *Nb*-only infected mice. Such intermediate responses are not intuitive when considering Th1-Th2 cross-regulation and the polarising effect of parasites. However, it should be noted that Th1/Th2 polarisation may well be compartmentalised to the lymph nodes at the site of infection (e.g mesenteric lymph nodes for gastro-intestinal *N. brasiliensis* infection) (Lamb, Graham et al. 2005), whereas serum antibody responses represent systemic effects. Ultimately, the intermediate Th1/Th2 bias observed in *Pcc-Nb* mice likely reflects the need to mount both Th1 and Th2 responses as a result of infection. The question of how the reduction in Th1 bias will affect control of *Plasmodium chabaudi* in co-infection will be the focus of the next chapter. Furthermore the antibody cross-reactivity observed in this system raises interesting biological, ecological and biochemical questions, which I aim to address throughout this thesis – Do these cross-reactive antibodies have functional relevance in co-infection? Is the production of cross-reactive antibodies a deliberate strategy of the host to conserve immune 'costs' of targeting diverse parasites? Is there a shared epitope on the antigens of these two distinct pathogens? These last two questions allude to adaptations of the host or parasite and the constraints that are placed on them. For example if cross-reactivity is a deliberate strategy of the host to combating diverse parasites this adaptation places a constraint on the parasites as they may fail to evade immune targeting. In contrast if parasites do indeed share an antigenic epitope, perhaps due to the parasites' adaptation to living in a particular niche within the host (e.g. circulatory system) this places a constraint on the host's ability to discriminate between these two parasites.

Chapter 3: Effect of co-infection on malaria parasitaemia and anaemia (host pathology).

3.1 Introduction

The geographical and socio-economic distribution of malaria overlaps with that of parasitic helminths and co-infections in these areas are common (Brooker, Akhwale et al. 2007; Mazigo, Waihenya et al. 2010). Co-infection with helminths has been shown to affect the severity of malaria in humans but the outcome is not consistent, as both amelioration and exacerbation have been observed (Nacher, Gay et al. 2000; Nacher, Singhasivanon et al. 2002; Le Hesran, Akiana et al. 2004; Lyke, Dicko et al. 2005). Perhaps this variation is not so unexpected in human populations where host heterogeneity, environment, species of co-infecting parasite, level of exposure and timing of infection may all contribute to outcome (Supali, Verweij et al. 2010). In addition immune pleiotropy may explain the heterogeneity of disease outcomes. For example the anti-inflammatory cytokine IL-10 and T-regulatory cells (T-regs) can impair control of parasites by reducing Th1 responses (Walther, Tongren et al. 2005) but may also serve to protect the host from excessive inflammatory reactions (Walther, Jeffries et al. 2009) that are associated with cerebral malaria (Awandare, Goka et al. 2006).

Different rodent malaria models accurately reflect many pathological aspects of the disease in humans, for example infection with *P. chabaudi* can be used as a model for uncomplicated malaria (parasite replication and associated anaemia), whilst *P. berghei or P. yoelii* are useful models of cerebral or complicated malaria (Li, Seixas et al. 2001). As discussed in the general introduction, exact mechanisms underlying the development of malaria pathology may differ between mice and humans. The parasitaemia associated with *P. chabaudi* is often much greater than that in human *Plasmodium* infection, where chronic low-level parasitaemia causes severe anaemia (Lamikanra, Brown et al. 2007). However there are important similarities in the immune mediated mechanisms that influence anaemia and asexual parasitaemia in mice and humans. Increased levels of pro-inflammatory cytokines (e.g TNFα) are

involved with suppression of erythropoiesis in mice (Li, Sanni et al. 2003) and an imbalance of the IL-10: TNFα ratio in Gambian children is associated with severe anaemia (Akanmori, Kurtzhals et al. 2000). Similarly the involvement of cytokines in Th1 cell-mediated control of parasitaemia is evident in mice and humans as is the protective function of cytophilic antibodies (Li, Seixas et al. 2001). Experimental animal models thus provide an opportunity to investigate potential mechanisms of how co-infection influences the outcome of malaria. As with humans, murine malaria-helminth co-infection results in differential effects on the control of parasite growth, or development of pathology associated with malaria infection. *S. mansoni* and *H. polygyrus* have been shown to exacerbate *P. chabaudi* and *P. yoelii* infection in terms of parasitaemia and host mortality (Helmby, Kullberg et al. 1998; Su, Segura et al. 2005; Helmby 2009; Sangweme, Shiff et al. 2009; Tetsutani, Ishiwata et al. 2009), whilst in other studies *S. mansoni* protects from cerebral malaria (Waknine-Grinberg, Gold et al. 2010) and reduces host mortality (Yoshida, Maruyama et al. 2000).

Many of these studies highlight the importance of immunological mechanisms in mediating the effect of co-infection on disease outcome. In murine models initial control of the erythrocytic stage of malaria parasites relies on the induction of a Th1 response via IFNγ and TNFα activation of macrophages (Stevenson, Huang et al. 1992; Li, Sanni et al. 2003). For the ultimate resolution and clearance of malaria infection B-cells and antibodies are absolutely required (von der Weid, Honarvar et al. 1996); in particular antibodies of the IgG2a (Th1) isotype are protective as they neutralise free merozoites and opsonise parasitised RBC for uptake by macrophages (Mota, Brown et al. 1998; Cavinato, Bastos et al. 2001). However, the protective pro-inflammatory Th1 response must be carefully controlled via the anti-inflammatory cytokines IL-10 and TGFβ to minimise the risk of immunopathology to the host (Stevenson, Huang et al. 1992; Li, Sanni et al. 2003). During co-infection helminths may negatively impact the response to malaria by weakening protective Th1 responses or positively influence the outcome by antagonising inflammatory responses. For example Su et al (Su, Segura et al. 2005) report that *H. polygyrus* infection leads to a reduction in IFNγ and impairment of the protective *P. chabaudi*-

specific IgG2a response. Infection with *S. mansoni* was associated with a switch to an anti-inflammatory Th2 environment and amelioration of cerebral pathology caused by *P. berghei* (Waknine-Grinberg, Gold et al. 2010). Helminth-induced dysregulation of the host's response to malaria has repercussions in terms of controlling parasite replication, which in addition to influencing host health/ disease outcome has implications in terms of parasite transmission and epidemiology because of effects on gametocytes (Noland, Graczyk et al. 2007).

I have shown that in the murine model of malaria-hookworm (*Pcc-Nb*) co-infection the *Pcc*-specific Th1 response is reduced, as indicated by analysis of antigen-specific antibody responses (see Chapter 2 results). In this chapter I focus on how this decrease in Th1 response affects the outcome of *Pcc-Nb* co-infection in terms of host pathology (anaemia, measured by loss of RBC density) and *Pcc* asexual parasitaemia. My hypothesis was that reduced IgG2a antibody production, indicative of reduced IFNγ levels, will increase the severity of *Pcc* infection in a manner similar to that reported by Su et al regarding the effect of *H. polygyrus* on *Pcc*, i.e. via increased peak parasite density (Su, Segura et al. 2005).

3.2 Experimental Design

Female BALB/c mice were infected on day 0 with *Pcc* at a dose of $1x10^5$ parasitised RBC with or without co-infection of 200 L3 *Nb* larvae. A group of mice infected with Nb only (200 L3 larvae s.cut) were also included in addition to a control group, which received nRBC ($1x10^5$) and PBS as sham injections for *Pcc* and *Nb* respectively. Bodyweight, RBC density (anaemia) and asexual parasite density (parasitaemia) were measured daily throughout the course of infection, which lasted a minimum of 20 days (see Materials and Methods chapter for details). Bodyweight was monitored as an indicator of host health and was primarily used to ensure that animals were fit to progress through infection in accordance with Home Office regulations. Animals lost weight both during peak of malaria infection and also co-incident with *Nb* larvae migrating through the lung {Hoeve, 2009 #433}. This data

was not formally analysed as due to the continued growth of the animals it was more variable and less informative as a measure of host pathology.

3.3 Statistical Analysis

The analysis of RBC density and asexual parasitaemia was performed on data compiled from 5 experiments (see Table 1 for details), and was carried out in the statistical package JMP 8.0 (SAS) using generalised linear models (GLM). Following the methods of Hoeve et al (Hoeve, Mylonas et al. 2009) the minimum RBC density data were analysed in two time frames, the first week post-infection (day 0-6 p.i inclusive) and the entirety of the infection. After exclusion of any mice with failed *Pcc* infection (peak parasite density < 0.05 pRBC 10^9/ml) the analysis focussed on 234 mice; n=65 *Pcc*-*Nb*, n=71 *Pcc*, n=62 *Nb* and n=36 controls. The main fixed effects of the model were worm presence or absence (*Nb*) and malaria presence or absence (*Pcc*) fitted as categorical variables. 'Experiment' was fitted as a random effect and the model was run with and without this factor to determine the difference between the -2 log likelihood measure for the two models. The significance of this difference was tested by Chi-square with 1 degree of freedom. The main effects and the interaction between them (*Nb***Pcc*) test whether the response variable (minimum RBC density) was affected by *Pcc* infection, *Nb* infection or both. To account for differences in initial RBC density, Day 0 values were included as a fixed covariate. Maximal models were first fit to the data and the minimal models were reached by removing non-significant terms ($p > 0.05$) beginning with interactions. Finally, significant pairwise differences ($p<0.05$) between groups were determined using the Tukey's All Pairs adjusted p-values for the most complex term in the minimal model.

Asexual parasitaemia was analysed only for animals experiencing *Pcc* infection (i.e. *Pcc*-*Nb* n=66, *Pcc* n=71). The effect of co-infection on *Pcc* peak parasite density was evaluated by GLM. Worm presence or absence (*Nb*) was included as a fixed categorical factor in the model. Experiment was again included as a random effect in the model (see above). Finally, significant pairwise differences ($p<0.05$) between

groups were determined using the Tukey's All Pairs adjusted p-values for the most complex term in the minimal model.

TABLE 1: Details of the number of infections from each of the 5 experiments that contribute to the host pathology data presented in this chapter. A note is also made of the results chapter in which corresponding immunology data can be found. Those experiments that only contribute to pathology data were initially conducted as part of a larger project to investigate cytokine responses.

EXPERIMENT	Infection group	Sample size	Other chapters contributed to
1	Nb	8	Chapter 2&5
	Pcc	8	
	Pcc-Nb	9	
	Control	4	
2	Nb	14	Chapter 2
	Pcc	9	
	Pcc-Nb	7	
	Control	8	
3	Nb	16	Chapter 2
	Pcc	30	
	Pcc-Nb	24	
	Control	10	
4	Nb	14	-
	Pcc	14	
	Pcc-Nb	16	
	Control	6	
5	Nb	10	-
	Pcc	10	
	Pcc-Nb	9	
	Control	8	

3.4 Results

Co-infection reduces peak Pcc *parasitaemia and the severity of associated anaemia.*

Asexual malaria parasitaemia was measured on a daily basis by microscopic examination of Giemsa stained blood smears. For both singly- (*Pcc*) and co-infected (*Pcc-Nb*) mice the peak of parasitaemia occurred on day 8 p.i. However, parasite density was significantly reduced in *Pcc-Nb* mice on this day (effect of *Nb* on peak parasite density $F_{1,127}$ =4.23, P=0.0417) (See Figure 1a and Table 2). Peak parasitaemia was associated with a loss in RBC density (anaemia) (effect of *Pcc* on minimum RBC density throughout infection $F_{1,226}$=2234.04, P<0.0001) in all animals infected with *Pcc* as asexual parasite replication within the RBC leads to cell rupture and the release of new merozoites resulting in a reduction in RBC numbers. However, the trough of anaemia on day 10 p.i was less severe in *Pcc-Nb* mice than in the singly infected animals (Tukey's pairwise analysis *Pcc-Nb* vs *Pcc* P=0.0230) (See Figure 1b).

Nb-*infection induces significant anaemia in the first week post-infection.*

Monitoring RBC density on a daily basis revealed not only the expected anaemia associated with the peak of *Pcc* infection but also a significant reduction in RBC density in *Nb*-infected mice in the early stages of infection (effect of *Nb* on minimum RBC density to day 7p.i $F_{1,225}$=15.77, P<0.0001; see Figure 1b and Table 2). This *Nb*-induced RBC loss was resolved over the course of infection in *Nb*-only mice but as noted above *Pcc-Nb* mice endured further *Pcc*-induced RBC loss albeit less severe than *Pcc*-only mice.

TABLE 2: Results of GLM for anaemia and malaria parasitology data. Non-significant terms come from last model before term was dropped and are shown in square brackets. F statistics and P values of significant terms (P<0.05) come from the minimal model with experiment fitted as a random effect. Significant pairwise differences were determined from the Tukey's adjusted P-values of the most complex significant term in the minimal model. Experiment was fitted as a random effect and the model was run with and without this factor to determine its significance. The test statistic in this case is the difference between the -2log likelihood measure for the two models and its significance was tested by Chi-square (Chi-square P value). All effect size estimates are taken from the minimal model with experiment included as a random effect.

Response Variable	Factor	df	Test statistic (F-ratio or Chi-square)	P value	Chi-square P value	Effect size estimates (mean ± SE)
RBC minima (to d6 p.i inclusive)						
	Day 0 RBC	$F_{1,225}$	62.87	<0.0001*	-	0.417±0.05
	Nb	$F_{1,225}$	15.76	<0.0001*	-	0.09±0.02
	[Pcc	$F_{1,224}$	0.13	0.7176]	-	
	[Pcc*Nb	$F_{1,223}$	1.05	0.3055]	-	
	Experiment	1	96.32	-	0.001	0.132±0.097
RBC minima (to end day)						
	[Day 0 RBC	$F_{1,223}$	2.35	0.1272]	-	
	Nb	$F_{1,226}$	3.05	0.0821	-	
	Pcc	$F_{1,226}$	2234.04	<0.0001*	-	2.056±0.04
	Pcc*Nb	$F_{1,226}$	3.62	0.0584	-	
	Experiment	1	62.75	-	0.001	0.193±0.143
Peak parasite density						
	Nb	$F_{1,127}$	4.23	0.0417*	-	0.046±0.02
	Experiment	1	98.9	-	0.001	0.149±0.108

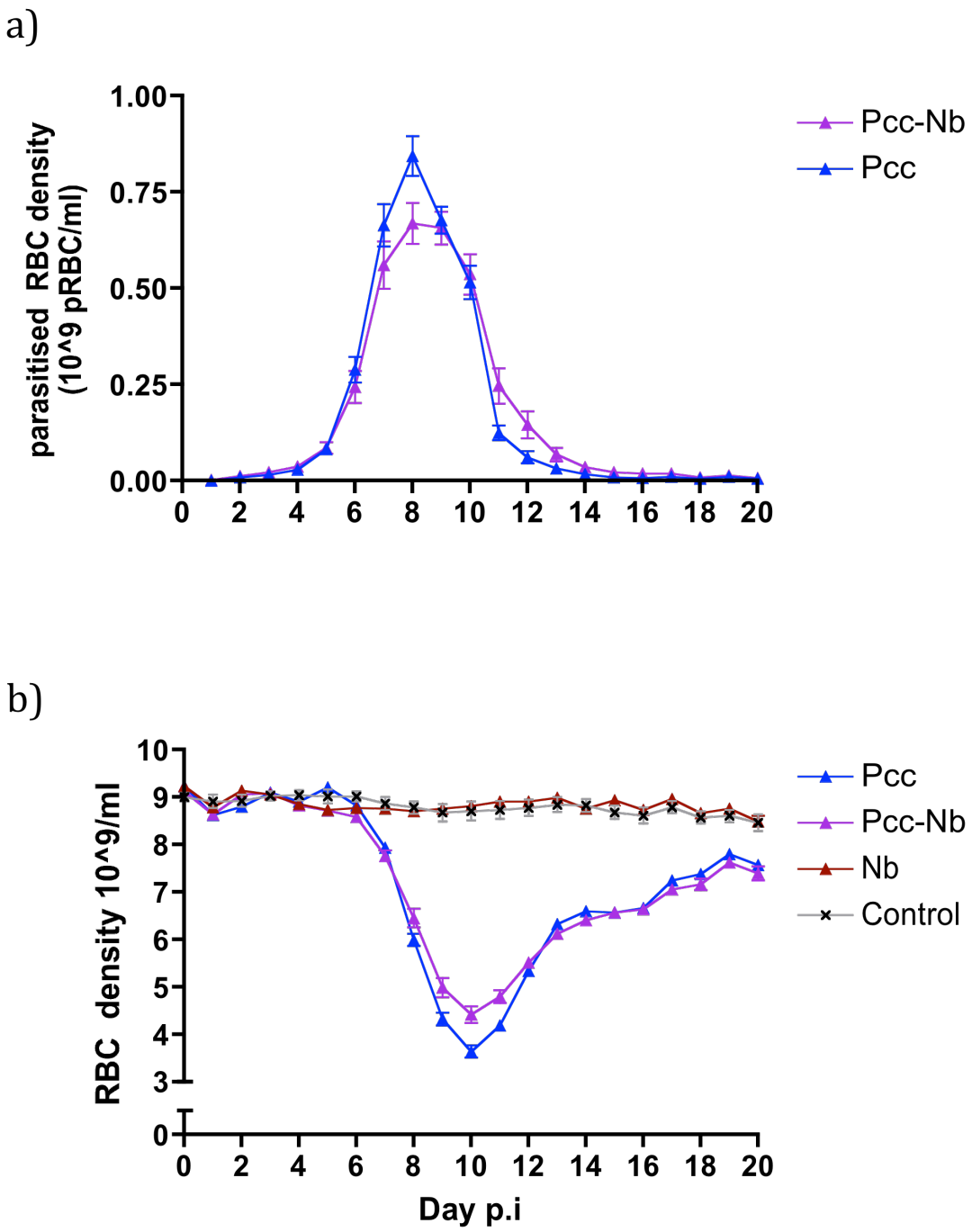


Figure 1: Malaria parasitaemia and anaemia timecourses.
Daily parasitised RBC density throughout infection a) *Pcc*-mice are shown in blue and co-infected (*Pcc-Nb*) mice in purple. Graph shows mean and standard error of n=66 (*Pcc-Nb*) and n=71 (*Pcc).* Daily RBC density throughout infection b) *Nb* mice are shown in red, *Pcc*-mice in blue, *Pcc-Nb* mice in purple and controls in grey. Graph shows mean and standard error of n=66 (*Pcc-Nb*), n=71 (*Pcc*), n=62 (*Nb*) and n=36 (control).

3.5 Discussion

Co-infection often exerts an influence on the host's immune response by altering the polarization of the Th1 and Th2 cell response, for example in various systems helminths have been shown to suppress Th1 responses (Brady, O'Neill et al. 1999; Tetsutani, Ishiwata et al. 2009; Kolbaum, Ritter et al. 2011). This helminth-induced reduction in Th1 responses can significantly impact disease outcome; for example exacerbation of the bacteria *Bordetella pertussis* was observed in mice co-infected with *Fasciola hepatica* (Brady, O'Neill et al. 1999), whilst infection with *Strongyloides ratti* reduced infection with *Trypanosoma brucei (Onah, Onyenwe et al. 2004)*. Of relevance to the co-infection model used here both amelioration and exacerbation of disease have also been observed in helminth-malaria co-infections (e.g. (Helmby, Kullberg et al. 1998; Yoshida, Maruyama et al. 2000; Legesse, Erko et al. 2004; Helmby 2009; Sangweme, Shiff et al. 2009; Waknine-Grinberg, Gold et al. 2010)).

Previously (Chapter 2 and (Fairlie-Clarke, Lamb et al. 2010)) I have shown that *Pcc-Nb* co-infection results in a reduced Th1 bias as measured by *Pcc*-specific IgG2a. As antibodies are absolutely required for the clearance of malaria parasites (von der Weid, Honarvar et al. 1996) and IgG2a in particular has been shown to be protective against the asexual stage of *Pcc* (Cavinato, Bastos et al. 2001; Mota, Brown et al. 2001) I hypothesised that malaria parasitaemia would be increased and the associated anaemia would be worse in co-infected mice. This prediction however was not borne out; co-infected mice showed a significant reduction in peak parasitaemia and a corresponding reduction in the trough of RBC loss. This suggests that despite the overall reduction in Th1 immune-bias the host is able to control *Pcc* infection as efficiently, if not better than a singly infected host. Co-infected mice most likely experienced less severe anaemia as they harboured fewer parasites and so suffered less destruction of RBC through parasite replication. It is also possible that a reduction in the pro-inflammatory Th1 response contributed to the effect if less cytokine (TNFα)-driven suppression of erythropoiesis (Li, Seixas et al. 2001)

enabled co-infected mice to compensate better for the parasite-induced anaemia. The reduction in malaria-specific Th1 response being associated with reduced peak parasitaemia is not easy to reconcile. A recent meta-analysis of the murine literature suggests that *Pcc*-helminth co-infection more commonly results in exacerbation of *Pcc* parasitaemia (Knowles 2011). For example, *S. mansoni* paired with *P. berghei, P. yoelii or P. chabaudi* resulted in increased peak parasitaemias (Helmby, Kullberg et al. 1998; Legesse, Erko et al. 2004; Sangweme, Shiff et al. 2009) as did the combination of *H. polygyrus* and *P. chabaudi* (Su, Segura et al. 2005). This increased parasitaemia was attributed to reduction in the Th1 response, in those studies where it was measured (Helmby, Kullberg et al. 1998; Su, Segura et al. 2005). However these studies used chronic helminth infections rather than acute infections such as *Nb* and in some cases the host genotype (C57BL/6) differed from that used here (BALB/c). Perhaps the Th2 response induced by chronic helminth infection is of greater magnitude than that of acute *Nb* infection and therefore results in greater down-regulation of the Th1 response to malaria leading to more severe parasitaemia. In other words the reduction in Th1 response during *Pcc-Nb* co-infection although statistically significant may not be biologically relevant. This however does not account for the fact that peak parasitaemia was significantly reduced. In humans there are reports that co-infection with *Schistosoma haematobium* reduces the parasite density of *Plasmodium falciparum* (Briand, Watier et al. 2005). This may be due to co-infection increasing the production of protective anti-malarial antibodies (Diallo, Remoue et al. 2010). Although I did not see an increase in *Pcc*-specific antibody in co-infected mice (Chapter 2 (Fairlie-Clarke, Lamb et al. 2010)) there is perhaps potential for cross-reactive antibodies induced by *Nb* to act in concert with the *Pcc*-induced response. Both IgG1 and IgG2a cross-reactive antibodies are induced by *Nb*-infection and although vaccine-induced IgG1 has been shown to be protective against malaria (Burns, Flaherty et al. 2004) it is the IgG2a isotype that is more commonly involved in antibody-mediated protection against *Pcc* (Cavinato, Bastos et al. 2001).

Of course the effect of co-infection is not limited to its influence on immune responses. Helminth infection can also limit RBC availability and this has been

shown to limit parasite replication (Lwin, Last et al. 1982). The reduction of RBC density induced by *Nb* in the early stages of infection (to d6 p.i) is likely caused by haemorrhaging, as the larvae migrate into the airspace of the lungs prior to being coughed up and swallowed (Marsland, Kurrer et al. 2008). Gross examination of the lungs of a cohort of mice from this experimental set-up that were culled at days 3, 5 and 7 p.i revealed substantial haemorrhaging and leakage of blood into the airways (Hoeve, Mylonas et al. 2009). RBCs are a key resource for *Pcc* as they provide the niche in which asexual parasite replication occurs and the availability of RBCs for new merozoites to invade is crucial in propagating this process (Hetzel and Anderson 1996). The timing of *Nb*-induced reduction in the availability of RBCs may therefore hinder the establishment and replication of *Pcc*-parasites culminating in reduced peak parasitaemia in co-infection. The transient *Nb*-induced anaemia may be too small to directly influence the actual number of RBCs available for *Pcc* invasion but it may alter their age-structure and reduce the number of mature RBCs that *Pcc* preferentially invades (Ott 1968).

Immune- and resource-mediated effects of co-infection are not necessarily mutually exclusive mechanisms (Graham 2008) but it would be interesting to quantify the role of each and determine which (if any) has the more potent effect. This is impossible with the infection regime used in this experiment, as I cannot distinguish the effect of *Nb*-induced RBC depletion early in infection from the down-regulation of the Th1 response and induction of cross-reactive antibodies. The next chapter aims to address this by manipulating the timing of *Nb*-infection -and thus the timing of *Nb*-induced anaemia- to separate resource- and immune-mediated effects.

Chapter 4: Untangling the influence of 'top-down' and 'bottom-up' mechanisms of regulation in co-infection.

4.1 Introduction

The terms 'top-down' and 'bottom-up' to describe interactions amongst organisms are borrowed from community ecology and are commonly used to describe the processes which govern ecosystem structure (Smith, Hunter et al. 2010). The classic example is the regulation of hare populations via food availability and predation by lynx, where 'bottom-up' interactions refer to the availability of plants and 'top-down' to predation levels (Stenseth, Falck et al. 1997). If we apply this analogy to host-pathogen interactions then immune-mediated pathogen killing takes on the role of predator (top-down) and host resources the plants (bottom-up). Both of these mechanisms can be influenced by co-infection. Parasites may compete with each other for host resources, such as the niche they occupy: in horses, competition for space in the caecum between two species of gastro-intestinal helminths reduced the number of the helminth species that in the absence of competition favoured this site (Stancampiano, Mughini Gras et al. 2010). Such resource-competition is also evident amongst polystome parasites of clawed toads and, depending on the composition of the competing species, can lead to the competitive-exclusion of one parasite species (Jackson, Pleass et al. 2006). Co-infecting species can also exert an effect on host immune responses; for example, an entomopathogenic fungus of leaf-cutter ants, releases toxins that inhibit immune responses and increase infection with a second fungal pathogen (Hughes and Boomsma 2004). In general, research on the within-host interactions that influence disease outcome focuses on the influence of one or other of these mechanisms, but seldom both.

The counter-regulatory nature of Th1 and Th2 responses that are required to combat microparasites and helminths (Mosmann and Sad 1996) makes the immunological setting of co-infection with these two types of parasite particularly interesting. Helminth infection is also associated with a general dampening of immune

responses, brought about by the induction of T-regulatory cells (T-regs), which suppress T-effector cells. This appears to be an adaptation of the helminth to achieve chronic infection in the host. Indeed, some species can persist for several years (Bradley and Jackson 2004; Zaccone, Fehervari et al. 2006; Figueiredo, Barreto et al. 2010; Taylor, Hoerauf et al. 2010). This helminth-induced regulation of immune responses via T-regs is also evident in mice (Maizels, Balic et al. 2004; Gillan and Devaney 2005; Grainger, Smith et al. 2010). The implications for helminth-induced reduction or suppression of immune responses that target micro-parasites has been the focus of several experimental studies in mice and reveals a whole range of effects on micro-parasite infection; amelioration (Waknine-Grinberg, Gold et al. 2010), exacerbation (Brady, O'Neill et al. 1999; La Flamme, Scott et al. 2002; Graham, Lamb et al. 2005; Helmby 2009) or even a neutral outcome (Yoshida, Maruyama et al. 1999; Liesenfeld, Dunay et al. 2004), have all been reported. A recent meta-analysis performed on helminth-microparasite co-infections in laboratory mice confirms these varying effects of helminth infection on microparasite growth (Graham 2008). More importantly, this study highlights the need to consider both top-down and bottom-up mechanisms of microparasite control when trying to predict the effect of co-infection (Graham 2008), as the relative influence of helminth infection on immune- or resource-mediated mechanisms will depend on the ecology of the microparasite it is paired with. The immuno-modulatory effects of helminths may be more detrimental to the host in the absence of resource-competition as microparasite growth is comparatively unchecked (Graham 2008).

Helminths can influence microparasite growth via bottom-up mechanisms if they compete for a shared resource. For example the asexual form of *Plasmodium* species is reliant on red blood cells for its replication, and various helminth species can affect the availability of red blood cells by causing anaemia through adults feeding or larvae migrating (Gilman 1982; Attout, Babayan et al. 2005; Marsland, Kurrer et al. 2008). This helminth-induced anaemia is significant (Robertson, Crompton et al. 1992) and has been linked to the severity of malarial disease in human populations although conflicting results are reported; the combined effect of hook-worm and *P. falciparum* on haemoglobin loss exacerbates severe malarial anaemia (Brooker,

Akhwale et al. 2007), whilst co-infection with soil-transmitted helminths (including hookworm) protects against haemoglobin reduction during *P. vivax* malaria attacks (Melo, Reyes-Lecca et al. 2010).

Immunological studies in laboratory mice rarely consider the resource-mediated effects of co-infection on disease outcome, and the difficulty of disentangling the influence of top-down and bottom-up mechanisms in natural populations means there is a paucity of information in this research area. Recent work has called for co-infection studies that consider within-host interactions in a community ecology framework (Pedersen and Fenton 2007; Graham 2008) and bring together insights from immunology and ecology. This study takes inspiration from that and, using the *P. chabaudi- N. brasiliensis* (*Pcc-Nb*) model of co-infection, attempts to untangle the influence of helminth-induced anaemia (bottom-up) and down-regulation of the *Pcc*-specific immune response (top-down) on *Pcc* disease outcome. In other words, can the apparent protection against *Pcc* that *Nb* infection afforded (Chapter 3) be explained by RBC limitation, changes in the immune response – such as reduced Th1 responses or the induction of cross-reactive antibodies – or both?

4.2 Experimental Design

In order to investigate whether resource- or immune-mediated effects of *Nb* co-infection are more influential in determining *Pcc* disease outcome, I manipulated the timing of *Nb*-infection so that *Nb*-induced anaemia was resolved before the introduction of *Pcc* (See Figure 1). On day 0 mice were infected with *Nb* (200 L3 s.cut) or a sham PBS injection, in the following week RBC density and bodyweight were measured. Once the *Nb*-induced anaemia had resolved, the mice were left for 1 month before being infected with *Pcc* ($1x10^5$ parasitised RBC i.p) or a sham injection of naïve RBC ($1x10^5$). Throughout *Pcc* infection RBC density, asexual malaria parasite density and bodyweight were measured on a daily basis. The experimental design also involved taking a 50µl blood sample for analysis of antibody responses prior to any infection (day -7) and 1 week prior to *Pcc* infection (day 28). Serum was also harvested on termination of the experiment, 55 days post-

Nb infection corresponding to 20 days post-*Pcc* infection. Mice that experienced *Nb* infection followed by *Pcc* infection are designated '*Nb--Pcc*' n=30, *Pcc* infection only '*Pcc*' n=30, *Nb* infection only '*Nb*' n=16 and mice receiving only sham injections 'control' n=15.

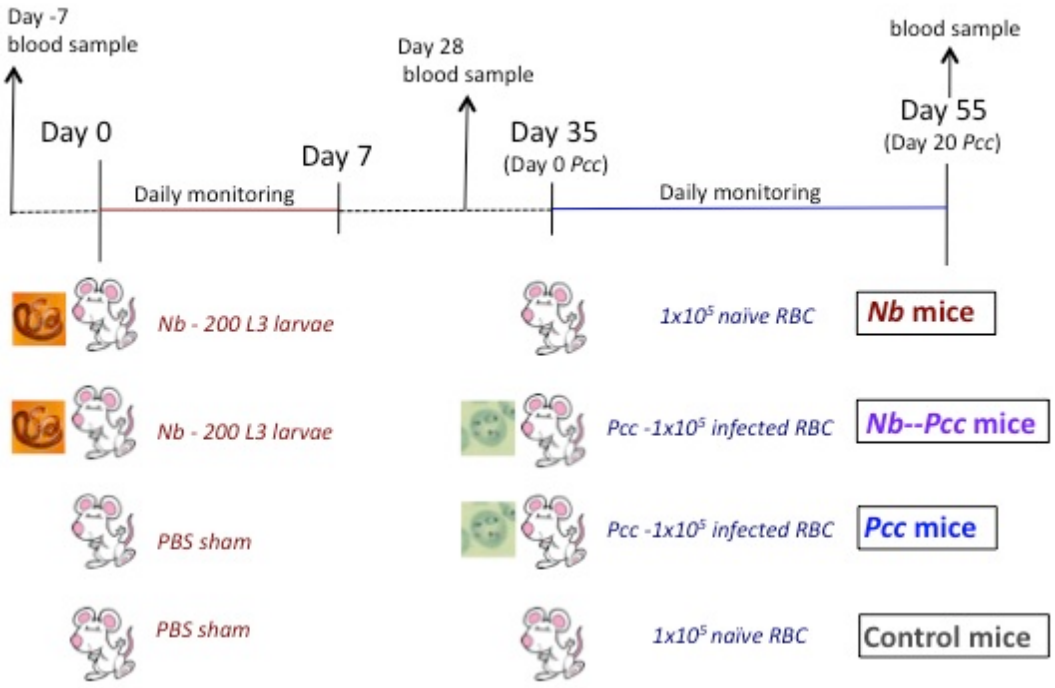


Figure 1: Infection regime detailing days when blood samples for antibody analysis were taken. Mice were infected at Day 0 with 200 L3 *Nb* larvae or received a sham PBS injection. The red line represents the typical duration of *Nb* infection in the host throughout which daily RBC density and bodyweight were measured. Mice were later (Day 35 p.i) infected with $1x10^5$ *Pcc*-infected RBC or received a sham injection of naive RBC. The blue line represents the duration of *Pcc* infection (20 days). At day -7 all mice were sampled to establish antibody levels pre-infection. Mice that received *Nb*-only are designated "*Nb*", mice that received *Nb* followed 35 days later by *Pcc* are designated "*Nb--Pcc*", mice that received *Pcc*-only are designated "*Pcc*" and those that received both sham injections "control". Blood samples for antibody analysis were taken pre-infection (day -7), 1 week prior to *Pcc* infection (day 28 p.i) and at the end of the experiment (day 55 p.i).

4.3 Statistical Analysis

The data reported here were compiled from 2 experiments, and analysed in the statistical package JMP 8.0 (SAS) using generalised linear models (GLM). RBC density was analysed in two time frames, the first 7 days representing the duration of *Nb*-infection and day 35 to 55p.i to capture the dynamics of *Pcc*-induced anaemia. Minimum RBC density in the first week was analysed with respect to *Nb* status (presence/ absence) fitted as a categorical variable. To account for differences among initial RBC density Day 0 values were included as a covariate. There were no differences between experiments regarding the effect of *Nb* status on RBC density. Including "experiment" as a factor in all the analyses controlled for any difference in the magnitude of RBC density between the experiments. Significant interactions with "experiment" were removed if they were purely quantitative and if their removal did not alter the significance of the main effects remaining in the model. Finally, significant pairwise differences ($p<0.05$) between groups were determined by Student's t-test for the most complex term in the minimal model. For analysis of minimum RBC density during *Pcc* infection the main effects of the model were worm presence or absence (*Nb*) and malaria presence or absence (*Pcc*) fitted as categorical variables. The main effects and the interaction between them (*Nb***Pcc*) test whether the response variable (minimum RBC density) was affected by *Pcc* infection, *Nb* infection or both. To account for differences among RBC density prior to the introduction of *Pcc* Day 35 values were included as a covariate. As detailed above, possible differences in the magnitude of the response between experiments were controlled for, by including 'experiment' as a factor. Minimal models were reached as described above and significant pairwise differences ($p<0.05$) among groups were determined using the Tukey's All Pairs adjusted p-values for the most complex term in the minimal model.

Asexual parasitaemia was analysed only for animals experiencing *Pcc* infection (i.e. *Pcc*-*Nb*, *Pcc*). The effect of co-infection on *Pcc* peak parasite density was evaluated

by GLM. Worm presence or absence (*Nb*) was included as a categorical factor in the model. The effect of co-infection on parasitaemia did not differ between experiments. Any differences in the magnitude of parasitaemia between the experiments were controlled for by including experiment as a factor in the model (see above). The interaction term "Nb*experiment" was removed if it was purely quantitative and if its removal did not alter the significance of the main effects remaining in the model. Finally, significant pairwise differences ($p<0.05$) between groups were determined using the Tukey's All Pairs adjusted p-values for the most complex term in the minimal model.

Antibody responses were measured on days -7, 28, and 55 to analyse responses prior to infection, following *Nb* infection (prior to *Pcc)* and following both *Nb* and *Pcc* infection respectively. Antibody titres were $\log_{10}$ transformed to satisfy the model assumptions of homogeneity of variance and normal distribution. In the first 28 days p.i mice were classified according to worm presence/ absence only (i.e *Nb*+ or *Nb*-). Antibody titres were calculated with respect to a cut-off value (mean +3 std. dev of *Nb*- mice) termed "zero". Significant production of antibody was then determined by comparison of the mean response of *Nb*+ mice to "zero" by a t-test. Day 55 responses were measured following the full course of both *Nb* and *Pcc* infection and responses were analysed by GLM. The main effects of the model were worm presence or absence (*Nb*) and malaria presence or absence (*Pcc*) fitted as categorical variables. The main effects and the interaction between them (*Nb***Pcc*) test whether the antibody responses are affected by *Pcc* infection, *Nb* infection or both. As detailed above, possible differences in the magnitude of the response between experiments were controlled for, by including 'experiment' as a factor. Minimal models were reached as described above and significant pairwise differences ($p<0.05$) among groups were determined using the Tukey's All Pairs adjusted p-values for the most complex term in the minimal model.

4.4 Results

Nb *infection induces anaemia in the first week post-infection.*

The aim of this experiment was to investigate whether anaemia or reduction of the *Pcc*-specific Th1 response induced by *Nb* were more influential in determining the outcome of *Pcc* infection. To confirm that *Nb*-induced anaemia was apparent in this experiment I measured RBC density on a daily basis for the first 7 days p.i. As observed in previous experiments, *Nb* infection resulted in a significant reduction (~3.5%) in the RBC minima experienced in the first week p.i (see Figure 2 and Table 1). This observation confirms that the effect of *Nb*-infection previously observed in the head to head co-infection regime is reproduced here. However in this experimental set-up *Nb*-induced anaemia was completely resolved prior to *Pcc* infection (on day 35p.i) so that competition for resources between the two parasites should not apply. In other words by manipulating the timing of *Nb*-infection (and so *Nb*-induced anaemia) we have removed the potential for this bottom-up mechanism of regulation to influence the outcome of *Pcc* infection.

TABLE 1: Results of GLM for anaemia data in first week p.i. Non-significant terms come from last model before term was dropped and are shown in square brackets. F statistics and P values of significant terms (P<0.05 denoted *) come from the minimal model. Significant pairwise differences were determined by Student's t-test of the most complex significant term in the model and are shown in Figure 2.

Response Variable	Factor	df	F ratio	P value	Effect size estimates (mean ± SE)
RBC minima (to d7 p.i)	Day 0 RBC density	$F_{1, 88}$	20.45	<0.0001*	0.315±0.07
	[Experiment	$F_{1, 87}$	2.93	0.0903]	
	Nb	$F_{1, 88}$	16.85	<0.0001*	

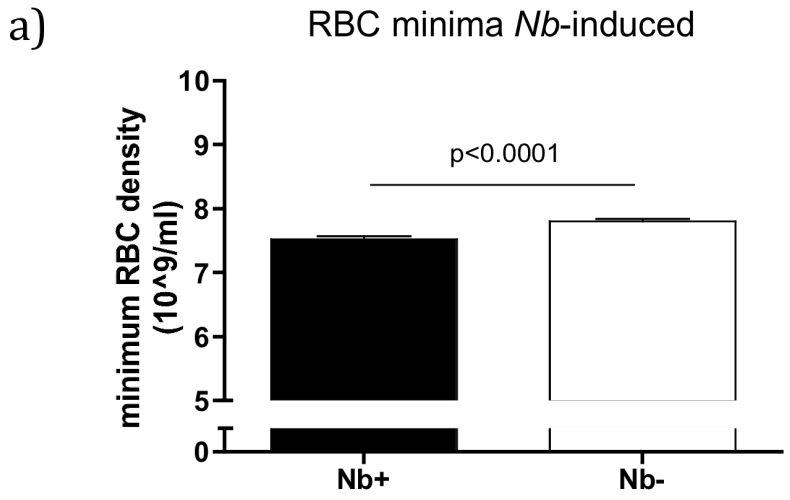


Figure 2: *Effect of* Nb *infection on RBC density*. Minimum RBC density in the first 7 days of infection (Nb-induced) is shown a) mice infected with *Nb* (Nb+) are shown in black bars and mice receiving sham injections (Nb-) are shown in white bars. Graph shows mean and standard error for 46 mice (Nb+) and 45 mice (Nb-). Groups were significantly different according to Student's T-test and this P-value is indicated.

Potential for Nb-*induced cross-reactive antibody responses to influence Pcc infection.*

The occurrence of cross-reactive antibodies in this *Pcc-Nb* co-infection model (Chapter 2 and (Fairlie-Clarke, Lamb et al. 2010)) raises the intriguing possibility that they may be functional and act in concert with *Pcc*-specific responses to control *Pcc* infection. In the experiments described here, significant levels of cross-reactive antibodies to *Pcc* antigens were also detected. To determine the level of cross-reactive antibodies that were induced by *Nb* infection antibody responses to the malaria antigens PcL and MSP-1_{19} were measured at day 28 p.i (1 week prior to *Pcc* infection). *Nb* infected mice (*Nb*+) produced titres of PcL IgG2a that were significantly greater than the levels detected in mice that had not experienced *Nb* infection (*Nb*-) (t_{44}= 3.93, P=0.0003; see Figure 3a). Similarly, the *Nb*-induced cross-reactive IgG2a response to the recombinant malaria antigen MSP-1_{19}, although low

in magnitude, was significantly greater than that of *Nb*- mice (t_{44}= 3.31, P=0.0019; see Figure 3b).

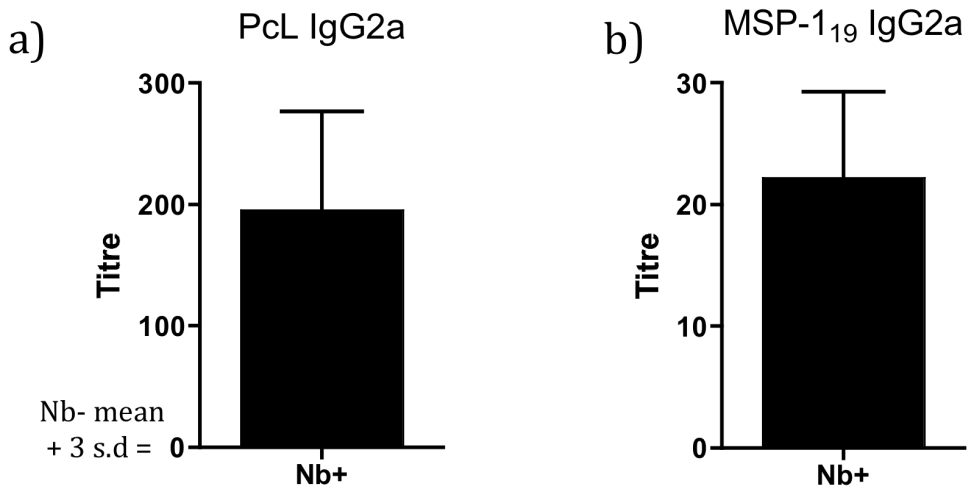


Figure 3: Nb-*induced cross-reactive responses to malaria antigens*.
Th1 (IgG2a) antibody responses to malaria antigens Pc Lysate (PcL) a) and MSP-1_{19} b) on day 28 p.i are shown. *Nb* infected mice (Nb+) are represented by black bars. Graph shows mean and standard error for 46 mice. The response observed in mice receiving sham *Nb* injection is shown as zero on the y-axis.

Effect of manipulating timing of Nb-*induced anaemia on* Pcc-*infection.*

Infecting hosts with *Nb* one month prior to introduction of *Pcc* allows the influence of bottom-up (resource) and top-down (immune)-mediated mechanisms of regulation to be disentangled as *Nb*-induced anaemia is resolved prior to *Pcc* infection. The influence of prior *Nb* infection on *Pcc* infection was assessed by measuring asexual parasitaemia by microscopic examination of blood smears on a daily basis throughout *Pcc* infection, accompanied by daily measurements of host RBC density. There was no significant difference in the peak of asexual parasite density between singly or 'co-infected' mice (Figure 4a and Table 2). In accordance with this there was no significant difference in the loss of RBC density at the trough of *Pcc* associated anaemia between singly or 'co-infected' mice (Figure 4b, 4c and Table 2). In other words the 'protective' effect of co-infection observed during simultaneous

Pcc-Nb co-infection (Chapter 3 and (Hoeve, Mylonas et al. 2009)) was not apparent when *Nb*-induced anaemia was resolved prior to *Pcc* infection.

TABLE 2: Results of GLM for malaria associated anaemia and parasitology data. Non-significant terms come from last model before term was dropped and are shown in square brackets. F statistics and P values of significant terms (P<0.05) come from the minimal model. Significant pairwise differences were determined from the Tukey's adjusted P-values of the most complex significant term in the model and are shown in Figure 4.

Response Variable	Factor	df	F ratio	P value	Effect size estimates (mean ± SE)
RBC minima (to d55 p.i)	Day 35 RBC density	$F_{1, 86}$	12.20	0.0008*	0.195±0.06
	[Experiment	$F_{1, 85}$	0.00	0.9412]	
	Pcc	$F_{1, 86}$	4336.2	<0.0001*	
	Nb	$F_{1, 86}$	3.72	0.0570	
	Nb*Pcc	$F_{1, 86}$	4.47	0.0374*	
Peak parasite density	Experiment	$F_{1, 57}$	53.26	<0.0001*	0.133±0.02
	Nb	$F_{1, 57}$	0.12	0.7301	

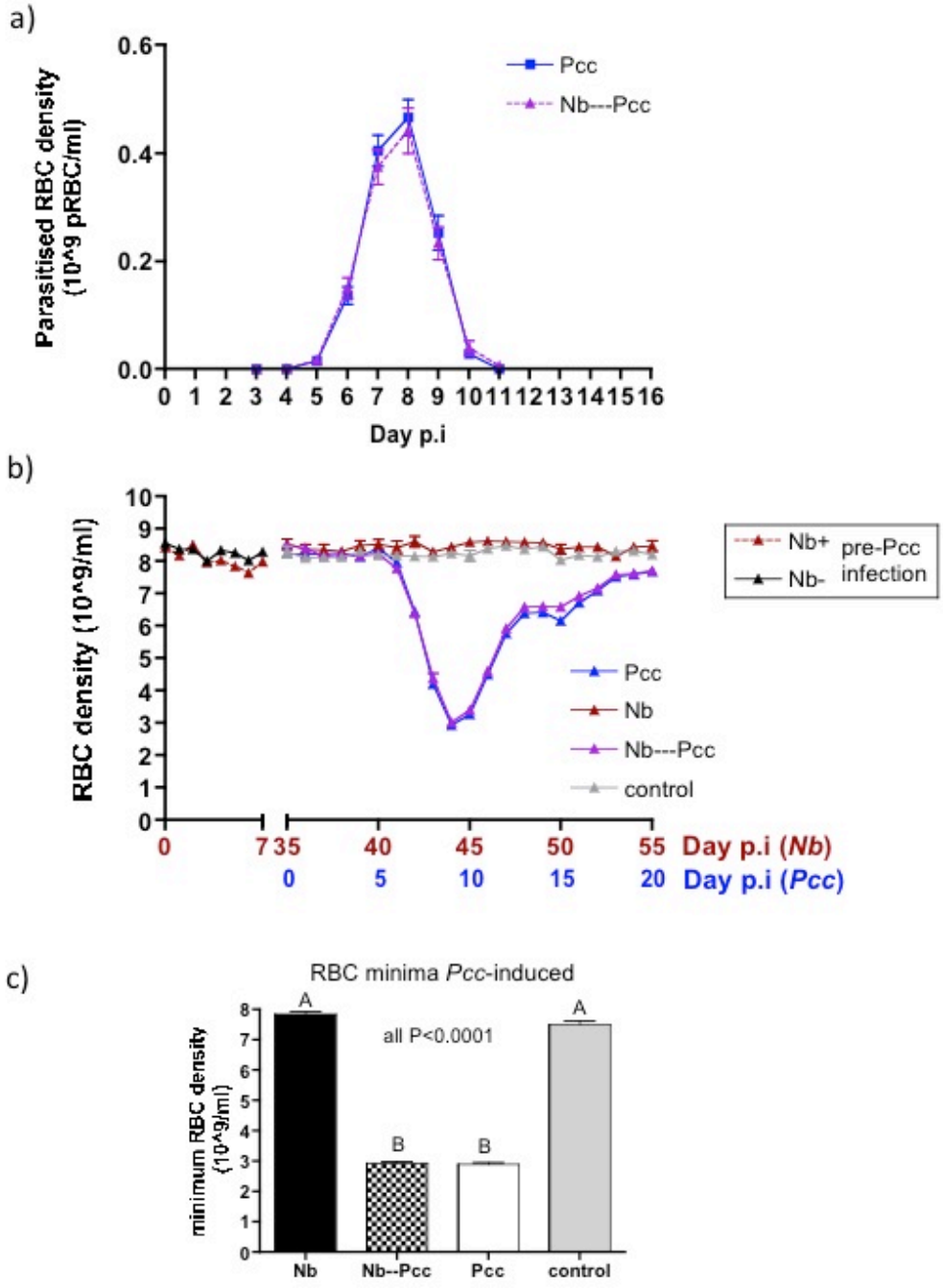


Figure 4: Daily parasitised RBC density throughout *Pcc* infection is shown a) *Pcc* mice are shown in blue and "co-infected" mice that previously experienced *Nb* infection (*Nb--Pcc*) are shown in purple. Daily RBC density throughout infection b) prior to *Pcc* infection (day 0-7 *Nb* infection) mice are designated Nb+ (n=46) shown in red or Nb- (n=45) shown in black. From day 35 p.i (Day 0 *Pcc* infection) Nb+ mice are subdivided into those with *Pcc* infection (*Nb--Pcc*; n=30) shown in purple and those that received naïve RBC (Nb; n=16) shown in red. *Pcc*-only mice (n=30) are shown in blue and controls (n=16) in grey. Minimum RBC density c). *Nb*-only mice are shown in black bars, *Pcc*-only mice in grey bars, *Nb--Pcc* mice in chequered bars and control mice in grey bars. Significant pairwise differences according to Tukey's post-hoc test are indicated by letters. Groups that do not share the same letters are significantly different and the Tukey's adjusted P-values are indicated. Graphs show mean and standard errors.

Altering the timing of Nb *infection did not affect the immune response in 'co-infected' mice.*

To ensure that immune responses of co-infected mice under this infection regime were consistent with those of the simultaneous co-infection used in prior experiments, the antibody responses in the mice were analysed at the end of infection (day 55 i.e. 20 days post-*Pcc* infection, Figure 1). In particular, it was important to establish that in accordance with previous observations (Chapter 2 and (Hoeve, Mylonas et al. 2009; Fairlie-Clarke, Lamb et al. 2010)) *Nb* given a month prior to *Pcc* was capable of exerting a down-regulatory effect on the *Pcc*-specific Th1 response. The *Pcc*-specific IgG2a response in 'co-infected' mice (*Nb*--*Pcc*) to the crude malaria antigen (PcL) was significantly reduced in comparison to *Pcc*-only mice (see Figure 5a and Table 3), confirming that *Nb* infection reduced the Th1 response. A similar though non-significant trend (P=0.1) was also observed for the anti-MSP-1_{19} IgG2a response in co-infected versus *Pcc*-only mice (see Figure 5b).

TABLE 3: Results of GLM for antigen-specific antibody responses at day 55 p.i. Non-significant terms come from last model before term was dropped and are shown in square brackets. F statistics and P values of significant terms (P<0.05 denoted *) come from the minimal model. Significant pairwise differences were determined from the Tukey's adjusted P-values of the most complex significant term in the model and are shown in Figure 5.

Response Variable	Factor	df	F ratio	P value	Effect size estimates (mean ± SE)
DAY 55:					
anti PcL IgG2a	Experiment	$F_{1, 72}$	48.75	<0.0001*	
	Infection	$F_{2, 72}$	14.94	<0.0001*	
anti MSP-1_{19} IgG2a	Experiment	$F_{1, 72}$	79.25	<0.0001*	0.55±0.06
	Infection	$F_{2, 72}$	96.82	<0.0001*	

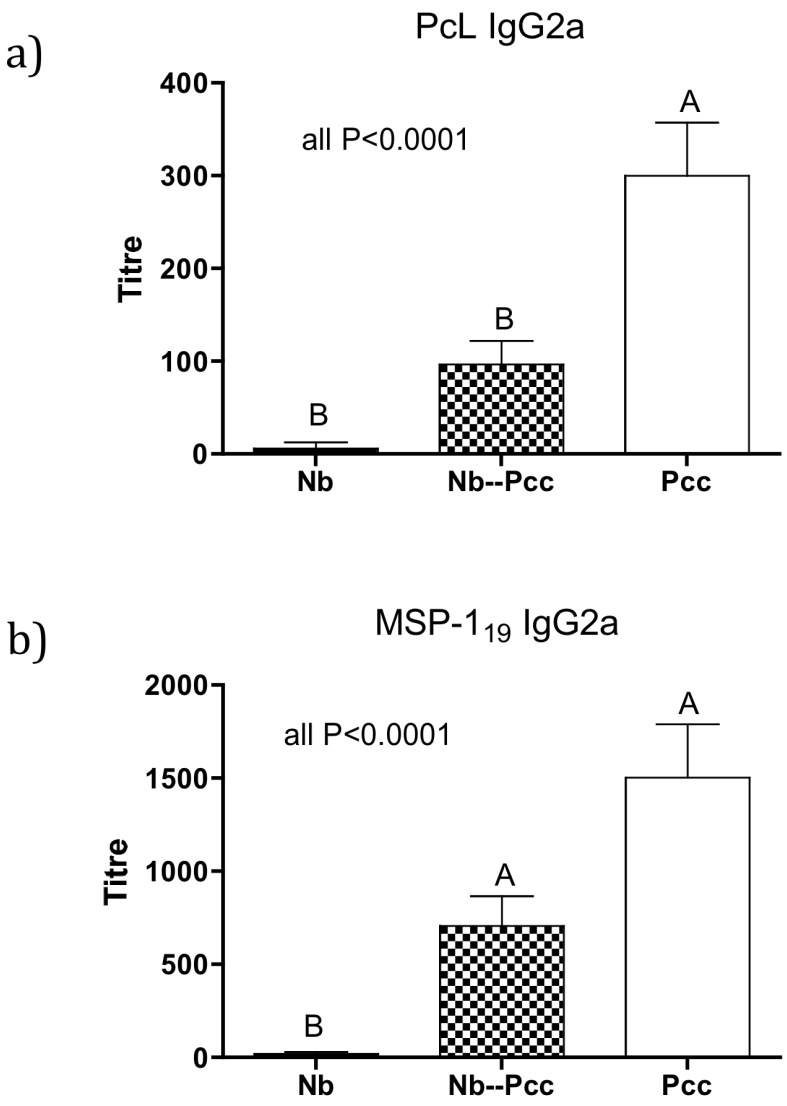


Figure 5: *Antibody responses to malaria antigens at day 55 p.i.*
IgG2a responses to crude malaria antigen PcL a) and recombinant malaria antigen MSP-1_{19} b) are shown. Mice infected with *Nb* (Nb) are shown in black bars, *Pcc* infected mice (*Pcc*) are shown in white bars and co-infected mice (*Nb--Pcc*) are shown in chequered bars. Graph shows mean and standard error for 30 mice per group *Pcc* and *Nb--Pcc* and 16 mice for the *Nb* group. Significant pairwise differences according to Tukey's post-hoc test are indicated by letters. Groups that do not share the same letters are significantly different and the Tukey's adjusted P-values are indicated.

The intermediate Th1/Th2 immune-bias seen previously in co-infected mice (Chapter 2 and (Fairlie-Clarke, Lamb et al. 2010)) is also replicated here; a reduced total IgE (Th2) response was detected in *Nb*--*Pcc* mice at d55 post-*Nb* infection (see Figure 6a and Table 4). A timecourse of the total IgE response was determined by measuring the antibody in serum taken at various timepoints throughout infection. Day -7 shows pre-infection responses did not differ amongst the mice (see Figure 6b and Table 4). As expected, *Nb* infection (Nb+) results in a significant increase in IgE prior to *Pcc* infection (Day 28 Figure 6b and Table 4) that decreased slightly over time (day 55 Figure 6b and Table 4). The cohort of '*Nb*+' mice that also experienced *Pcc* infection (*Nb*--*Pcc*) showed a further reduction in the IgE (Th2) response at day 55 (Figure 6a, 6b and Table 4).

TABLE 4: Results of GLM for total IgE responses taken at day -7, day 28 p.i and day 55 p.i. Non-significant terms come from last model before term was dropped and are shown in square brackets. F statistics and P values of significant terms (P<0.05 denoted *) come from the minimal model. Significant pairwise differences were determined from the Tukey's adjusted P-values of the most complex significant term in the model and are shown in Figure 6.

Response Variable	Factor	df	F ratio	P value	Effect size estimates (mean ± SE)
IgE day -7	Experiment	$F_{1, 87}$	341.19	<0.0001*	-0.211±0.01
	Nb	$F_{1, 87}$	1.83	0.1792	
IgE day 28	Experiment	$F_{1, 86}$	117.01	<0.0001*	-0.196±0.02
	Nb	$F_{1, 86}$	282.69	<0.0001*	
IgE day 55	Experiment	$F_{1, 85}$	188.73	<0.0001*	-0.202±13.74
	Pcc	$F_{1, 85}$	6.46	0.0129*	
	Nb	$F_{1, 85}$	184.57	<0.0001*	
	Nb*Pcc	$F_{1, 85}$	2.15	0.1464	

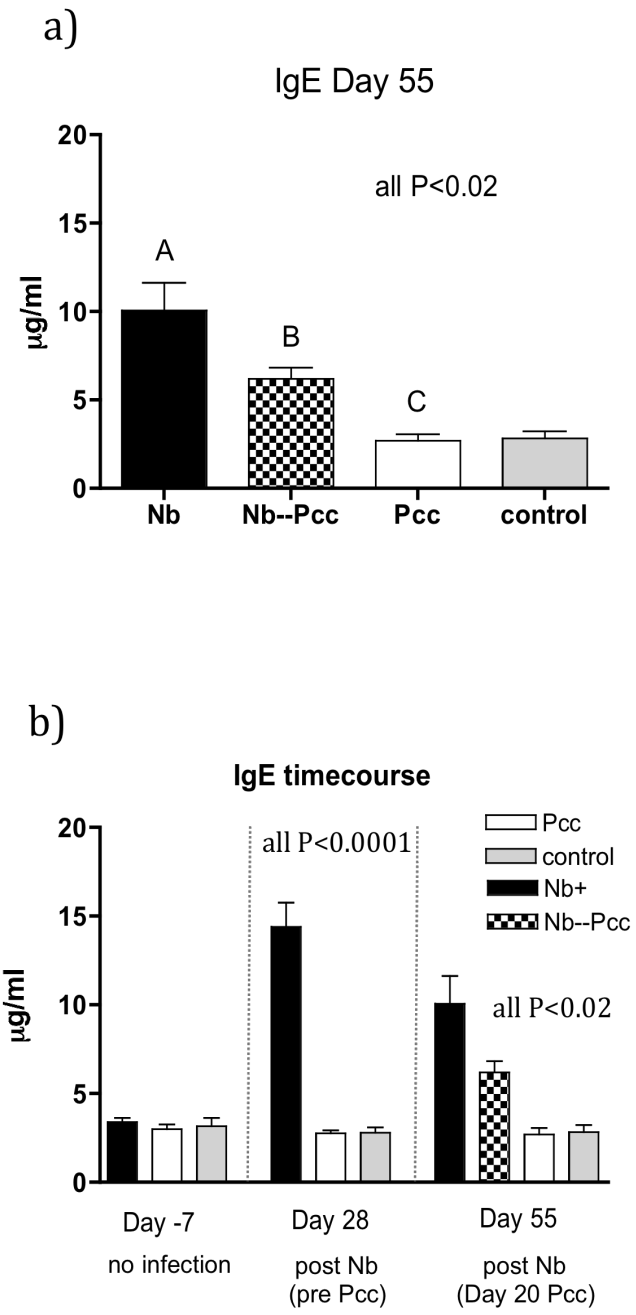


Figure 6: *Total IgE responses throughout infection.*
Total IgE responses (µg/ml) at the end of infection (day 55) are shown a) *Nb*-only mice are represented by black bars, *Pcc*-only mice by white bars, "co-infected" mice (*Nb--Pcc*) by chequered bars and control mice by grey bars. Graph shows mean and standard error for 30 mice per group *Pcc* and *Nb—Pcc*, 16 mice for the *Nb* group and 15 mice for the control group. Significant pairwise differences according to Tukey's post-hoc test are indicated by letters. IgE responses on day -7, day 35 p.i and day 55 p.i are plotted together to show the change in IgE concentration over time b) prior to *Pcc* infection at day -7 and day 28p.i Nb+ mice are shown in black bars. Nb- mice destined to receive *Pcc* at day 35 p.i are shown in white bars. Significant pairwise differences (within day)were analysed by Tukey's post-hoc test, Groups that do not share the same letters are significantly different and the Tukey's adjusted P-values are indicated.

Nb-*induced antibody responses are not increased by exposure to* Pcc.

The infection regime used in these experiments is analogous to a vaccination protocol; mice were 'immunised' with *Nb* and then subsequently 'challenged' with *Pcc*. This provides an opportunity to test whether responses to *Nb* antigen are 'boosted' by exposure to *Pcc,* which I would predict if *Pcc* displayed antigens that are shared with *Nb*. *Nb*-specific IgG responses measured at day 55 were not significantly higher in mice that were infected with *Nb* and subsequently challenged with *Pcc* (*Nb--Pcc*). In fact, responses in *Nb--Pcc* mice were significantly reduced in comparison to *Nb* mice and no different to the cross-reactive response observed in *Pcc*-only mice (see Figure 7a and Table 5). Th1 responses are more typically induced by *Pcc* infection so I investigated whether the IgG2a response to NbA was 'boosted' in *Nb--Pcc* mice at day 55 p.i. Although anti-NbA IgG2a responses in *Nb--Pcc* were increased in comparison to *Nb* mice they were not significantly different to the cross-reactive response induced by *Pcc* infection alone (See Figure 7b and Table 5).

TABLE 5: Results of GLM for antigen-specific antibody responses at day 55 p.i. Non-significant terms come from last model before term was dropped and are shown in square brackets. F statistics and P values of significant terms (P<0.05 denoted *) come from the minimal model. Significant pairwise differences were determined from the Tukey's adjusted P-values of the most complex significant term in the model and are shown in Figure 7.

Response Variable	Factor	df	F ratio	P value	Effect size estimates (mean ± SE)
DAY 55:					
anti-NbA IgG2a	Experiment	$F_{1, 72}$	64.09	<0.0001*	0.54±0.07
	Infection	$F_{2, 72}$	78.28	<0.0001*	
anti-NbA IgG	Experiment	$F_{1, 72}$	42.56	<0.0001*	0.58±0.09
	Infection	$F_{2, 72}$	8.73	0.0004*	

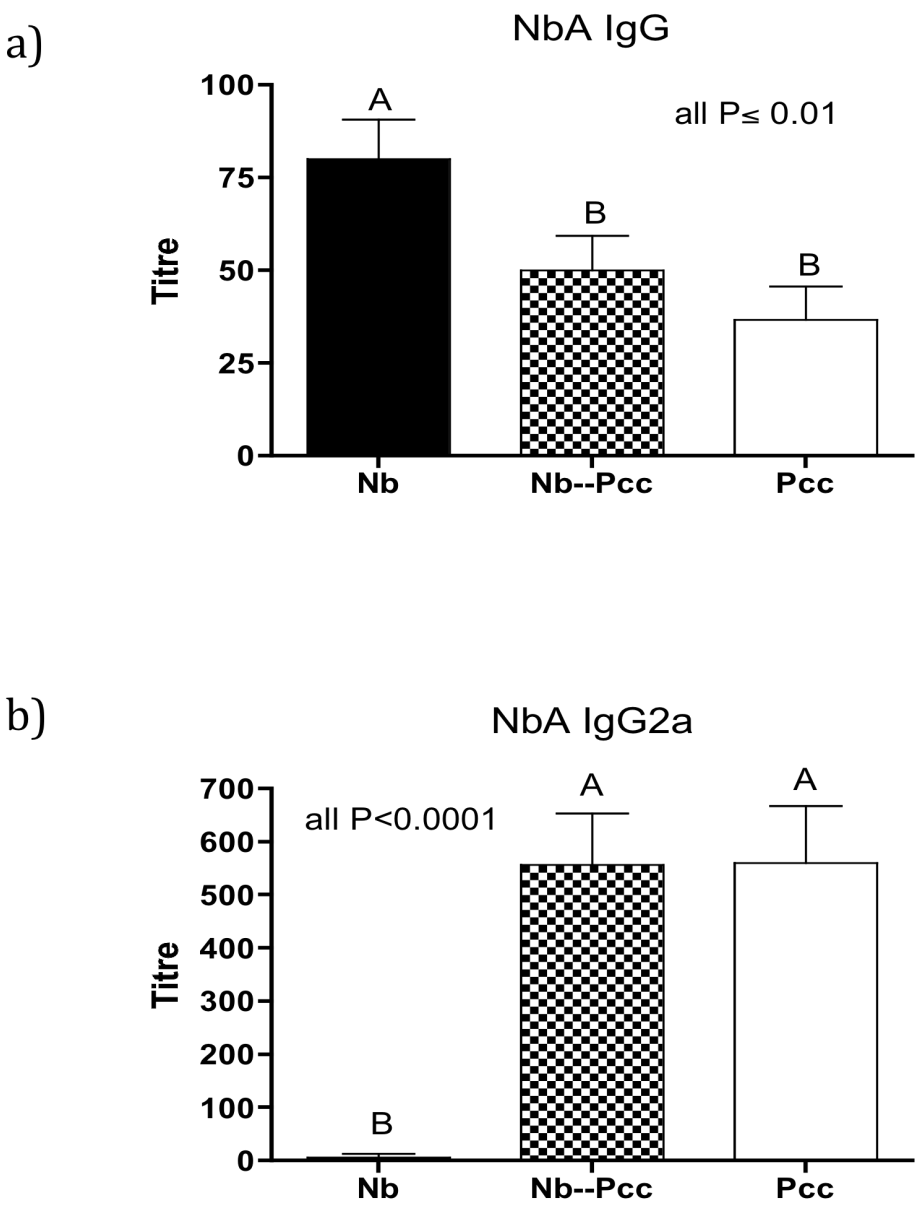


Figure 7: *Antibody responses to* N. brasiliensis *antigen at day 55 post-infection.* Total IgG responses to *Nb* antigen (NbA) a) and Th1 antibody responses (IgG2a) to NbA b) were measured at day 55 p.i. *Nb*-only mice are represented by black bars, *Pcc*-only mice by white bars and "co-infected" mice (*Nb--Pcc*) by chequered bars. Graph shows mean and standard error for 30 mice per group *Pcc* and *Nb—Pc*c and 16 mice for the *Nb* group. Significant pairwise differences according to Tukey's post-hoc test are indicated by letters. Groups that do not share the same letters are significantly different and the Tukey's adjusted P-values are indicated.

4.5 Discussion

Considering the host as an ecosystem, and the host-parasite interactions that govern within-host infection dynamics in a community-ecology framework, is useful for understanding the complexity of these interactions in the context of co-infection (Pedersen and Fenton 2007). Community ecologists take into account the potential for both top-down (predator) and bottom-up (resource) interactions to regulate the numbers or diversity of species (Burkepile and Hay 2006; Elmhagen and Rushton 2007). For an infection, top-down regulation refers to the influence of parasite-killing mechanisms and bottom-up to resource availability. It is important to note that immune-mediated mechanisms of parasite control can act via top-down mechanisms (i.e. direct, immune-mediated killing) or via bottom-up mechanisms (i.e. a starvation strategy to kill parasites). For example, direct killing of malaria parasites can be antibody mediated (Cavinato, Bastos et al. 2001), whereas 'bystander killing' of uninfected RBC by macrophages (Tippett, Fernandes et al. 2007) or retention of RBCs in the spleen (Buffet, Safeukui et al. 2009) are immune-mediated bottom-up control mechanisms that reduce availability of RBC for parasite invasion. In co-infections, one parasite species may influence another through bottom-up mechanisms by directly competing for resources (Lwin, Last et al. 1982), or via top-down mechanisms by influencing the host immune response (Supali, Verweij et al. 2010). In helminth-malaria co-infection, host red blood cells (RBCs) are the most significant shared resource: malaria parasites require RBCs for asexual parasite replication (Paul and Brey 2003) and parasitic helminths consume RBC when feeding as adults or indirectly deplete RBCs by causing haemorrhaging and tissue damage as larval stages migrate (Gilman 1982; Attout, Babayan et al. 2005; Marsland, Kurrer et al. 2008). Helminths also have the potential to moderate the immune response to malaria as the Th2 response induced by helminths has a counter-regulatory affect on the protective Th1 response to malaria (Mosmann and Sad 1996; Su, Segura et al. 2005).

These mechanisms are by no means mutually exclusive and may have differential effects depending on the context of co-infection (Graham 2008). In previous experiments (see Chapter 3) simultaneous infection of *Nb* and *Pcc* revealed a

'protective' effect of co-infection in terms of reduced peak *Pcc* parasite density and associated anaemia. The potential for both bottom-up and top-down mechanisms to have a role in this outcome is evident in the *Nb*-induced depletion of RBC density in the early stages of infection (Chapter 3 and (Hoeve, Mylonas et al. 2009)) and the reduction of *Pcc*-specific Th1 antibody responses (Chapter 2 and (Fairlie-Clarke, Lamb et al. 2010)). Here, my aim was to investigate whether top-down or bottom-up mechanisms of regulation were more important for determining malaria (*Pcc*) disease outcome in the context of *Nb* co-infection. A distinct advantage of using an experimental murine model of co-infection to address this question is the ability to manipulate the timing of *Nb* infection (and so *Nb*-induced anaemia) to untangle the relative effects of resource- and immune-mediated control of *Pcc* parasite numbers. When *Nb*-induced anaemia was resolved well before the introduction of *Pcc* there was no reduction in asexual parasite density or *Pcc* associated anaemia, suggesting that in this co-infection model resource availability (rather than an immune-mediated mechanism) is more influential in the control of *Pcc* parasite replication. However, it is impossible with this experimental design to rule out the possibility that *Nb*-induced changes in the immune response (e.g. production of cytokines, activation of macrophages) did not affect *Pcc* parasite density in simultaneous infection. As by altering the timing of *Nb*-induced anaemia (relative to *Pcc* infection) I have also altered the timing of these immune mechanisms. Interestingly, the reduced *Pcc*-specific Th1 response in *Nb--Pcc* mice (as indicated by antibody isotype bias) was able to control parasite numbers as efficiently as the 'full-blown' response in *Pcc*-only mice.

The anaemia detected in the early stages of *Nb* infection is likely to have resulted from tissue damage as the larvae migrate from the circulation into the airways of the lung (Marsland, Kurrer et al. 2008; Hoeve, Mylonas et al. 2009). When this depletion of RBCs occurs just prior to the detection of asexual parasites in the blood, as is the case in simultaneous *Pcc*-*Nb* infection, it is possible that reduced availability of RBCs slows the propagation of asexual malaria parasites. Indirect support for this hypothesis exists as blood transfusion to alleviate anaemia and supplement RBC availability led to a prolonged period of patency in *Pcc* infected mice (Yap and

Stevenson 1994). It is also apparent from experiments involving immunodeficient mice that RBC availability alone can influence parasitaemia; in T-cell deficient mice, control of the initial peak of parasitaemia was attributed to an influx of reticulocytes, which are not the preferred host cell for *Pcc* (Podoba and Stevenson 1991). It should be noted that the reduction in RBC density is small (approx 3.5%) and transient, so that the simple explanation of reduced RBC availability may not be satisfactory to explain the subsequent effect on peak *Pcc* parasite density. Indeed it is not obvious from the dynamics of *Pcc* parasite density that establishment of asexual parasites in co-infection is negatively affected by *Nb*-induced changes early in infection (see Chapter 2 Figure 1a). It may be that more subtle effects on the age structure of RBCs are important. Following reduction in RBC density erythropoiesis is induced to redress the balance and results in an influx of reticulocytes (immature red blood cells)(Savill, Chadwick et al. 2009). *P. chabaudi* shows a preference for the invasion of normocytes (mature red blood cells) early in infection (Ott 1968), so if *Nb*-induced anaemia increases the proportion of reticulocytes this may reduce parasite numbers. However, the preference of *P. chabaudi* for normocytes is just that- a preference - and not an absolute requirement as *P. chabaudi* can also successfully invade reticulocytes (Jarra and Brown 1989) and some research suggests they may even replicate more effectively within them (Mideo, Barclay et al. 2008). A full understanding of the changes in RBC age structure brought about by *Nb* infection and the potential for this to affect *Pcc* parasite replication will require integration of data – of the sort I have collected – with sophisticated mathematical modelling (e.g (Mideo, Barclay et al. 2008; Savill, Chadwick et al. 2009; Miller, Raberg et al. 2010)). Some (qualitative) insight to the proposed *Nb*-induced change in RBC population age-structure may be gained by counting infected/ uninfected reticulocytes in *Pcc* versus *Pcc-Nb* mice in the model of simultaneous co-infection or transfusing mice with reticulocytes (to mimic the proposed change in RBC age-structure) and assessing changes in *Pcc* parasitaemia. To definitively address the role of resource limitation in this model it would be crucial to directly manipulate this parameter. For example an experimental group in which erythropoietin was used to increase RBC availability could be included.

The results presented here are contrary to the findings in some other malaria-helminth co-infection systems where reduction of the Th1 immune response results in exacerbation of malaria parasitaemia (Helmby, Kullberg et al. 1998; Su, Segura et al. 2005; Tetsutani, Ishiwata et al. 2009). *Nb* infection is short-lived (i.e., acute), with worms being expelled from the host (BALB/c) within a week p.i, which contrasts with the chronic helminth infections used in the other studies. Remarkably, the *Nb*-induced reduction in IgG2a *Pcc*-specific responses is still evident 7 weeks after the parasite has been cleared (day 55 p.i). It is possible that chronic helminth infections exert a greater down-regulatory effect on Th1 responses than acute *Nb* infection, which results in exacerbation of *Pcc* parasitaemia. There are many other potential explanations for the contrasting affects of co-infection on malaria disease outcome, such as: host heterogeneity, environment, species of co-infecting parasite, level of exposure, and timing of infection (Hartgers and Yazdanbakhsh 2006; Supali, Verweij et al. 2010). Given that the reduced Th1 response to *Pcc* seen here and in simultaneous infection with *Nb* and *Pcc* (Chapter 2) did not adversely affect the control of *Pcc* parasitaemia, it is interesting to speculate that the ancient relationship between helminths and the vertebrate immune system has led to the evolution of an immune system which functions optimally in the context of helminth infection (Jackson, Friberg et al. 2009). Under this hypothesis, the reduction of the Th1 response to *Pcc* in co-infected mice actually reflects the status quo for a host whose ancestors are unlikely to have evolved without worms and thus would not be considered impaired in this more natural setting (co-infection).

Although the main focus of manipulating the timing of *Nb*-infection was to elucidate the relative influence of bottom-up/ top-down mechanisms of regulation on *Pcc* infection, this experimental design also allowed further investigation of the significance and development of cross-reactive antibody responses. As seen previously (Chapter 2), *Nb*-induced infection resulted in production of cross-reactive antibodies to *Pcc* antigens. However the fact that there was no 'protective' effect of co-infection in this model suggests that *Nb*-induced cross-reactive antibodies do not have a functional role in *Pcc* parasite clearance in-vivo.

The infection regime used in this study is analogous to a vaccination protocol: mice were 'immunised' with *Nb* and then subsequently 'challenged' with *Pcc*. If as is suggested by *Nb*-induced cross-reactivity (Fairlie-Clarke, Lamb et al. 2010) these parasites share epitopes, then the antigen-specific response induced by *Nb* should be 'boosted' on exposure to *Pcc* antigens, as they should stimulate the B-cells that were induced during *Nb*-infection. However no such 'boosting' of the anti-NbA response was observed in *Nb--Pcc* mice. Responses were not significantly different to the cross-reactive response induced by *Pcc* alone. The fact that *Pcc*-only mice recognise (bind to) NbA *in-vitro* (Figure 7) supports the notion that these parasites have shared epitopes. Why then was 'boosting' of the NbA response not evident in *Nb--Pcc* mice (Figure 7)? There is clear evidence that the passage of *Nb* larvae through the lung causes persistent immunological and physiological changes to the pulmonary environment (Marsland, Kurrer et al. 2008; Reece, Siracusa et al. 2008) and it has been suggested that an antigen-depot may be responsible for sustaining the inflammatory infiltrate associated with these changes (Marsland, Kurrer et al. 2008). If antigen does persist long-term in the lungs of *Nb*-infected mice, perhaps due to dead or dying larvae, then there is potential for B-cells to be continually stimulated by worm antigen. This on-going active response to *Nb* antigen may reduce the possibility of these B-cells being stimulated by *Pcc* antigens so that the proposed 'boosting' effect of *Pcc* infection would not be observed. It is also likely that only a small proportion of *Nb* and *Pcc* antigens cross-react so that it may be difficult to detect a 'boost' by measurement of antibody titre to a crude homogenate of *Nb* (NbA).

In summary, manipulating the timing of *Nb* infection – and so *Nb*-induced anaemia- with respect to *Pcc* infection resulted in the loss of the 'protective' effect on peak parasitaemia and severity of anaemia that was observed when these infections were administered simultaneously. This regime did not alter the reduction in *Pcc*-specific Th1 antibody responses and so strongly suggests that bottom-up mechanisms (i.e., resource-mediated) of *Pcc* parasite control are more influential than top-down mechanisms (i.e., immune-mediated parasite killing) in this model of malaria-hookworm co-infection. However, it should be noted that some *Nb*-induced immune

responses are now also removed in time from *Pcc* infection and further investigation of the influence of these mechanisms would be required to establish the relative contribution of these top-down mechanisms in disease outcome. This approach to understanding within-host dynamics of co-infection is not new (Pedersen and Fenton 2007; Graham 2008), but this study is one of the few that addresses the role of top-down/ bottom-up mechanisms by explicitly measuring them. This study demonstrates the importance of bringing together immunology and ecology in future research aimed at understanding how co-infection affects disease ecology and epidemiology. Important interactions between immunological and ecological factors make it hard to understand mechanisms driving infection dynamics while ignoring one of these elements. Finally, this study also serves to highlight the importance of the timing of co-infection on disease outcome and attempts to identify the mechanisms that underpin these effects.

Chapter 5: Why do antibodies induced by malaria and helminths cross-react?

5.1 Introduction

In theory a diverse repertoire of T and B cell receptors enables the adaptive immune system to recognise (bind to) every potential parasite antigen (Pancer and Cooper 2006). Unlike T-cell receptors, which recognise peptide antigens, B-cell receptors and so antibodies recognise conformational epitopes. Having bound to their cognate antigen, B-cells proliferate and undergo rounds of somatic hypermutation, which result in changes to the antigen-binding site of the receptor (and thus the secreted antibody). These altered receptors are then retested for their ability to bind antigen and those with the greatest avidity (strength of binding to antigen) are selected, culminating in the production of highly specific antibodies (Tarlinton and Smith 2000). However, this exquisite specificity is not observed in reality, as cross-reactivity (binding to more than one antigen) is common (Naus, Jones et al. 2003; Endy, Nisalak et al. 2004; Losada, Chacon et al. 2005; Casadevall and Pirofski 2007; Nguyen, Zemlin et al. 2007; Nagao, Kimura-Sato et al. 2008).

Cross-reactive antibodies as effector molecules of the adaptive immune response have the potential to influence disease outcome or susceptibility to re-infection. This has been particularly well studied for viral infections. Indeed, the generation of cross-reactive responses that recognise antigenic variants of hemagglutinin or neuraminidase is key to the success of influenza vaccination programs (Sandbulte, Jimenez et al. 2007; Levie, Leroux-Roels et al. 2008). The importance of understanding the influence of cross-reactive antibody responses is particularly acute in the case of dengue virus; cross-reactive antibodies induced by infection with one of the four serotypes can be protective against secondary infection (Endy, Nisalak et al. 2004) but can also facilitate invasion of cells resulting in exacerbation of disease to dengue haemorrhagic fever (Balsitis, Williams et al. 2010). The outcome seems to depend on the order in which the host is exposed to the different dengue serotypes

(Rothman 2004).

Cross-reactivity also occurs between pathogen species and is particularly evident amongst parasitic helminths; for example antibodies induced by the human filarial parasite *Wuchereria bancrofti* recognise surface antigens of adult *Brugia pahangi* worms (Maizels, Sutanto et al. 1985) and sera from individuals living in an area endemic for *Necator americanus* cross-reacts with antigens from *Oesophagostomum bifurcum* which is non-endemic (Pit, Polderman et al. 2001). Such cross-reactivity between helminth species can be beneficial as demonstrated by *Fasciola hepatica* infection protecting mice against subsequent infection with *Schistosoma mansoni*, in this case a common antigen "FhSmIII" seems to be responsible for the induction of cross-immunity (Hillyer 1985). Similarly *S. mansoni* infection has been shown to reduce the number of *Strongyloides venezuelensis* larvae that survive migration to the gut and this is most likely due to cross-reactive antibody-dependent cell-mediated cytotoxicity (Yoshida, Maruyama et al. 1999). The occurrence of cross-reactive antibodies that recognise different strains of the same parasite species, or indeed different species of the same phylum, are intuitive to understand in systems where similar effector mechanisms are required to control or eliminate parasites.

Perhaps more remarkable is the cross-reactivity that arises between taxonomically distinct parasites such as protozoa and helminths. Immunisation of mice with *Trichinella spiralis* antigens resulted in the production of antibodies that recognise *Leishmania infantum* (Dea-Ayuela, Rama-Iniguez et al. 2007). Antibody cross-reactivity to *Leishmania major* was also demonstrated in mice infected with the filarial parasite *Litomosoides sigmodontis* (Lamb, Graham et al. 2005). Similarly cross-reactivity between *Litomosoides sigmodontis* and *Plasmodium chabaudi* has been reported (Fairlie-Clarke, Lamb et al. 2010). Of particular interest to the study of malaria-helminth co-infection is the cross-reactivity that has been reported between *Schistosoma mansoni* and *Plasmodium falciparum* in human populations (Naus, Jones et al. 2003). Further research in this area led to the identification of a novel Schistosome antigen (SmLRR) that has 57% similarity to a putative gene product in *P. falciparum* and was recognised by sera from individuals who had only

experienced *P. falciparum* infection (Pierrot, Wilson et al. 2006). In areas co-endemic for these two parasites cross-reactive antibodies may influence disease severity, as exposure to malaria and subsequent induction of the cross-reactive IgG3 response seems to increase the risk of developing hepatosplenomegaly in schistosome infected individuals (Mwatha, Jones et al. 2003).

The question as to why antibodies induced by malaria and helminths cross-react can be answered from a biochemical, physiological or evolutionary perspective. For example, the biochemical composition of the cross-reacting antigens can be important in determining the level of cross-reactive antibody binding that occurs. Broadly speaking antigens are made up of protein and carbohydrate moieties and often cross-reactive antibodies target carbohydrates (van Remoortere, Bank et al. 2003; Paschinger, Fabini et al. 2005). Whether cross-reactive antibodies target the protein or carbohydrate moiety of an antigen can be determined experimentally by periodate treating antigens to disrupt the carbohydrate epitopes and then assessing whether antibody binding is conserved (Xu and Powell 1991; Dell, Haslam et al. 1999). In this context IgG3 is often analysed, as in mice this isotype has been shown to be involved in carbohydrate-recognition (Snapper, McIntyre et al. 1992; Dea-Ayuela, Martinez-Fernandez et al. 2000). For malaria and nematodes, there is potential for carbohydrate induced cross-reactive antibodies to arise because the outer layer (cuticle) of nematodes, which forms the interface between host and pathogen, is heavily glycosylated as are many of the excretory/ secretory antigens (Dell, Haslam et al. 1999). Similarly, protozoan parasites express carbohydrates on the cell surface and on secreted molecules (Mendonca-Previato, Todeschini et al. 2005) although this is restricted to expression of glycosylphosphatidyinositol (GPI) anchors in *Plasmodium* species (von Itzstein, Plebanski et al. 2008). This is discussed further in the following section. The carbohydrate moieties of antigens may be more likely to be the target of cross-reactive antibodies if they are conserved amongst parasites but there is also evidence that cross-reactive antibodies can target protein antigens (Pierrot, Wilson et al. 2006). In this chapter I use periodate treatment to explore whether the cross-reactivity I observed in the *N. brasiliensis- P. chabaudi* model (see Chapter 2 and (Fairlie-Clarke, Lamb et al. 2010)) is directed

toward carbohydrate or protein antigens. Determining whether proteins or carbohydrates drive certain isotype responses may also be important in vaccine design and diagnostic serology.

Antibody cross-reactivity could also be accounted for if there is a physiological constraint or limit to the specificity of antigen-antibody binding. For example, in a host with a reduced number of lymphocytes the full repertoire of receptor diversity may not be expressed as discussed for T-cell receptors (Regner 2001). In this case cross-reactive responses may compensate for reduced lymphocyte numbers by providing recognition of several antigens – a "two for the price of one" offer on antigen-recognition, if you will! Similarly if there were a limit to the number of rounds of somatic hypermutation that a B-cell undergoes (perhaps under nutritional restriction; e.g. (Martin, Navara et al. 2008)), then perfect antigen-specificity may not be achieved. Indeed, the relative abundance of lymphocytes versus antigen may shape the specificity that develops. The potential for increasing availability of antigen to affect the specificity of antibody is explored in the next chapter.

Given that mounting an immune response is energetically costly (Demas, Chefer et al. 1997; Derting and Compton 2003; Martin, Scheuerlein et al. 2003) and that a host will have limited resources to invest, due to the energy demands of growth and reproduction, it is possible that the production of cross-reactive antibodies is an evolutionary adaptation to offset some of the immune costs involved in combating multiple pathogens. Testing the concepts that cross-reactive responses may arise through physiological constraints or evolutionary adaptation is complicated. The data presented here focuses on ruling out one potential biochemical reason for the cross-reactivity observed in the *Pcc-Nb* model, namely that these antibodies target conserved carbohydrates on the parasite antigens.

5.2 Experimental Design

The data presented here and in chapter 2 was generated from the same experiment (i.e., "Experiment 1"). Female BALB/c mice were infected with *Pcc* at a dose of

$1x10^5$ parasitised RBC (i.p) with or without co-infection of 200 *Nb* L3 larvae (s.cut) on day 0. A group of mice infected with Nb only (200 L3 larvae s.cut) were also included in addition to a control group, which received nRBC ($1x10^5$) and PBS as sham injections for *Pcc* and *Nb* respectively. Bodyweight, RBC density and asexual parasite density were measured daily throughout the course of infection (data reported in Chapter 3). Serum was collected on day 20 p.i and ELISA was used to measure antibody binding to *Nb* crude antigen (NbA), crude malaria antigen (pRBC) and recombinant malaria antigen MSP-1_{19} (data also reported in Chapter 2). For details of how antigens were generated and antibody titres were calculated see Materials and Methods chapter. In parallel to this standard ELISA, antibody binding to periodate treated antigens was also analysed (see Materials and Methods chapter). Periodate oxidises carbohydrate to aldehydes and so disrupts any carbohydrate epitopes on the antigen. By comparing antibody titres measured in ELISA to periodate treated and untreated antigens I was able to determine whether antibodies targeted the protein or carbohydrate moiety of the antigen.

5.3 Statistical Analysis

The analyses of antibody titres at day 20 p.i included 24 mice (n=8 per infection group). All analyses were carried out in the statistical package JMP 8.0 (SAS) using generalised linear models (GLM). The generation of antibody titre creates ordinal data that were $\log_{10}$ transformed to satisfy the model assumptions of homogeneity-of-variance and normal distribution. Infection status (*Nb*, *Pcc* or *Pcc*-*Nb*) and treatment (periodate or control) were included as fixed categorical factors and their ability to predict antibody titre was formally evaluated. Mouse identity was fitted as a random effect to account for non-independence of titres (with and without periodate treatment) measured for a given mouse. The maximal model was fitted first and minimal models were reached by sequentially removing non-significant terms (P-value > 0.05), beginning with interactions. Finally, whenever a factor was significant ($P < 0.05$), an All Pairs Tukey post-hoc test was carried out to identify which groups of mice differed significantly in antibody titre, with respect to infection status and/or periodate treatment.

5.4 Results

IgG3 antibody responses indicate binding to the carbohydrate moiety of Pcc *and* Nb *antigens.*

In mice, the antibody isotype IgG3 is associated with binding to carbohydrates (Snapper, McIntyre et al. 1992; Dea-Ayuela, Martinez-Fernandez et al. 2000) and was detected in sera of infected mice against the malaria antigens MSP-1_{19} and pRBC (Figure 1a, 1b and Table 1). Responses in mice that had experienced *Pcc* infection (i.e. *Pcc* and *Pcc-Nb*) mounted significantly greater responses than *Nb* mice (Figure 1a, 1b and Table 1). IgG3 responses were also detected against *Nb* antigen (NbA), interestingly the IgG3 response to this antigen was greater in co-infected (*Pcc-Nb*) than *Nb* mice (Figure 1c and Table 1). However, there was no significant difference between *Nb* and *Pcc* singly infected mice, which suggests that in co-infection *Pcc* effected the induction of the IgG3 isotype. This is consistent with the effect that malaria induced IFNγ may have on the production of IgG3 (Snapper, McIntyre et al. 1992). Disruption of carbohydrates, via periodate treatment of antigens, significantly reduced IgG3 recognition of the *Pcc* antigens but responses to NbA were unchanged (Figure 1 and Table 1). This may reflect incomplete disruption of carbohydrate epitopes on the helminth antigen. However T-independent type 2 antigens that due to highly repetitive structures are able to cause activation by cross-linking multiple B-cell receptors {Murphy, 2008 #472} also induce the IgG3 isotype {Snapper, 1992 #249}. This mechanism of IgG3 induction may be more likely to occur in the presence of large multicellular organisms such as helminths. Although not definitively associated with carbohydrate binding the occurrence of significant IgG3 titres to the parasite antigens and the effect of periodate on the detection of these antibodies led me to further investigate IgG1 and IgG2a recognition of the carbohydrate versus protein moieties of these antigens.

TABLE 1: Results of GLM for antigen-specific IgG3 antibody responses at day 20 p.i. F statistics and P values of significant terms (P<0.05) come from the minimal model. Significant pairwise differences were determined from the Tukey's adjusted P-values of the most complex significant term in the model and are shown in Figure 1.

Response Variable	df	Factor	F ratio	P value
Anti-MSP-1_{19} IgG3	$F_{2,20}$	Infection	84.92	<0.0001*
	$F_{1,22}$	Periodate	13.46	0.0013*
	[$F_{2,20}$	Infection*Periodate	1.85	0.1834]
Anti-pRBC IgG3	$F_{2,20}$	Infection	6.47	0.0068*
	$F_{1,20}$	Periodate	9.90	0.0018*
	$F_{2,20}$	Infection*Periodate	3.43	0.0525
Anti-NbA IgG3	$F_{2,20}$	Infection	10.42	<0.0007*
	[$F_{1,22}$	Periodate	0.34	0.5657]
	[$F_{2,20}$	Infection*Periodate	1.30	0.2946]

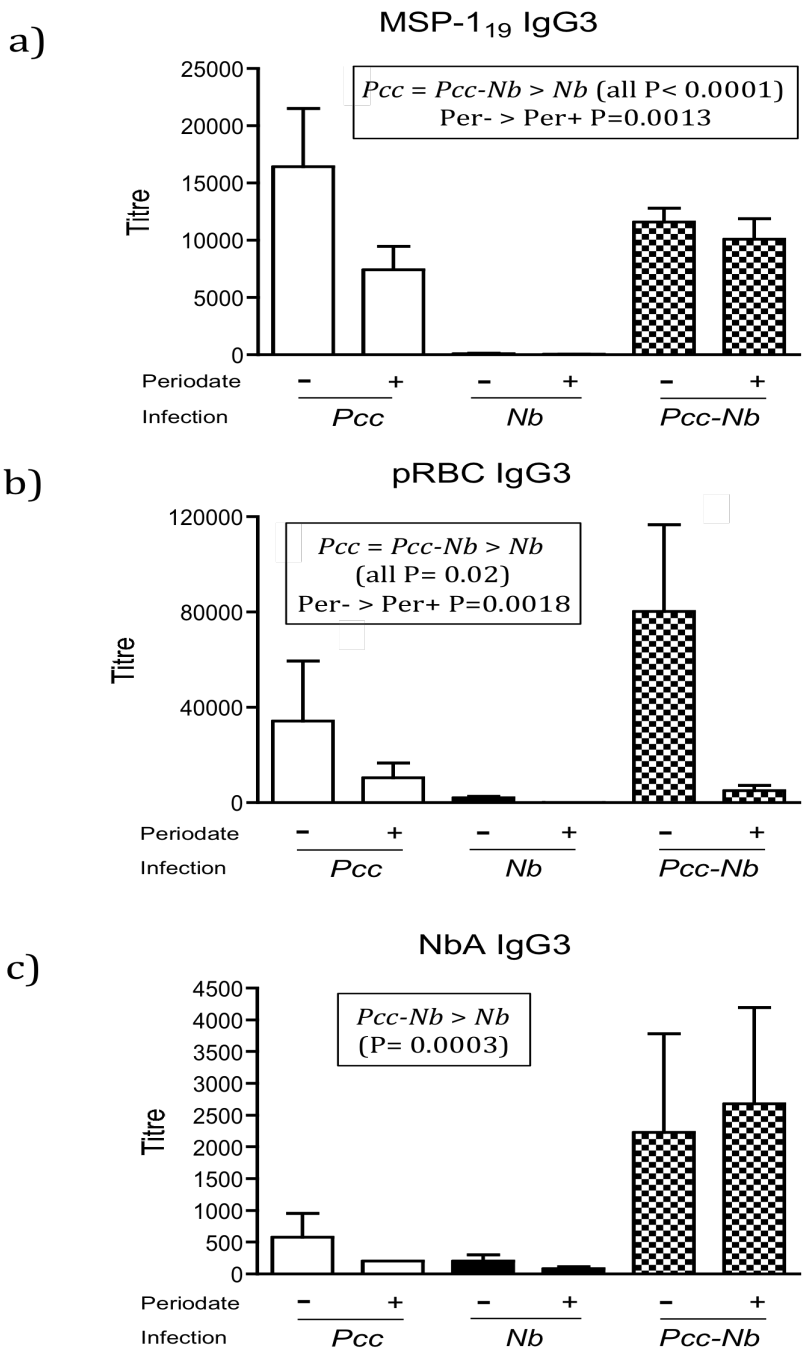


Figure 1*:* Comparison of IgG3 antibody responses to parasite antigens treated (or not) with periodate. Mice were infected with 200 *Nb* L3 larvae and/or 1x10^5 *Pcc*-infected RBCs. Serum antibody titres were measured at day 20 p.i to malaria antigens MSP-1$_{19}$ a) pRBC b) and worm antigen NbA c). IgG3 responses to untreated antigen (periodate -) and treated antigen (periodate +) are shown. All titres are above the mean +3 standard deviations of control mice at serum dilution of 1/200 represented as 0 on the y-axis. Single *Pcc* infections are shown in white bars, single *Nb* infections in black bars and co-infected mice (*Pcc-Nb*) are shown in the chequered bars. Graph shows mean and SEM of 8 mice per group. Significant pairwise differences according to Tukey's post-hoc test and the corresponding P-values are summarised in the text boxes.

Th2 antigen-specific and cross-reactive responses to Nb antigens target carbohydrates.

Analysis of the Th2 antibody response (IgG1) to malaria antigens was unaffected by periodate treatment (Figure 2a, 2b and Table 2), suggesting that both *Nb*-induced cross-reactive and *Pcc*-specific antibodies recognise a protein moiety of these antigens. In contrast there was a significant reduction in the IgG1 response to the helminth antigen NbA following periodate treatment (Figure 2c and Table 2). The reduction in the antigen-specific response of *Nb* infected mice lends support to the idea that the heavily glycosylated worm cuticle is the target of host immune responses (Dell, Haslam et al. 1999) (Figure 2c). Interestingly the *Pcc*-induced cross-reactive response to NbA was completely ablated when the antigen was treated with periodate, indicating that the atypical IgG1 response in *Pcc*-infected mice recognises carbohydrates (Figure 2c).

This has significance for the interpretation of immune bias in co-infected mice (*Pcc-Nb*); as the anti-NbA IgG1 response to periodate treated antigen was solely driven by *Nb* infection, the significant difference between *Nb*-only and *Pcc-Nb* mice must reflect the influence of *Pcc* infection on the bias of the *Nb*-specific response. In summary, Th2 antibody responses to the helminth antigen, both cross-reactive and antigen-specific, were mainly targeted to carbohydrates whereas the Th2 response to malaria antigens recognised proteins.

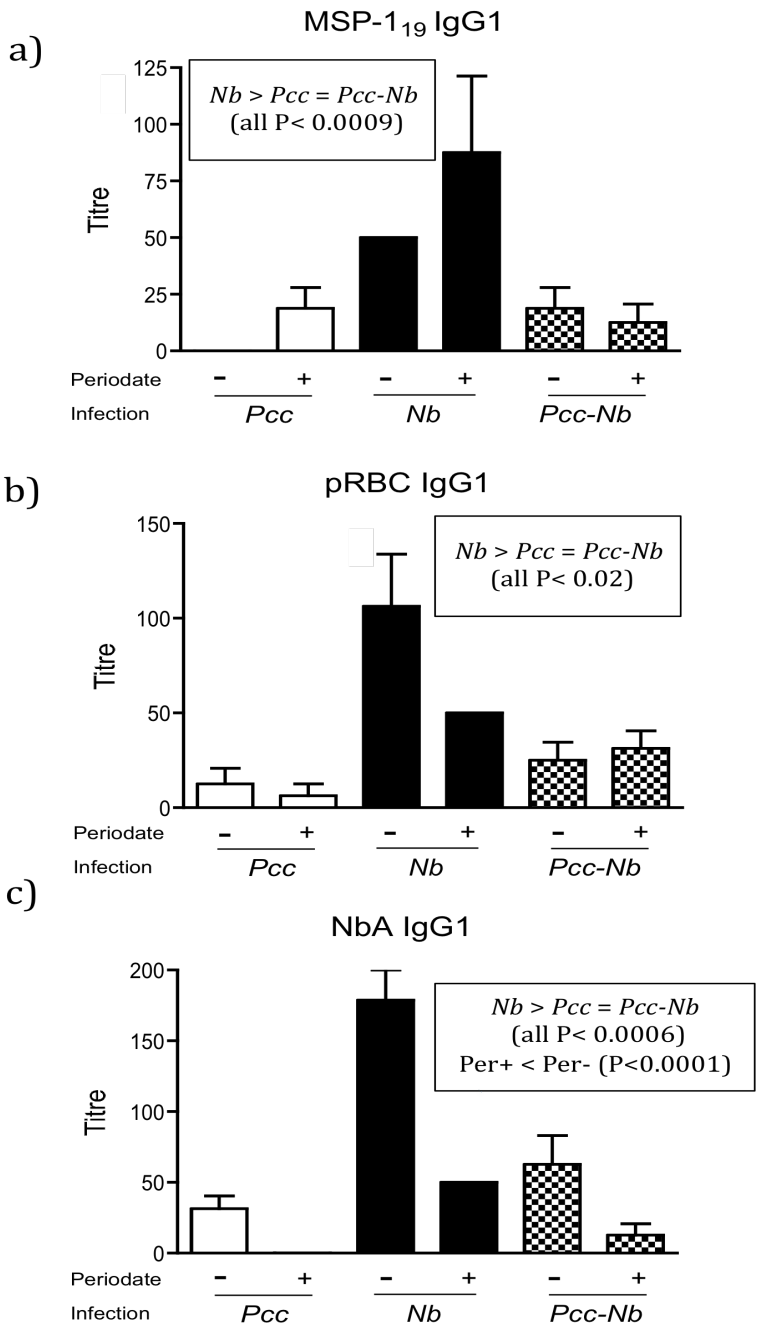


Figure 2: Comparison of Th2 antibody responses to parasite antigens treated (or not) with periodate. Mice were infected with 200 *Nb* L3 larvae and/or 1x10^5 *Pcc*-infected RBCs. Serum antibody titres were measured at day 20 post-infection to malaria antigens MSP-1$_{19}$ a) pRBC b) and worm antigen NbA c). IgG1 responses to untreated antigen (periodate -) and treated antigen (periodate +) are shown. All titres are above the mean +3 standard deviations of control mice at serum dilution of 1/200 represented as 0 on the y-axis. Single *Pcc* infections are shown in white bars, single *Nb* infections in black bars and co-infected mice (*Pcc-Nb*) are shown in the chequered bars. Graph shows mean and SEM of 8 mice per group. Significant pairwise differences according to Tukey's post-hoc test and the corresponding P-values are summarised in the text boxes.

TABLE 2: Results of GLM for Th2 antibody responses at day 20 p.i measured before and after treatment of antigens with periodate. F statistics and P values of significant terms (P<0.05) come from the minimal model. Significant pairwise differences were determined from the Tukey's adjusted P-values of the most complex significant term in the model and are shown in Figure 2.

Response Variable	df	Factor	F ratio	P value
Anti-MSP-1_{19} IgG1	$F_{2,21}$	Infection	10.53	0.0007*
	[$F_{1,23}$	Periodate	0.0002	0.9883]
	[$F_{2,21}$	Infection*Periodate	2.37	0.1176]
Anti-pRBC IgG1	$F_{2,21}$	Infection	14.52	<0.0001*
	[$F_{1,23}$	Periodate	0.24	0.6268]
	[$F_{2,21}$	Infection*Periodate	0.90	0.4214]
Anti-NbA IgG1	$F_{2,21}$	Infection	19.94	<0.0001*
	$F_{1,23}$	Periodate	34.47	<0.0001*
	[$F_{2,21}$	Infection*Periodate	1.61	0.2215]

Th1 responses recognise the protein moiety of malaria antigens.

Overall there was a significant effect of periodate on the detection of IgG2a responses (Th1) to malaria antigens pRBC and MSP-1_{19} (see Table 3). Within an infection group, responses following treatment with periodate were either significantly increased – e.g. anti-MSP-1_{19} in *Pcc-Nb* mice (Figure 3a) and anti-pRBC in *Pcc* mice (Figure 3b) – or showed a trend in this direction (anti-MSP-1_{19} in *Pcc* and anti-pRBC in *Pcc-Nb* mice - Figure 3a and 3b respectively). In each case this indicates that IgG2a antibodies recognised the protein component of malaria antigens. In other words both a lack of change in response or an increase following periodate treatment indicated binding to the protein moiety. Increases in response following periodate-treatment of the malaria antigens suggest that protein epitopes may have been masked by glycosylation. *Plasmodium* species lack the glycosyltransferases required for glycosylation of antigens with the exception of GPI anchors (von Itzstein, Plebanski et al. 2008) but expression of the recombinant antigen in the *Pichia* system may have resulted in inappropriate glycosylation of the antigen (von Itzstein, Plebanski et al. 2008). In other words periodate treated

antigens may more accurately reflect those that are seen in the context of *Pcc* infection. It is interesting that detection of *Nb*-induced cross-reactive responses (Figure 3a and 3b) was also unaffected by periodate treatment, suggesting that these too target the protein portion of the antigen. This is in contrast to my proposal that carbohydrates, with the potential to be conserved across parasite species, would be the targets of cross-reactive responses.

Investigating responses to periodate treated antigens has again proved useful in determining the effect of co-infection on immune bias. For untreated-pRBC IgG2a responses there was no significant difference amongst infection groups (Figure 3b and Chapter 2 Fig 1c). Following periodate treatment, however, the antigen binding by serum of *Pcc* infected mice was significantly greater than that of *Nb* mice (Figure 3b) such that at least some of the binding of periodate-treated antigen by sera from *Pcc-Nb* mice can be attributed to *Pcc*-specific responses.

Cross-reactive Th1 responses to Nb *antigen recognise proteins whereas antigen-specific responses target carbohydrates.*

Detection of anti-NbA IgG2a responses following periodate treatment was reduced in *Nb* and *Pcc-Nb* mice, although this was only statistically significant in *Nb* mice (Figure 3c). Interestingly the response in *Pcc* mice was unaltered, suggesting that cross-reactive responses recognise the protein moiety of this crude antigen preparation.

Prior to periodate treatment there was no difference in the cross-reactive responses of *Pcc* mice and the antigen-specific response of *Nb* mice to *Nb* antigen (Figure 3c and Chapter 2, Fig 1e). After periodate treatment of antigens however a significant difference between *Pcc* and *Nb* mice was apparent (Fig 3c) due to the ablation of the antigen-specific response (*Nb*-mice). This result is crucial for the interpretation of immune-bias in co-infection (*Pcc-Nb*) as the apparent increase in Th1 response in comparison to *Nb* mice can be attributed to *Pcc*-induced cross-reactivity rather than any real effect of bias on the antigen-specific response.

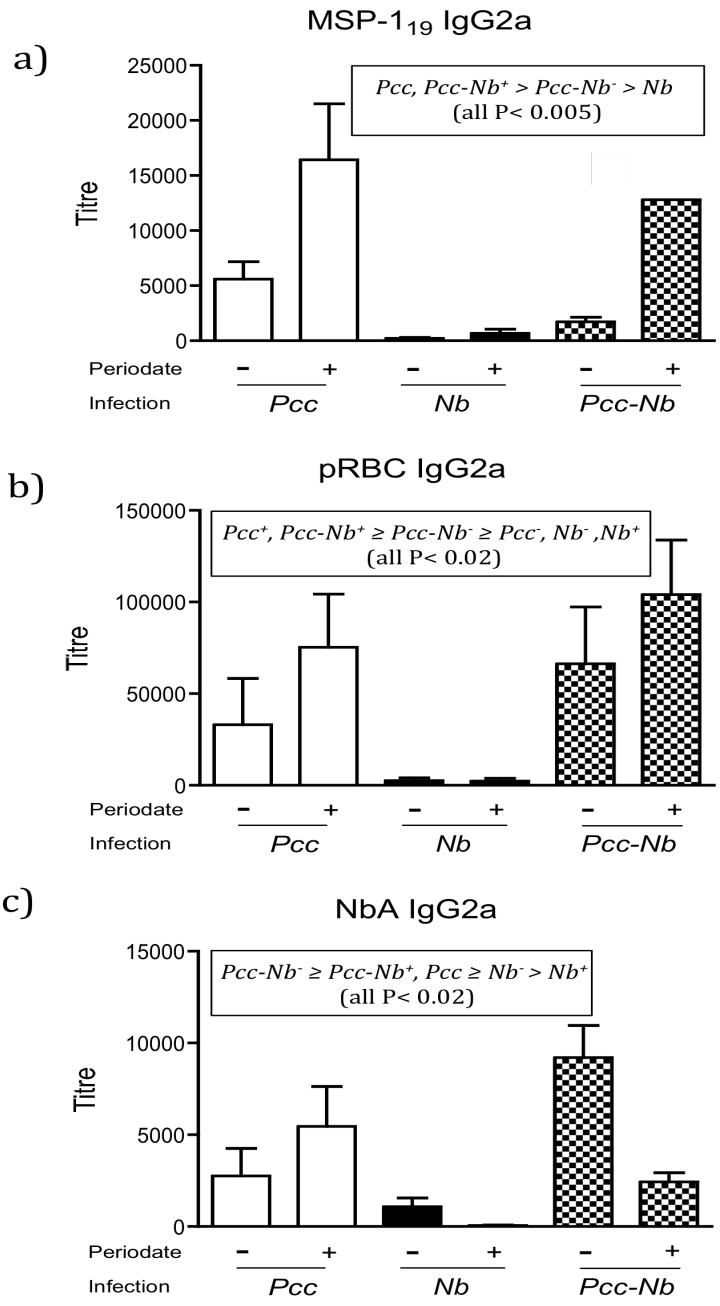


Figure 3: Comparison of Th1 antibody responses to parasite antigens treated (or not) with periodate. Mice were infected with 200 *Nb* L3 larvae and/or 1x10^5 *Pcc*-infected RBCs. Serum antibody titres were measured at day 20 post-infection to malaria antigens MSP-1$_{19}$ a) pRBC b) and worm antigen (NbA) c). IgG2a (Th1) responses to untreated antigen (periodate -) and treated antigen (periodate +) are shown. All titres are above the mean +3 standard deviations of control mice at serum dilution of 1/200 represented as 0 on the y-axis. Single *Pcc* infections are shown in white bars, single *Nb* infections in black bars and co-infected mice (*Pcc-Nb*) are shown in the chequered bars. Graph shows mean and SEM of 8 mice per group. Significant pairwise differences according to Tukey's post-hoc test and the corresponding P-values are summarised in the text boxes.

TABLE 3: Results of GLM for Th1 antibody responses at day 20 p.i measured before and after treatment of antigens with periodate. F statistics and P values of significant terms (P<0.05) come from the minimal model. Significant pairwise differences were determined from the Tukey's adjusted P-values of the most complex significant term in the model and are shown in Figure 3.

Response Variable	df	Factor	F ratio	P value
Anti-MSP-1_{19} IgG2a	$F_{2,21}$	Infection	56.48	<0.0001*
	$F_{1,21}$	Periodate	37.64	<0.0001*
	$F_{2,21}$	Infection*Periodate	6.14	0.0079*
Anti-pRBC IgG2a	$F_{2,21}$	Infection	10.50	<0.0001*
	$F_{1,21}$	Periodate	11.65	0.0026*
	$F_{2,21}$	Infection*Periodate	8.92	0.0016*
Anti-NbA IgG2a	$F_{2,21}$	Infection	22.65	<0.0001*
	$F_{1,21}$	Periodate	12.69	0.0015*
	$F_{2,21}$	Infection*Periodate	6.56	0.0051*

5.5 Discussion

The cross-reactive antibody responses that are observed in the *N. brasiliensis* and *P. chabaudi* co-infection model (Chapter 2 and (Fairlie-Clarke, Lamb et al. 2010)) are not a novel finding but rather add to a body of evidence that suggests the host immune system 'perceives' certain helminth and malaria antigens as similar. The most well characterised cross-reactivity occurs between *S. mansoni* and *P. falciparum* and has been attributed to the protein SmLRR (Naus, Jones et al. 2003; Pierrot, Wilson et al. 2006). Antibody recognition of this *S. mansoni* protein is induced both by *P. falciparum* infection in humans and *P. berghei* infection in rats (Pierrot, Wilson et al. 2006). Although Helmby et al (Helmby, Kullberg et al. 1998) report that infection of mice with *P. chabaudi* does not elicit cross-reactive responses to Schistosome egg antigen (SEA), my own observations in a pilot experiment indicate that *P. chabaudi* infected mice do mount sizeable responses to SEA (data not shown). This discrepancy may be due to a difference in host genotype: Helmby et al (Helmby, Kullberg et al. 1998) infected C57BL/6 mice with *P. chabaudi* whereas I used BALB/c mice. Reciprocal cross-reactive antibody responses are also evident in the *P. chabaudi – L. sigmodontis* co-infection model (Fairlie-Clarke,

Lamb et al. 2010). The potential for these cross-reactive responses to be of biological importance in co-infection warrants further understanding of why they might arise. In this co-infection model the precise antigen that the cross-reactive antibody response is targeting has not yet been characterised. In order to infer the biological relevance of the observed cross-reactive response it will be important to ensure that host-proteins or bacterial contaminants in the antigen preparations are not responsible. For the recombinant MSP-1_{19} protein however this is unlikely.

The question of why antibodies induced by malaria and helminths cross-react is particularly interesting from an immunological perspective as, in theory, the adaptive immune response should be capable of mounting a specific response to every potential antigen (Pancer and Cooper 2006). The occurrence of cross-reactive antibodies indicates that this exquisite specificity is not always realised. It can be difficult to predict which antigens antibodies may cross-react with as the antibody recognises conformational epitopes, the structure of which is not easy to determine from amino-acid or protein sequences (Smith, Lapedes et al. 2004). Many parasite antigens are glycosylated (Dell, Haslam et al. 1999; Mendonca-Previato, Todeschini et al. 2005) and display carbohydrates on their surface, the conformation of which could be conserved across species and so provide a source of cross-reactivity. In this chapter I used periodate treated antigens to explore whether the carbohydrate moieties of *Pcc* and *Nb* antigens were the target of cross-reactive antibodies. It should be noted that periodate is not the only way in which carbohydrate epitopes could be disrupted, and indeed there are caveats to this method that should be acknowledged. For example oxidation of carbohydrates to aldehydes by periodate might not completely disrupt all carbohydrate epitopes and could potentially alter the protein moiety of the antigen. An alternative approach would be to use enzymatic cleavage of the carbohydrates from the protein backbone, although this too could alter binding to the protein moiety. The *Pcc*-induced cross-reactive Th1 response (IgG2a) to NbA recognised proteins, as did anti-*Pcc* IgG2a antibodies induced by *Nb*. Similarly *Nb*-induced Th2 (IgG1) antibodies recognised the protein moiety of *Pcc* antigens. The only cross-reactive response that recognised carbohydrate was the *Pcc*-induced IgG1 response to NbA. On the whole, cross-reactive antibodies in the

Pcc-Nb model recognise the protein moiety of antigens, which is in line with the findings from the *S. mansoni* and *P. falciparum* study, where the SmLRR protein was conserved between parasites (Pierrot, Wilson et al. 2006). Cross-reactivity in that case is therefore not a failure to discriminate between antigens but recognition of a common epitope expressed by both parasites. Determining if this is also true for *Nb* and *Pcc* will require in depth proteomic analysis, but as a starting point I was able to visualise helminth proteins that are recognised by *Pcc* anti-sera via Western Blot (see results section Chapter 6).

The experimental focus of this chapter has been biochemical, in that I have addressed the following question: "would disruption of carbohydrate epitopes reduce cross-reactivity?" This question is based on the hypothesis that the glycosylation of antigens is more likely to be conserved amongst parasite species. Yet the occurrence of cross-reactive responses raises questions as to how accurate the fine-tuning of antibody-antigen specificity during the adaptive response actually is. As outlined in the introduction, there are various points in the process of producing highly-specific antibody that could be constrained in such a way that cross-reactive antibodies would result. Initially the number of B-cells will determine the repertoire of B-cell receptor (BCR) diversity that is available to bind antigen. In a host with fewer B-cells, a constraint on the B-cell repertoire may necessitate the ability to bind more than one antigen (cross-reactivity). This proposed constraint can be somewhat counteracted by B-cell somatic hypermutation and the re-binding of antigen to select for a more specific BCR (and antibody). The potential for antigen availability to constrain antibody specificity (and thus increase cross-reactivity) is explored further in the following chapter.

It is of course possible that cross-reactive antibodies do not result from constraints that are imposed on the immune system but that the host adopts a strategy to maintain a level of cross-reactivity that is beneficial. By employing cross-reactive responses a host may be able to invest less in immune responses whilst still achieving good coverage of antigenic space (Fairlie-Clarke, Shuker et al. 2009). This last proposal is the hardest to test experimentally but some knowledge as to the

extent to which this mechanism might be operating could be gleaned – for example, by investigating whether responses in individuals who are energetically challenged are more cross-reactive, or whether individuals that have evolved under the selection force of exposure to multiple parasites have more cross-reactive responses than those exposed to single parasites.

Chapter 6: "Who goes there?" How antigen dose informs the specificity and isotype bias of the antibody response during co-infection.

6.1 Introduction

Th1/ Th2 polarisation is ultimately determined by the cytokine milieu and and varying antigen dose has been shown to affect this (Constant and Bottomly 1997). Although the effect of varying antigen dose is often explored in model systems the findings are pertinent to natural infections where parasite load (dose) will be anything but uniform. Despite helminth infections characteristically inducing Th2 responses and intracellular parasites Th1, in both the *Trichuris muris* and *Leishmania major* system low doses of parasite can induce Th1 cell mediated responses whereas high doses can lead to a Th2 response (Menon and Bretscher 1998; Bancroft, Else et al. 2001). In *L. major* infection this dose-dependent bias in Th1/Th2 polarisation was also apparent in the isotype of antibody produced by the B-cell population. Mice infected with a low dose of parasites produced predominantly IgG2a whereas those infected with high dose favoured IgG1 (Kaur, Kaur et al. 2008). From these and other studies (Caulada-Benedetti, al-Zamel et al. 1991; Wakelin, Goyal et al. 1994; Power, Wei et al. 1998) it is tempting to surmise that as the dose of parasite moves from low to high so immune bias shifts from Th1 to Th2. However, the effect of antigen dose on the immune response is not straightforward and there is certainly no general rule that low dose antigen equals Th1 and high dose Th2. In fact the exact opposite has been demonstrated where low doses of Hen Egg Lysozyme (HEL), Keyhole Limpet Haemocyanin (KLH) and Toxic Shock Syndrome Toxin (TSST) all elicit Th2 responses (Cho, Chang et al. 2000; Brandt, van der Bosch et al. 2002; Barwig, Raker et al. 2010). The reasons for this discrepancy are not clear but it is worth noting that in the majority of cases where low dose elicits Th1 the host was exposed to live infection with a parasite, as opposed to immunisation with a discrete soluble antigen. Although not explicitly tested in the studies of parasite infection described here it is possible that for the immune system a 'low dose' of parasite

broken down into its component antigens and presented to T and B cells may in fact constitute a high dose in terms of immune stimulation (Constant and Bottomly 1997). This may be further compounded by parasites that replicate within the host.

Thus, when trying to understand how antigen dose will affect immune bias the complexity of the antigen and the context in which it is seen are important. Consideration must be given to the fact that infection with parasitic organisms will cause tissue damage as they invade, expose the immune system to multiple complex antigens, potentially from different parasite life cycle stages and that molecules produced through parasite metabolism or reproduction could all influence the environment in which the immune response is elicited. For instance, parasite infection is associated with 'danger signals', both endogenous resulting from tissue damage and inflammation and exogenous, in the form of pathogen-associated molecular patterns (PAMPS). These danger signals in themselves can influence immune bias by signalling through different pattern recognition receptors known as Toll-like receptors (TLRs) on dendritic cells and B-cells (Xu, Liu et al. 2004). PAMPs are typically associated with bacterial infection but there are also a number of protozoan products that have been found to act as TLR ligands (Gazzinelli and Denkers 2006). The effect of TLR stimulation on immune bias is likely to be exaggerated with increased parasite dose. Greater numbers of parasites will cause more tissue damage and display more PAMPs, intuitively this increase in endogenous and exogenous TLR ligands will result in increased TLR ligation. Exposure to different antigens associated with development of the parasite within the host may also influence immune bias; in Schistosome infection progression through initial infection with the larval stage (cercariae) to the onset of egg production by mature adults results in a switch from Th1 to Th2 responses, suggesting that the egg antigens interact with immune cells in a way that drives the production of cytokines that skew the immune bias toward Th2 (Pearce and MacDonald 2002).

I am interested in the effect of varying the inoculating dose of *Pcc* on immune bias and I hypothesise that increasing the dose of this particular parasite will skew immune bias toward Th1. Acute malaria infection induces a potent Th1 response

characterised by the secretion of the pro-inflammatory cytokines IL-12, TNF-α and IFN-γ. The initial induction of these cytokines has been attributed to the direct interaction of schizonts and merozoites with TLR-9 on dendritic cells (Seixas, Cross et al. 2001; Wu, Gowda et al. 2010). Similarly haemazoin, a by-product of haemoglobin digestion by malaria parasites, has been shown to stimulate TLR9 on dendritic cells (Engwerda and Good 2005). The Th1 cytokine response initiated early in infection will influence the bias of the adaptive response as IFN-γ drives production of IgG2a antibody from murine B-cells (Collins and Dunnick 1993). In addition to this cytokine-mediated antibody production the direct stimulation of TLR9 on B-cells can result in the production of IgG2a (Jegerlehner, Maurer et al. 2007). It is feasible that the TLR9 ligand haemozoin produced during malaria infection could also interact with B-cells in this way to further skew the antibody response toward Th1 (IgG2a). The recognition of protein antigens by T and B-cells is important for determining parasite-specific responses and there is evidence that protein antigens also carry motifs that preferentially induce Th1 or Th2 responses (Guy, Krell et al. 2005). For example the *P. falciparum* antigen MSP-2 has an epitope that induces IgG2b (Th1) antibody production in mice (Tongren, Corran et al. 2005). In summary, infection with *Pcc* induces a heavily Th1 biased environment and I would envisage that increasing the dose of this parasite would only exacerbate this skew in immune bias, reflecting increased exposure to the Th1 inducing malaria antigens and the potential adjuvant effect of haemozoin and malaria parasite DNA via TLR signalling.

Mounting an appropriate immune response to an invading parasite is not simply a case of managing Th1/ Th2 (or Treg/Th17) immune bias. Discrimination of one parasite from another and a specific targeted effector response are also crucial. In theory a diverse repertoire of B cell receptors enables the adaptive immune system to recognise (bind to) every potential parasite antigen (Pancer and Cooper 2006). On recognition of antigen the B-cell proliferates and subsequent targeting of the parasite is achieved through the production of parasite-specific antibodies. Antigen dose may affect this process, as higher doses of antigen will increase the chance of a B-cell encountering its antigen and proliferating.

The relationship between antigen dose and antibody specificity is of further importance as B-cell receptors that have bound their antigen undergo rounds of somatic hypermutation to become even more specific to that antigen. This mechanism involves mutation of the antigen-binding site of the B-cell receptor and re-binding of antigen to select for the cell bearing the receptor with the greatest affinity, culminating in the release of highly specific antibody molecules by plasma cells (Tarlinton and Smith 2000). Given that antigen is required for this process it is reasonable to predict that when there is more antigen available the B-cell could undergo more rounds of somatic hypermutation producing antibodies of even greater specificity to the antigen in question. One way to investigate how antibody-specificity is affected by antigen-dose is to assess the potential of the antibody to bind other antigens, for which it has not been selected, in other words test for antibody cross-reactivity. Antibody cross-reactivity arises when the immune system fails to discriminate between antigens. I am proposing that this could be overcome when there is the opportunity to gather more information on one of those antigens (information being provided in the form of increased antigen dose), such that hosts exposed to greater amounts of antigen produce more specific and consequently less cross-reactive antibodies. It is of course possible that two taxonomically different parasites actually share epitopes (Pierrot, Wilson et al. 2006) in which case dose would not affect the immune system's ability to differentiate between them.

Affinity refers to the strength of a single bond between antibody and antigen, whereas avidity refers to the combined strength of multiple bonds and in its simplest terms can be thought of as the sum of the affinities. Affinity (and so overall avidity) is a property that is selected for, as the antigen-binding site of the B-cell receptor becomes a more perfect fit to the antigen through rounds of somatic hypermutation. This being the case increasing antigen availability (by increasing dose) should allow for further somatic hypermutation and selection of antibodies with greater avidity for their antigen. Comparing the avidity of antigen-specific versus cross-reactive antibody responses will help to elucidate how information provision (antigen) affects the immune systems ability to fine-tune its response to a particular antigen/

pathogen. For example if even after selection for its cognate antigen by somatic hypermutation an antibody was still able to bind another antigen it may only do so with weak avidity. Therefore, in the presence of its cognate antigen, for which it has high avidity, it is unlikely to bind to the other (cross-reactive) antigen and the immune system could be viewed as having successfully fine-tuned its response.

As controlling infection with intracellular parasites often relies on Th1 responses and the clearance of nematodes Th2 responses, the effect of antigen dose on immune bias has serious implications for the host in terms of susceptibility to disease (Bretscher, Wei et al. 1992; Bancroft, Else et al. 2001). The potential for differing antigen dose to affect the bias and specificity of the immune response may also be particularly relevant in the control of co-infection, with parasites such as malaria and helminths, that require the host to mount Th1 and Th2 responses respectively. The fact that Th1/Th2 immune responses are counter-regulatory (Abbas, Murphy et al. 1996) prohibits the host simultaneously mounting a strong response of each type; instead the host is required to manage these opposing responses in an attempt to reach some optimal immune bias (Fenton, Lamb et al. 2008). This suggests that the immune system must 'decide' which parasite poses the greatest threat in order to focus the immune response to that parasite and implies that the host in some way assesses parasite load. Deenick et al (Deenick, Hasbold et al. 2005) report on a hierarchical order for cytokine-induced isotype class switching, where IFNγ is dominant over IL-4 and suggest that this is an adaptation to focussing the immune response toward the more 'virulent' parasite in a mixed Th1/Th2 cytokine setting such as co-infection. The assumption being that rapidly replicating parasites (e.g. malaria or viruses) pose a greater threat to the host than helminths and are associated with increased IFNγ levels. It is interesting to speculate that identifying the greatest parasite threat could be achieved through quorum sensing of immune cells. If for example, a high dose of one parasite antigen relative to another indicates increased parasite load (or uncontrolled parasite growth) and results in greater proliferation of B-cells specific for that parasite (versus those specific for another) then antigen dose could provide the immune system with a proximate measure of parasite load. If this is the case

increasing the dose of malaria in co-infection should result in immune responses more comparable to those observed in malaria only infected hosts.

The *Pcc* and *Nb* model of co-infection provides a useful tool for investigating the effect of antigen dose on immune bias, antibody specificity/ cross-reactivity and avidity (strength of binding) as the initial (inoculating) dose of *Pcc* parasites can be readily manipulated. I propose the following hypotheses:

i) Increasing the dose of *Pcc* will further skew immune bias toward Th1.
ii) Increasing the dose of *Pcc* will decrease cross-reactivity to *Nb* antigen.
iii) Increasing the dose of *Pcc* will increase antibody avidity to *Pcc* antigen.

The rationale behind these hypotheses centres round the interaction of B-cells with malaria antigens and how this is affected by antigen availability. Low dose limits antigen availability meaning that there is less opportunity for B-cell proliferation to be initiated via antigen binding. When proliferation does occur somatic hypermutation ensues, as each round of somatic hypermutation requires that the new B-cell receptor be selected for by antigen binding, low levels of antigen may cap the number of rounds of somatic hypermutation that occur, which has implications for refinement of antibody specificity and avidity. Using a low inoculating dose of malaria parasites may not induce the same level of inflammatory cytokines as high dose and as with antigen availability I predict that parasite associated TLR ligands will also be fewer at low dose. Thus the environment in which the immune response is elicited at low dose is likely to be less Th1 biased, this combined with reduced B-cell proliferation at low dose should be reflected in the antibody response as a lower magnitude of the IgG2a isotype.

6.2 Experimental Design

Female BALB/c mice were infected with a range of inoculating doses of *Pcc*-infected RBC ($1x10^3$, $1x10^4$, $1x10^5$ and $1x10^6$) with or without co-infection of *Nb* (200 L3) on day 0 (Figure 1). A group of mice were also infected with *Nb* alone (200

L3 larvae s.cut). In addition a group of control mice were included which received nRBC ($1x10^5$) and PBS as sham injections for *Pcc* and *Nb* respectively. There were 8 mice per infection group and the experiment was performed twice.

As the inoculating dose of *Pcc* increases by each order of magnitude the peak of asexual parasitaemia occurs one day earlier (Timms, Colegrave et al. 2001). Terminating the experiment on day 11 p.i should therefore result in the mice being exposed to different numbers of malaria parasites and by proxy, antigen. Bodyweight, RBC density and asexual parasite density were measured daily throughout the course of infection. Serum was collected on day 11 p.i and ELISA was used to measure antigen-specific antibody responses of both mixed and high avidity. For mixed avidity responses standard antigen-specific ELISA was performed (see Materials and Methods chapter). To measure high avidity responses the standard ELISA protocol was followed with an additional step introduced following serum incubation, in which the plates were washed with 6M Urea, a hydrogen-bond dissociating agent. This step removed antibodies that had bound weakly (with low avidity) to the antigen (for detailed protocol see Materials and Methods chapter). The antibody titre calculated after Urea treatment was divided by the antibody titre without Urea treatment to determine the 'avidity index', where a higher score corresponds to higher avidity. It should be noted that some of the values exceed 1, when higher titres of antibody were measured following elution of the low avidity antibodies by urea. This could be explained by steric interference if low avidity antibodies crowded around high avidity antibodies prevented binding of the secondary (detection) antibody in the ELISA reaction.

6.3 Statistical Analysis

The analyses presented here are from the combined data of two experiments (n=16 per infection group). The analysis focussed on 153 mice, because 7 mice were excluded due to failed *Pcc* infection (peak parasite density < 0.05 pRBC 10^9/ml). All analyses were carried out in the statistical package JMP 8.0 (SAS) using generalised linear models. Antibody titre and the 'avidtiy index' data were $\log_{10}$

transformed to satisfy the model assumptions of homogeneity-of-variance and normal distribution. All other data (parasite density, day of peak parasite density and RBC minima) did not require transformation. Main effects were worm presence or absence (Nb), and malaria inoculation dose $1x10^3$ – $1x10^6$ (*Pcc* dose) fitted as categorical variables. The main effects and the interaction between them (Nb**Pcc* dose) test whether the response variable (antibody titre) is affected by changes in *Pcc* dose or infection status or both. To address the question of whether *Pcc* dose was significant *Nb*-only mice were excluded, the final analysis of antibody titre was therefore carried out on 121 mice. The effects of dose and infection status were consistent between experiments. Including "experiment" as a factor in all the analyses controlled for any differences in the magnitude of the responses between the 2 experiments. Maximal models were first fit to the data and the minimal models were reached by removing non-significant terms ($p > 0.05$) beginning with interactions. Significant interactions with "experiment" were removed if they were purely quantitative and if their removal did not alter the significance of the main effects remaining in the model. Finally, significant pairwise differences ($p < 0.05$) between groups were determined using the Tukey's All Pairs adjusted p-values for the most complex term in the minimal model.

Figure 1: *Model of* P. chabaudi- N. brasiliensis *co-infection.*
BALB/c mice inoculated on Day 0 with 4 different doses of *Pcc* ($1x10^3$, $1x10^4$, $1x10^5$ and $1x10^6$ *Pcc*-infected RBC) are denoted as *Pcc3, Pcc4, Pcc5 and Pcc6* respectively. Their co-infected counterparts are denoted *NbPcc3, NbPcc4, NbPcc5* and *NbPcc6*. Singly infected mice were given a sham injection of naïve RBC or PBS to mimic the missing *Pcc* or *Nb* inoculation respectively. Control mice received both sham injections. Daily sampling for anaemia, bodyweight and *Pcc* parasitaemia was undertaken until Day 11 p.i when mice were sacrificed for collection of sera.

6.4 Results

Varying the inoculating dose of Pcc *exposes the host to different levels of antigen.*

Previous experiments in C57BL/6 mice infected with *P. chabaudi* genotypes CW and BC, revealed that increasing the inoculating dose of *P. chabaudi* resulted in the highest dose reaching the peak of asexual parasitaemia first (Timms, Colegrave et al. 2001). This result was replicated here using BALB/c mice and the *P. chabaudi* genotype AS. A decrease in dose corresponded to a delay in peak parasitaemia of one to two days per order of magnitude difference (Figure 2a and Table 1). The dynamics of parasitaemia were not significantly different in co-infected mice (Table 1, data not shown). Even though the kinetics of infection was altered there was no significant difference in parasite density at its peak between the groups (see Table 1). The minimum RBC density, which is associated with peak parasitaemia, however did differ in severity in a dose-dependent manner, as higher doses of *Pcc* caused more severe anaemia. There was a trend, although non-significant, for co-infected mice to suffer less severe anaemia than *Pcc*-only infected mice (Figure 2b and Table 1). The rationale that terminating the experiment on day 11 p.i would result in exposure to different levels of antigen (parasites) amongst the groups was borne out as the cumulative parasite density throughout infection differed significantly between the groups. Co-infected mice showed a reduction in cumulative parasite density that bordered on significance (Figure 2c and Table 1). The overall effect of dose on cumulative parasite density was largely driven by differences between the highest and lowest dose of *Pcc*.

TABLE 1: Results of GLM for malaria parasitology and anaemia data. Non-significant terms come from last model before term was dropped and are shown in square brackets. F statistics and P values of significant terms (P<0.05) come from the minimal model. Significant pairwise differences were determined from the Tukey's adjusted P-values of the most complex term in the model and are shown in Figure 2.

Response Variable	Factor	df	F ratio	P value	Experiment effect size estimates (mean ± SE)
Peak Parasite Density					
	Experiment	$F_{1,116}$	8.97	0.0034*	-0.054 ± 0.018
	Pcc Dose	$F_{3,116}$	1.94	0.1274	
	[Nb	$F_{1,115}$	1.05	0.3084]	
	[Nb*Pcc Dose	$F_{3,112}$	0.99	0.4022]	
RBC minima					
	Experiment	$F_{1,116}$	1.10	0.1602	
	Pcc Dose	$F_{3,116}$	39.25	<0.0001*	
	[Nb	$F_{1,115}$	2.31	0.1312]	
	[Nb*Pcc Dose	$F_{3,112}$	2.07	0.1081]	
Cumulative Parasite Density					
	Experiment	$F_{1,116}$	12.87	0.0008*	-0.152 ± 0.044
	Pcc Dose	$F_{3,116}$	5.98	0.0009*	
	[Nb	$F_{1,115}$	3.55	0.0622]	
	[Nb*Pcc Dose	$F_{3,112}$	2.63	0.0536]	
Day of Peak Parasite Density					
	Experiment	$F_{1, 116}$	15.80	0.0001*	0.228±0.057
	Pcc Dose	$F_{3, 116}$	262.76	<0.0001*	
	[Nb	$F_{1, 115}$	0.24	0.6278]	
	[Nb*Pcc Dose	$F_{3, 112}$	1.85	0.1423]	

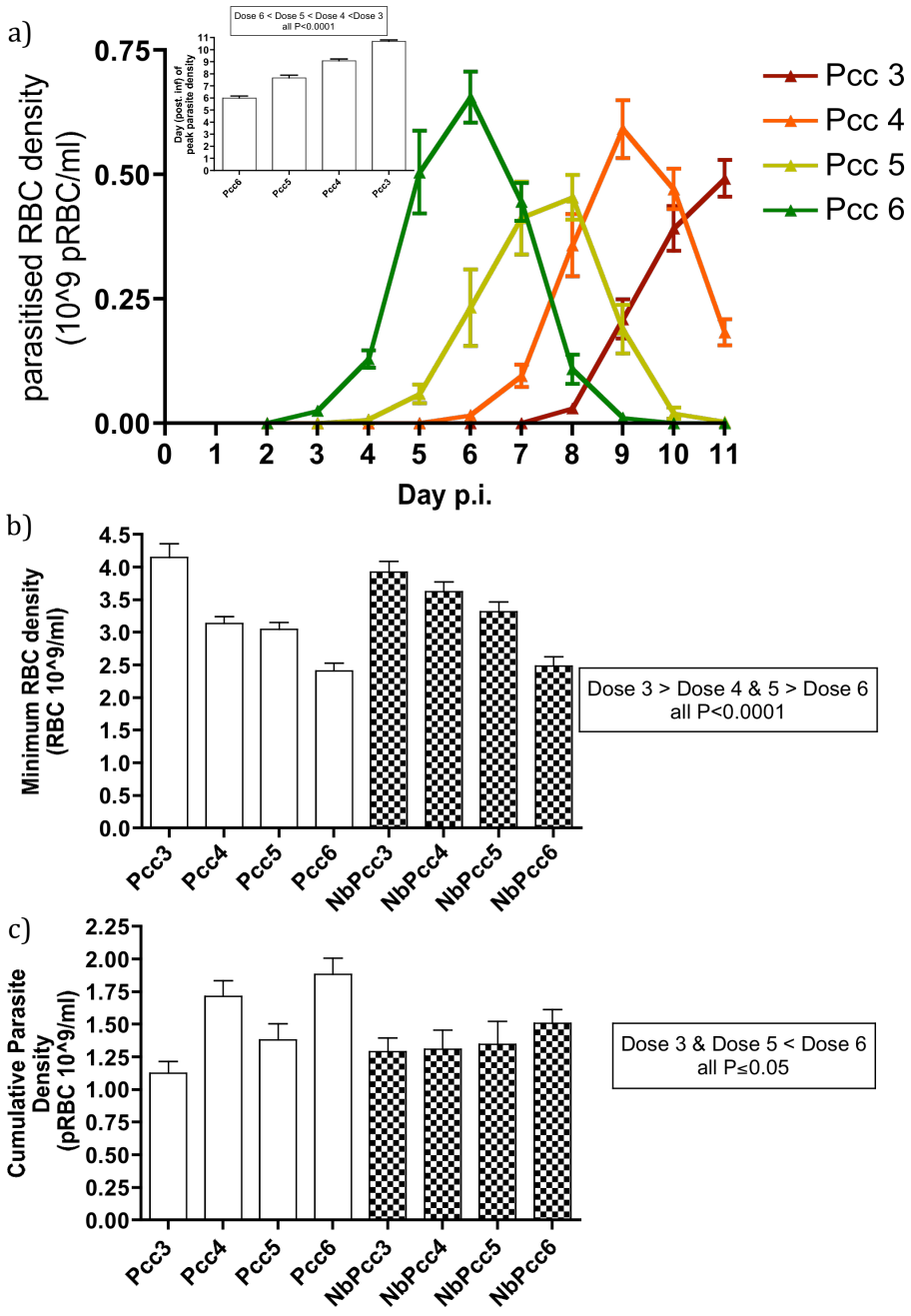


Figure 2: *Malaria parasitaemia and anaemia data. Pcc* was administered at 4 different inoculating doses with or without 200 L3 *Nb* larvae. *Pcc* inocula doses of $1x10^3$, $1x10^4$, $1x10^5$ and $1x10^6$ *Pcc*-infected RBC are denoted as *Pcc3, Pcc4, Pcc5 and Pcc6* respectively. Their co-infected counterparts are additionally denoted with *Nb.* Dynamics of *Pcc* parasite density throughout infection (a) *Pcc* single infections are shown as solid lines or as white bars in inset graph. Co-infection with *Nb* did not significantly alter infection dynamics (data not shown). The RBC minima experienced in single and co-infected mice are shown (b) and the cumulative parasite density experienced throughout infection (c). Single *Pcc* infections are shown in white bars and co-infected mice in chequered bars (mean and SEM n=16 per group). Significant pairwise differences according to Tukey's post-hoc test and the corresponding P-values are summarised in the text boxes.

Increasing Pcc *dose increases the Th1 bias of the immune response.*

To investigate how antigen dose affected Th1/Th2 immune-bias ELISA was used to measure antibody responses at day 11 p.i. Focussing initially on *Pcc*-only infected mice, Th1 (IgG2a) antibody responses to the malaria antigens Pc Lysate and MSP-1_{19} increased as *Pcc*-dose increased (Figure 3a, 3b and Table 2). Alongside this increase in Th1 antibody response was a significant dose-dependent decrease in the IgG1 response to Pc Lysate (see Figure 3d and Table 2), although this was not apparent for anti-MSP-1_{19} (Fig 3e and Table 2). Overall this pattern of antibody isotype production indicated that the immune-bias was skewed toward Th1 as the host was exposed to increasing doses of *Pcc*. It should be noted that this observation was largely driven by pairwise comparisons to the lowest *Pcc* dose (*Pcc* 3). For *Pcc*-only mice, titrating in malaria further skews the immune bias toward Th1.

In order to determine if this dose-dependent Th1 skew in immune bias is also evident when a Th2 inducing worm is on the scene I investigated the response of co-infected mice (Nb*Pcc*) to increased doses of *Pcc*. Although IgG1 responses on the whole were greater in co-infected mice than *Pcc*-only mice (see Figure 3d, 3e, 3f and Table 2) a reduction in the Th2 response as *Pcc* dose increased was evident for anti-Pc Lysate IgG1 and anti-NbA IgG1 responses (Figure 3d, 3f and Table 2). This decrease in Th2 response corresponded with an increase in the Th1 (IgG2a) response to the malaria antigen MSP-1_{19} and worm antigen NbA (See Figure 3b, 3c and Table 2). In contrast to *Pcc* only mice, dose did not affect the IgG2a response to Pc Lysate in co-infected mice (Figure 3a and Table 2). The overall shift in immune bias toward Th1 in co-infected mice is supported by the analysis of total IgE antibody responses. Nb infection induced IgE antibodies characteristic of a Th2 response (Nb, $F_{10, 141}$ =460.88, $p<0.0001$) that were decreased during co-infection with *Pcc* in a dose-dependent manner (Nb**Pcc* Dose, $F_{10, 141}$ =4.29, p=0.0026)(Figure 4). Again the effect of dose was largely driven by significant differences between the dose of $1x10^3$ *Pcc*-infected RBC (*Pcc*3) and $1x10^6$ *Pcc*-infected RBC (*Pcc*6).

TABLE 2: Results of GLM for antibody responses of all avidities. Non-significant terms come from last model before term was dropped and are shown in square brackets. F statistics and P values of significant terms (P<0.05) come from the minimal model. Significant pairwise differences were determined from the Tukey's adjusted P-values of the most complex term in the model and are shown in Figure 3 and Figure 5.

Response Variable	df	Factor	F ratio	P value	Experiment effect size estimates (mean ± SE)
anti-NbA IgG1					
	[$F_{1, 116}$	Experiment	1.31	0.2557]	
	$F_{1, 116}$	Nb	20.58	<0.0001*	
	$F_{3, 116}$	*Pcc* Dose	7.61	0.0001*	
	[$F_{3, 112}$	Nb**Pcc** Dose	1.25	0.2952]	
anti-Pc Lysate IgG1					
	$F_{1, 115}$	Experiment	6.34	0.0132*	0.207±0.082
	$F_{1, 115}$	Nb	6.61	0.0114*	
	$F_{3, 115}$	*Pcc* Dose	6.77	0.0003*	
	[$F_{3, 112}$	Nb**Pcc** Dose	1.19	0.3164]	
anti-MSP-1_{19} IgG1					
	$F_{1, 115}$	Experiment	7.21	0.0083*	0.236±0.088
	$F_{1, 115}$	Nb	28.27	<0.0001*	
	[$F_{3, 115}$	*Pcc* Dose	0.50	0.6835]	
	[$F_{3, 112}$	Nb**Pcc** Dose	0.42	0.7425]	
anti-NbA IgG2a					
	[$F_{1, 115}$	Experiment	3.43	0.0666]	
	$F_{1, 116}$	Nb	3.47	0.0649	
	$F_{3, 116}$	*Pcc* Dose	12.37	<0.0001*	
	[$F_{3, 112}$	Nb**Pcc** Dose	1.29	0.2829]	
anti-Pc Lysate IgG2a					
	$F_{1, 112}$	Experiment	63.17	<0.0001*	0.373±0.047
	$F_{1, 112}$	Nb	0.29	0.5913	
	$F_{3, 112}$	*Pcc* Dose	23.06	<0.0001*	
	$F_{3, 112}$	Nb**Pcc** Dose	6.77	0.0003*	
anti-MSP-1_{19} IgG2a					
	$F_{1, 115}$	Experiment	7.65	0.0066*	0.081±0.029
	$F_{1, 115}$	Nb	3.60	0.0602	
	$F_{3, 115}$	*Pcc* Dose	24.46	<0.0001*	
	[$F_{3, 112}$	Nb**Pcc** Dose	1.01	0.3903]	
anti-LsA IgG2a					
	[$F_{1, 29}$	Experiment	2.33	0.1377]	
	$F_{1, 30}$	Pcc Dose	18.66	0.0002*	
anti-HpA IgG2a					
	[$F_{1, 29}$	Experiment	0.33	0.5708]	
	$F_{1, 30}$	Pcc Dose	76.54	<0.0001*	

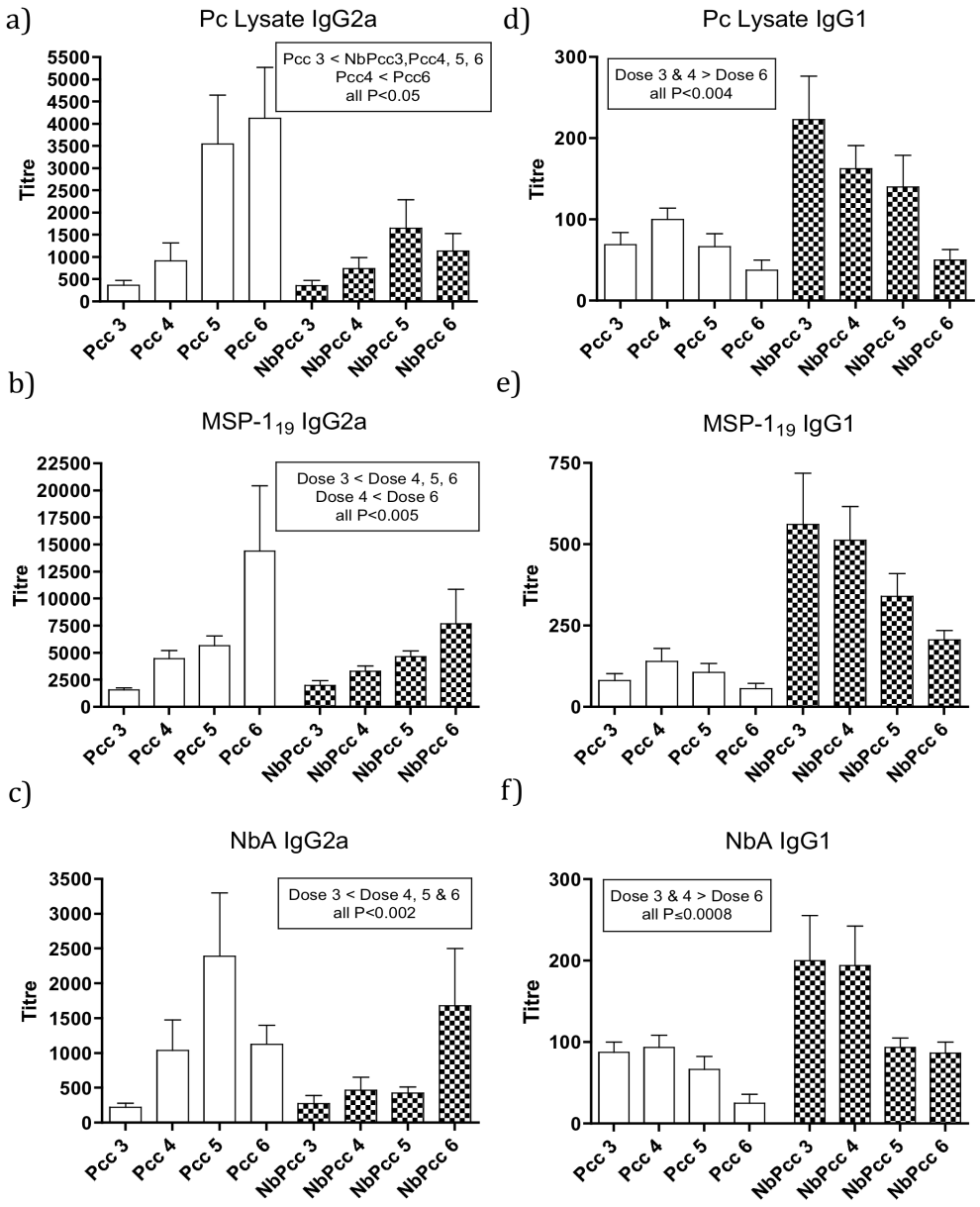


Figure 3: *Antigen-specific antibody responses in malaria and co-infected mice.*
Antibody titres to worm antigen (NbA) and malaria antigens (MSP-1_{19} and Pc Lysate), Th1 responses indicated by IgG2a and Th2 responses by IgG1. All titres are above the mean +3 standard deviations of control mice at serum dilution of 1/200. This cutoff is represented as 0 on the y-axis. *Pcc* doses of $1x10^3$, $1x10^4$, $1x10^5$ and $1x10^6$ *Pcc*-infected RBC are denoted as *Pcc3, Pcc4, Pcc5 and Pcc6* respectively and are shown in white bars. Their co-infected counterparts are additionally denoted with *Nb* and are shown in chequered bars. n=16 per infection group. Graph shows mean and standard errors of 16 mice per group. Significant pairwise differences according to Tukey's post-hoc test and the corresponding P-values are summarised in the text boxes.

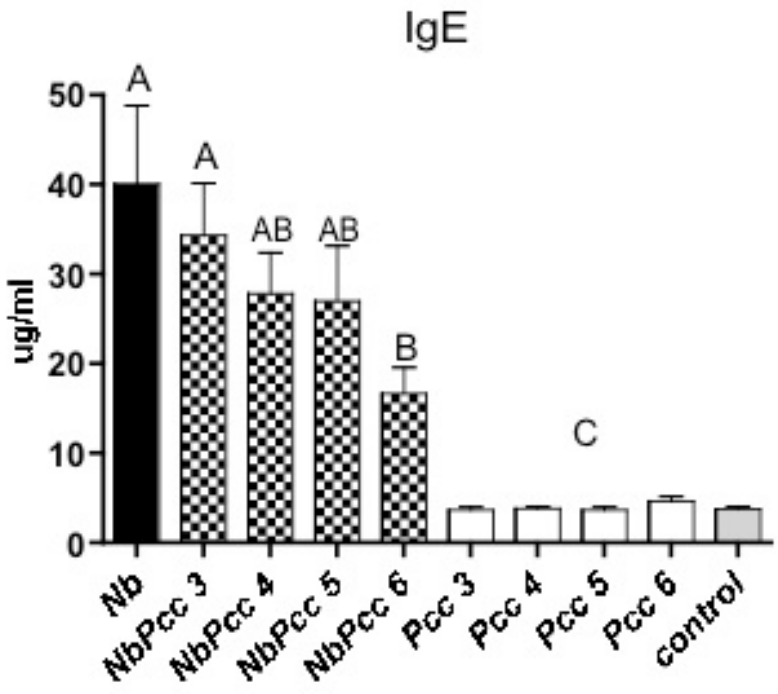


Figure 4: *Total IgE responses in malaria and co-infected mice.* IgE antibody concentration is shown. *Pcc* inocula doses of $1x10^3$, $1x10^4$, $1x10^5$ and $1x10^6$ *Pcc*-infected RBC are denoted as *Pcc3, Pcc4, Pcc5 and Pcc6* respectively and are shown in white bars. Their co-infected counterparts are additionally denoted with *Nb* and are shown in chequered bars. *Nb*-only mice are shown in black and control mice are shown in grey. Graph shows mean and standard errors of 16 mice per group. Significant pairwise differences according to Tukey's adjusted P-values are indicated by letters. Groups that do not share letters are significantly different ($P< 0.01$ for all comparisons).

Increasing Pcc *dose increases both antigen-specific and cross-reactive IgG2a antibody responses.*

Pcc antigen exposure was manipulated in order to investigate how this would affect targeting of the immune response to a particular antigen (parasite). This tested the hypothesis that increasing *Pcc* dose would result in the immune response focussing on malaria antigens. This targeting of the immune response would be indicated by increased production of *Pcc*-specific antibodies and, as a consequence of this selection for antibodies of greater specificity, a decrease in cross-reactivity (ability to

bind other antigens). As reported above infection with *Pcc* results in a Th1 biased immune environment and a dose-dependent increase in the production of malaria-specific IgG2a antibodies, supporting the first part of my hypothesis. To address the second part of the hypothesis cross-reactivity to NbA was assessed in *Pcc*-only mice that had not been exposed to worm antigens. The *Pcc*-induced cross-reactive IgG2a response to NbA increased in a dose-dependent manner (Figure 3c and Table 2).

I wanted to investigate whether this *Pcc*-induced cross-reactive response to *Nb* was extended to other nematodes and so I performed ELISA assays with the same sera using *Litomosoides sigmodontis* antigen (LsA) and *H. polygyrus* antigen (HpA). IgG2a responses to these nematode antigens also increased with dose of *Pcc* (Figure 5a, 5b and Table 2). Western blots were also performed in order to visualise which antigens of the crude preparations (HpA, NbA, LsA and Pc lysate) the *Pcc* anti-sera bound to. Western blot data supported the cross-reactivity to nematode antigens observed in ELISA; serum from *Pcc6* mice recognised (bound to) several distinct bands in the *H. polygyrus, L. sigmodontis* and *N. brasiliensis* crude antigen preparations (Figure 6). Western blots from individual *Pcc6* mice are shown in Appendix Figure 2. Importantly, the binding of antibodies in serum from control mice was negligible (data not shown). The magnitude (titre) of the cross-reactive response to other helminths was less pronounced than that seen for NbA (Figure 5c). The prediction that increased *Pcc*-dose would result in decreased cross-reactivity to worm antigens was thus not borne out. Interestingly the change in immune bias toward Th1 with increasing *Pcc*-dose discussed earlier for antigen-specific responses is extended to the cross-reactive response of *Pcc* mice to worm antigen (NbA). The increase in cross-reactive IgG2a and decrease in cross-reactive IgG1 are shown in Figure 3c and 3f respectively (see Table 2 for statistics).

Germinal centres are the site of T and B-cell interaction and B-cell proliferation during infection. We used fluorescence labelling of splenocytes to measure the size of germinal centres in *Pcc* infected mice (see Materials and Methods section). Representative images are shown in Appendix Figure 1. The increased production of serum antibodies with increased dose was reflected in the increased size of germinal

centres in the spleens of *Pcc6* mice (see Appendix Figure 2a). Similarly the proportion of IgG positive plasma cells increased with dose, driven by the significant difference between control and *Pcc6* mice (see Appendix Figure 2b). It is possible that the increase in both antigen-specific and cross-reactive serum antibodies is simply due to an overall increase in total antibody production. However, measuring total IgG antibodies in serum revealed no significant differences amongst the groups (data not shown).

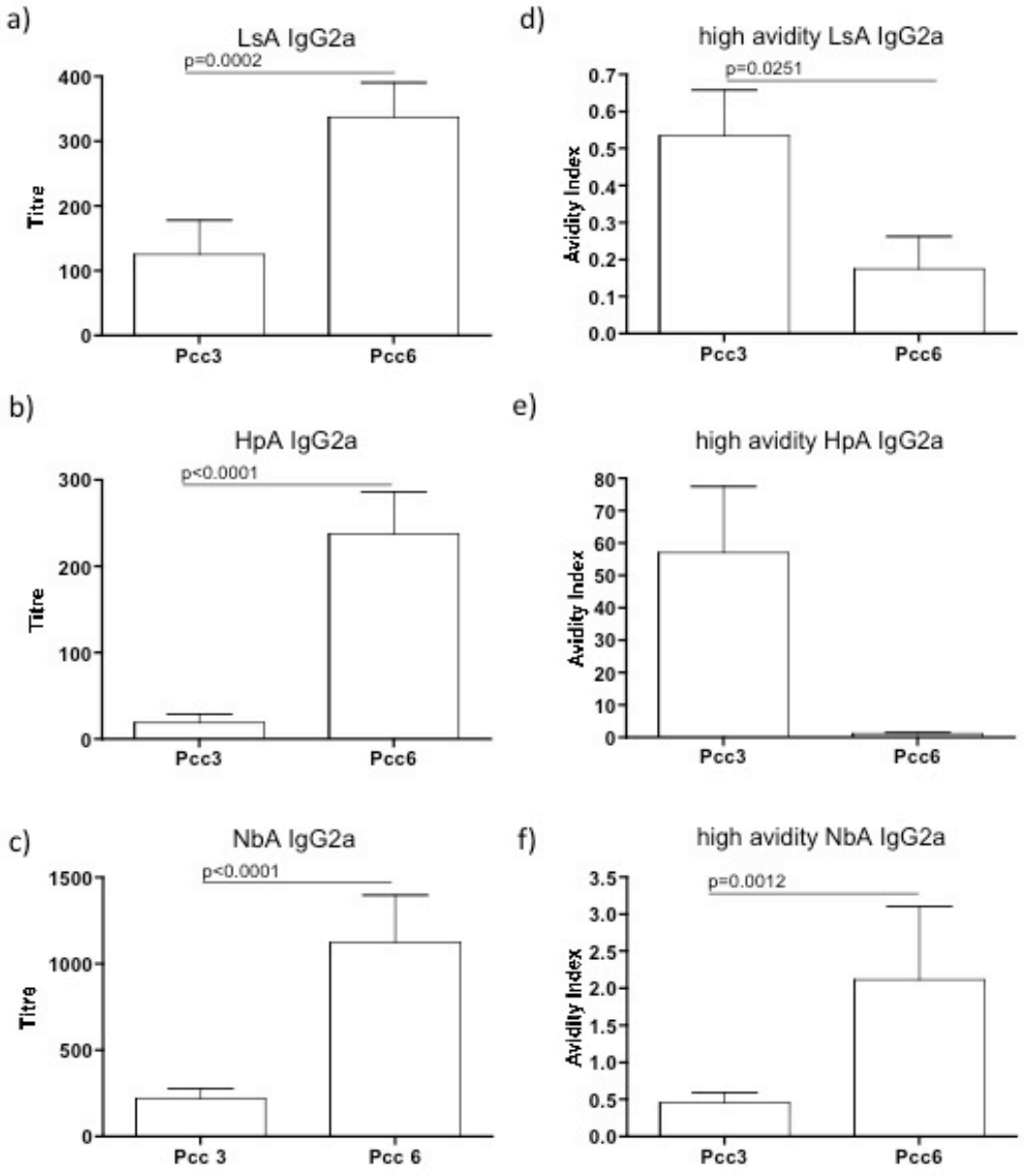


Figure 5: *Malaria-induced cross-reactive antibody responses to nematode antigens.* Th1 (IgG2a) cross-reactive responses induced by *Pcc* infection at low and high dose (Pcc3 and Pcc6) to *L. sigmodontis*, *H. polygyrus* and *N. brasiliensis* worm antigens (LsA, HpA and NbA) are shown (a,b and c). All titres are above the mean +3 standard deviations of control mice at serum dilution of 1/200. This cutoff is represented as 0 on the y-axis. The fraction of the antibody response that is high avidity is presented as the avidity index in figures d, e and f (y-axis). Graph shows mean and standard errors of 16 mice per group. P-values indicate significant pairwise differences according to Tukey's post-hoc test.

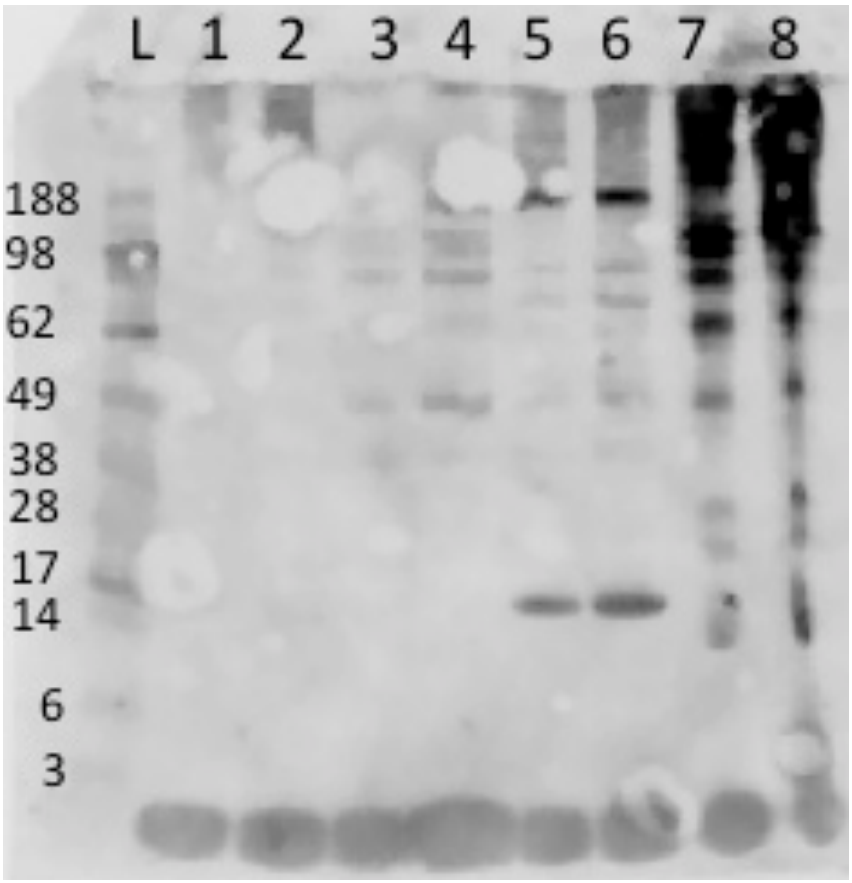


Figure 6: *Western blot of nematode antigens probed with anti-sera from a pool of mice infected with malaria (1x10^6 Pcc-infected RBC).* Antibody (IgG) binding to *H. polygyrus* antigen (HpA) at two concentrations (25ug and 50ug) is shown lanes 1 and 2, to *L. sigmodontis* antigen (LsA) at two concentrations (25µg and 50µg) is shown lanes 3 and 4, to *N. brasiliensis* antigen (NbA) at two concentrations (25ug and 50ug) is shown lanes 5 and 6 and to *P. chabaudi* lysate (PcL) in lanes 7 and 8. The size of the bands (kDA) is indicated by reference to the protein standard (L).

TABLE 3: Results of GLM for antibody responses to nematode antigens. Non-significant terms come from last model before term was dropped and are shown in square brackets. F statistics and P values of significant terms (P<0.05) come from the minimal model. Significant pairwise differences were determined from the Tukey's adjusted P-values of the most complex term in the model and are shown in Figure 5 and Figure 7.

Response Variable	df	Factor	F ratio	P value
High avidity anti-LsA IgG2a				
	[$F_{1, 29}$	Experiment	2.45	0.1284]
	$F_{1, 30}$	Pcc Dose	5.56	0.0251*
High avidity anti-HpA IgG2a				
	[$F_{1, 29}$	Experiment	0.46	0.5048
	$F_{1, 30}$	Pcc Dose	2.15	0.1530
High avidity anti-NbA IgG2a				
	[$F_{1, 116}$	Experiment	0.21	0.6489]
	[$F_{1, 115}$	Nb	0.04	0.8517]
	$F_{3, 117}$	Pcc Dose	5.06	0.0013*
	[$F_{3, 112}$	Nb*Pcc Dose	0.27	0.8475]
High avidity anti-NbA IgG1	[$F_{1, 116}$	Experiment	2.08	0.1518]
	[$F_{1, 115}$	Nb	0.73	0.3931]
	$F_{3, 117}$	Pcc Dose	2.82	0.0422*
	[$F_{3, 112}$	Nb*Pcc Dose	1.44	0.2351]

Increasing Pcc *dose differentially affected the avidity of cross-reactive responses to helminth antigens.*

Even though the ability of *Pcc*-induced antibodies to bind worm antigens was not decreased by increasing *Pcc*-dose, it is possible that the avidity (strength of binding) of these antibodies was altered. For example, mutation of the antigen binding-site through somatic hypermutation could result in antibodies that bind more strongly to their cognate antigen and less strongly to other antigens. Modifying the ELISA protocol to remove weakly bound (low avidity) antibodies enabled the avidity index for each antibody response to be measured (see Materials and Methods). The decreased avidity of IgG2a responses to LsA (Figure 5d Table 3) is in line with the hypothesis that increased *Pcc* dose results in decreased avidity of cross-reactive responses. Although not statistically significant this trend was also seen for IgG2a responses to HpA (Figure 5e Table 3). Contrary to hypothesis iii), the avidity of

cross-reactive responses to NbA (IgG2a and IgG1) increased with increased dose of *Pcc* (Figure 5f, Figure 7 and Table3). This effect of dose also applied to co-infected mice (Table 3) but conclusions regarding cross-reactive responses are drawn from the response induced in *Pcc*-only mice. There was no change in the avidity of responses to malaria antigens with *Pcc* dose (data not shown).

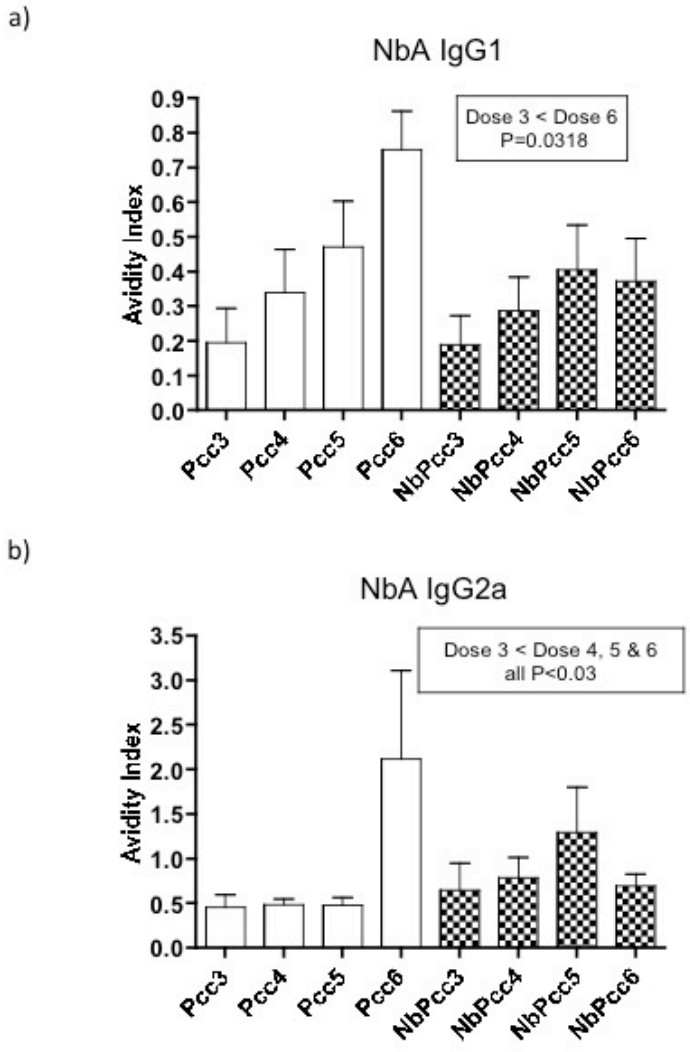


Figure 7: *High avidity antibody responses in malaria and co-infected mice.*
The antibody response to NbA that is of high avidity is presented as the avidity index (y-axis). Th2 responses indicated by IgG1 (a) and Th1 responses by IgG2a (b) Single *Pcc* infections are shown in white bars with the different *Pcc* inocula doses of $1x10^3$, $1x10^4$, $1x10^5$ and $1x10^6$ *Pcc*-infected RBC denoted as *Pcc3, Pcc4, Pcc5* and *Pcc6* respectively. Co-infected mice are additionally denoted with 'Nb' and are shown in the chequered bars. Graph shows mean and standard errors of 16 mice per group. Significant pairwise differences according to Tukey's post-hoc test and the corresponding P-values are summarised in the text boxes.

For several of the antibody responses measured the observed variation was explained by *Pcc* dose (Table 2 and 3). I sought to determine if some factor associated with dose such as cumulative parasite density could account for this effect. Controlling for cumulative parasite density in the statistical model did not help to explain the effect of dose on antibody responses with the exception of IgG2a responses to MSP-1_{19} (Cumulative Density, $F_{9, 111}$=4.49, p=0.0363). This suggests that some property of dose other than cumulative parasite density is important. Although earlier I reasoned that differences in cumulative density would equate to differences in antigen exposure it seems that this is insufficient to explain the dose-dependent effect. Cumulative density as a measure of antigen exposure may be too simple; perhaps, for example, the point at which parasite density exceeds a certain threshold could be key. This alternative explanation could also be dose-dependent; the change in the kinetics of parasitaemia observed for the different doses (Figure 2a) could result in the highest dose of *Pcc* exceeding the threshold first, so that induction of the antibody response occurs earlier and maturation of the antibody response takes place over an extended period.

6.5 Discussion

"Who goes there?" is a simple question that illustrates the immune system's need to gather information to recognise when a host is under attack and from what. This information processing occurs at the molecular level via T- and B-cell receptors binding antigen. It is not enough to simply register infection: successfully combating infection requires the immune system to 'make decisions' regarding what type of effector response (e.g. Th1 or Th2) should be deployed and precise targeting of the immune response (pathogen-specificity) requires discrimination of different pathogens. When faced with co-infection these decisions are potentially further complicated by the need to manage counter-regulatory responses and prioritise immune targeting of the predominant pathogen. A better informed immune system should help ensure the most appropriately balanced outcome. Indeed B-cells through production of affinity matured specific antibody display 'immunological learning'

when provided with information (antigen) (Rajewsky 1996). I increased the inoculating dose of *Pcc* (antigen dose) as a means of increasing information provision to assess the effect this would have on antibody responses in both single and co-infecion. Immune bias, antibody specificity/ cross-reactivity and avidity for antigen were used to determine whether the opportunity to gather more information on the *Pcc* antigen would improve immune targeting of this parasite.

The dogma that increasing parasite dose causes a switch from Th1 to Th2 does not always hold true.

The patterns of cytokine production that ultimately determine Th1/Th2 immune bias can be influenced by many factors such as the route of infection, antigen affinity and antigen dose (Constant and Bottomly 1997; Cho, Chang et al. 2000; Zinkernagel 2000; Kaur, Kaur et al. 2008). In many model systems of parasite infection as antigen dose increases the response switches from Th1 to Th2 characterised by the production of IFNγ or IL-4 respectively (Menon and Bretscher 1998; Ismail and Bretscher 1999; Bancroft, Else et al. 2001; Shata, Tricoche et al. 2003). The effect of dose on Th1/ Th2 bias can also be defined by the antibody isotype produced (Fairlie-Clarke, Lamb et al. 2010). Characterisation of immune bias in this way reveals the same dichotomy for infectious agents and soluble antigens discussed previously; where low or high dose of the parasites *L. donovani* and *T. muris* results in production of IgG2a (Th1) or IgG1 (Th2) respectively (Bancroft, Else et al. 2001; Kaur, Kaur et al. 2008) whereas a shift from IgG1 to IgG2a is seen with increasing dose of the soluble antigen HEL (Cho, Chang et al. 2000). The induction of Th1 responses at low dose is supported by studies of malaria infection in which T-cells exclusively produced IFNγ during sub-patent infections (Pombo, Lawrence et al. 2002).

Experimental models that manipulate the dose of an antigen or pathogen aim to capture the variation in dose that arises through natural exposure to pathogens, as ethical constraints often restrict research in humans. Thus observations from ‘natural experiments’ are extremely valuable, for example a human population with natural

variation in malaria endemicity revealed increasing cumulative exposure to antigen resulted in a gradual skew toward IgG1 (Th1) antibody production (Tongren, Drakeley et al. 2006). Interestingly, infection of human volunteers with sub-patent doses of *P. falciparum* resulted in exclusive production of IFNγ (Pombo, Lawrence et al. 2002). Taken together, these data suggest that malaria elicits Th1 responses that increase with dose. My experimental set-up reflected changes in cumulative antigen exposure, albeit over a very short timescale, as introducing malaria infection with a range of inoculating doses and terminating the infection at day 11 capitalised on the shift in malaria parasite growth dynamics such that mice were exposed overall to different parasite densities. The results reported in this chapter support the findings of Tongren et al (Tongren, Drakeley et al. 2006) as the data indicates Th1 bias is more pronounced with increased *Pcc* dose. Of course it is important to acknowledge the limitations of extrapolating animal models to human populations as how the pathogen is introduced (e.g. route of administration, life cycle stage) may not accurately reflect induction of, for example, malaria-specific immune responses following the bite of an infectious vector. Nonetheless, the study of these dynamics in animal models can give us fundamental information that may help assess observations available from human studies.

It is generally thought that the dichotomy of how antigen dose affects immune bias is due to the type of antigen used (Constant and Bottomly 1997; Cho, Chang et al. 2000). Thus the results presented here (and those of Tongren et al (Tongren, Drakeley et al. 2006)) are contrary to the current thinking that increasing the dose of parasite antigen (as opposed to soluble antigen) will result in a Th2 biased environment. I would argue that attempting to apply this generalisation to the vast array of parasites and associated antigens that a host may encounter is unrealistic. Indeed there is evidence that this 'rule', which applies to *T.muris* (Bancroft, Else et al. 2001) does not hold for other helminths; infection with as little as 6 *N.brasiliensis* larvae induces a potent Th2 response that is only enhanced at higher doses (Lawrence, Gray et al. 1996). Similarly, low or high dose infection of BALB/c mice with the liver fluke *F. hepatica* induces a Th2 response. Interestingly, infection of an alternative host (C57BL/6 mice) with this parasite does induce a switch from Th1 to

Th2 with increasing dose (O'Neill, Brady et al. 2000), suggesting host genotype can also be an important consideration in attributing an effect of dose on immune bias. Other factors such as antigenic differences between parasites may also exert an influence over cytokine induction, this is elegantly demonstrated by infection with *Trichinella spiralis* or the subspecies *T. pseudospiralis,* which induce Th1 and Th2 biased responses respectively (Wakelin, Goyal et al. 1994). Understanding the effect of dose on immune-bias is certainly not straightforward and demonstrates the need for further research in this area.

Co-infection with malaria and helminths can influence immune bias due to the counter-regulatory nature of the Th1 and Th2 response induced by each parasite (Abbas, Murphy et al. 1996). Here I investigated how increasing the dose of *Pcc* could further influence immune bias and investment in Th1 immune responses during co-infection. In co-infected mice as *Pcc* antigen dose increased the magnitude of the IgG2a (Th1) response increased with a corresponding decrease in the IgG1 (Th2) response. This bias toward Th1 was further supported by the dose-dependent reduction in total IgE responses in co-infected mice. On a proximate scale this shift in immune bias could be a consequence of increased *Pcc* parasite load (relative to Nb), registered via stimulation of TLRs and T-cell receptors. Several studies have shown that activation of TLR9 on innate immune cells (e.g. dendritic cells) by merozoites or hemazoin results in IL-12 induction by antigen presenting cells (e.g. dendritic cells) and subsequent production of IFNγ from T-cells (Coban, Ishii et al. 2005; Franklin, Parroche et al. 2009). The TLR-9 driven production of these cytokines increases with pathogen dose and may increase Th1 cell differentiation (Wu, Gowda et al. 2010). Due to activation by high levels of *Pcc* antigens these Th1 cells are likely be *Pcc*-specific and will in turn drive the production of Th1 *Pcc*-specific antibodies from B-cells. Such an antigen-dose-dependent increase in antibody production was observed by Coban et al (Coban, Igari et al. 2010) who also noted the adjuvant effect that TLR-9 stimulation had on this response. Increased production of Th1 antibodies can also be driven by direct stimulation of TLR-9 on B-cells (Jegerlehner, Maurer et al. 2007; Eckl-Dorna and Batista 2009). The proximate cause of increased Th1 antibody production in this system is therefore increased dose

of *Pcc* antigen and in all likelihood the associated TLR ligands but the ultimate cause is to assess which parasite poses the greater threat and to mount an appropriate response to ensure survival of the host.

Increasing Pcc *dose does not reduce cross-reactive responses suggesting* Nb *and* Pcc *share antigens.*

For a host to successfully target a particular parasite relies on the adaptive immune system discriminating between invading pathogens by having a receptor to match every potential antigen. In theory this is possible as the process of somatic hypermutation results in a staggering degree of B-cell receptor diversity (Pancer and Cooper 2006). However, antibody cross-reactivity suggests that there is some limitation imposed on the system (Fairlie-Clarke, Shuker et al. 2009), and there are many cases of antibody recognising epitopes on two different pathogens (Xu and Powell 1991; Naus, Jones et al. 2003; Casadevall and Pirofski 2007; Nguyen, Zemlin et al. 2007). In earlier chapters and (Fairlie-Clarke, Lamb et al. 2010) I have demonstrated that this cross-reactivity is also observed in the *Pcc*-*Nb* model. It is possible that some degree of cross-reactivity is maintained when fewer rounds of somatic hypermutation are undergone, which might be the case when antigen is limiting. If cross-reactivity is simply a result of the immune system not having the opportunity of 'getting to know' the antigen then in this model increasing *Pcc* antigen should enable antibody to become specifically tuned to *Pcc* antigens and (cross-reactive) recognition of nematode antigens, for example, would diminish. As predicted antigen-specific IgG2a antibody responses increased as *Pcc* dose increased however the suggested trade-off in ability to bind other parasite's antigens was not evident; the cross-reactive IgG2a response of *Pcc*-mice to nematode antigens NbA, LsA and HpA also increased with *Pcc* dose. Both antigen-specific and cross-reactive IgG1 responses decreased in a dose-dependent manner, which reflects the dose-dependent increase in Th1 bias discussed earlier.

Increased specificity for an antigen is also reflected in the strength of antibody binding (avidity) and this can also be improved through rounds of somatic

hypermutation. At higher levels of antigen there is greater potential for cells to undergo more rounds of somatic hypermutation, each round being interspersed with the need to test antigen-binding (Gonzalez-Fernandez and Milstein 1998). Antibodies that have undergone somatic hypermutation and affinity maturation in the context of malaria infection should be selected for improved specificity (binding) to *Pcc* antigens. This leads to the prediction that increasing *Pcc* dose will increase the avidity of antibody for *Pcc* antigen and reduce avidity to nematode antigens. The proportion of antibodies that bound with high avidity was determined by comparing antigen binding in ELISA in the presence and absence of a hydrogen-bond dissociating agent (urea). Low avidity antibodies that rely more heavily on hydrogen bonds are preferentially dissociated from the antigen by this method (Bjorkman, Naslund et al. 1999; Yasodhara, Ramalakshmi et al. 2001).

The avidity of cross-reactive antibodies to NbA in the *Pcc* mice increased with dose. This was true of both the IgG1 and IgG2a response. In contrast antibody binding to LsA and HpA was mostly of low avidity. It should be noted that there is some evidence that high doses of antigen in contrast to selecting for highly specific antibodies actually enhance selection of cross-reactive or low avidity antibodies. The idea behind this is that at lower doses when antigen is limited high affinity antibodies bind to antigen more rapidly and prevent the antigen-dependent selection of other less-specific B-cell clones (Gonzalez-Fernandez and Milstein 1998; Eisen and Chakraborty 2010). Although we observed increased cross-reactivity at higher doses our data does not support this theory, as better avidity was not selected for at low dose. The propensity for this competitive exclusion of low-avidity antibodies to occur at low dose may depend on the nature of the selecting antigen (Eisen and Chakraborty 2010). A complex mixture of antigens such as a parasite inoculate may be more likely to select for antibodies capable of binding a variety of epitopes (with variable avidities), whereas a defined antigen such as MSP-1_{19} may select for highly specific antibodies of uniform avidity. In support of this titrating in malaria results in a more linear increase in the antibody response to MSP-1_{19} than to PcL.

The fact that cross-reactive responses to NbA increased in avidity with increasing dose strongly suggests that *Pcc* and *Nb* have shared antigens. In other words there is an epitope (antibody binding site) that *Pcc*-induced antibodies are capable of recognising on both *Pcc* and *Nb*. This is supported by western blot data, which shows that *Pcc* anti-sera binds to antigens present in both NbA and PcL. Such shared antigens have previously been identified for *S. mansoni* and *P. falciparum*; a putative *P.falciparum* protein showed 57% amino acid identity to the 64kDa protein "SmLRR" from *S. mansoni* and this protein was bound by antibodies induced by both infections (Pierrot, Wilson et al. 2006). Often such cross-reactivity is found to be due to conserved carbohydrate moieties (Xu and Powell 1991; van Remoortere, Bank et al. 2003) but I have shown this is not the case in the recognition of NbA by *Pcc*-induced antibody (Chapter 5 and (Fairlie-Clarke, Lamb et al. 2010)). In the case of LsA and HpA it is feasible that cross-reactivity may be due to recognition of conserved carbohydrates and may offer an explanation as to why the avidity with which *Pcc*-induced antibodies bound these antigens decreased with increased *Pcc* dose. This begs the question as to why malaria-induced antibodies maintain the ability to recognise *N. brasiliensis* and not other nematodes (i.e. *L. sigmodontis* and *H. polygyrus*)? The asexual stages of *Pcc* infect erythrocytes and circulate in the blood and migration of *Nb* larvae causes significant haemorraghing in the lungs (Hoeve, Mylonas et al. 2009), as does adult feeding on the gut wall (Variyam and Banwell 1982). In contrast *H. polygyrus* resides in the gut lumen and *L. sigmodontis* in the lymphatics. Perhaps the propensity for both *Nb* and *Pcc* antigens to be encountered in the circulatory system has led to the selection of cross-reactive antibodies. It is possible that if a shared antigen does exist between these parasites its function may relate to the adaptation of these parasites to survive in the blood for periods of their life cycle. Interestingly *S. mansoni*, which resides in the hepatic portal vein is recognised by *P.falciparum* induced cross-reactive antibodies, lending some support to the idea that a shared niche may be important for the maintenance of cross-reactive antibodies to these taxonomically distinct parasites.

Earlier I hypothesised that cross-reactivity might be overcome with the opportunity to gather more information about one of those antigens. This appears not to be the

case for NbA and *Pcc*, rather the information given to the immune system (regardless of dose) is that these 2 antigens (NbA and PcL) are similar. As shown by the persistence of cross-reactive responses at high dose and the western blot data that revealed proteins common to both parasites. However, when we consider cross-reactivity to the other nematode antigens (LsA and HpA) increasing information provision (*Pcc* dose) reduced the avidity of cross-reactive responses to these antigens so I would argue that the immune system is capable of discriminating between malaria and these other nematode antigens. Yet cross-reactivity, albeit of low avidity, persists at high *Pcc*-dose. It is interesting to speculate that maintaining some degree of cross-reactivity may benefit the host if it allows recognition of more than one antigen by fewer B-cells, this may be particularly important for a resource limited host (Fairlie-Clarke, Shuker et al. 2009; Eisen and Chakraborty 2010).

What underlies the dose-dependent effect on antibody responses?

As *Pcc* dose was found to have a significant impact on antibody production I sought to determine if cumulative parasite density, which differed significantly with dose was responsible. For all responses, except anti-MSP-1 IgG2a, cumulative density did not explain the observed variation in antibody response. This suggests that the dose-dependent increase in Th1 antibody responses was not due to cumulative antigen exposure (cumulative parasite density). In trying to understand what factor may be responsible for the dose-dependent effect observed it is important to acknowledge the caveats of the experimental design used. Namely those dose-dependent effects that result in differences in cumulative parasite density are inextricably linked to changes in the kinetics of infection. Previous experiments (see Chapter 2 Figure 3 & Chapter 3 Figure 1a) have shown that antibody production initially occurs approximately 2 days after asexual parasites are measurable by microscopy in blood smears, indicating that antibody production may occur after some critical parasite density is exceeded. If the period of time available for B-cells to proliferate and differentiate into plasma cells, after stimulation by a critical level of antigen, has more influence on antibody production than stimulation by increased antigen exposure then the dose-dependent effect on infection kinetics may be crucial. In this

experimental design mice that experienced the highest dose of *Pcc* could have produced antibody from day 5 p.i onwards whereas those mice experiencing the lowest dose may only have begun producing antibody at day 9 or 10 p.i. Thus at higher dose the magnitude of the antibody response may not only be greater but exhibit enhanced specificity/avidity due to extended opportunity for affinity maturation. To untangle the effect of parasite-dose and changes in infection kinetics, inoculation with different concentrations of whole parasite antigen rather than live parasites could be used.

In summary increasing the dose of *Pcc* antigen resulted in antibody responses that were increasingly Th1 biased in both single (*Pcc*) and co-infection. Increasing *Pcc* antigen dose did not result in the reduction of cross-reactive responses to *Nb* antigen. Antibody avidity to *Pcc* antigen was not changed by dose but avidity to *Nb* antigen was increased at higher doses. Increasing the level of information provision to the immune system by altering antigen dose resulted in immune responses that were increasingly tailored to targeting malaria in terms of immune bias (Th1). However the anticipated trade off in reduced recognition of *Nb* antigen was not evident suggesting that cross-reactivity in this system was due to these antigens having shared epitopes.

Outlook:

Understanding how pathogen dose affects immune responses has important implications for disease outcome and health management. Dose-induced changes in immune bias can mean the difference between resistance/ susceptibility to disease (Menon and Bretscher 1998). The design of effective vaccine strategies often relies on the induction of Th1 or Th2 responses and dose is an important means of manipulating the desired response (Power, Wei et al. 1998). These issues have received due attention in the literature but whilst the influence of co-infection on immune bias and disease outcome is reported (Spiegel, Tall et al. 2003; Sokhna, Le Hesran et al. 2004; Lyke, Dicko et al. 2005; Ezenwa, Etienne et al. 2010) there is a paucity of data of the type reported here that investigates the effect of pathogen dose

on immune responses during co-infection, with the exception of (Montano M 2005). As animal models strive to reflect natural infection, incorporating co-infection and differential exposure to pathogens (dose) is important for capturing the situation in natural populations where co-infection is the norm and exposure to parasites is anything but uniform.

To my knowledge this study is the first to show *Pcc*-induced cross-reactivity to *Nb* by Western blot and this data highlights the potential for interactions between these two pathogens to be mediated by immune targeting of cross-reactive antigens. However, the functional consequences of these cross-reactive responses have yet to be elucidated. From an evolutionary standpoint targeting cross-reactive antigens may be beneficial to the host if it enables recognition of various pathogens to be achieved by fewer B-cells. It would be interesting to determine if individuals with natural variation in their B cell population varied accordingly in production of cross-reactive antibodies. Genetic variants exist that result in a reduced response to stimulation via the B cell receptor (Rieck, Arechiga et al. 2007), potentially cross-reactive binding could ameliorate this effect by engaging more B cell receptors. Investigating the potential for this could aid understanding of the benefits of cross-reactivity to the host.

Appendix

Materials and Methods

Immunocytochemistry

Spleen sections from control, *Nb*, *Pcc3*, *Pcc6*, *NbPcc3* and *NbPcc6* mice (see Chapter 6 for nomenclature) were stained with fluorescent antibodies to identify germinal centres and IgG or IgM positive plasma cells.

Tissue sectioning

Whole spleens removed post-mortem from BALB/c female mice were placed in moulds and immediately covered in Tissue Tek O.C.T^{TM} compound on dry ice. These frozen blocks were stored at -80^{o}C prior to being cut into 5µ sections by Cryostat and mounted on microscope slides at the Queen Margaret Research Institute histology department (University of Edinburgh). Sections were left to air dry at room temperature for 1 hour and then stored at -20^{o}C until use.

Fixing tissue sections

Sections were removed from -20^{o}C and allowed to reach room temperature whilst remaining in a sealed box to prevent condensation forming. Sections were then submerged in acetone at 4^{o}C for 20 mins to fix the sections. The sections were stored at -20^{o}C until required for immuno-staining with fluorescent antibody.

Staining sections

Sections were removed from -20^{o}C and allowed to reach room temperature whilst remaining in a sealed box to prevent condensation forming. Sections were then submerged in a blocking solution of 1% BSA: sterile PBS for 15 mins at room temperature. Primary antibodies were all used at 1:100 dilution in 1%BSA:PBS; PNA- Fluorescein Peanut Agglutinin (5mg/ml Vector laboratories FL-107), IgM- Goat anti-mouse IgM conjugated to Texas Red (1mg/ml Southern Biotech 1021-07) and IgG- Goat anti-mouse IgG conjugated to Biotin-SP (1.4mg/ml Jackson

ImmunoResearch laboratories 115-066-008). 50µl of this antibody cocktail was added to each section and incubated at room temperature for 2 hours in a hydrating chamber protected from light. The sections were then washed 3 times by quenching the slides in PBS for 5 mins. A secondary antibody Streptavidin Alexafluor 350 (1mg/ml Invitrogen S11249) was added to label the primary IgG antibody (others were directly conjugated). 50µl of a 1:100 dilution of antibody: 1% BSA:PBS was added to each section and incubated at room temperature for 1 hour in a hydrating chamber protected from light. Sections were washed 3 times in PBS as before. Sections were then covered with a cover slip secured by Mowiol mountant medium with 2.5% DABCO (fluorescence stabilizer) added. Sections were stored at 4^{o}C in the dark.

Imaging

Immuno-staining of the tissue sections was visualized by fluorescence microscopy x10 magnification. PNA labeled green by FITC stained germinal centers, B-cells and IgM positive plasma cells were stained in red by Texas Red and IgG positive B-cells and plasma cells were labeled blue by Alex Fluor 350. Individual photographs were taken for each stain and then merged to give a 3-colour image using Improvision Openlab 5.5.1 software.

Image quantification

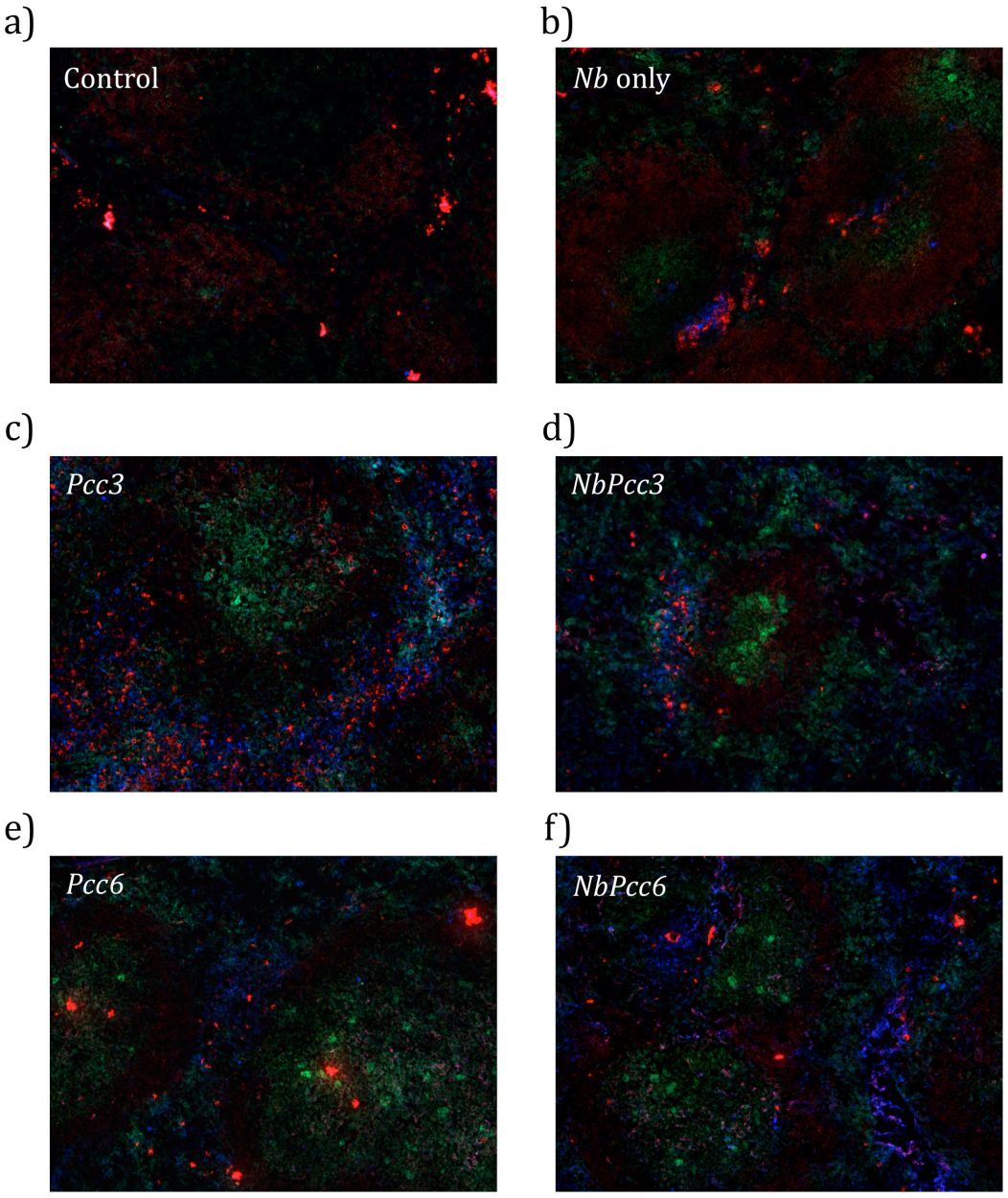
ImageJ software version 10.2 (developed by NIH for the public domain) was used to quantify the size of the germinal centre and the proportion of IgG positive cells in a representative area of the tissue section. The germinal centre, identified by PNA staining, was outlined and the software calibrated so that the area of the germinal centre was recorded in mm^2.

Rather than counting individual cells the proportion of IgG postive cells in a representative microscope field was calculated. Fluorescence falling within the range 87 to 255 units indicated IgG positive plasma cells.

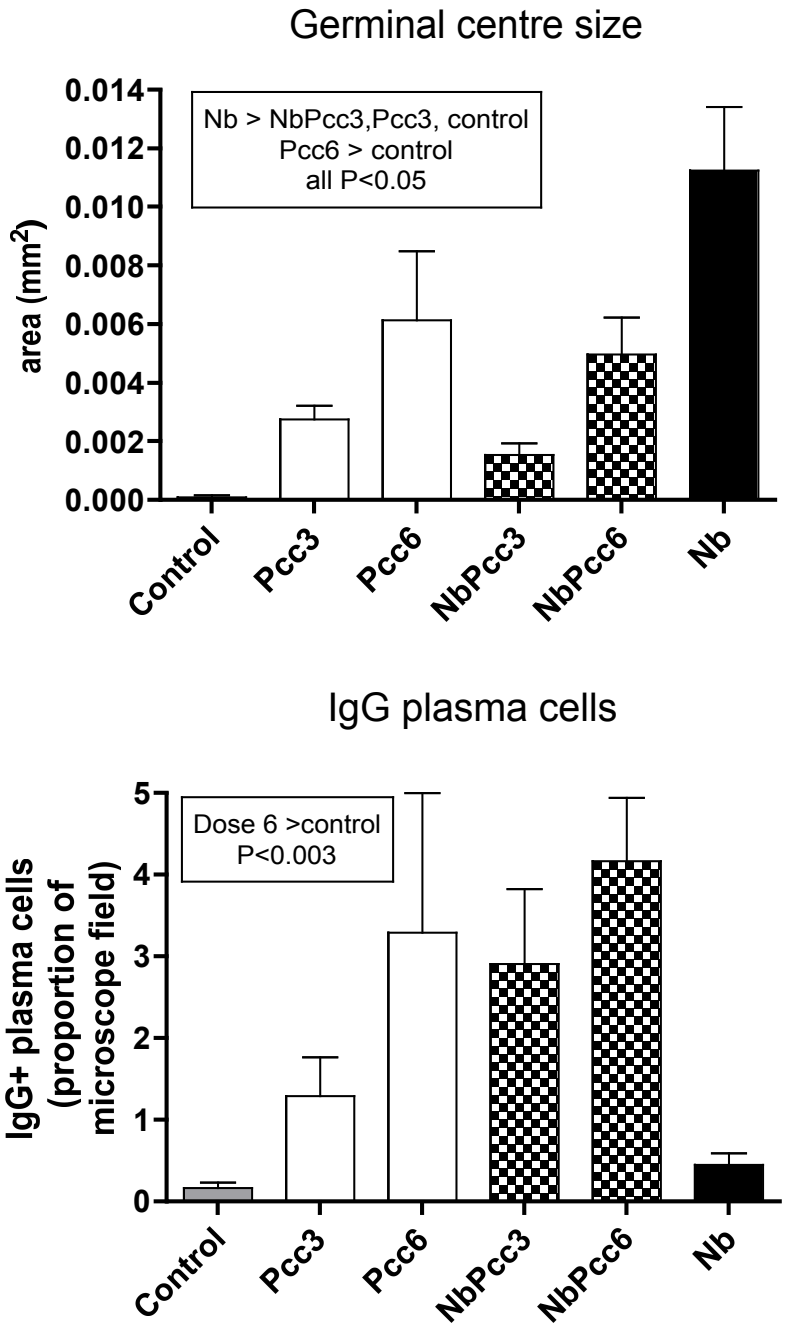
Statistical Analysis

The analyses presented here relate to a subset of mice from a single experiment. Female BALB/c mice were infected with two different inoculating doses of *Pcc* parasitised RBC ($1x10^3$and $1x10^6$) with or without co-infection of *Nb* (200 L3) on day 0. A group of mice infected with *Nb* only and control mice, which received nRBC ($1x10^5$) and PBS as sham injections for *Pcc* and *Nb* respectively, were also included. There were 8 mice per infection group.

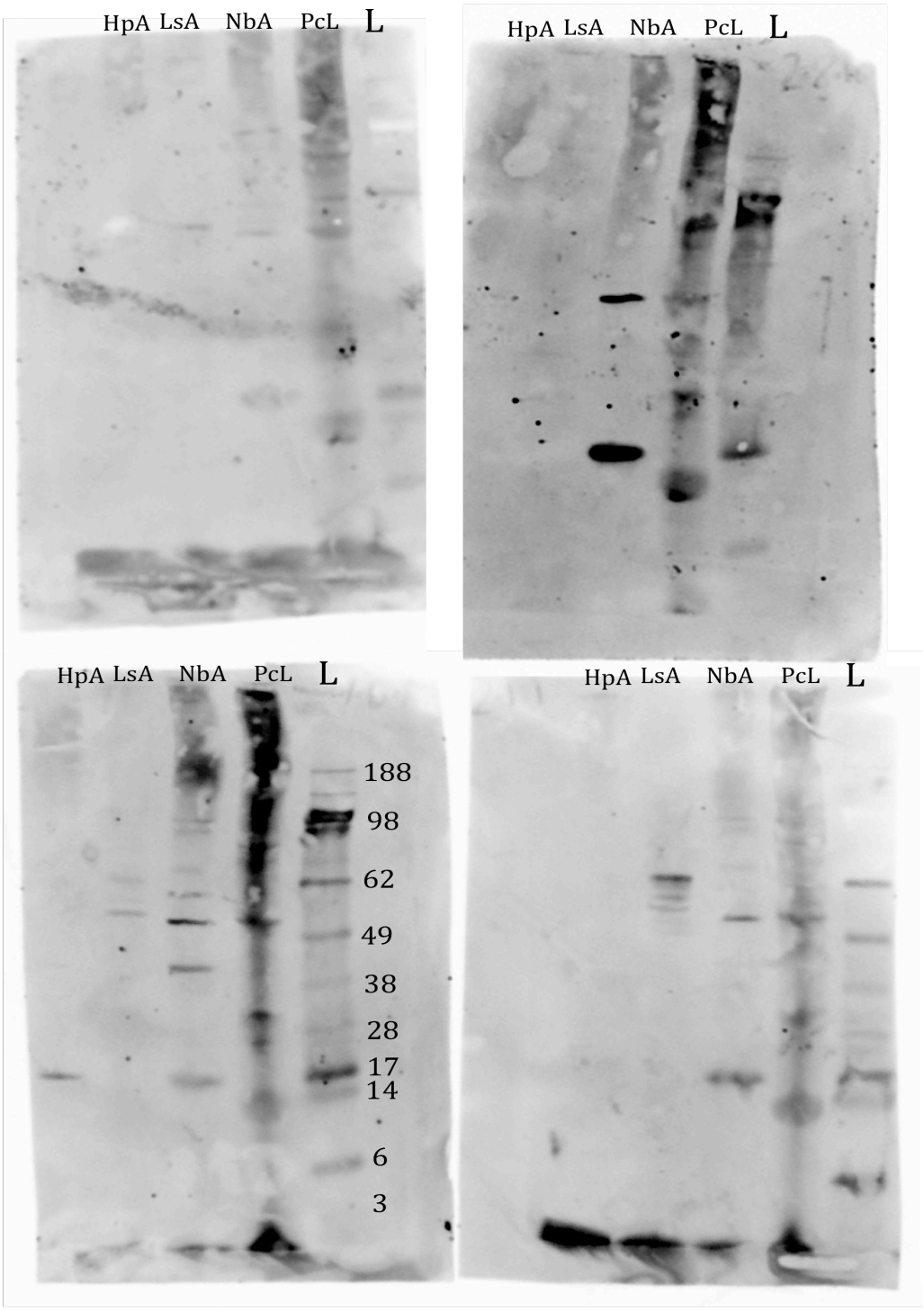
The analysis focussed on 48 mice. All analyses were carried out in the statistical package JMP 8.0 (SAS) using GLM. Main effects were worm presence or absence (Nb), and malaria inoculation dose $1x10^3$ or $1x10^6$ (*Pcc* dose) fitted as categorical variables. The main effects and the interaction between them (Nb**Pcc* dose) test whether the response variables (germinal centre size and proportion of IgG positive plasma cells) are affected by changes in *Pcc* dose, *Nb* status or both. Maximal models were first fit to the data and the minimal models were reached by removing non-significant terms ($p > 0.05$) beginning with interactions. Finally, significant pairwise differences ($p<0.05$) between groups were determined using the Tukey's All Pairs adjusted p-values for the most complex term in the minimal model.Results See Chapter 6 for description and discussion of results.



APPENDIX FIGURE 1: Representative spleen sections stained for germinal centres, IgG and IgM positive plasma cells. Section from control a) *Nb* infected b) $1x10^3$ *Pcc*-infected c) $1x10^3$ *PccNb* infected d) $1x10^6$ *Pcc*-infected e) and $1x10^6$ *PccNb* infected f) are shown. Germinal centres are stained green, IgM positive plasma cells red and IgG positive plasma cells blue.



APPENDIX FIGURE 2: The size of germinal centres a) and the proportion of IgG positive plasma cells b) for mice infected with different doses of *Pcc* with or without *Nb* infection are shown. Control mice are represented by the grey bars, *Nb* only mice by the black bars, singly infected *Pcc* mice in white bars and co-infected mice (*Pcc-Nb*) by chequered bars. Pcc dose is denoted *Pcc3* or *Pcc6* corresponding to inoculating dose of $1x10^3$ or $1x10^6$ respectively. Graph shows mean and standard errors of 8 mice per group. Significant pairwise differences were determined from the Tukey's adjusted P-values of the most complex term in the model and are summarised in the text boxes.



APPENDIX FIGURE 3: Western blot of nematode antigens (25µg) probed with anti-sera from individual mice infected with *Pcc* ($1x10^6$ *Pcc*-infected RBC). Antibody binding (IgG) to *H. polygyrus* antigen (HpA), *L. sigmodontis* antigen (LsA), *N. brasiliensis* antigen (NbA) and *P. chabaudi* lysate (PcL) is shown. The size of the bands (kDA) is indicated by reference to the protein standard (L).

TABLE 1: Results of GLM for germinal centre size and proportion of IgG positive plasma cells. Non-significant terms come from last model before term was dropped and are shown in square brackets. F statistics and P values of significant terms (P<0.05) come from the minimal model. Significant pairwise differences were determined from the Tukey's adjusted P-values of the most complex term in the model and are shown in Figure 1.

Response Variable	Factor	df	F ratio	P value
Germinal centre size				
	Pcc Dose	2,29	2.80	0.0773
	Nb	1,29	3.61	0.0672
	Nb*Pcc Dose	2,29	9.44	0.0007*
IgG positive cells (proportion)				
	Pcc Dose	2,40	6.43	<0.0038*
	[Nb	1,37	1.83	0.1844]
	[Nb*Pcc Dose	2,37	0.38	0.6876]

Chapter 7: General Discussion

The underlying motivation for this thesis was to understand how a host preserves its own fitness when exposed to infection with an unpredictable diverse array of parasites each of which has evolved complex strategies to exploit the host. This is compounded by the likelihood that the host will be co-infected. To tackle the complexity implicit in this question I have used a murine model of malaria-hookworm co-infection to investigate host-parasite interactions that may influence disease outcome. Utilising a mouse model means that variation in exposure to parasites, infection history, variation in host and parasite genotype can all be controlled, which in a natural population would be impossible. Of course it is important to acknowledge that the use of animal models also has its limitations in terms of the generalisation of my findings to other systems, in particular human populations. The specific findings of each set of experiments (Summarised in Figure 1) are discussed in detail at the end of each chapter. Here I will address my general findings and how they relate to the concept of a host evolving an optimal strategy to contend with co-infection and the importance of considering host-parasite interactions within a community ecology framework.

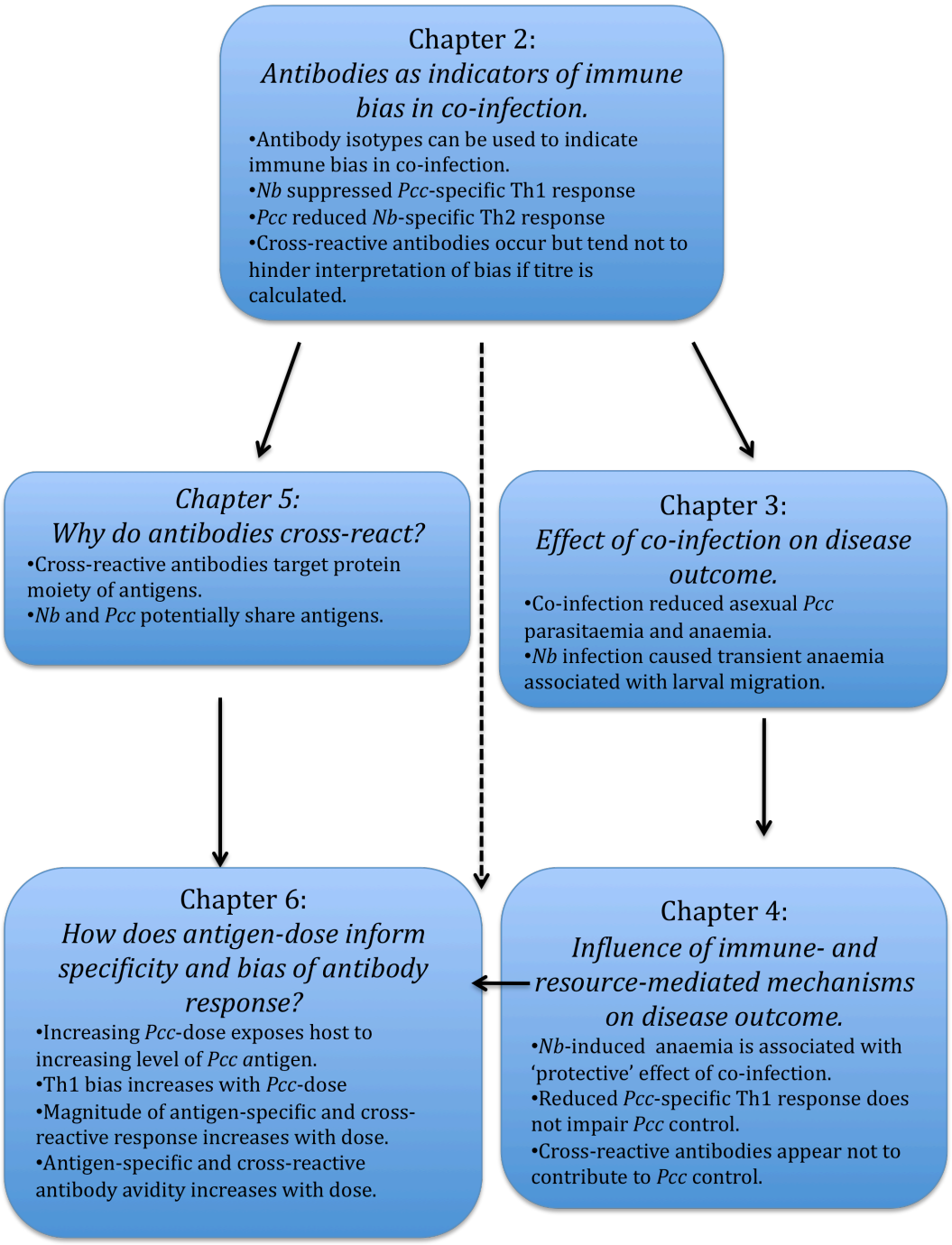


Figure 1: *Summary of the specific findings of each results chapter*. Arrows indicate chapters that are linked by themes or findings of previous chapters.

7.1 Do immune responses in co-infection represent an optimal strategy?

The key role of the immune system is to defend the host from infection. This presents an enormous challenge when you consider the vast diversity of pathogens it could be exposed to. To efficiently combat infection the host must distinguish between different pathogens in order to mount appropriate effector responses and failure to do this can have serious health consequences (Mwatha, Jones et al. 2003; Balsitis, Williams et al. 2010). Pathogen infections can be broadly categorised as 'microparasites' (e.g fungi, viruses, bacteria, intracellular parasites) and large extracellular parasites (e.g helminths). The effector responses that are required to control these different parasites also fall broadly into two categories; Th1 responses to 'microparasites' and Th2 responses to helminths (Abbas, Murphy et al. 1996; Ezenwa, Etienne et al. 2010; Kolbaum, Ritter et al. 2011). This is an oversimplification of the different arms of the immune system that extend to Th17 and regulatory (T-reg) responses with the prospect of more on the horizon (Locksley 2009). However it is a useful simplification for studies that address the question of immune-bias in 'microparasite'-helminth co-infection. The focus on Th1 and Th2 responses in this malaria- helminth (*P. chabaudi (Pcc)- N. brasiliensis (Nb)*) co-infection study is further justified, as Th1 effector responses are crucial for the control of *Pcc* infection (Cavinato, Bastos et al. 2001; Stevenson and Riley 2004). Similarly Th2 responses are the main effector response initiated in response to *Nb* (Nel, Hams et al. 2011) whereas the involvement of T-regs in this acute helminth infection may be limited (Smits, Everts et al. 2010).

A theme that runs throughout this thesis is the idea that hosts faced with co-infection by diverse parasites are required to mount optimal immune responses. During co-infection due to the counter-regulatory nature of Th1 and Th2 responses it is not possible for a host to mount a strong response of both types (Mosmann and Sad 1996), such that a host is required to balance these responses to arrive at some optimal immune bias (Fenton, Lamb et al. 2008). Why Th1 responses predominate over Th2 or vice-versa during co-infection is open to debate but it has been suggested that the optimal immune response may reflect the need to prioritise

defence against the most virulent parasite (that which causes most harm to the host) (Graham 2001). Increased parasite abundance is one mechanism by which parasites may cause harm to a host. Increased parasite load will be associated with an increase in the levels of antigen the immune system is exposed to. This could therefore provide a proximate measure of parasite abundance and ultimately lead to immune targeting of the more predominant parasite. This study explores these concepts in the *Pcc*-*Nb* model of co-infection by measuring immune bias (Th1/ Th2 balance) and how this is affected by parasite dose.

This thesis provides two key results relevant to better understanding immune bias in co-infection. Firstly, helminth (*Nb*) co-infection resulted in a reduction in the malaria-specific Th1 antibody response, whilst *Pcc* infection negatively impacted the Th2 antibody response to *Nb*. This confirms the cytokine production observed in spleen cell culture (Hoeve, Mylonas et al. 2009) thus demonstrating that using a different sampling strategy (serum for antibody ELISA), more accessible to field biologists provides a robust measure of Th1/ Th2 bias. Secondly, increasing *Pcc* dose was associated with an increase in malaria-specific Th1 responses. Taken together these results support the idea that the host manages the Th1/ Th2 bias in order to simultaneously combat these diverse parasites and that the immune system responds to antigen (parasite) load by preferentially targeting the parasite that poses the greatest threat. Further investigation of this result is warranted in other murine models of co-infection. In particular understanding how immune bias is shaped by chronic -as opposed to acute- helminth infection or variation in dose of both helminths and malaria may help to determine the mechanisms that underlie the diverse effect of co-infection on disease outcome.

In the course of analysing antibody isotype production to determine bias in the immune response I discovered that singly infected hosts produced cross-reactive antibodies that recognised antigens from parasites they had never been exposed to. The production of cross-reactive antibodies that convey protection against antigenic variants of the influenza virus (Sandbulte, Jimenez et al. 2007; Straight, Ottolini et al. 2008) or certain combinations of serotypes of Dengue (Endy, Nisalak et al. 2004)

can be viewed as an optimal host strategy. By mounting an antibody response capable of targeting different pathogen strains the host maximises the benefit, whilst conserving the cost, of mounting an immune response. This idea could be tested via nutritional restriction of laboratory mice. Admittedly, it stretches the imagination to consider this antibody 'multi-tasking' applied to parasites as diverse as malaria and helminths. Indeed cross-reactivity is viewed by some as nothing more than 'background' binding as a result of non-specific interactions between hydrophobic ligands and antigen binding sites (hydrophobic stickiness) (Padlan 1994). However, the ability of antibodies to bind unrelated antigens through specific hydrogen bonds and by displaying conformational binding-site diversity has been shown (James, Roversi et al. 2003; James and Tawfik 2003). This in addition to the magnitude of the cross-reactive titres observed in this model implying immune investment lends weight to the argument that production of cross-reactive antibodies may be a deliberate strategy of the host to increase the antibody repertoire (and so recognition of antigens/ parasites) at reduced 'cost'; the ultimate in 'two for one' offers.

It is of course possible that the production of cross-reactive antibodies is not a deliberate strategy of the host but rather arises because of some constraint on the immune process that leads to increased antigen-specificity and avidity (strength of binding to antigen) i.e. somatic hypermutation. For example reduced rounds of somatic hypermutation due to limited antigen availability may mean that, even if it is advantageous, the host is not able to produce antibodies that distinguish between *Pcc* and *Nb* antigens. The attempt to determine if this was the case did not support the idea that constraints on immune specificity resulted in cross-reactive antibodies; at higher *Pcc* doses (increased antigen availability) antibodies of greater avidity to *Pcc* antigens were selected, however these antibodies also bound *Nb* antigens more avidly.

Importantly cross-reactive binding to different parasite antigens could occur because two different antigens share an epitope, as is the case for *S. mansoni* and *P. falciparum* (Pierrot, Wilson et al. 2006). In this sense antibodies are only cross-reactive in that they bind (recognise) phylogenetically different parasites. In the co-

infection model studied here *Pcc*-induced cross-reactive antibodies bound to *Nb* antigens but whether these antibodies exhibit binding because the antigens share an epitope or because flexibility in the antigen-binding site of the antibody permits binding of unrelated antigens is yet to be determined. Antigen cross-absorption assays of the kind described by (Naus, Jones et al. 2003) could clarify if the same antibody molecule is binding to both *Nb* and *Pcc* antigens. Western blot assays suggest that there may be a shared antigen as proteins of similar molecular weight were bound in malaria and *Nb* antigen preparations. Further proteomic analysis (e.g. mass spectrometry) of the *Nb* and *Pcc* antigens that are recognised by *Pcc* anti-sera in Western blots could identify shared antigens. If these (putative) shared antigens represent an adaptation of the parasite to a particular niche within the host (e.g. the circulatory system) then immune-targeting of these conserved antigens, rather than being a strategy of the host, is due to a constraint imposed on blood-borne parasites.

Although it appears that the host is managing the immune response in such a way as to combat the parasite that poses the greatest threat during co-infection this can only be considered an optimal strategy if it conveys some fitness benefit to the host. In the *Pcc*-*Nb* model helminth co-infection afforded protection from malaria indicated by reduced parasitaemia and less severe anaemia. Of course the underlying mechanism for this cannot be inferred without consideration of other factors such as the ecology of the co-infecting parasites.

7.2 Considering within-host interactions in a community ecology framework.

Community ecologists appreciate the importance of considering both top-down and bottom-up mechanisms of regulation in determining the numbers or biodiversity of species (Burkepile and Hay 2006; Elmhagen and Rushton 2007). These mechanisms are also relevant to the structuring of parasite communities within a host (Bradley and Jackson 2004; Jackson, Pleass et al. 2006; Stancampiano, Mughini Gras et al. 2010; Telfer, Lambin et al. 2010). However few studies consider that both 'top-down' and 'bottom-up' mechanisms of regulation may play a role in determining disease outcome and those that do e.g. (Pedersen and Fenton 2007; Graham 2008)

are a meta-analysis and review of the available literature. There is a paucity of data in which both factors have been measured explicitly, using the *Pcc-Nb* model meant that this was possible. To assess the influence of top-down regulation on disease outcome the potential for 'bottom-up' regulation via competition for red blood cells was alleviated by manipulating the timing of *Nb*-induced anaemia. Hosts in which resource (red blood cell)- competition was alleviated but effects of co-infection on immune response were maintained were not 'protected' from malaria peak parasitaemia or anaemia. Thus it seems that resource-mediated mechanisms were more influential than immune-mediated mechanisms in determining disease outcome, a finding that could easily have been overlooked if within-host interactions had not been considered from a community ecology perspective.

7.3 How applicable are the findings to other co-infection studies?

It is important to emphasise that animal models such as the one used in the experiments reported here are just that 'models' of infection in natural populations. Inevitably there are limitations to extrapolating the findings in animal models to humans particularly when there are known differences in the mechanisms that underlie disease pathology (as discussed in the General Introduction). Rather than dwell on these caveats I would like to highlight how the findings of this thesis relate to studies of co-infection in general.

Perhaps the most basic finding of this thesis was the ability to discern immune bias via the analysis of Th1 and Th2 antibody isotypes provided that antibody titre is calculated. Importantly, this method of measuring bias should be applicable to all mammalian models of co-infection as the impact of cytokine bias on antibody class switching is a trait that is shared amongst species (e.g. lab mice, sheep, goats, rabbits). There is a growing interest in incorporating immune measures to ecological studies (Bradley and Jackson 2008) and I would advocate the use of antibodies for the following reasons. The immunological assays used to measure these molecules are technically simple and as such are accessible to ecologists who may not have access to the equipment needed for analysis of other immune molecules such as

cytokines e.g. tissue culture facilities. In addition, the sample volume required to measure antibodies is much smaller than that needed for cytokine analysis and this makes antibody analysis an attractive option for studies that aim to follow the course of infection as it negates the need for untimely sacrifice of individuals. I acknowledge that the application of reagents designed for use with *Mus musculus* to wild rodents such as *Apodemus* and *Peromyscus* may not be straightforward. However the manufacture of reagents tailored to specific species is becoming more affordable and in a pilot experiment I had some success in measuring total IgG in sera from wild rodents with laboratory reagents (data not shown).

Just as disease ecologists can gain from investigating the immunological effects of co-infection on host-parasite interactions. So immunologists would do well to consider the potential for resource-mediated mechanisms to influence co-infection. In other words adopting a community ecology approach to the investigation of co-infection may help to understand its diverse effects on disease outcome e.g. exacerbation or amelioration of malaria by helminth infection (Helmby, Kullberg et al. 1998; Yoshida, Maruyama et al. 2000). Two recent meta-analyses of experimental 'micro-parasite'-helmimth co-infection predicted that the ecology of co-infecting parasites would be particularly relevant to disease outcome when parasites competed for resources (Graham 2008; Knowles 2011). Importantly the analysis performed by Graham revealed that immunological interactions only took effect once resource-mediated interactions were accounted for (Graham 2008). The current finding that *Nb*-induced anaemia played a critical role in mediating the 'protective' effect of co-infection on malaria disease outcome strengthens the argument for considering resource-mediated interactions in co-infection studies. This result was discovered when helminth infection occurred prior to *Pcc* and highlights how the timing of infection can also result in differential effects on disease outcome.

The final set of experiments involved manipulating the inoculating dose of *Pcc*, which can be likened to variability in exposure to parasites and so enhances the link between this experimental model and natural systems. Increasing the dose of *Pcc* parasites increased the Th1 bias of the antibody response; a result which adds to a

body of literature (Lawrence, Gray et al. 1996; O'Neill, Brady et al. 2000; Eschbach, Klemm et al. 2010), which is in contrast to the accepted dogma that increasing parasite dose results in a shift from Th1 to Th2 immune responses.

The findings in this murine model indicate that changes in Th1/ Th2 immune-bias, the occurrence of cross-reactive antibodies, resource-mediated interactions and the timing of infection should all be considered when attempting to discern the influence of co-infection on disease outcome. Whilst this is achievable in animal models, the application of this to human studies is challenging. Both technical and ethical constraints mean that the influence of these different factors may prove extremely difficult to dissect. Nevertheless it is something we should aim for. Obviously it is not possible to deliberately infect individuals with parasites to assess how changes in the immune response may manifest so we must take advantage of the opportunity that 'natural experiments' afford e.g. differences in exposure or endemicity of helminths in malaria endemic areas. Measuring antibody responses in individuals infected or not with helminths could provide information on how this parasite affects immune responses to other parasites/ antigens. As previously discussed measuring antibody response may be more logistically feasible than cytokine analysis for field immunology. With regard to how helminth-induced changes may affect malaria outcome it is the change in cytophilic (Th1) antibody responses that is particularly relevant. Admittedly finding the appropriate controls in order to assess cross-reactive responses is an additional problem. However differences in exposure to parasites on a micro-geographical scale within a community (Booth, Vennervald et al. 2004) may mean that 'controls' could be selected from within the population. In addition monitoring RBC / haemoglobin levels in helminth infected or non-infected individuals may allow information to be gathered regarding the potential for resource-mediated mechanisms to be playing a role. I do not say any of this lightly, realising what a massive undertaking this would be for any field study and even then the inherent variation (genetic and environmental) in these individuals may prevent clear conclusions being reached.

As discussed, the constraints of human studies mean that in-depth investigation of the mechanisms underlying these outcomes may necessitate the use of an animal model. The findings described in this thesis; co-infection affecting the antibody bias of antigen-specific responses and the occurrence of cross-reactive antibodies are most similar to the findings of Mwatha et al in human populations exposed to *S. mansoni* and *P. falciparum* (Mwatha, Jones et al. 2003). In this study more severe *S. mansoni*-induced hepatosplenomegaly was correlated with an increase in *P. falciparum* antibodies (Th1 isotype) that cross-reacted with *S. mansoni* antigens. However, it was unclear whether the effect on hepatosplenomeglay was due to concomitant malaria-Schistosome infection or an effect of *P. falciparum* on the quality/ quantity of the anti-Schistosome response. More recently the exacerbation of hepatosplenomegaly in children co-exposed to *S. mansoni* and *P. falciparum* has been linked to amplification of pro-inflammatory cytokines (Wilson, Jones et al. 2009). As the cellular source of plasma cytokines is unknown it is impossible to say if the enhanced pro-inflammatory response is a result of cross-reactive stimulation or due to the additive effect of anti-Schistosome inflammation. A mouse model in which malaria infection was administered prior to Schistosome infection may help to elucidate whether concomitant malaria infection per se or its effect on immune responses are more influential. Similarly manipulations such as serum transfers, whereby malaria-induced cross-reactive antibodies were transferred to a naïve mouse that was subsequently challenged with a Schistosome infection, could address the role of cross-reactivity in this system.

7.4 Implications/ Outlook

The results of this thesis have implications that relate to both evolutionary and applied biology. Here I discuss the potential for co-infection to influence, the evolution of both host and parasite traits, and vaccine design.

One of the main themes of this thesis relates to the discovery of cross-reactive antibodies and the idea that these responses may either be beneficial to the host or result from constraints imposed on the immune system or the parasite its fighting. If

these cross-reactive antibodies are functional they could play a role in parasite clearance. However these antibodies could still be beneficial even if they are not functional as they may enable initial recognition of diverse parasites for less 'immune cost'. These ideas are difficult to test experimentally but some insight could be gained by investigating whether energetically challenged hosts mount more cross-reactive responses. Similarly hosts that evolved under the selective force of multiple parasites may have more cross-reactive responses. If cross-reactive responses are beneficial to the host this could have implications for the evolution of parasites attempting to evade immune recognition.

Helminth co-infection also has the potential to influence the evolution of virulence in malaria parasites. Virulence, defined as harm to the host following infection, is thought to be an unavoidable side effect of host exploitation. Such virulence is thought to evolve as it increases parasite abundance within hosts and thus increases fitness through enhanced transmission. However, a proposed trade-off between the costs (e.g., host mortality) and benefits (e.g., increased transmission) of increased virulence may limit virulence evolution (reviewed in (Alizon, Hurford et al. 2009)). Virulence in malaria can be both parasite and immune-mediated (Long and Graham 2011), which is an important consideration when addressing how helminth co-infection may effect virulence evolution in malaria parasites. For example if helminth infection moderates the pro-inflammatory responses, which contribute significantly to the immunopathology associated with malaria (Li, Seixas et al. 2001; Dodoo, Omer et al. 2002), this could have a positive impact on host health and thus alleviate the negative selection pressure (of costly host mortality) imposed on virulence. Thus malaria parasites would evolve to be more virulent (in line with the evolutionary consequences of other disease-minimising mechanisms; (Alizon, Hurford et al. 2009)). The potential for this to occur in helminth-infected hosts has serious implications regarding the administration of anthelmintics. If more virulent parasites evolved in a population of helminth-infected hosts but were transmitted to helminth-free hosts, the consequences for those individuals could be devastating. This scenario may readily occur if there is only partial coverage of anthelmintic treatment in the wider community. Another possibility is that any helminth-induced reduction of anti-malarial Th1 responses could reduce effector efficacy and lead to

an explosion in malaria parasite growth, which could increase host-mortality. Consequently these malaria parasites would pay the cost of virulence and would ultimately evolve to be less virulent.

One of the major applied implications of the findings in this thesis relates to parasite control programs. Drug-resistance is a growing concern for the control of infectious diseases such as malaria and helminthiases (Sargison, Scott et al. 2010; Petersen, Eastman et al. 2011; Vercruysse, Behnke et al. 2011). The ultimate goal is to vaccinate individuals against these debilitating diseases. The success of vaccines often relies on the induction of Th1 or Th2 responses, and antigen dose can play an important role in determining this bias (Power, Wei et al. 1998). The current dogma suggests that increasing parasite dose increases the Th2-ness of the immune response. The findings reported here do not support this and reveal that for a range of *Pcc* dose Th1 responses predominate. It is important to note that this is not an isolated finding (Lawrence, Gray et al. 1996; O'Neill, Brady et al. 2000; Eschbach, Klemm et al. 2010) and is pertinent to vaccine design. If, for example, an anti-helminth vaccine designed to induce a Th2 response were introduced to a population where exposure to malaria is high the Th1 bias in the host may reduce its efficacy.

7.5 Concluding remarks

Investigating the effect of co-infection on disease outcome in natural populations is complicated by variation in host and parasite genotype, unknown infection history and variable exposure to parasites. These variables can be compounded by host age and sex differences. Utilising an animal model of malaria-helminth co-infection controlled for much of this variation but I was keen to preserve some of this complexity in my study to increase the usefulness of the main findings. Consequently when I discovered cross-reactive antibodies I chose to pursue a line of questioning/ experimentation that incorporated the idea that they may be a deliberate strategy of the host to increase the antibody repertoire whilst conserving 'immune costs'. Similarly incorporating a range of parasite doses and investigating immune responses under such conditions relates to natural variation in exposure to parasites.

In addition considering both immune- and resource-mediated mechanisms of regulation acknowledges the importance of variation in parasite ecology. I would hope that this thesis goes some way to bridging the gap between immunological and ecological approaches to understanding disease outcome in co-infection. Or at the very least highlights the importance of doing so!

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THESIS APPENDIX: PUBLICATIONS

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RESEARCH ARTICLE Open Access

Antibody isotype analysis of malaria-nematode co-infection: problems and solutions associated with cross-reactivity

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Abstract

Background: Antibody isotype responses can be useful as indicators of immune bias during infection. In studies of parasite co-infection however, interpretation of immune bias is complicated by the occurrence of cross-reactive antibodies. To confidently attribute shifts in immune bias to the presence of a co-infecting parasite, we suggest practical approaches to account for antibody cross-reactivity. The potential for cross-reactive antibodies to influence disease outcome is also discussed.

Results: Utilising two murine models of malaria-helminth co-infection we analysed antibody responses of mice singly- or co-infected with *Plasmodium chabaudi chabaudi* and *Nippostrongylus brasiliensis* or *Litomosoides sigmodontis*. We observed cross-reactive antibody responses that recognised antigens from both pathogens irrespective of whether crude parasite antigen preparations or purified recombinant proteins were used in ELISA. These responses were not apparent in control mice. The relative strength of cross-reactive versus antigen-specific responses was determined by calculating antibody titre. In addition, we analysed antibody binding to periodate-treated antigens, to distinguish responses targeted to protein versus carbohydrate moieties. Periodate treatment affected both antigen-specific and cross-reactive responses. For example, malaria-induced cross-reactive IgG1 responses were found to target the carbohydrate component of the helminth antigen, as they were not detected following periodate treatment. Interestingly, periodate treatment of recombinant malaria antigen Merozoite Surface Protein-1_{19} (MSP-1_{19}) resulted in increased detection of antigen-specific IgG2a responses in malaria-infected mice. This suggests that glycosylation may have been masking protein epitopes and that periodate-treated MSP-1_{19} may more closely reflect the natural non-glycosylated antigen seen during infection.

Conclusions: In order to utilize antibody isotypes as a measure of immune bias during co-infection studies, it is important to dissect antigen-specific from cross-reactive antibody responses. Calculating antibody titre, rather than using a single dilution of serum, as a measure of the relative strength of the response, largely accomplished this. Elimination of the carbohydrate moiety of an antigen that can often be the target of cross-reactive antibodies also proved useful.

Background

The geographical and socio-economic distribution of malaria overlaps with areas in which a number of helminth parasites are also endemic. It is the norm in these areas for co-infection to occur and a growing body of literature reflects this [1-12]. The influence of co-infection on the immune response may result in either exacerbation or amelioration of disease [13-15]. It is therefore crucial to understand the host-parasite relationship in the context of multiple infections, if vaccine design and drug administration programmes are to be managed effectively [16]. Animal models accurately reflect many pathological aspects of malaria-helminth co-infection with regard to impact on disease outcome and also provide the opportunity to further examine immunological mechanisms in detail [17-20].

We previously undertook an investigation to assess the impact of a pre-existing chronic nematode infection on

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malaria-related pathology, utilising the rodent malaria *Plasmodium chabaudi chabaudi (Pcc)* and the rodent filarial nematode *Litomosoides sigmodontis (Ls)* [21]. We found that co-infected mice (*Pcc-Ls*), particularly those that did not have blood microfilaremia, had exacerbated immunopathology. This was associated with increased interferon-gamma (IFN-γ) responsiveness but was independent of *Pcc* parasitemia [21]. One of the primary objectives in our previous malaria-nematode co-infection studies was to gather antigen-specific T-cell data to determine whether nematode infection could alter the cytokine bias of the *Pcc*-specific T lymphocyte response towards Th1 and conversely, whether a potent Th1 response could alter the Th2 bias of the nematode-specific response.

Cytokine production by antigen specific T-cells can be difficult to assess during malaria, due to immune suppression associated with the peak of infection and apoptosis of splenocytes [22]. Additionally, the complex nature of the target antigen (*Pcc*-infected red blood cells) is a further complicating factor. Thus, gathering antigen-specific T-cell data remains a technical challenge of studying immunity to malaria particularly in human studies where there is the additional challenge of obtaining and maintaining lymphocytes in the field.

Here we focus on the dissection and interpretation of parasite antigen-specific antibody responses as an alternative to T-cell analysis. Antibodies of the IgG2a isotype are mainly produced by B cells in response to IFN-γ in mice [23-25] whereas the Th2 cytokine IL-4 switches B cells to produce IgG1 [24,26]. Although the generation of IgG1 as a marker for Th2 cells is less definitive than IgG2a as a marker of a Th1-type response, the ratio of IgG1 to IgG2a provides a powerful indicator of immune bias [27-30]. Measurement of antibodies can also be achieved with smaller sample volumes and poses fewer technical challenges than T-cell recall assays. Furthermore, antibody analysis can provide information on the fuller history of infection as it reflects cumulative immunological activity, whereas cytokine responses of T-cells are an *ex-vivo* 'snapshot' that can more readily be altered by changes in the timing of sampling both *in vivo* and *in vitro*. Antibody analyses of co-infected animals might therefore provide evidence of overall Th1-Th2 cell cross-regulation even when cytokine analyses may not.

In addition to their use as indicators of cytokine bias during infection, antibody isotypes have direct functional relevance to disease severity in helminth-malaria co-infection. Antibodies are absolutely required for the ultimate clearance of malaria parasites [31]. In mice, antibodies of the cytophilic isotype IgG2a have been shown to recognise infected erythrocytes [32] and facilitate their destruction by phagocytes [33]. Similarly, in humans IgG1 and IgG3 are associated with enhanced parasite clearance [34]. If helminth co-infection alters antibody class-switching and consequently the production of malaria-specific cytophilic antibodies then the resolution of malaria infection may be affected. Indeed, co-infection with the gastro-intestinal nematode *Heligomosoides polygyrus* reduced *Pcc*-specific IgG2a responses and resulted in exacerbated malaria parasitemia [18]. There are also important implications for vaccine efficacy and administration. For example, immunisation that protected mice from malaria failed to do so in mice that also harboured a nematode infection [35].

In this study, the characterisation of antibody isotype responses as an indicator of cytokine bias during co-infection has proved unexpectedly challenging due to the production of cross-reactive antibodies induced by single-species infection. To establish the real effect of co-infection on the Th1/Th2 immune bias from non-specific reactivity to antigen we needed to determine how robust the cross-reactive responses were in comparison to the antigen-specific. We demonstrate that a combination of calculating antibody titre, from a dilution series of test sera, and periodate treatment of the parasite antigens can control for most cross-reactivity. The magnitude and robustness of some cross-reactivity, however merits further investigation to explore the potential function of these responses during co-infection.

Methods

Hosts, parasites and experimental infection

Specific pathogen free, 8-10 week old female BALB/c mice (Harlan, UK) were maintained in individually ventilated cages on diet 41b *ad lib* in a 12 h:12 h light-dark cycle. All experiments were carried out in accordance with the animals (Scientific Procedures) Act 1986, and were approved by the UK Home Office inspectorate and institutional review committee.

Pcc clone AS was originally isolated from thicket rats (*Thamnomys rutilans*) and was cloned by serial dilution and passage [36]. Parasites were recovered from frozen blood stabilates by passage through donor mice. Experimental parasite inoculations were prepared from donor mice by diluting blood in calf serum solution (50% heat-inactivated foetal calf serum, 50% Ringer's solution [27 mM KCl, 27 mM CaCl2, 0.15 M NaCl, 20 units heparin per mouse]). Each mouse received 0.1 ml of inoculum intraperitoneally (i.p) corresponding to an infective dose of 1×10^6 or 1×10^5 parasitized red blood cells (RBC), depending on the experiment. An inoculum of naïve RBC was given as a control for erythrocyte proteins.

The filarial nematode *Ls* was maintained by cyclical passage between gerbils (*Meriones unguiculatus*) and mites (*Ornithonyssus bacoti*) as described previously [37]. Infection was initiated by subcutaneous (s.c)

injection of 25 infective (L3) larvae. For co-infection experiments in which the influence of malaria on chronic nematode infection was addressed, 1×10^6 *Pcc* parasitized RBC were introduced i.p on Day 60 of an established *Ls* infection and mice were sacrificed on day 20 post-*Pcc* infection, as described previously [21]. Whole blood was collected from the brachial artery and serum recovered after clotting at room temperature.

Nb worms were maintained by serial passage through Sprague-Dawley rats. L3 larvae were obtained by culturing the faeces of infected rats at 26°C for a minimum of 5 days [38]. For acute nematode-malaria co-infection, infection was initiated by s.c injection of 200 infective (L3) larvae on the same day that *Pcc* was introduced by inoculation i.p of 1×10^5 parasitized RBC. Mice were sacrificed on Day 20 post-infection under terminal anaesthesia. Whole blood was collected from the brachial artery and was separated using Sera Sieve (Hughes & Hughes Ltd).

Antigens

Two malaria antigens were used in this study: a recombinant protein and a crude antigen homogenate prepared from parasitized erythrocytes. The recombinant Merozoite Surface Protein-1_{19} (MSP-1_{19}) was originally sequenced, cloned and expressed from *Pcc* AS clone, as described previously [39]. In brief, the MSP-1_{19} nucleotide sequence was inserted into *Pichia pastoris* vector pIC9K and protein expression carried out in *Pichia pastoris* strain SMD1169. This antigen was used in ELISA at a concentration of 1 μg/ml.

The crude malaria homogenate - lysed *Pcc* parasitized red blood cell extract (pRBC) - was prepared from whole blood of mice with a parasitemia in excess of 20%. Mice were bled by cardiac puncture with a heparinised syringe and blood stored at -80°C prior to 3 rounds of freeze-thaw to lyse the parasitized red blood cells. The lysed cells were sonicated, on ice, twice for 30 sec at 10 Amp and centrifuged at 16060 g for 10 min. The supernatant was stored at -80°C. Similarly, a naïve red blood cell extract (nRBC) was prepared as a control for RBC proteins; responses to this antigen amongst infected mice were indistinguishable from naïve (data not shown). In the *Ls* experiments this antigen was used in ELISA at 0.5 μg/ml and in the *Nb* experiments at 5 μg/ml.

Ls and *Nb* extracts (LsA and NbA) were prepared by homogenisation of adult nematodes in PBS. The somatic extracts were centrifuged at 1000 g for 20 mins and the pellet discarded. The extract was stored at -20°C. LsA was used in ELISA at 0.5 μg/ml and NbA at 5 μg/ml.

Antibody detection

ELISA was used to measure antigen-specific IgG antibodies in the serum of nematode-infected, *Pcc*-infected or co-infected mice. In the *Pcc-Ls* study, sera were added in a serial dilution 1/100 - 1/400 and a dilution was then chosen whereby all samples fell in the linear range of the curve; for IgG1, a dilution of 1/200 and for IgG2a 1/100. For the subsequent *Pcc-Nb* study, serum samples were added in a serial dilution 1/50 - 1/819200. Antibody titres were calculated as the reciprocal of the greatest dilution at which optical density (O.D) was greater than the mean plus 3 standard deviations of the O.D values observed for control mouse sera at 1/200 dilution.

Antibody responses to MSP-1_{19}, pRBC, NbA or LsA were determined for IgG isotypes IgG1, IgG2a, and IgG3. 96 well maxisorp immunoplates (Nunc) were coated at 4°C overnight with either recombinant or crude antigens at the concentrations indicated (see Antigens section) in 0.06 M carbonate buffer (0.04 M $NaHCO_3$, 0.02 M $NaCO_3$, pH9.6) in a final volume of 50 μl per well. Non-specific binding was blocked with 5% FCS in carbonate buffer (200 μl/well) for 2 hours at 37°C. Wells were washed three times in Tris buffered saline with 0.1% Tween (TBST) after each step. Serum samples were added in serial dilutions as indicated using TBST as a diluent, in a final volume of 75 μl per well and incubated for 2 hours at 37°C. Isotype specific detection antibodies were diluted in TBST in a final volume of 50 μl per well. For IgG1, HRP conjugated goat anti-mouse IgG1 (Southern Biotech 1070-05) was used at 1/6000, HRP conjugated goat anti-mouse IgG2a (Southern Biotech 1080-05) at 1/4000 and HRP conjugated goat anti-mouse IgG3 (Southern Biotech 1100-05) was used at 1/1000. Plates were incubated for 1 hour at 37°C. An additional wash in distilled water was carried out before developing with ABTS peroxide substrate (Insight Biotechnology), 100 μl per well, at room temperature for 20 minutes. O.D was read at 405 nm using a spectrophotometer.

Polyclonal IgE levels were determined by sandwich ELISA. 96 well maxisorp immunoplates (Nunc) were coated overnight at 4°C with 100 μl of IgE capture antibody (2 μg/ml; clone R35-72 Pharmingen) diluted in carbonate buffer. Plates were blocked with 5% non-fat skimmed milk in carbonate buffer for 2 hr at 37°C. Plates were washed 5 × in TBST before addition of sera at 1/10 and 1/20 dilutions in a final volume of 50 μl/well and left overnight at 4°C. For the standard curve two- fold serial dilutions of purified mouse IgE, κ monoclonal isotype standard (Pharmingen) were used. After 5 washes in TBST, 100 μl of biotinylated detection antibody (2 μg/ml; clone R35-118 Pharmingen) diluted in TBST with 5% FCS was added and plates left at 37°C for 90 mins. Plates were washed 5 × in TBST prior to incubation with ExtrAvidin peroxidase (SIGMA), diluted 1:8000 in TBST with 0.5% FCS, for 30 mins at 37°C.

After a final wash in distilled water, plates were developed with 100 μl TMB microwell peroxidase substrate system (Insight Biotechnology Ltd) and read at 650 nm.

In order to determine the extent to which carbohydrate or protein moieties contributed to the antibody response the antigens were pre-treated with periodate. Antigen-specific IgG1, IgG2a and IgG3 antibodies were measured in response to antigens treated with periodate. The ELISA was carried out as detailed for the *Nb* co-infection experiment with untreated antigens but the following additional steps were included after blocking with 5% FCS: carbonate buffer, prior to sample addition. TBST wash (×3) was followed by the addition of 10 mM sodium (meta) periodate diluted in 50 mM sodium acetate in a final volume of 100 μl/well. Plates were incubated at 37°C for 1 hour and then washed in 50 mM sodium acetate. To stop the activity of periodate, 100 μl of 50 mM sodium borohydride solution was added to each well.

Statistical Analysis

General linear statistical models allowed us to frame and test questions such that we could determine whether differences in infection status and/or presence of carbohydrate antigen explained the observed variation in antibody responses. For more detailed explanation of the statistical methods employed see Grafen and Hails [40]. Infection status and treatment with periodate (or not) were included as categorical factors and their ability to predict antibody response was formally evaluated via Analysis of Variance (ANOVA). The serial dilution of sera in an ELISA produces ordinal data, which were $\log_{10}$ transformed prior to analysis to ensure the data were approximately normally distributed, in accordance with the requirements of linear models. Analyses were carried out using the statistical package JMP 5.1 (SAS). The maximal model was fitted first and minimal models were obtained by sequentially removing non-significant terms (P-value > 0.05), beginning with interactions. Finally, whenever a factor was significant ($P < 0.05$), an All Pairs Tukey post-hoc test was carried out to identify which groups of mice differed significantly in antibody induction, with respect to infection status or periodate treatment.

Results

Antibody isotype responses are skewed by malaria-filaria co-infection but cross-reactivity confounds data interpretation

We had previously used a model of *Pcc*-nematode co-infection in which *Pcc* is introduced into mice with pre-existing chronic *Ls* infection to investigate the dynamics of infection with regard to parasitological outcome and cytokine bias [21]. In the *Pcc-Ls* model the peak of malaria parasitemia was controlled by day 10 and resolved by day 14 post Pcc-infection. For this study, we envisaged that analysis of the antibody isotype response from these mice would provide a method to rapidly and quantitatively assess immune bias. We thus asked whether we could use antibody isotype ELISA for IgG2a and IgG1 to address whether nematode infection would skew the Th1 cell response to *Pcc* to a more Th2 cell biased response. Conversely, we wished to address whether the powerful Th1 cell response induced by malaria would have the capacity to alter an established IgG1 response to a nematode infection. As is common practice in many studies [41-48], especially with large sample sizes as in this study, the ELISAs were performed with a fixed serum concentration derived from the linear point in a dilution series.

As expected from previous studies [49-51], we were able to detect an IgG1 response against LsA in *Ls* mice. Co-infected (*Pcc-Ls*) mice also produced LsA-specific IgG1, but it was reduced in magnitude compared to the *Ls* mice (Fig 1Ai). Thus co-infection with *Pcc* appeared to down-regulate the anti-*Ls* specific IgG1 response. It also appeared that responses in *Pcc-Ls* mice were further biased toward a Th1 cell response through the induction of IgG2a to LsA (Fig 1Aii). Also as expected [32,52], *Pcc* mice mounted highly biased Th1-cell responses as indicated by the predominance of *Pcc* (pRBC)-specific IgG2a over IgG1 (Fig 1B). Once again, *Pcc-Ls* mice appeared to alter this bias by increasing the amount of pRBC-specific IgG1 in comparison to the *Pcc* mice (Fig 1Bi).

At first glance, the results strongly suggested that the isotype and hence cytokine bias of each single-species infection was significantly impacted by co-infection. In support of this, polyclonal IgE was highest in *Ls* mice and absent in *Pcc* mice with *Pcc-Ls* mice exhibiting an intermediate level (Fig. 1C) although *Pcc-Ls* mice did not differ statistically from *Ls* mice in this polyclonal analysis. However, it was apparent that *Pcc* mice were exhibiting sizable antibody responses to LsA (X_1 in Fig 1Aii) and conversely *Ls* mice were exhibiting strong antibody responses to pRBC (X_2 in Fig 1Bi). Western blot analysis confirmed that these *Ls*-induced cross-reactive responses were directed against the parasite rather than the RBC (data not shown). We thus had to ask whether the shift away from LsA-specific IgG1 toward IgG2a responses in the *Pcc-Ls* mice was due to the influence of IFN-γ on the *Ls*-induced response or simply reflected the presence of cross-reactive *Pcc*-induced IgG2a responses to LsA. Conversely, was the apparent increase in pRBC-specific IgG1 responses in *Pcc-Ls* mice due to *Ls*-induced antibodies that cross-reacted with infected red blood cells? Because serum titres had not been determined in this study, we could not assess the relative strength of cross-reactive versus antigen-specific

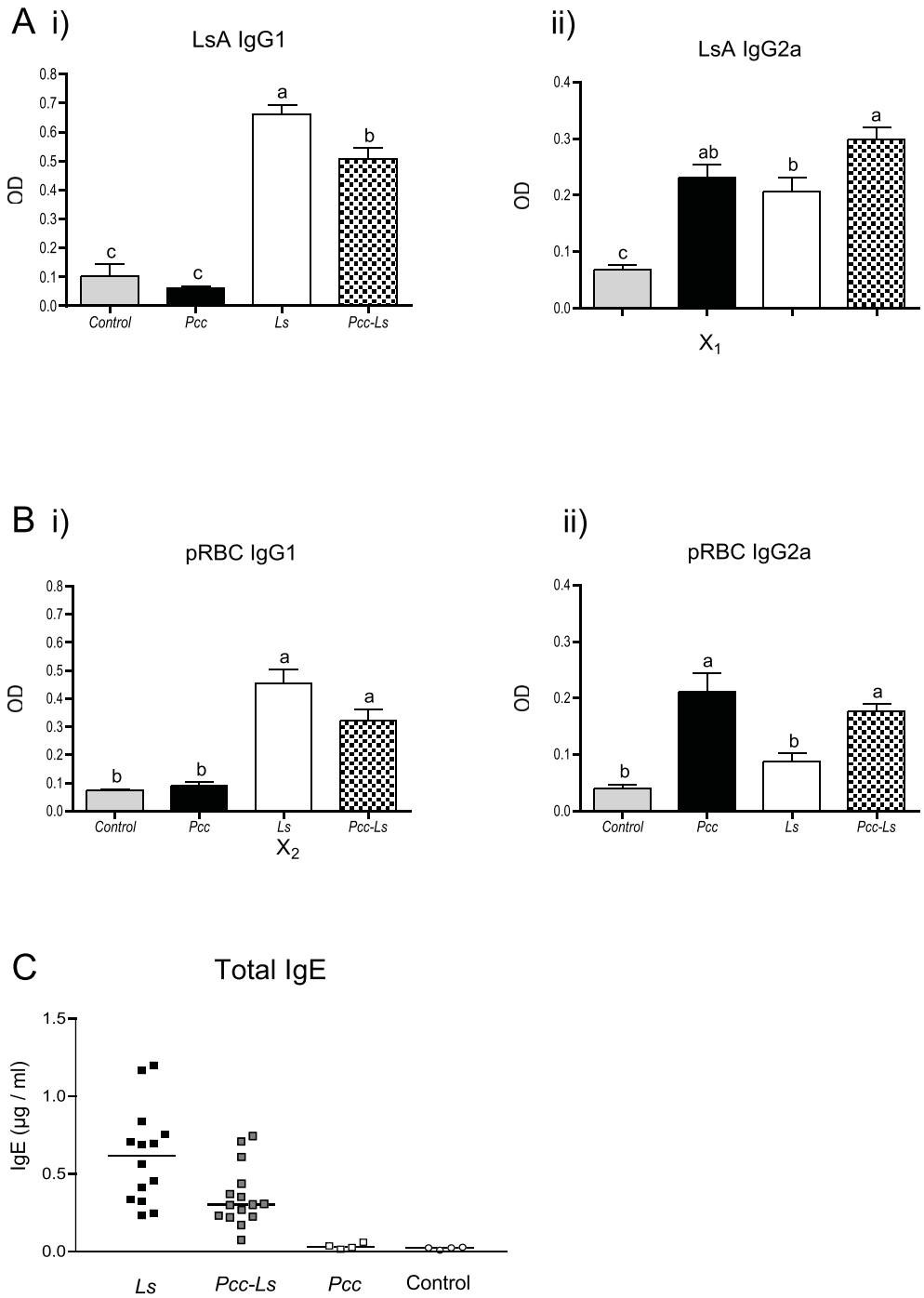

Figure 1 Antibody isotype responses in infection and co-infection with *Litomosoides sigmodontis* and malaria. Mice were infected with 25 *Ls* L3 larvae on day 0 and/or 10^6 *Pcc*-infected RBCs on day 60 post-*Ls* infection. Serum antibody responses measured by ELISA at day 80 post-*Ls* infection to (A) *Ls* antigen (LsA) and (B) crude *Pcc* infected RBC antigen (pRBC). Th2 isotype IgG1 is shown in (Ai) and (Bi) whilst the Th1 associated isotype IgG2a is shown in (Aii) and (Bii). Grey bars represent control mice, black bars represent *Pcc* mice, white bars the *Ls* mice and the chequered bars co-infected mice (*Pcc-Ls*). The letter X highlights those responses that are cross-reactive. Groups not connected by the same letter denote pairs that are significantly different according to Tukey's Pairwise analysis while those that share the same letter do not differ significantly. (C) Polyclonal IgE responses. Black squares represent *Ls* mice, white squares represent *Pcc* mice and grey squares represent co-infected (*Pcc-Ls*) mice. White circles indicate control animals. Data shown in A & B are compiled from 3 experiments (control mice n = 13, Ls mice n = 37, Pcc mice n = 14, Pcc-Ls mice n = 41).

responses. Our observation that there was significant cross-reactivity at the sera dilution tested thus confounded our ability to interpret any changes in immunological bias during *Pcc-Ls* co-infection.

Cross-reactive antibody responses are also observed during malaria-*Nippostrongylus brasiliensis* co-infection

In order to address the utility of antibody isotype responses further, we embarked on co-infection experiments with *Pcc* and the nematode *Nb*. Because *Pc-Nb* is an acute model, whereby the nematode is cleared by day 7 and the peak of malaria parasitemia is controlled by day 10, the antibody data could be collected after only 20 days of co-infection, a more practical time frame than the 80 days required for the *Pcc-Ls* experiments. This also allowed us to address whether our observations of antibody cross-reactivity were a more general feature of *Pcc*-nematode infection. Given the apparent cross-reactivity observed at a fixed dilution of sera in the *Pcc-Ls* ELISA we used endpoint titres derived from a serial dilution (1:50 - 1:819200) in the *Pcc-Nb* assays to address whether this readout would overcome cross-reactivity problems. To determine if the specificity of the assay could be improved with the use of recombinant antigens we also included the malaria protein, MSP-1_{19} [39] not available to us for the *Ls* studies.

The antibody responses we observed on Day 20 of *Pcc-Nb* co-infection (Fig 2) paralleled those we had seen in the *Pcc-Ls* experiments at Day 80. For example, as seen in Fig 2Aiii, *Nb* mice made IgG1 biased responses against NbA, and responses in *Pcc-Nb* mice were intermediate between *Nb* and *Pcc* mice. In addition, *Pcc* mice mounted a strong MSP-1_{19}-specific IgG2a response that was reduced in *Pcc-Nb* mice (Fig 2Bi). As before, levels of polyclonal IgE in *Pcc-Nb* mice were intermediate (data not shown). We again observed cross-reactivity, whereby *Nb* mice mounted detectable IgG1 and IgG2a responses to both recombinant and crude malaria antigens (indicated by $X_{1\&2}$ in Fig 2A and $X_{4\&5}$ in Fig 2B, respectively). The magnitude of the *Nb*-induced IgG2a cross-reactive response is particularly striking with titres against crude and recombinant malaria antigens reaching 2500 and 200 respectively. Similarly, *Pcc* mice mounted responses to NbA (X_3 in Fig 2A and X_6 in Fig 2B). It is important to note that these titres although low are markedly greater than background responses (mean plus 3 standard deviations of serum responses from control mice), which are represented as zero on the y-axis. The immune bias that is apparent in serum antibody isotype responses is fully supported by cytokine responses in the lymph nodes of *Pcc-Nb* infected mice as we have recently described [53]. Of interest, no cross-reactivity was observed at the T-cell level.

Cross-reactive IgG1 responses of malaria-infected mice to NbA are lost at higher dilutions but IgG2a responses remain

The analysis of both *Pcc-Ls* and *Pcc-Nb* co-infection indicates that the issue of cross-reactivity is a factor investigators are likely to routinely encounter. Determining the qualitative and quantitative aspects of the cross-reacting antibody responses are not only important for the practical analysis of immune deviation but could be of real biological relevance during co-infection.

As expected, antibody responses were biased, in terms of isotype, by infection status. The bias in isotype due to a particular infection (Th2 associated IgG1 induced during *Nb* infection, for example) was extended to non-specific antigens, as seen in the IgG1 response of *Nb* mice to both MSP-1_{19} and pRBC (X_1 and X_2 in Fig 2Ai + 2Aii). However, *Pcc*-specific IgG2a titres in *Pcc* mice were significantly higher than the cross-reactive response induced in *Nb* mice (Fig 2Bi). Thus, although *Nb* mice made cross-reactive IgG2a responses, these were no longer detectable with increasing dilution of sera (Fig 2Bi). In this case capitalising on the differences in strength of antigen-specific and cross-reactive responses clarified interpretation of immune bias in co-infected mice. Similarly, IgG1 responses to NbA were significantly higher in *Nb* mice than the cross-reactive response induced by *Pcc* mice (Fig 2Aiii). However, titre of *Pcc*-induced cross-reactive IgG2a to NbA did not differ significantly from *Nb* mice (X_6 in Fig 2Biii).

We can conclude from this analysis that cross-reactive IgG1 responses to NbA were only detectable at dilutions less than 1:100 and thus higher dilutions may be used to avoid cross-reactivity when assessing antigen-specific antibody isotype profiles for the purpose of interpreting immune bias. However, increasing sera dilution did not always overcome the cross-reactivity observed, as IgG2a responses to NbA in *Pcc* mice were still observed at 1:2500. This cross-reactivity warrants further investigation, as it is likely to be important biologically. Indeed even cross-reactive responses detectable only at high serum concentrations may still have functional relevance *in vivo*.

Cross-reactivity appears to lie predominantly with carbohydrate epitopes and can be largely eliminated by periodate treatment

Antibody cross-reactivity in a broad range of systems can be attributed to reactivity with carbohydrate determinants [54,55]. Additionally, the IgG3 isotype is often associated with recognition of carbohydrates [56] and we observed cross-reactive IgG3 antibody responses during *Pcc-Nb* co-infection (Fig. 3). *Nb* mice mounted IgG3 responses to both MSP-1_{19} and pRBC antigens, achieving titres of 200 and 3200 respectively (Fig 3Ci

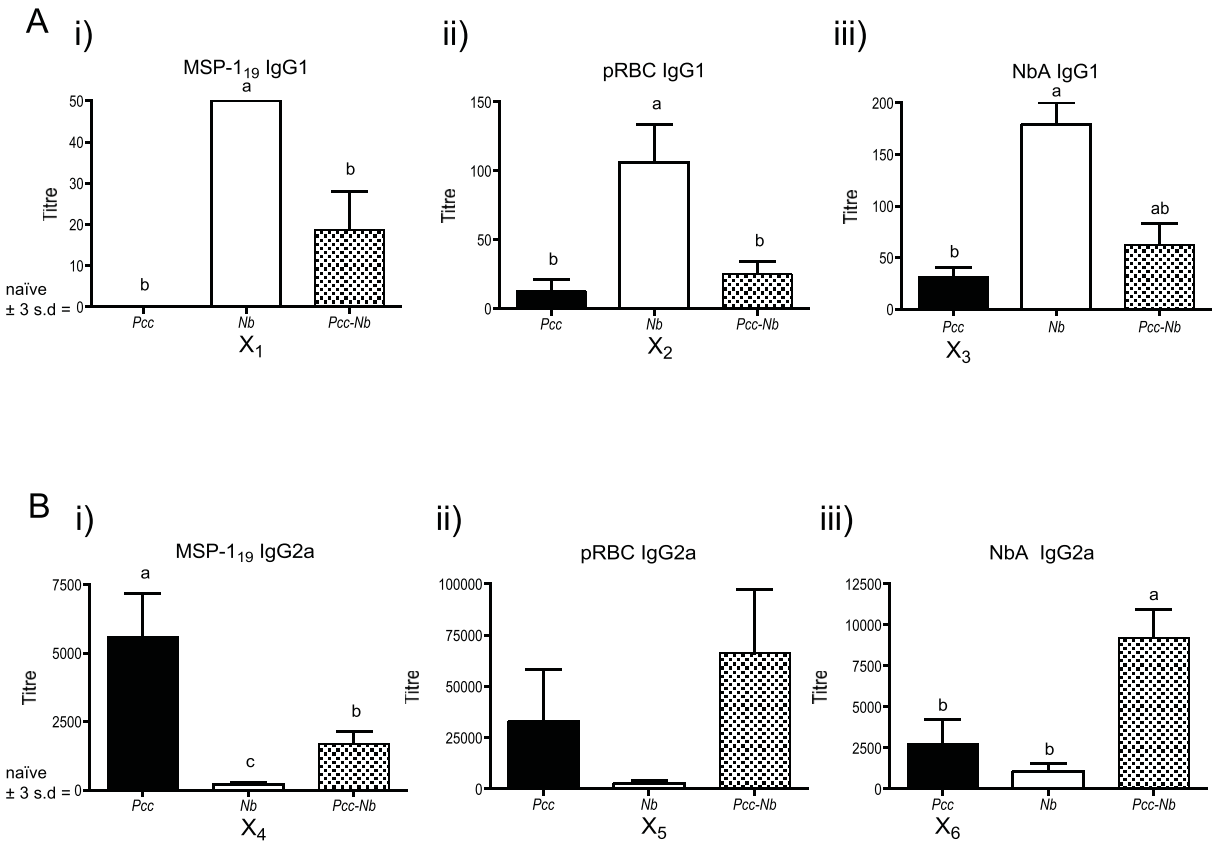

Figure 2 Antibody isotype responses in infection and co-infection with *Nippostrongylus brasiliensis* and malaria. Mice were infected with 200 *Nb* L3 larvae and/or 10^5 *Pcc*-infected RBCs on day 0. Serum antibody titres (A) IgG1 (B) IgG2a to recombinant *Pcc* antigen MSP-1_{19} (i), crude *Pcc* antigen (*pRBC*) (ii) and crude *Nb antigen* (*NbA*) (iii) were measured at day 20 post-infection for 8 mice per infection group. Black bars represent the *Pcc* mice, white bars the *Nb* mice and the chequered bars the co-infected mice (*Pcc-Nb*). Antibody titres are shown on the y-axis and represent the reciprocal of the greatest dilution at which O.D was greater than the mean plus 3 standard deviations of the O.D values observed for control mouse sera at a 1/200 dilution. The letter X highlights those responses that are cross-reactive. Groups not connected by the same letter denote pairs that are significantly different according to Tukey's Pairwise analysis.

and 3Cii). *Pcc* mice mounted similar cross-reactive IgG3 responses to NbA (Fig 3Ciii). We thus chose to assess whether cross-reactivity in our *Pcc-Nb* co-infection system could be overcome by periodate treatment of the parasite antigens. Periodate oxidises carbohydrate to aldehydes, thus disrupting carbohydrate epitopes, which allowed us to distinguish if cross-reactive responses target the carbohydrate or protein moiety of an antigen. This could be of particular importance where detection of cross-reactive responses was not overcome by increasing serum dilution. In addition to clarifying the interpretation of shifts in immune bias, determining whether induction of specific isotype responses is driven by protein or carbohydrate recognition has important implications for vaccine design and diagnostic serology.

For the pRBC and MSP-1_{19} antigens, periodate treatment did not significantly affect recognition by IgG1 antibodies ($P \geq 0.7$). Periodate treatment of NbA however, significantly reduced anti-NbA IgG1 titres across all infection groups (Fig 3Aiii), suggesting common recognition of a carbohydrate moiety. In particular, the cross-reactive recognition of NbA, by IgG1 antibodies from *Pcc* mice, was ablated (X_7 in Fig 3Aiii).

Treatment of MSP-1_{19} antigen with periodate resulted in a significant increase in IgG2a detected in sera from *Pcc-Nb* mice. Detection of IgG2a in singly-infected mice also followed this trend but was not statistically significant (Fig 3Bi). These results suggest recognition of a protein epitope on the recombinant antigen previously masked by glycosylation. *Plasmodium* species lack the glycosyltransferases required for any glycosylation other than attachment of GPI anchors [57,58]. However, inappropriate glycosylation of the recombinant protein can occur in the *Pichia* expression system [57]. The increase in protein-specific responses, following periodate treatment of the recombinant antigen (MSP-1_{19}), may thus

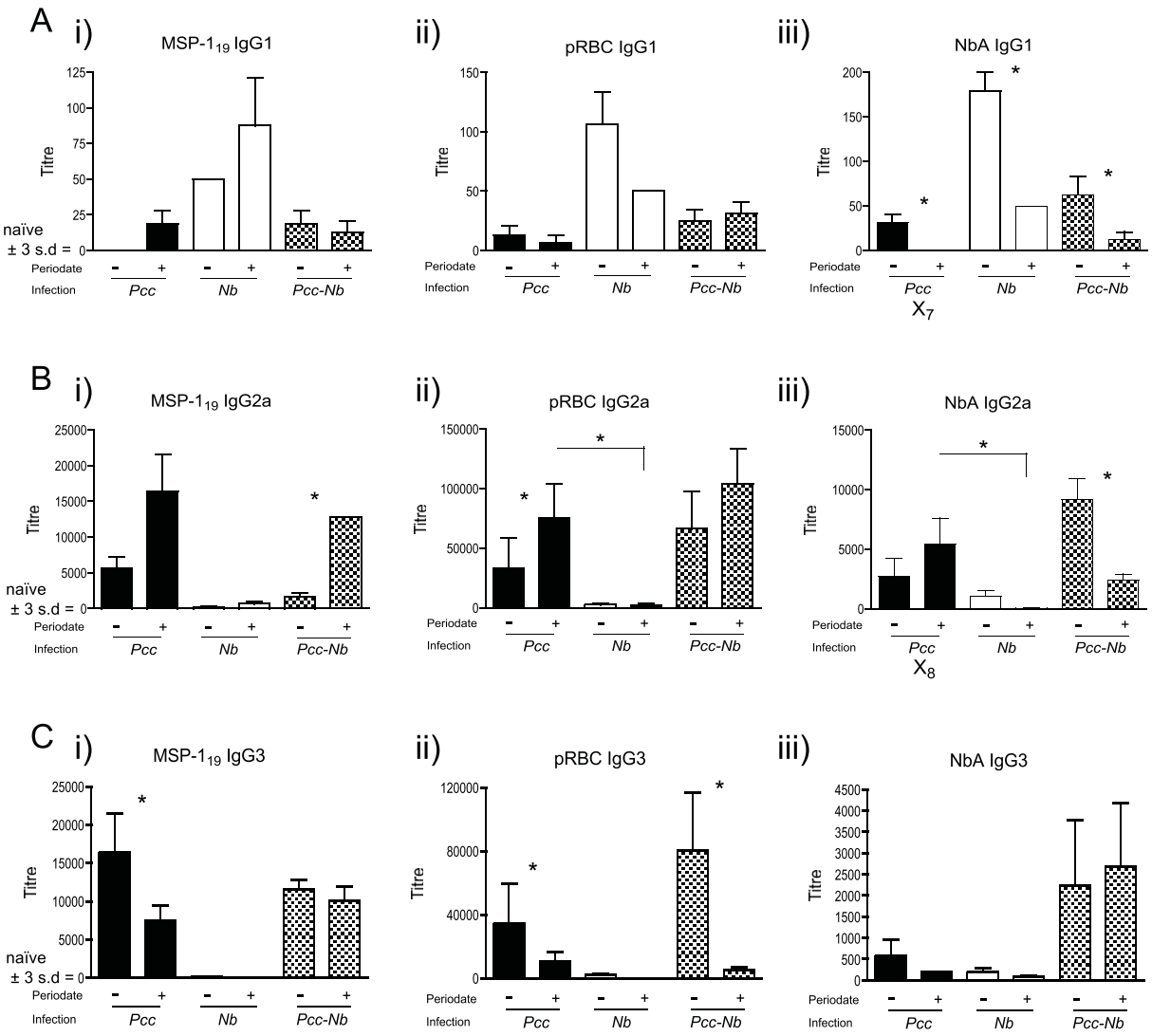

Figure 3 Antibody responses to protein epitopes in infection and co-infection with *Nippostrongylus brasiliensis* and malaria. The parasite antigens used in ELISA were pre-treated with periodate to disrupt carbohydrate epitopes, in order to determine if cross-reactive responses target the carbohydrate or protein moiety of an antigen. Mice were infected with 200 *Nb* L3 larvae and/or 10^5 *Pcc*-infected RBCs on day 0. Serum antibody titres (y-axis) were measured at day 20 post-infection for 8 mice per infection group and represent the reciprocal of the greatest dilution at which O.D was greater than the mean plus 3 standard deviations of the O.D values observed for control mouse sera at 1/200 dilution. Antibody isotypes (A) IgG1 (B) IgG2a (C) IgG3 to recombinant *Pcc* antigen MSP-1_{19} (i), crude *Pcc* antigen (*pRBC*) (ii) and crude *Nb antigen* (*NbA*) (iii) before (-) and after (+) treatment with periodate are shown. Black bars represent *Pcc* mice, white bars the *Nb* mice and the chequered bars the co-infected (*Pcc-Nb*) mice. (*) denotes pairs which are significantly different according to Tukey's pairwise analysis.

reflect the response to the natural non-glycosylated parasite protein seen during infection. IgG2a recognition of periodate treated pRBC antigen was enhanced in sera from *Pcc* mice, though not in the *Pcc-Nb* or *Nb* mice (Fig 3Bii). This suggests the cross-reactive IgG2a response is not targeting carbohydrates, which could potentially be conserved amongst parasite antigens.

For NbA, it was only after treatment with periodate that differences amongst infection groups become apparent, whereby significantly greater amounts of (cross-reactive) IgG2a antibodies were detected in *Pcc* mice in comparison to *Nb* mice (X_8 in Fig 3Biii). Thus, the *Pcc*-induced cross-reactive IgG2a response to NbA (X_6 in Fig 2Biii) that was not lost with serial dilution was also maintained following periodate treatment (X_8 in Fig 3Biii). In contrast, the *Nb*-specific IgG2a response appears to target carbohydrate as periodate treatment reduced recognition of NbA in both *Nb* and *Pcc-Nb*

mice (Fig 3Biii) although only statistically significant in the *Pcc-Nb* mice. This may indicate that atypical Th1-type responses to helminth antigens are driven by carbohydrate.

Disruption of carbohydrates via periodate treatment significantly affected the recognition of *Pcc* antigens by IgG3 antibodies. In particular, recognition of periodate treated pRBC was reduced in serum from all mice that had experienced *Pcc* infection; this was most evident in the *Pcc-Nb* mice (Fig 3Cii). The recognition of periodate treated MSP1-$_{19}$ was also significantly reduced in *Pcc* mice (Fig 3Ci). The more pronounced reduction in IgG3 response to the treated pRBC antigen may reflect the greater proportion of carbohydrate components in this crude antigen preparation in comparison to the single recombinant protein.

Discussion

Antibody analysis should be able to provide critical information on changes in cytokine bias due to co-infection. This is particularly important in human studies where serum may be the only reagent available for immunological analysis. Whilst we acknowledge that there is a need to confirm the relationship between splenic or serum cytokines and antibody responses in co-infection if this strategy is to be used in human studies, our focus is on the interpretation of antigen-specific Th1/Th2 bias based on antibody isotype, which was complicated by cross-reactivity in the two co-infection models studied here. It is worth noting that cross-reactive responses were observed regardless of whether recombinant or crude antigens were used. We primarily address technical strategies that will enable us, and others, to draw conclusions regarding the influence of a co-infecting parasite on immune bias using serum antibodies. However, the functional implications of cross-reactive responses are also discussed.

Murine models that aim to dissect the real effect of a co-infecting parasite on immune bias must use large numbers of animals to detect significant differences in antigen-specific responses between single and dual infection. Thus for antibody analysis of the large sample size (see legend Fig 1 for details) in our *Pcc-Ls* study of co-infection we chose a fixed serum concentration, previously determined to fall within the linear range of the dilution curve. Although this saved time and reagents, in retrospect, it provided insufficient information for our purposes: it did not allow us to distinguish the relative strengths of cross-reactive versus antigen-specific responses.

When antibody titres were calculated in the *Pcc-Nb* study, we were able to determine whether apparent alterations in antibody isotype profile on co-infection were due to actual changes in parasite-specific responses or reflected a cross-reactive response. For example, determining that cross-reactive IgG2a antibody titres in *Nb* mice (X_4 in Fig 2Bi) were significantly lower than the antigen-specific response of *Pcc* mice meant that cross-reactivity was unlikely to influence the titre observed in *Pcc-Nb* mice. This allowed us to conclude that the reduction in Th1 type antibody in *Pcc-Nb* mice was probably due to suppression of *Pcc*-specific Th1 responses by nematode infection. Further to this, had we not calculated titre and relied on optical density data derived from a single dilution of sera we may not have observed the difference between *Pcc* and *Pcc-Nb* mice and thus incorrectly concluded that there was no effect of co-infection on Th1 responses. Similarly, analysis of antibody titre enabled us to detect the reduction in anti-NbA IgG1 antibody in *Pcc-Nb* mice (Fig 2Aiii), which suggests a *Pcc*-mediated bias toward a Th1 cell response. In other cases, cross-reactivity was observed even at high dilutions with *Pcc* mice achieving IgG2a titres equivalent to or greater than *Nb* mice (X_6 in Fig 2Biii). In this case, calculation of titre did not help to unravel potential cytokine influences and the enhanced IgG2a response in co-infected mice may be due to increased Th1 cytokines during co-infection and/or the presence of cross-reactive antibody (Fig 2Biii).

Nematode surface antigens and the excretory/secretory products from these parasites are heavily glycosylated [59]. Similarly, *Plasmodium* species express glycoconjugates on their surface and have abundant glycophosphatidylinositol anchors [60]. In other co-infection systems cross-reactive epitopes have been shown to derive from carbohydrate structures [61]. The sensitivity of the carbohydrate component of an antigen to periodate treatment [59] has been beneficial in interpreting our results. In particular, treatment of *Pcc* antigens demonstrated that cross-reactive nematode-induced IgG3 responses were largely attributed to the carbohydrate component. Interestingly, periodate treatment also reduced apparent cross-reactivity by exposure of protein epitopes, previously masked by carbohydrate, which enhanced the detection of antibodies from mice that had been exposed to the antigen during infection (e.g., anti-pRBC in Fig 3Bii). This allowed us to conclude that levels of anti-pRBC IgG2a antibody in *Pcc-Nb* mice are solely induced by the *Pcc* parasite. Further to this, detection of cross-reactive protein-specific antibodies enabled responses, previously indistinguishable in magnitude between singly-infected groups, to be differentiated. For example, periodate treatment of NbA enhanced detection of *Pcc* induced cross-reactive IgG2a antibodies (X_8 in Fig 3Biii) whilst the antigen-specific response of *Nb* mice was ablated. This indicates that the level of *Nb*-specific IgG2a observed in *Pcc-Nb* mice is due to *Pcc* driving a cross-reactive IgG2a Th1 type response.

We have demonstrated that the use of serial dilutions and periodate treatment of the parasite antigens can help overcome cross-reactivity for the purposes of analysing and interpreting Th1/Th2 cell immune bias. However, some 'true' cross-reactivity remained (i.e. *Pcc*-induced IgG2a responses to NbA (Fig 2Biii/Fig 3Biii)), and the induction of these antibodies has important implications with regard to biological function. For example the immune responses to nematode infection are typically characterised by a Th2 type (IgG1) response, as we observed for *Nb*-induced responses to the nematode antigen (NbA). The propensity for *Pcc* mice to induce atypical IgG2a antibody isotypes to nematode antigen is likely due to the malaria parasite promoting Th1 cytokines in the environment where the antibody response is established [45]. The biological consequences of the *Pcc* driven IgG2a response to the nematode antigen and the less pronounced IgG1 response of *Nb* mice to *Pcc* antigens remain to be investigated. In *Trichuris muris* infection, manipulation of the immune environment to a Th1 type setting, characterised by elevated IgG2a and IFNγ, was shown to enhance chronicity of this intestinal helminth [62]. *Pcc*-induced IgG2a to nematode antigens may thus have real consequences in terms of disease outcome. Effects of nematode co-infection on the malaria parasite are also evident; *Pcc-Nb* mice have reduced levels of malaria parasitemia in comparison to *Pcc* mice [53] and it is interesting to consider the possibility that cross-reactive IgG2a antibodies induced by the nematode infection may act in concert with the antigen-specific response to control malaria parasites. The potential for cross-reactive responses to have a functional role during infection raises the intriguing possibility that their production is a deliberate strategy of the host to combat diverse parasites [63]. To fully understand the relative contribution of cross-reactive antibodies in parasite control would require passive antibody transfer experiments.

Although schistosome parasites are phylogenetically distinct from nematodes, helminth co-infection studies that investigate *Schistosoma mansoni* provide evidence that cross-reactivity is relevant in other co-infection systems and can have a strong impact on disease severity. Naus et al [43] report the induction of cross-reactive IgG3 antibodies that recognise both *Plasmodium falciparum* and *S. mansoni* antigens. Pierrot et al extended this study, identifying the *S. mansoni* antigen (SmLRR) that is recognised by both malaria and *S. mansoni* singly-infected hosts. Interestingly, as we observed in our *Pcc-Nb* model of co-infection, the two infections induce different antibody isotypes to antigen: cross-reactive malaria driven IgG3 and helminth driven IgG4 [45]. In areas co-endemic for these two parasites, exposure to malaria and subsequent induction of the cross-reactive IgG3 response seems to increase the risk of developing hepatosplenomegaly in schistosome infected individuals [44].

Conclusions

In summary, our data illustrate that whilst cross-reactivity may confound observations of interest, it can largely be overcome by a combination of increasing sera dilution and pre-treatment of antigens with periodate. Adopting such strategies will enable antibody isotypes to be used as an indicator of cytokine bias and clarify interpretation of when Th1-Th2 cell shifts have occurred as a result of co-infection. Arising from this analysis is the opportunity to dissect antigen-specific from cross-reactive responses and thus obtain information pertaining to the relative strength of these responses and recognition of carbohydrate versus protein epitopes. This will provide the foundation on which to base more detailed characterisation of the antibody responses during co-infection in order to investigate their functionality.

Abbreviations

Ls: (*Litomosoides sigmodontis*); *Nb*: (*Nippostrongylus brasiliensis*); *Pcc*: (*Plasmodium chabaudi chabaudi*); LsA: (*Litomosoides sigmodontis* crude antigen); NbA: (*Nippostrongylus brasiliensis* crude antigen); pRBC: (*Pcc* parasitized red blood cell crude antigen); MSP-1_{19}: (Merozoite Surface Protein -1_{19}); RBC: (Red Blood Cell); Th: (T helper); pi: (post-infection).

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Authors' contributions

KFC participated in the design of the study, conducted the *Pcc-Nb* experiments and immunoassays, performed the statistical analysis and helped to draft the manuscript. TL conducted the *Pcc-Ls* experiments and immunoassays. JL provided recombinant MSP-1_{19}. ALG conceived of the study and participated in its design, was involved in all co-infection experiments and helped to draft the manuscript. JEA also conceived of the study, participated in its design and helped to draft the manuscript. All authors read and approved the manuscript.

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PERSPECTIVE ARTICLE

Why do adaptive immune responses cross-react?

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Abstract

Antigen specificity of adaptive immune responses is often in the host's best interests, but with important and as yet unpredictable exceptions. For example, antibodies that bind to multiple flaviviral or malarial species can provide hosts with simultaneous protection against many parasite genotypes. Vaccinology often aims to harness such imprecision, because cross-reactive antibodies might provide broad-spectrum protection in the face of antigenic variation by parasites. However, the causes of cross-reactivity among immune responses are not always known, and here, we explore potential proximate and evolutionary explanations for cross-reactivity. We particularly consider whether cross-reactivity is the result of constraints on the ability of the immune system to process information about the world of antigens, or whether an intermediate level of cross-reactivity may instead represent an evolutionary optimum. We conclude with a series of open questions for future interdisciplinary research, including the suggestion that the evolutionary ecology of information processing might benefit from close examination of immunological data.

Introduction

One of the most startling and impressive features of the vertebrate adaptive immune system is its ability to recognize and bind diverse parasite antigens. As part of this process, the immune system is able to generate extraordinary specificity of antibodies to particular antigens. This specificity is an axiomatic feature of the adaptive immune system, but it is also an incomplete picture. Cross-reactivity of lymphocyte receptors and antibodies to parasite antigens is common, with important consequences for both host and parasite, in terms of host health (e.g., Fesel et al. 2005; Urbani et al. 2005), antigenic variation (Lipsitch and O'Hagan 2007), parasite strain structure (e.g., Recker and Gupta 2005; Koelle et al. 2006a), and epidemiological dynamics (e.g., Adams et al. 2006; Koelle et al. 2006b; Wearing and Rohani 2006). However, the extent to which we should **expect** to see cross-reactivity of adaptive immune responses has not been fully explored, especially for antibodies.

In this perspective, we consider whether cross-reactivity is an evolved trait of the immune system, driven by conflicting costs and benefits of antigen specificity, or whether it is an inescapable side-effect of the problem of recognizing and binding to an enormous range of putative antigens. Throughout, we will use 'parasite' in a general sense, to include all infectious disease agents, and we define 'specificity' as the ability of the immune system to discriminate among antigens and 'cross-reactivity' as the absence of discrimination, in accordance with general (Janeway et al. 2001) as well as evolutionary (Frank 2002) immunological usage. Cross-reactivity is also known as 'heterologous immunity' (Page et al. 2006) or, in some contexts, by the more colorful term 'original antigenic sin' (e.g., Liu et al. 2006). Here we use 'cross-reactivity' to cover all cases. We would also stress that specificity and cross-reactivity should be considered endpoints of a spectrum rather than strict alternatives, and we would hope that our approach encourages thinking about quantitative predictions for the level of cross-reactivity we might expect lymphocytes or antibodies to exhibit.

To address whether cross-reactivity of adaptive immune responses is an evolved trait or a side-effect of biological or chemical constraints, we explore the

problem facing the immune system in terms of the information it needs in order to function correctly. First, the immune system must obtain information about the parasites attacking it as efficiently as possible, in order to rapidly combat infection. Thus we expect T- and B-cell repertoires, in terms of their overall size and the binding specificities of component cell lineages, to have been influenced by natural selection in their ability to search 'antigenic space' with a certain degree of cross-reactivity. However, the immune system then faces a second problem: how specific should antibodies be, to achieve a major ultimate aim of the immune system, the destruction of parasites? For both of these problems, we consider the theoretical work to date on cross-reactivity, and we then review empirical data on the costs and benefits of specific versus cross-reactive antibodies. We start by introducing parasite detection as an information problem.

The immune system as an information gatherer and processor

Precise phenotypic adaptation to environmental conditions requires that organisms process information about their surroundings in order to make appropriate context-dependent decisions (Dall et al. 2005). Optimal foraging decisions, for example, depend upon the ability of a forager correctly to perceive the relative resource value of different patches of food, in light of associated costs of foraging such as threats of predation (Stephens et al. 2007). Optimal offspring sex ratios for a given intensity of local mate competition require that female parasitoid wasps accurately perceive the number of other females laying eggs on a patch (Shuker and West 2004; Burton-Chellew et al. 2008). The mammalian immune system must similarly tailor action to context by processing information about the world of antigens: in the face of unpredictable exposure to diverse parasites, a host must perceive infections, identify parasites, and then mobilize the appropriate mechanisms to kill those parasites. In each of these examples of phenotypic adaptation, understanding the mechanisms by which information is gathered and translated to action – i.e., information processing – can help to explain why organisms may fail to be perfectly adapted to their environments (West and Sheldon 2002; Shuker and West 2004; Dall et al. 2005). For the immune system, is the apparent imperfection in discrimination of parasite antigens (manifested as cross-reactivity) a deliberate strategy to fight parasites across antigenic space with cross-reactive antibodies, or merely an information constraint imposed by the task faced by the immune system?

The antigen recognition task of the adaptive immune system is not easy: it must distinguish self from nonself, and one parasite from the next, in a sea of molecules. The innate immune system drives the process of sifting through this antigenic information (Janeway and Medzhitov 2002), but it is the adaptive immune system, via T and B cells, that possesses the remarkable machinery necessary for posing 'search terms' over antigenic space, and for recognizing matches to those terms (Fig. 1). We thus consider that the immune system gathers information by binding to parasite antigens, with a failure to obtain that information (a failure to recognize and bind to a parasite antigen) posing a serious risk to the organism's health (and we also note that avoiding being observed by immune systems is a legitimate and not uncommon strategy of parasites (Maizels et al. 2004; Tortorella et al. 2000)). The capacity of lymphocyte receptors to recognize antigen is in theory infinite (Pancer and Cooper 2006). However, this initial searching of antigenic space is only the first step taken by the immune system (Fig. 1). Via somatic hypermutation, B cells generate more specific receptors for a given antigen, which can be construed as a form of 'local searching' of antigenic space, or gaining very specific information about the antigen to inform further action, which in this case is the generation of antibodies by plasma cells (Fig. 1). B cells may provide a more focused information-gathering capacity, as they provide very fine-grained information about a certain part of antigenic space.

Despite this sophistication, antibodies often do cross-react with, and take action against, antigens displayed by parasite strains or species other than the one that induced the initial response. Is this cross-reactivity a deliberate feature of the overall strategy of the immune system, or an unselected constraint posed by the realities of antigenic variation? To address this, we first turn our attention to the initial searching problem faced by T and B cells.

How should the immune system search antigenic space?

Given the huge range of possible antigens that an immune system might have to recognize, how best should the immune system cover, or search, antigenic space? In particular, in terms of the adaptive immune system, how specific should the T and B cell repertoire be?

Energetic and other constraints affect many aspects of immunological function (Viney et al. 2005; Martin et al. 2007), and the degree of antigen specificity is probably no exception. Hosts may be constrained by lymphocyte numbers as well as the need to avoid self-damaging responses in their search of antigenic space. For example, the lymphocyte pool of each person bears millions of different T-cell receptors and billions of different B-cell receptors, but every mammalian cell may display 10^{12} potential

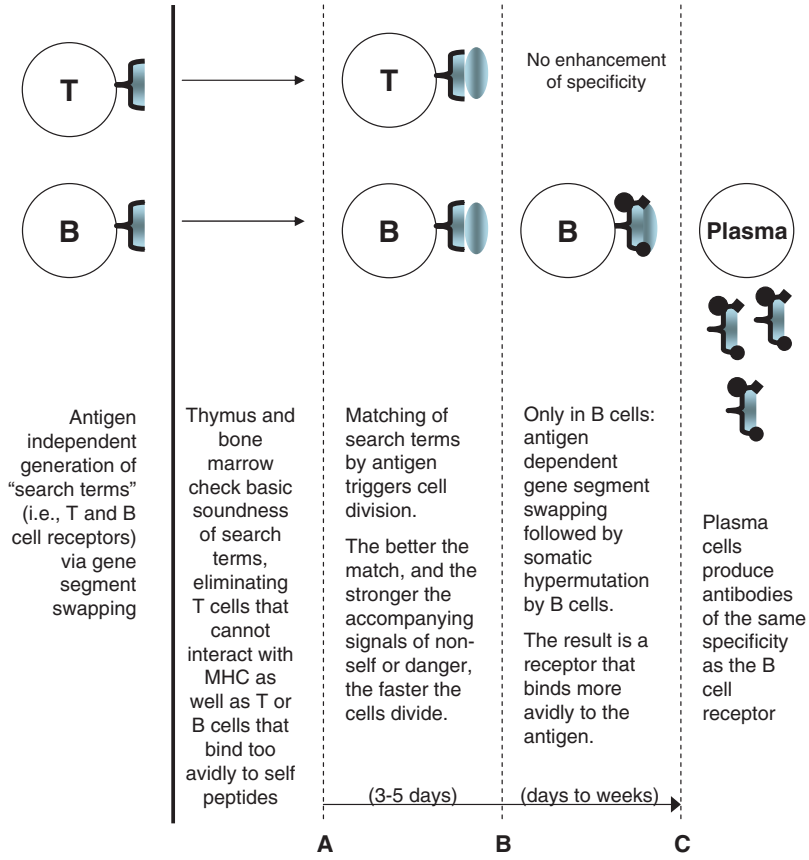

Figure 1 How the mammalian adaptive immune system explores antigenic space with lymphocyte receptors. The receptor of each clonal T or B cell lineage determines the antigens it can bind. Receptor repertoire formation involves the generation of 'search terms' – i.e., T- or B-cell receptors – via gene segment swapping. Cells with receptors that meet basic criteria shown to left of filter A are released into circulation. When the receptor matches antigen encountered in the body (filter A), cell division is triggered at a rate influenced by binding kinetics, co-receptors, and co-stimulatory signals. When a B cell encounters its antigen, a second process of diversity generation takes place: somatic hypermutation, a form of 'local searching' in which point mutations are generated (filter B). The resulting receptor is again tested against antigen. B cells that bind more avidly are selectively favored and thereby more likely to contribute to the antibody repertoire, once cells of the lineage differentiate into plasma cells (filter C). See Janeway et al. (2001) for further details. Although the generation of receptor diversity is largely somatic, there is strong potential for genetically encoded regulatory genes to act at many steps along the way (e.g., filters A–C). Thus the regulatory aspects of antigen specificity, at least, should be accessible to natural selection.

protein antigens on its surface (Sun et al. 2005), and parasites of mammals span a huge range of biological (and probably antigenic) diversity – from archaea (Lepp et al. 2004) to metazoa (Maizels et al. 2004). Given these constraints, attempts have been made to predict the information-gathering potential of lymphocytes. Empirically grounded theoretical work suggests that, prior to exposure to antigen, a certain degree of cross-reactivity in the lymphocyte search algorithm is essential (Langman and Cohn 1999). Indeed, hosts may ensure recognition of a large parasite set, or a rapidly evolving parasite set of any size, by coarse-graining antigen recognition (Oprea and Forrest 1998), enabling production of antibody libraries that are strategically placed to generalize over antigenic space (Oprea and Forrest 1999). Moreover, the optimal level of cross-reactivity increases with decreases in repertoire size – i.e., fewer lymphocyte receptors must cross-react more, to cover antigenic space – but that strategy risks autoimmunity (Borghans et al. 1999). To balance these factors for the size range of the human repertoire, a low degree of cross-reactivity is optimal for both T (van den Berg et al. 2001; Borghans and De Boer 2002) and B cells (Louzoun et al. 2003).

Theory further suggests that a receptor's cross-reactivity should be adapted to the portion of antigenic space in which it binds (Fig. 2). By this logic, receptors very unlikely to bind self antigens should have wide circles of reactivity. In studies comparing fixed low cross-reactivity

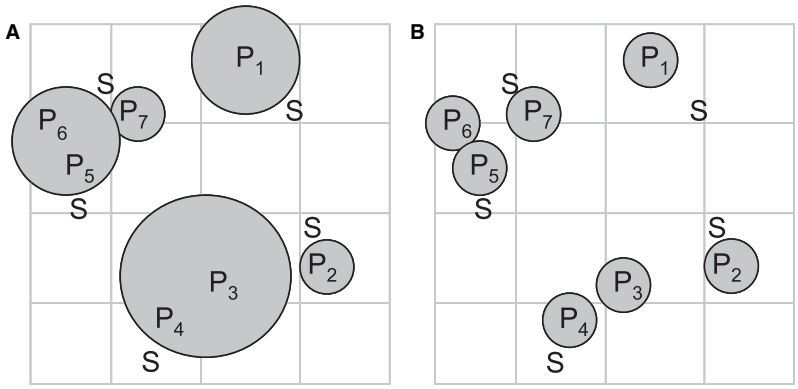

Figure 2 Contrasting degrees of cross-reactivity over two-dimensional antigenic space. Seven parasite antigens ($P_{1–7}$) and five self antigens (S) are represented on a grid. The size of the filled circle represents the range of cross-reactivity of a given lymphocyte receptor or antibody. The host in (A) plays a more cross-reactive strategy than the host in (B). Both avoid self-reactivity and respond to all parasite antigens, but (A) covers more antigenic space with fewer lymphocyte lineages. Is that a good thing? The answer probably depends upon context. For example, imagine both hosts are sequentially exposed first to P_3 and then P_4. If P_3 and P_4 were different strains or species of malaria, the host using strategy (A) would likely benefit from cross-protection (e.g., Mota et al. 2001). If P_3 and P_4 were different serotypes of dengue virus, however, the strategy depicted in (A) could be lethal (e.g., Goncalvez et al. 2007). Figures are modified from Scherer et al. (2004), based on shape-space tools for immunological reactivity developed by Perelson and colleagues (e.g., Smith et al. 1997).

with plastic cross-reactivity set by proximity to self antigens, both strategies eliminated self-reactivity but the latter achieved greater coverage of antigenic space, including the space near self peptides (van den Berg and Rand 2004; Scherer et al. 2004). If cross-reactivity is good for the information gathering phase of an immune response, what about the next phase?

Fine-grained information: the problem of discrimination

If lymphocytes search antigenic space efficiently by being initially cross-reactive, then discriminating between closely related antigens is not a problem for them. However, the task assigned to antibodies in the immune response is one that may require discrimination, if antigen-specificity is advantageous. Discrimination is a key component of information processing theory (reviewed by Stephens 2007); also see Fig. 3). From this theory, we should expect the binding specificity of a given antibody to be a function of the difference between two antigens (i.e., the target and any nontarget antigens) and the relative costs and benefits of specificity versus cross-reactivity. First we will consider how easy or difficult it might be to discriminate between antigens using the concept of antigenic distance.

If we are going to predict when cross-reactivity will occur, when it will help or hinder the host, or to identify the optimal degree of cross-reactivity for a given context, we need to understand the antigenic distance between parasites: how different do different parasites appear from

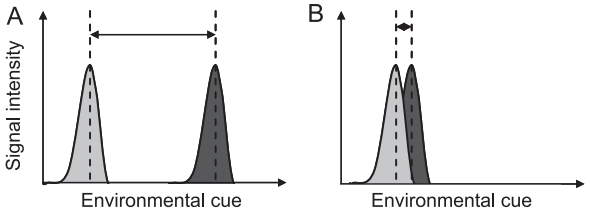

Figure 3 Optimal discrimination among environmentals, depending upon the perceived magnitude of the difference between cues, as well as the benefits of the ability to perceive the difference. For example, if the *x*-axis represents a cue that a forager can perceive regarding the food quality of a patch, then low versus high food quality may be more easily discriminated in (A) than in (B). Still, if there are great rewards for perceiving the difference in (B), then optimal discrimination may have the relatively high resolution depicted in (B).

the perspective of the immune system? The analogy from behavioral ecology is working out what an animal can perceive, in order to make sense of behavioral responses to environmental change (Boomsma et al. 2003; Shuker and West 2004). The problem of antigenic distance is a difficult one, and not just for the immune system. In this era of whole-genome sequencing of parasites, it has become clear that antigenic distance can bear a decidedly nonlinear relationship to phylogenetic distance (Gog and Grenfell 2002), partly because the recognition of antigen can be as much about physical conformation as about amino acid sequence (e.g., Donermeyer et al. 2006), and partly because antigens can be conserved across taxa. For example, cross-reactivity can occur between antibodies induced by parasites with rather distant phylogenetic

relationships, such as helminths and malaria (Mwatha et al. 2003; Naus et al. 2003). Within parasite species, phylogenies may largely parallel antigenic distances over long genetic distances (Frank 2002), but a stepwise and nonlinear relationship between genetic and antigenic change may become evident when examined at higher resolution. In influenza, for example, silent mutations may move a parasite to new regions of antigenic space that are realized with the occurrence of one last mutation; such a mechanism can account for the way in which a single amino acid change can release a strain from immune pressure while the preceding 19 changes led to little antigenic change (Koelle et al. 2006a). The functional form of the relationship between genetic and antigenic change is likely to shape parasite strain structure (Adams and Sasaki 2007) and epidemiology (Gog and Grenfell 2002; Adams et al. 2006; Koelle et al. 2006b) as well as the efficacy of vaccines (Gupta et al. 2006) and memory responses (Deem and Lee 2003).

Various methods can be used to quantify antigenic distance. Much of the work in this area has been on influenza, because annual attempts are made to match vaccine antigens with antigens of the strain that caused the preceding year's outbreak. Hemagglutination inhibition assays, for example, measure the ability of ferret antibodies induced by one strain of influenza A to block agglutination of red blood cells by another strain; if strong cross-reactivity is evident, a small antigenic distance is inferred (Smith et al. 2004; Koelle et al. 2006a). Such measurements sometimes successfully predict the efficacy of vaccines, but predictions can be improved by knowledge of the antigenic distance between the dominant antibody binding sites rather than whole viruses (Gupta et al. 2006). Another way of assessing antigenic distance is to measure the dilution of serum at which cross-reactivity disappears (K. J. Fairlie-Clarke, T. J. Lamb, J. Langhorne, A. L. Graham, and J. E. Allen, unpublished data). If cross-reactivity persists at million-fold dilutions (and it can), then the antigenic distance is small.

Such methods can be used to compare antigens from different parasite lineages, as well as antigen samples from a single lineage over time, and are necessary if we are to understand whether cross-reactivity is something that cannot be escaped by antibodies – two antigens are just too alike to be separated, even if they are from very different strains (or kingdoms) of parasites – or whether cross-reactivity is a deliberate, selectively advantageous strategy. If antigenic distances were measured among a wide array of parasite taxa, the data would enable assessment of how fully or evenly occupied parasite antigenic space may be. The data might also clarify how many cases of apparent cross-reactivity are due to specific molecular recognition of antigens that are conserved across parasite taxa. Mapping antigenic space (*sensu* Smith et al. (2004), but applied across a much wider set of parasites) would therefore be extremely useful for understanding the causes of antibody cross-reactivity and host–parasite interactions more generally.

Differences among antigens might not be the only constraint on antibodies, however, because the mechanics of the immune system may also be important. For example, the persistence of cross-reactivity once antigenic information is available (i.e., after filter A of Fig. 1) may be explained by lymphocyte limitation in some contexts. The clonal lymphocyte lineage whose receptor best binds a given antigen replicates more rapidly than other clones (Janeway et al. 2001), such that the best-matched lymphocyte lineage wins by competitive exclusion (Scherer et al. 2006). This process tends to favor specificity, but when lymphocytes are limiting, cross-reactivity may result. For example, if B cells undergo fewer rounds of cell division and somatic hypermutation when a host is resource-limited, the antibodies produced may fall short of the maximal possible specificity. Furthermore, lymphocyte dynamics during memory responses may constrain the development of specific responses to new antigens. For example, cross-reactive antibodies are produced in preference to specific antibodies during secondary exposure to dengue because memory B cells are so rapidly activated and thus outcompete cells that are more specific to the new virus (Rothman 2004), a phenomenon observed in memory responses to various other viruses (e.g., Brehm et al. 2002; Liu et al. 2006). The mechanisms whereby the immune system permits recognition of all possible antigens with limited lymphocyte numbers may therefore constrain its ability to match antibody perfectly to antigen. Given these possible constraints, what are the possible costs and benefits of cross-reactive antibodies?

Is cross-reactivity of antibodies a deliberate strategy?

Models have suggested that cross-reactivity at the lymphocyte level is an effective strategy, but what about at the level of antibodies? For antibody cross-reactivity to be favored by natural selection, the costs of cross-reactivity need to be balanced by the benefits. The typical textbook view is that antibody specificity is a good thing, and indeed fine discrimination of parasite antigens can bring fitness benefits to hosts. When a host precisely targets antigen with specific antibodies, it is often rewarded with efficient clearance of infection. For example, antigen-specific antibodies, but not antigen-induced, cross-reactive antibodies, protect mice against parasites such as the intracellular bacteria *Nocardia brasiliensis* (Salinas-Carmona and Perez-Rivera 2004) or lymphocytic chorio-

meningitis virus (Recher et al. 2004). Furthermore, specific antibodies are produced more rapidly when memory B cells encounter the exact same antigen in a subsequent infection – a major benefit of immunological memory (Ahmed and Gray 1996), provided the parasites are identical to those previously encountered.

When subsequently infected with antigenically different parasites, however, those same antibodies can actually promote parasite replication. These apparent failures of specificity can have health consequences. A classic case is the enhancement of dengue virus replication by cross-reactive antibodies, alluded to above. Antigen-specific antibodies provide long-lasting protection against reinfection with the same serotype (Sabin 1952, cited by Goncalvez et al. 2007), but cross-reactive antibodies are associated with dengue hemorrhagic fever during subsequent infection with a different serotype, and the severity of disease varies with the combination and order of appearance of serotypes (Endy et al. 2004; Rothman 2004). Unable to neutralize the virus, the cross-reactive antibodies instead facilitate viral uptake to cells (Goncalvez et al. 2007). The antibodies are specific enough to bind but not to kill parasites. Costs of cross-reactive responses are also observed across parasite species. For instance, cross-reactive responses induced by influenza A exacerbate liver disease due to hepatitis C virus (Urbani et al. 2005).

Balanced against these benefits of specificity and costs of cross-reactivity, it is apparent that cross-reactive immune responses can, in some contexts, simultaneously protect hosts against a wide array of parasites, a possibility that has not been lost on vaccinologists (Nagy et al. 2008). Indeed, cross-reactive antibodies induced by infection or immunization can protect hosts against other infections. For example, mice experimentally infected with a single malaria clone make cross-reactive antibodies that can bind to antigens of other parasite clones (displayed on the surface of infected red blood cells) and lead to their phagocytosis by macrophages *in vitro* (Mota et al. 2001). Similarly, cross-reactive antibodies from a person infected with *Plasmodium vivax* can inhibit the growth of *Plasmodium falciparum in vitro* (Nagao et al. 2008). More importantly, cross-reactive antibodies benefit human hosts living in areas of multi-strain or multi-species malaria transmission in nature (Fesel et al. 2005; Haghdoost and Alexander 2007). Benefits of cross-reactive antibodies are also observed amongst flaviviruses: St. Louis encephalitis virus and Japanese encephalitis (JE) vaccine both induce cross-reactive antibodies to West Nile virus that ameliorate the disease in hamsters (Tesh et al. 2002). The induction of cross-reactive antibodies to West Nile by JE vaccine was corroborated in humans (Yamshchikov et al. 2005), though whether the antibodies are protective remains to be seen. In the case of influenza, cross-reactive responses induced by immunization with one virus can protect hosts against other viral genotypes (Sandbulte et al. 2007; Levie et al. 2008; Quan et al. 2008). Cross-reactive antibodies have also been implicated in protection against fungal infection (Casadevall and Pirofski 2007).

Imprecision of antibody responses can therefore benefit the host in some contexts. Ideally, the degree of cross-reactivity would match the infections at hand (see Fig. 2; Scherer et al. 2004; van den Berg and Rand 2007). Variation in the activation thresholds of individual cells (van den Berg and Rand 2007) or tuning mechanisms such as the immunomodulatory molecules employed by regulatory T cells (Carneiro et al. 2005) should allow precise targeting when needed and cross-reactivity when needed. Recognizing need, however, would require lymphocytes to gather information on the relatedness of parasite antigens – e.g., during co-infections, or comparing remembered to current antigens – to generate the optimal imprecision for a given context. The likelihood of such additional information processing ability is unclear, but even if the immune system could not manage by itself, biomedicine could potentially promote cross-reactive responses (i.e., help the immune system to see two parasites as related), if the context were right. Predicting when imprecisely targeted immune responses will occur, and when they will be to the detriment or benefit of hosts, is therefore of clear biomedical relevance, for vaccination programs and other medical interventions.

Outlook

Why, then, do adaptive immune responses cross-react? While we cannot give a definitive answer to this question, we suggest that the answer is likely to depend on context. In some cases, the true antigenic distance between phylogenetically distant parasites may be very small, such that specificity becomes a biochemical impossibility (and the 'information' cannot be discerned by the immune system). In other cases, strict constraints such as the physical limits of binding strengths or physiological constraints such as lymphocyte limitation may operate. We do not currently know how common these constraints on the immune system actually are. However, we also do not yet know exactly how natural selection operates on the specificity of adaptive immune responses, though we do know that the effects of cross-reactive antibodies on host fitness are context-dependent. Would natural selection always favor greater specificity, but constraints intervene? Or might variability in exposure to parasites over space and time, for example, impose fluctuating selection on the specificity of immune responses? We do at least know that the genetic variation that selection could act upon to effect

evolutionary change is present in the immune system (Frank 2002). For example, thresholds for B-cell activation or the number of rounds of somatic hypermutation, and thus the timing of plasma cell differentiation, may be polymorphic (Fig. 1). Hosts are known to be heterogeneous in the specificity of the antibodies that they make to a given antigen (e.g., Lyashchenko et al. 1998; Sato et al. 2004). What remains to be done is to measure the selective consequences of variation in cross-reactivity in the different components of the immune system.

One intriguing possibility, given the antigenic diversity of parasites as well as the uncertainty of exposure to those parasites, is that imprecision in antigen recognition might ultimately be to the benefit of hosts. Might cross-reactive antibodies represent an adaptation to an unpredictable wide world of antigenic exposures? It has been suggested that imprecision in the waggle dance of honeybees is an adaptation that spreads foragers over an optimal patch size: natural selection is proposed to have tuned the amount of error in the waggle dance, to balance the benefits of known nectar sources against benefits of wider searching (Weidenmuller and Seeley 1999; Gardner et al. 2007); but see Tanner and Visscher (2006). An alternative analogy from evolutionary ecology is that of 'bet-hedging,' whereby life history decisions (such as how much energy to invest in offspring, or where to lay eggs) are deliberately variable, to try to cater for uncertainty in the future environment (Seger and Brockman 1987). Bet-hedging has had its conceptual problems over the years (e.g., Grafen 1999, 2006), but it can be favored under a range of circumstances (e.g., King and Masel 2007), and it would be interesting to explore further the evolution of imprecise antibodies in this context.

We envision several further potential contributions that evolutionary ecologists could make towards understanding and controlling the antigen-specificity of immune responses. For example, evolutionary ecological analyses could aid identification of contexts in which hosts would do well to hedge their bets and make cross-reactive antibodies, or clinics would do well to administer gamma globulin shots. As epidemiologists are often able to characterize exposure risks on local geographical scales, we could combine such information with data on antigenic distances and the relative efficacy of antigen-specific responses to allow evolutionary optimization models to advise which specificity strategy best suits a given setting. Thus quantitative evolutionary ecology could enhance the potential for biomedicine to tailor treatments to epidemiological settings.

Another important issue for the attention of evolutionary ecologists is that biomedical success in generating cross-reactive immune responses with vaccines (Nagy et al. 2008) is likely to feed back on the structure of parasite populations (Restif and Grenfell 2007). Calculation of the co-evolutionary risks of altered antigen-specificity of immune responses is therefore essential; might cross-reactive vaccines impose strong selection for escape mutants to make larger antigenic, and perhaps more virulent, leaps than they do naturally? It will also be critical to identify the role of parasite strategies in promoting cross-reactivity of immune responses. The theory reviewed here (e.g., van den Berg and Rand 2004; Scherer et al. 2004) suggests that the closer the antigenic distance between self and parasite antigens, the less likely that infection will promote cross-reactive antibodies. Do parasites that mimic host molecules select for antigen-specific immunity? These questions are amenable to both theoretical and, more importantly, experimental study.

Finally, we suggest that evolutionary ecology might also gain tremendous insights from the immunological data itself. In particular, interactions between the mammalian immune system and parasites present a rare and useful combination of traits for studies of information processing and adaptation. For a start, the molecular details of the antigens and antibodies or receptors are either known or knowable (Boudinot et al. 2008). Thus the information-gathering system is likely to be better characterized than is usually possible in behavioral ecology systems. Such data might be powerfully combined with quantitative tools such as statistical decision theory, an increasingly important component of studies of information processing (Dall et al. 2005). Statistical decision theory is based on Bayesian approaches, and the parallels between an organism making decisions based on updated knowledge of the environment (formalized as 'prior' and 'posterior' distributions, before and after information acquisition) and the workings of the adaptive immune system, with its updatable immunological memory, are striking. Further, the functional consequences of changes in specificity of immunological recognition can often be measured in exquisite detail. Thus in the immune system, as perhaps in few others, one might be able to discover whether there are limits to the benefits of perfect knowledge of the environment.

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Research article

Plasmodium chabaudi limits early *Nippostrongylus brasiliensis*-induced pulmonary immune activation and Th2 polarization in co-infected mice

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Abstract

Background: Larvae of several common species of parasitic nematodes obligately migrate through, and often damage, host lungs. The larvae induce strong pulmonary Type 2 immune responses, including T-helper (Th)2 cells as well as alternatively activated macrophages (AAMϕ) and associated chitinase and Fizz/resistin family members (ChaFFs), which are thought to promote tissue repair processes. Given the prevalence of systemic or lung-resident Type 1-inducing pathogens in geographical areas in which nematodes are endemic, we wished to investigate the impact of concurrent Type 1 responses on the development of these Type 2 responses to nematode larval migration. We therefore infected BALB/c mice with the nematode *Nippostrongylus brasiliensis*, in the presence or absence of *Plasmodium chabaudi chabaudi* malaria parasites. Co-infected animals received both infections on the same day, and disease was assessed daily before immunological measurements were taken at 3, 5, 7 or 20 days post-infection.

Results: We observed that the nematodes themselves caused transient loss of body mass and red blood cell density, but co-infection then slightly ameliorated the severity of malarial anaemia. We also tracked the development of immune responses in the lung and thoracic lymph node. By the time of onset of the adaptive immune response around 7 days post-infection, malaria co-infection had reduced pulmonary expression of ChaFFs. Assessment of the T cell response demonstrated that the Th2 response to the nematode was also significantly impaired by malaria co-infection.

Conclusion: *P. c. chabaudi* co-infection altered both local and lymph node Type 2 immune activation due to migration of *N. brasiliensis* larvae. Given recent work from other laboratories showing that *N. brasiliensis*-induced ChaFFs correlate to the extent of long-term lung damage, our results raise the possibility that co-infection with malaria might alter pulmonary repair processes following nematode migration. Further experimentation in the co-infection model developed here will reveal the longer-term consequences of the presence of both malaria and helminths in the lung.

Background

Many prevalent species of parasitic nematodes - such as *Ascaris lumbricoides*, which infects over a billion people [1], or *Necator americanus*, the most geographically widespread of the human hookworms [2] - migrate through host lungs as larvae. Lung tissue is ruptured as the larvae burst out of the blood vessels to enter the alveolar spaces. Although this process is typically asymptomatic in humans, it can also be associated with acute respiratory distress or longer term complications [3]. For example, infection with lung-migrating helminths has been associated with bronchial hyper-reactivity and other asthma symptoms among children in China [4] and Brazil [5].

The rodent parasite *Nippostrongylus brasiliensis* (*Nb*) has proven a valuable laboratory model for nematode migration through the host body. In mice, L3 larvae injected into the skin migrate via the lungs to the small intestine, where the parasites develop into adults [6]. Peak abundance of *Nb* larvae in the lung occurs around 2 days post-infection (pi) in many strains of mice [7]. The lung migratory stage of *Nb* is associated with a strong local Type 2 inflammatory response that includes T-helper (Th)2 cells, eosinophils and basophils [8,9]. Alternatively-activated macrophages (AAMϕ) have also been identified as a major component of the pulmonary response to *Nb* infection [10,11]. AAMϕ are characterised by IL-4/IL-13-dependent production of chitinase and Fizz/resistin family members (ChaFFs) including RELMα (also known as Fizz1), the chitinase-like protein Ym1, and Arginase-1 [12-15], and all three proteins are consistently observed in the *Nb* infected lung [10,11,16-18]. Arginase-1 is the counter-regulatory enzyme to iNOS and can thus act to suppress NO production and Type 1 effector function. Arginase-1 also has well documented roles in tissue repair [19,20] and has recently been implicated as an anti-nematode effector molecule [21]. The functions of RELMα and Ym1 are less well understood but, like Arginase-1, they have been strongly implicated in the response to injury [22-24] and have putative roles in the repair process, including extracellular matrix deposition and angiogenesis [25,26]. However, recent data have shown that RELMα and macrophage-derived arginase can also negatively regulate Th2 effector responses and thus limit the pathology associated with overzealous repair [27-29].

Although not formally proven, the association of Arginase-1, RELMα, and Ym1 with the tissue repair process suggests that in the context of nematode infection, ChaFFs, potentially produced by AAMϕ, may be required to orchestrate the repair of damage caused by larval migration in order to restore lung integrity. Two recent papers have highlighted the potential for *Nb* migration to damage the lung with potentially long term consequences [16,18]. Both studies document haemorrhaging of lung tissue and sustained increases in airway hyper-responsiveness. A striking novel observation in these studies is that *Nb* causes disruption of the alveolar architecture that is consistent with pulmonary emphysema many weeks after infection. Dysregulated, AAMϕ-mediated repair of the damage caused by the nematodes may be responsible for such detrimental outcomes [16].

Helminths with lung migratory stages are often co-endemic with Type 1-inducing parasites such as malaria [30-32]. Given the potential for cross-regulation between Type 1 and Type 2 immune responses, we wished to use mouse models to investigate the consequences of co-infection for the pulmonary Type 2 immune responses induced by nematode migration. We chose to focus on *Nb* and a rodent malaria, *Plasmodium chabaudi chabaudi* (*Pcc*), that induces a potent Type 1 immune response and non-lethal infection [33]. We challenged hosts simultaneously with these two acute infections, thus demanding polarized, conflicting immune responses at the same point in time. In addition, we expected *Nb-Pcc* co-infection to induce conflicting responses in the same anatomical location, because malaria-infected red blood cells (RBCs) of many species, including *Pcc*, adhere to endothelial cells of the microvasculature of the lung [34-36]. Furthermore, malaria itself has been shown to cause lung injury [37,38]. Thus, we expected the lung and draining (thoracic) lymph nodes to be potential sites of strong interactions between *Nb* and *Pcc*. The idea that helminth-malaria co-infection may impose Type 1-Type 2 immunological conflict is not new [30], nor is the idea that parasitic co-infection may alter the severity of pulmonary disease [39,40], but our emphasis on the consequences of malaria for pulmonary Type 2 responses has not previously been explored.

Using these model systems, we assessed production of the ChaFFs, RELMα and Ym1 as primary read-outs of the Type 2 effector response in the lung. We also examined thoracic lymph node (TLN) cytokine profiles, parasitology and systemic pathology, to set the co-infected lung in its whole-organism context. By 7 days pi, malaria infection had significantly reduced the expression of ChaFFs in the lungs of co-infected animals relative to those with *Nb* only. This reduction correlated with changes in Th2 cytokines in the TLN, with co-infected mice producing significantly less IL-13, IL-10 and IL-5 than mice infected with *Nb* only. *Pcc* co-infection thus reduced the extent of pulmonary Type 2 activation and Th2 polarisation in response to *Nb*. Future long-term experiments (up to a year in duration [16]) in the co-infection model established here will explore how helminth migration may interact with malaria infection to affect chronic lung pathology.

Results

Nb infection caused loss of both body mass and RBC density but ameliorated Pcc infection

To investigate pulmonary immune polarization during acute helminth-malaria co-infection, on day 0 we infected female BALB/c mice with 200 *Nb* L3 larvae, in the presence or absence of co-infection with 10^5 *Pcc*-infected RBCs. Our first goal in developing this model was to characterise the systemic pathology induced by each infection and co-infection. We thus measured body mass (to the nearest 0.1 g), RBC density (billions/mL), and malaria blood parasitaemia daily. We also assessed the presence of malaria parasites in lung tissue of mice culled at 3 or 7 days pi.

Consistent with previous reports in rats [41], we found that *Nb* infection induced loss of body mass in mice during the first week of infection: *Nb*-infected mice reached a significantly lower minimum body mass than mice without *Nb* (Fig. 1A; $F_{1,110} = 21.1$; $P < 0.0001$). This amounted to a mean loss of 0.7 g, or ~3% of body mass, and was observed regardless of malaria co-infection. No further changes in body mass among groups achieved statistical significance, though *Pcc*-infected mice showed an expected dip in weight around day 10 that was unaltered by *Nb*.

In addition to weight loss, *Nb* infection caused RBC densities to be reduced by ~5% (Fig. 1B; effect of *Nb* on minimum RBC density to 7 days pi: $F_{1,110} = 8.8$; $P = 0.0037$). Unsurprisingly [33], *Pcc* also caused loss of RBCs by 7 days pi ($F_{1,110} = 40.2$; $P < 0.0001$). Between days 7 and 20 pi, *Nb*-induced RBC loss resolved, but *Pcc* induced further loss of RBCs - up to 60% of original density (Fig. 1B; $F_{1,110} = 385.4$; $P < 0.0001$). However, this was slightly (~5%) but significantly ameliorated by *Nb* co-infection (Fig. 1B; $t_{110} = 4.2$; $P = 0.0003$).

Consistent with this slight protective effect of *Nb* on RBC loss during peak *Pcc* infection, *Nb* co-infection was associated with a modest reduction in *Pcc* blood parasitaemia, as determined by microscopic examination of blood films (Fig. 2A; maximum parasitaemia $F_{1,62} = 4.13$; $P = 0.0465$).

By real-time PCR, the number of *Pcc* genome copies per 75 mg lung homogenate sample was assessed in *Pcc*-infected versus co-infected mice. Malaria parasites were present in the lung of most animals examined at 3 days pi and all animals at 7 days pi (Fig. 2B). At 3 days pi, which is shortly after *Nb* parasites have migrated through the lung [7] and coincident with the loss of body mass in *Nb*-infected mice (Fig. 1A), co-infected mice had more *Pcc* genome copies per lung sample (Fig. 2B; $P = 0.010$). This could be due to enhanced *Pcc* adherence to the lung endothelia [34] during *Nb* co-infection. However, by gross examination we observed substantial haemorrhaging in all *Nb*-infected mice at this time point and thus it is also possible that leakage of blood into the lung tissue increased the number of *Pcc* parasites in day 3 samples. At 7 days pi there was no significant difference between the *Pcc* and the *Nb*+*Pcc* groups.

Gut nematode burden at 3 days pi varied between experiments: for example, *Nb*+*Pcc* and *Nb* mice bore 56+10 and 35+9 adult nematodes, respectively, in experiment one, versus 6+4 and 1+1 nematodes in experiment two. Such variation has been previously reported [7] and in the present study is likely to be due to the very rapid infection kinetics typically observed for our *Nb* strain, which is not mouse-adapted (e.g., no nematodes remain in the gut at 5 days pi). A difference of a few hours in *Nb* injection times on day 0 and/or in gut sampling times on day 3 could therefore lead to the differences in nematode burden that we observed. Indeed, two lines of evidence suggest that the number of *Nb* larvae moving through the lung was much more consistent than the observed gut burdens. First, there were no significant differences among experiments in the amount of *Nb*-induced weight loss ($P \sim 0.2$) nor RBC loss ($P \sim 0.4$). Furthermore, Type 2 immunological readouts were extremely consistent among experiments. For example, TLN production of IL-4, IL-5, and IL-13 in response to *Nb* infection did not differ significantly among experiments ($P \sim 0.9$, 0.8, and 0.9, respectively). Still, in order to be certain that experimental variations were not confounding any of our conclusions, we controlled for *experiment* in all statistical analyses of combined data (as described in Methods).

Nb-induced ChaFFs in the lung peaked around 5-7 days pi

Before we undertook studies of Type 2 immune responses in the lung during co-infection, we assessed the time course of *Nb*-induced pulmonary expression of ChaFFs. Previous work has mainly focused on ChaFF mRNA expression in the lungs [10,11,16,17]. We wished to also ascertain protein expression *in situ* after *Nb* infection, to more closely determine the location of these proteins *in vivo*. Female BALB/c mice were infected with 200 *Nb*-L3s, or injected with PBS as a control, and RELMα and Ym1 protein levels were determined in BALF (via Western blots) and lungs (via IHC) at days 3, 5, 7, 15, 20 and 26 pi, to reflect the early events, peak Th2 time point and resolution stages of *Nb* infection [9].

RELMα and Ym1 were both detected in BALF at 3 days pi, and rose to a peak around 5-7 days pi (Fig. 3A, B). Expression of both proteins dropped off by days 20-26 pi. Histological analysis of Ym1-stained lung sections from infected mice illustrated this peak, with an increase in the intensity and area of anti-Ym1 staining at 5-7 days pi (Fig. 3E, F). These representative micrographs also show the

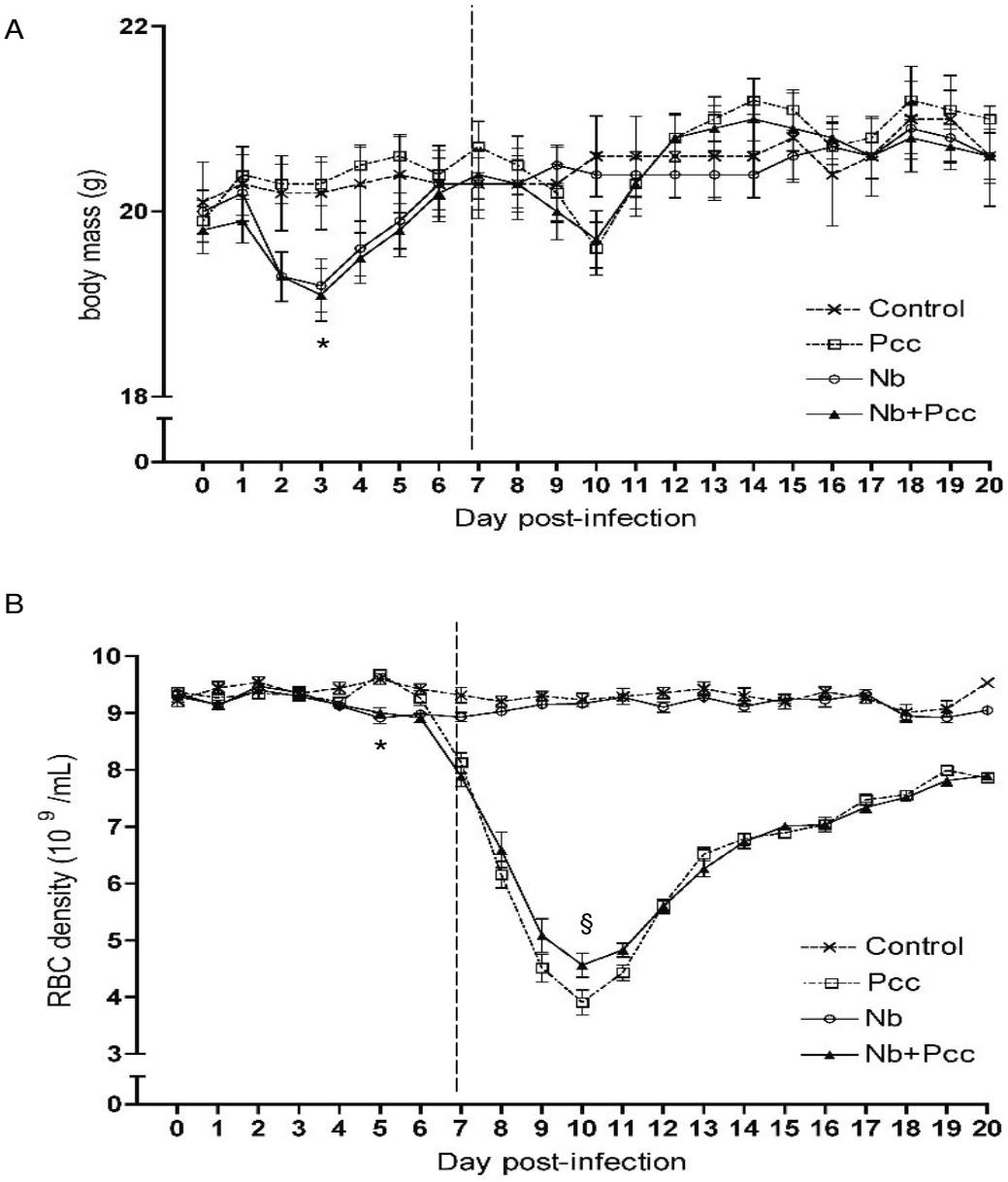

Figure 1

Pathology, measured as loss of body mass (A) and red blood cell density (B). Mice were infected with 200 *Nb* L3 larvae and/or 10^5 *Pcc*-infected RBCs on day 0, then weighed daily to the nearest 0.1 g. Daily samples of blood were taken for flow cytometric analysis of RBC density. (A) *Nb* infection induced significant loss of body mass during the first week of infection (lower minimum body mass than mice without *Nb*; $P < 0.0001$). (B) *Nb* also induced significant loss of RBCs that week (lower minimum RBC density to 7 days pi than mice without *Nb*; $P = 0.0037$), whereas *Pcc*-induced RBC loss was mainly apparent during the second week of infection (lower minimum RBC density to 20 days pi than mice without *Pcc;* $P < 0.0001$). *Nb* co-infection significantly ameliorated this (higher minimum RBC density in co-infected compared to *Pcc*-infected mice; $P = 0.0003$). Means ± SEM from combined results of 3 independent experiments (each with 4-9 mice per group per time point) are shown. The vertical dashed line indicates the two time periods for which minimum weight and RBC density were analysed: up to 7 days pi, and 7-20 days pi, as described in Methods. The symbol * indicates statistical significance of the *Nb* main effect, and § indicates significance of the difference between co-infected and *Pcc* mice.

influx of Ym1-positive inflammatory cells into the lung tissue that was evident by day 7 - e.g., alveolar and peribronchial inflammation. Of note, although infiltrating cells were RELMα$^+$ (data not shown) and Ym1$^+$, epithelial cells also appeared to be a major source of these molecules. Reece et al. (2006) clearly demonstrate macrophages as sources of these proteins in the lung during *Nb* infection but do not mention epithelial cells [11]. Our data are more consistent with a recent study in which RELMα was localized primarily to epithelial cells in *Nb*-infected mice [29]. Further, several reports on inflamed allergic (asthma model) and fibrotic (bleomycin- or gammaherpes virus-induced) rodent lungs, as well as our own unpublished data, demonstrate expression of both Ym1 and RELMα by epithelial cells [26,42,43]. By 15-26 days pi (Fig. 3G, H, I), Ym1 protein expression had returned to near-background (Fig. 3C), with reduced inflammatory influx and resolution of the thickened and disrupted epithelial layer that was apparent at earlier time points. These data are supportive of the idea that pulmonary activation of AAMϕ is a highly dynamic process [44].

Pcc changed the dynamics of expression of Nb-induced ChaFFs, especially 7 days pi

To assess the effect of *Pcc* co-infection on the dynamics of *Nb*-induced pulmonary AAMϕ and Type 2 epithelial cell activation, we next analysed both mRNA and protein expression of two ChaFFs, RELMα and Ym1, at a series of time points during co-infection. We also measured local mRNA expression of iNOS as a marker for Type 1 macrophage activation [19], which might be expected during malaria [45]. Furthermore, we measured mRNA of Type 1 cytokines (IL-12p40, TNF-α, and IFN-γ), as well as mRNA of IL-13, a key cytokine likely to drive production of Type 2 effector molecules such as the ChaFFs [12-15]. We chose to examine the effect of malaria infection around the peak of larvae-induced damage (i.e., ∼day 3 pi) [7] and the time of transition to adaptive Type 2 responses (i.e., ∼days 5-7 pi) [9,11]. We also assessed a later time point well into the adaptive immune phase: 20 days pi.

Analysis of ChaFF mRNA expression at 3, 5, 7 and 20 days pi suggested that the strongest interactions between *Nb* and *Pcc*-induced responses in lung tissue occurred 5-7 days pi (Fig. 4A, B). Indeed, because days 5-7 pi represented a time of strong ChaFF expression in *Nb* lung (Fig. 3) and day 7 pi coincided with the presence of *Pcc* there (Fig. 2B), it is perhaps unsurprising that days 5-7 pi could be the time of maximum effect of *Pcc* co-infection. At 3 days pi, there was a transient elevation of RELMα mRNA in co-infected compared to *Nb*-infected mice ($P = 0.0257$) that was reversed over the next few days: differences between the groups were not significant at 5 days pi, but RELMα gene expression was significantly reduced in co-infected mice at 7 days pi (Fig. 4A; $F_{1,25} = 7.5$, $P = 0.0113$, for combined analysis of experiments). Expression of Ym1 mRNA was also significantly lower in co-infected than *Nb* mice at days 5 and 7 pi (Fig. 4B: day 5 $P = 0.0442$; day 7 $F_{1,25} = 9.5$, $P = 0.005$, for combined analysis of experiments). In agreement with these observations, day 7 expression of Arginase-1 mRNA was significantly lower in the lungs of co-infected mice than in mice that had *Nb* only (data not shown; $F_{1,24} = 7.7$, $P = 0.0104$). Furthermore, ChaFF expression is known to be driven by engagement of IL-4Rα [24] by IL-4 and/or IL-13; accordingly, IL-13 mRNA expression in the lungs of co-infected mice was significantly reduced compared to *Nb*-infected mice at 5 days pi (Fig. 4C: $P = 0.0169$). This suggests that suppression of IL-13 by *Pcc* may be responsible for the reduced ChaFFs in co-infected mice. At 20 days pi, mRNA expression for RELMα ($P = 0.0101$) was slightly elevated in co-infected relative to *Nb*-only mice, but ChaFF and IL-13 mRNA expression had otherwise largely returned to background levels. Throughout this time course, *Pcc*-infected and uninfected animals expressed little or no mRNA for the ChaFFs.

In support of the mRNA data, when we analysed protein expression in lung BALF using Western blotting, we saw a significant day 7 pi reduction of both RELMα (Fig. 5A; $F_{1,25} = 8.7$, $P = 0.0067$, for analysis of combined experiments) and Ym1 (Fig. 5B; $F_{1,25} = 7.7$, $P = 0.0104$, for analysis of combined experiments) in co-infected mice compared to *Nb*-infected mice. By day 20 pi, the pattern had reversed, with co-infected mice expressing more RELMα ($P = 0.0398$) and Ym1 ($P = 0.0171$) than *Nb* mice. ChaFF expression in *Pcc*-infected mice did not differ from near-null expression in uninfected control mice (Fig. 5A, B). Importantly, BAL cell counts and cellular composition did not differ significantly among groups at any timepoint. There was a non-significant elevation in total numbers of cells in co-infected mice at 3 days pi and in *Nb* mice at 5 days pi, which might partly explain the elevation in ChaFFs at these early timepoints. However, any apparent differences were entirely absent from the 7 and 20 day BAL samples. Therefore, the observed effects on ChaFF protein expression are unlikely to be due to differences in cellular makeup alone, particularly at 7 and 20 days pi. Further, the inflammatory cells may not be the major source of CHaFF proteins in lavage fluid. The pattern and intensity of epithelial cell staining (see Fig 6C) suggests that these cells may be largely responsible for the changes in mRNA and BALF protein.

IHC scoring of lung sections further confirmed these dynamics. At both day 5 pi and day 7 pi, the intensity of RELMα (Fig 6A) and Ym1 (Fig 6B) staining in the lungs of co-infected animals was reduced compared to *Nb* mice, though the RELMα difference did not achieve statistical significance at day 7 (day 5: RELMα $P = 0.0056$; Ym1 $P =$

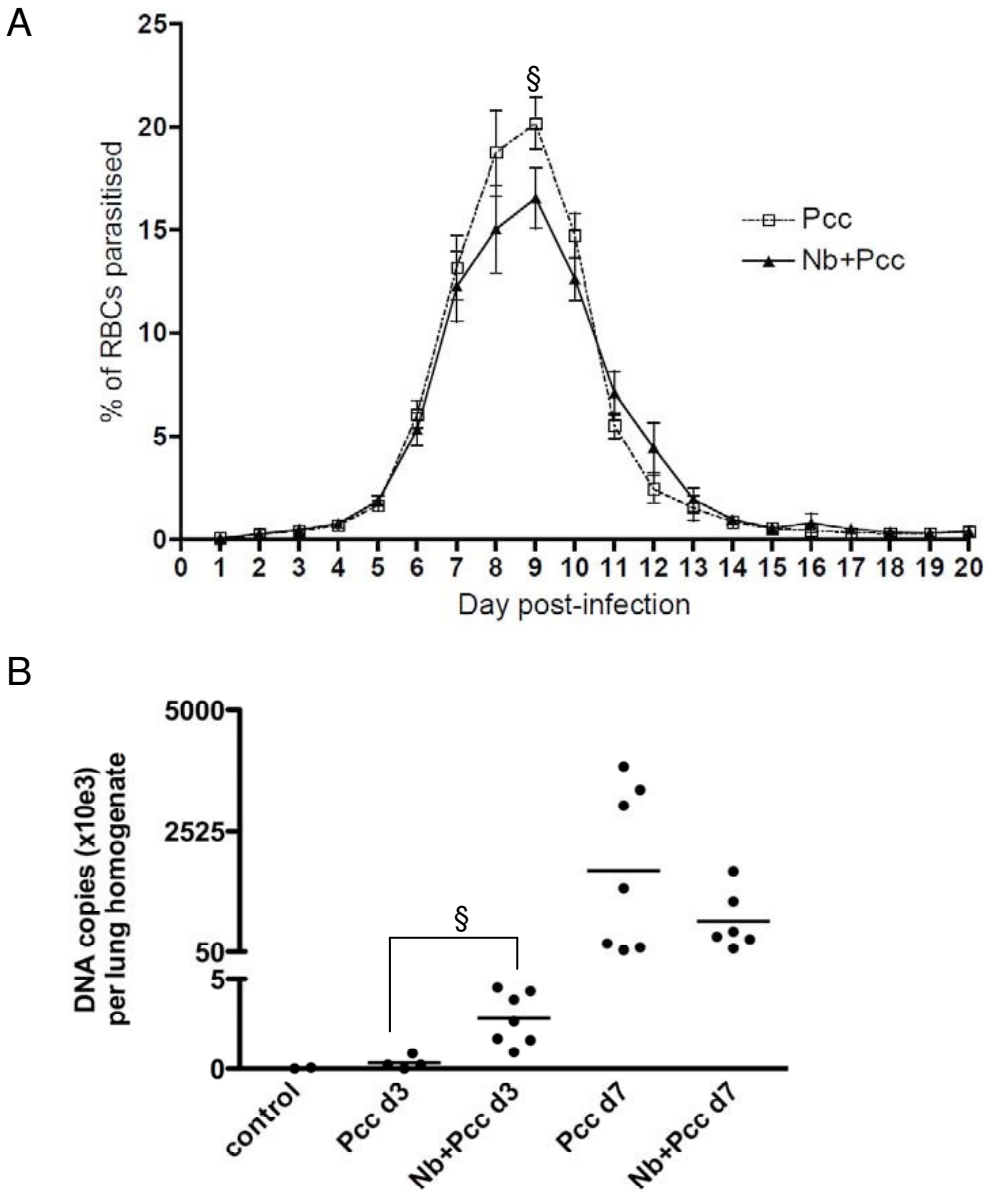

Figure 2
Malaria parasites in the blood (A) and lungs (B) of *Pcc*-infected and co-infected mice. (A) Co-infected mice (*Nb*+*Pcc*) had a lower peak proportion of parasitized RBCs than *Pcc*-infected mice did ($P = 0.0465$), as determined by 1000× microscopic examination of Giemsa-stained thin blood films. Means ± SEM from combined results of 3 independent experiments (each with 4-9 mice per group per time point) are shown. (B) Co-infected mice harboured greater numbers of *Pcc* genome copies per lung sample than *Pcc* mice at 3 days pi ($P = 0.01$), as assessed by real-time PCR of lung homogenates (derived from 75 mg lung) using MSP-1 specific primers. Genome copies were apparent in both *Pcc* infected groups at 7 days pi and the copy numbers no longer differed significantly between groups. This experiment included 4-7 mice per group. The symbol § indicates significance of the difference between co-infected and *Pcc* mice.

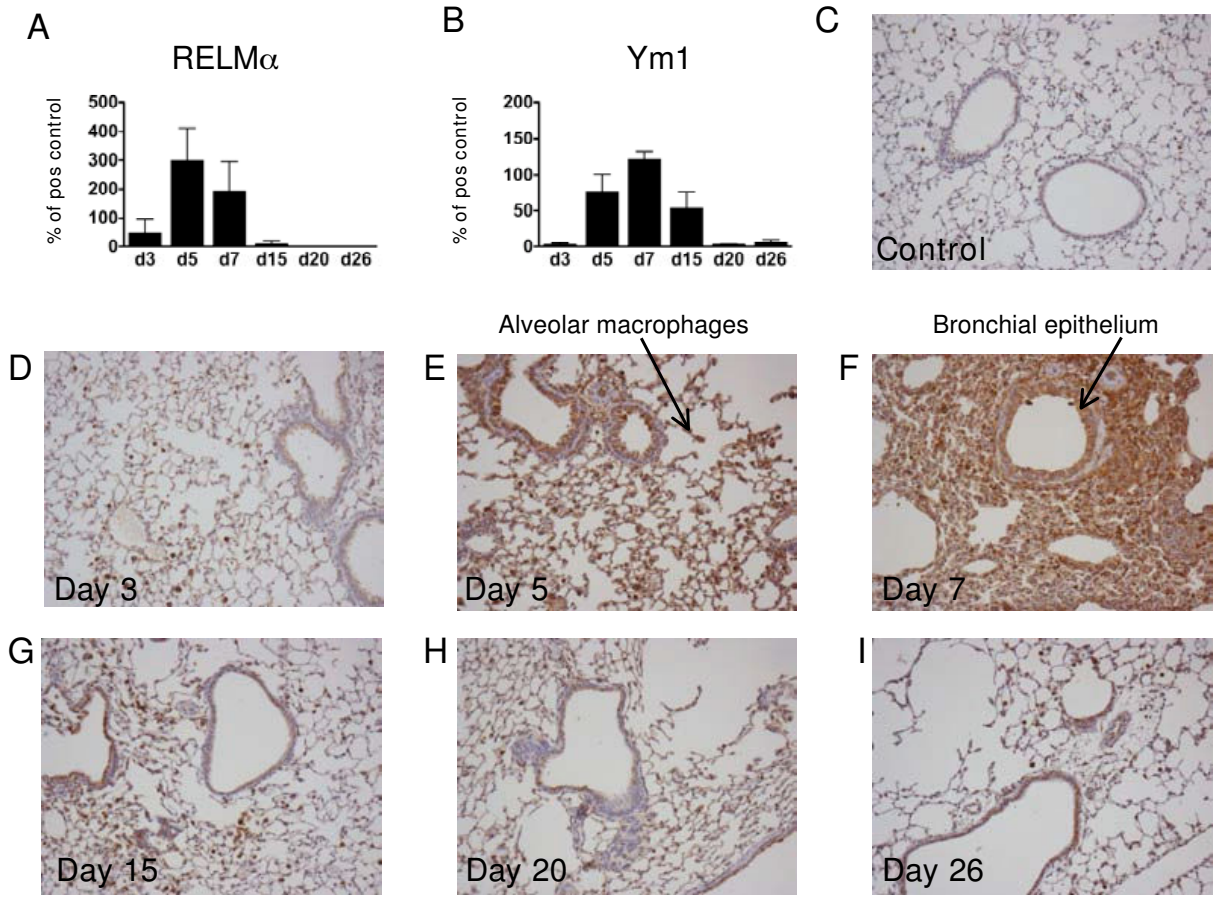

Figure 3

Time course of protein expression of ChaFFs in *Nb* infected lungs. Western blot analysis of lung lavages harvested at different time points post *Nb* infection (4 *Nb* mice per timepoint) suggested that the secretion of ChaFFs RELMα (A) and Ym1 (B) peaked around 5-7 days post infection. Data are expressed as percentage of a positive control sample (pooled lung lavages of three *Nb*-infected mice). Representative photomicrographs from *Nb*-infected lung tissue sections stained histologically for Ym1 at the indicated time points, plus an uninfected control lung (C), illustrate peak expression of brown Ym1 staining of epithelial cells and macrophages (E-F).

0.0153; day 7: RELMα $P = 0.1011$; Ym1 $P = 0.0171$). Representative micrographs from day 7 pi show the reduced intensity of RELMα staining in co-infected animals relative to *Nb* mice and no RELMα staining in *Pcc* mice (Fig. 6C). No differences among groups were detected by IHC at days 3 or 20 pi. Therefore, the expression pattern of ChaFF protein *in situ* was largely in agreement with mRNA in whole lung tissue (Fig. 4) and protein expression in the BALF (Fig. 5). As discussed above for the time course of *Nb* infection (Fig. 3), although Ym1$^+$ and RELMα$^+$ macrophages were present in lung IHC sections, the predominant cell type expressing these molecules appeared to be epithelial cells. Furthermore, lung macrophages in this system do not appear to become classically activated, as assessed by lung mRNA for iNOS and IL-12p40, which were not detectable in any mice at any time point, and TNF-α, which was detectable but at low levels that did not differ among groups (data not shown). However, IFNγ was elevated in the lung tissue of all *Pcc* infected mice, regardless of *Nb* co-infection, at day 7 pi (Fig. 7).

Cytokine production in local LN largely mirrored pulmonary ChaFF expression

To assess whether pulmonary ChaFF patterns corresponded to immune responses in the draining thoracic lymph node (TLN), we performed *in vitro* culture of TLN

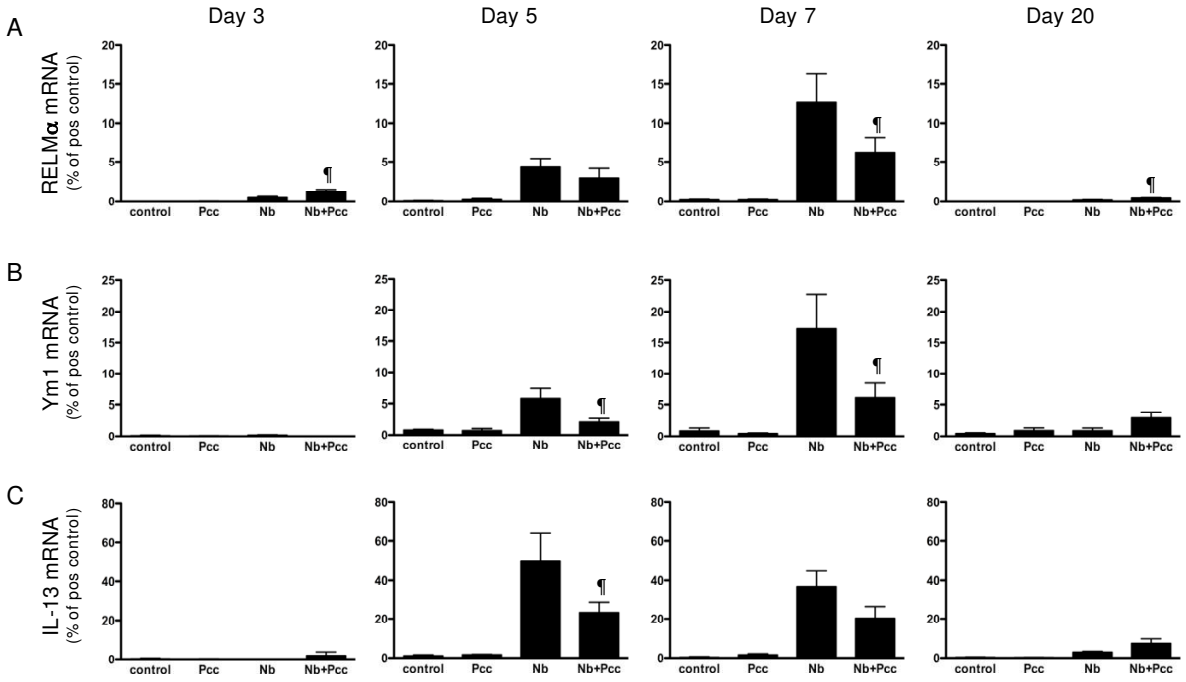

Figure 4

ChaFF and IL-13 mRNA expression in the lungs of *Nb, Pcc* and co-infected mice. Real-time RT-PCR of mRNA from lung tissue harvested at 3, 5, 7 or 20 days pi showed that the effect of *Pcc* on *Nb*-induced expression of RELMα (A) and Ym1 (B) changed over time. RELMα was transiently elevated in co-infected (*Nb+Pcc*) compared to *Nb* mice at 3 days pi ($P = 0.0257$), but expression of mRNA for RELMα (A), and Ym1 (B) was then found to be significantly lower in the lungs of *Nb+Pcc* mice than in *Nb* mice by 7 days pi (RELMα, $P = 0.0113$; Ym1, $P = 0.005$). IL-13 mRNA expression (C) largely mirrored this pattern, as it was significantly reduced in *Nb+Pcc* mice compared to *Nb* mice at 5 days pi ($P = 0.0169$). At 20 days pi, however, RELMα (A) was again significantly elevated in *Nb+Pcc* relative to *Nb* mice. ($P = 0.01$) Data are expressed as percentage of positive control samples (for RELMα and Ym1, peritoneal macrophages of a mouse implanted with *Brugia malayi* adult parasites for 3 weeks [72]; for IL-13, spleen of an MHV-68 infected IFNγR-KO mouse [63]). Bars indicate mean ± SEM of 2 combined independent experiments, each with 4-9 mice per group per timepoint. The symbol ¶ indicates significance of the difference between co-infected and *Nb* mice (*P* values included above and in Results text).

cells, in media alone or with antigen or ConA. At 3 and 5 days pi, there were no significant differences in supernatant cytokine concentrations among infection groups. However, by 7 days pi, differences among the groups were apparent, as outlined below. No antigen-specific responses were observed (i.e., no cytokines in excess of spontaneous secretion in media alone) until 20 days pi (discussed below). Spontaneous and ConA-induced cytokines exhibited identical patterns, though spontaneous responses were of lower magnitude.

At 7 days pi, *Nb* infection in singly as well as co-infected mice was associated with significantly elevated ConA-induced production of IL-4, IL-13, IL-5, and IL-10 compared to uninfected and *Pcc*-infected mice (Fig. 8A, B, C, D; all *P* for *Nb* main effect < 0.0001). Of interest, IL-6 and sTNFR1 were also associated with *Nb* but not *Pcc* infection (Fig. 8E, F; both *P* for *Nb* main effect <0.0001). TNF-α production did not vary among infection groups (Fig. 8G), but *Pcc* infection was associated with significantly elevated IFN-γ production compared to uninfected and *Nb*-infected mice (Fig. 8H; *P* for *Pcc* main effect <0.0001). To our knowledge, TLN polarization by systemic malaria infection has not been previously reported. The Th1 bias of the observed response to *Pcc* was unsurprising.

Co-infected mice differed from singly-infected mice in ConA-induced production of some of these cytokines. For example, IL-13, IL-5, and IL-10 production were significantly reduced in co-infected compared to *Nb*-infected animals (Fig. 8B, C, D; $t_{61} = 7.3$, 18.8, 8.1 and $P = 0.011$, 0.0001, 0.007, respectively), while IFN-γ production was

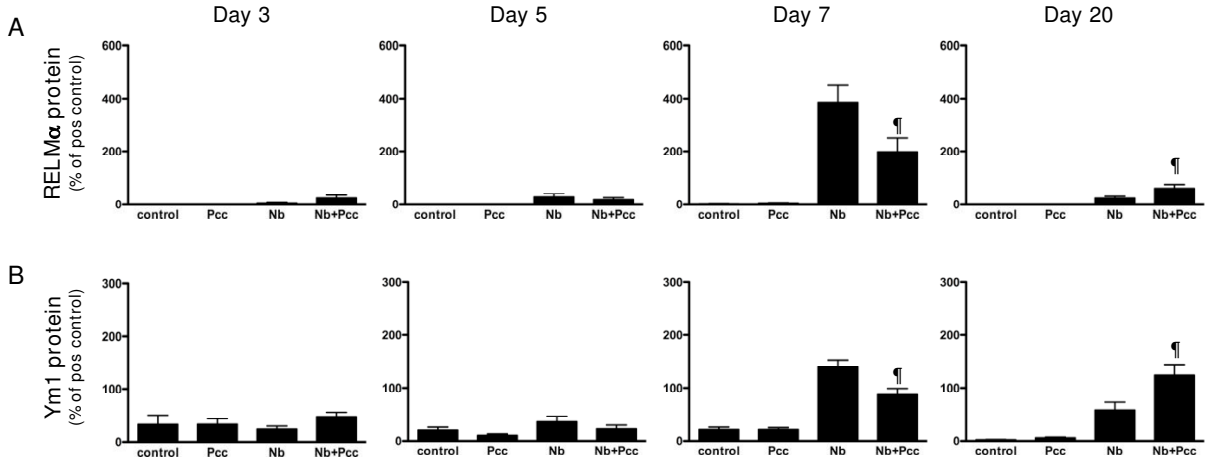

Figure 5

Western blot analysis of ChaFF protein expression in BALF of *Nb, Pcc* and co-infected mice. Lung lavages harvested at days 3, 5, 7 and 20 pi indicated that the ChaFF proteins RELMα (A) and Ym1 (B) exhibited temporal dynamics that were similar to those observed for mRNA expression in lung tissue (Fig. 4). In particular, expression of both proteins was significantly lower in the lungs of co-infected (*Nb*+*Pcc*) than in *Nb* mice at 7 days pi (RELMα $P = 0.0067$ and Ym1 $P = 0.0104$). This pattern had reversed by 20 days pi, with increased ChaFF protein expression in *Nb*+*Pcc* compared to *Nb* mice (RELMα $P = 0.0398$ and Ym1 $P = 0.0171$). Data are expressed as percentage of a positive control sample (pooled lung lavages of three *Nb*-infected mice). Bars indicate mean ± SEM of combined results of 2 independent experiments, each with 4-9 mice per group per timepoint. The symbol ¶ indicates significance of the difference between co-infected and *Nb* mice (*P* values included above and in Results text).

reduced in co-infected compared to *Pcc*-infected animals (Fig. 8H; $t_{61} = 8.4$ and $P = 0.006$). These results provide evidence of cross-regulation between Th1 and Th2 immune responses in the local lymph node during the first week of co-infection. This finding is consistent with a wide range of studies of murine co-infection (reviewed in [30,46]).

By 20 days pi, the overall strength of TLN cytokine responses had waned, but antigen-specific responses were detectable. Correcting for background (spontaneous) secretion of cytokines in wells with media alone, antigen-specific responses to crude helminth (*Nb* Ag) and recombinant malaria (MSP-1_{19} Ag) antigens were each observed for a subset of the cytokines measured (Fig. 9). For example, *Nb* infection was associated with strong *Nb* Ag-specific IL-13 responses (Fig. 9A: $P < 0.0001$), regardless of co-infection. Co-infection, however, had a significant boosting effect on *Nb* Ag-specific IL-5 (Fig. 9B: $P = 0.0025$ for the comparison with *Nb* mice; $P < 0.0001$ for the comparison with *Pcc* mice). Co-infection was also associated with significantly elevated MSP-1_{19}Ag-specific IL-6 (Fig. 9C: $P = 0.0163$ for the comparison with *Nb* mice; $P = 0.0112$ for the comparison with *Pcc* mice). TNF-α production, though greater than background, did not differ significantly among groups (Fig. 9D).

Discussion

Our primary aim in this study was to address the interplay of two acute infections that place conflicting demands on the host immune response, particularly in the lung. We wanted to focus on Type 1-Type 2 cross-regulation rather than any effects of regulatory T cells, so we opted for a model of acute rather than chronic helminthiasis. In addition, although anatomical compartmentalization does not preclude immunological interaction - for example, gut-restricted helminths can induce a strong systemic Th2 bias [47] - compartmentalization can buffer the effects of co-infection [48]. We thus chose murine infection models that would pose an immunological conflict in anatomical space as well as time post-infection (pi). The dynamics we were investigating may have real life corollaries, because nematode migration occurs in the lungs of over a third of the world's human population [1,2], many of whom are co-infected with malaria [31,32]. However, because both of our murine models (*Pcc* and *Nb*) produce self-resolving infections, the effects of co-infection on anaemia and on pulmonary immunology reported here cannot be firmly

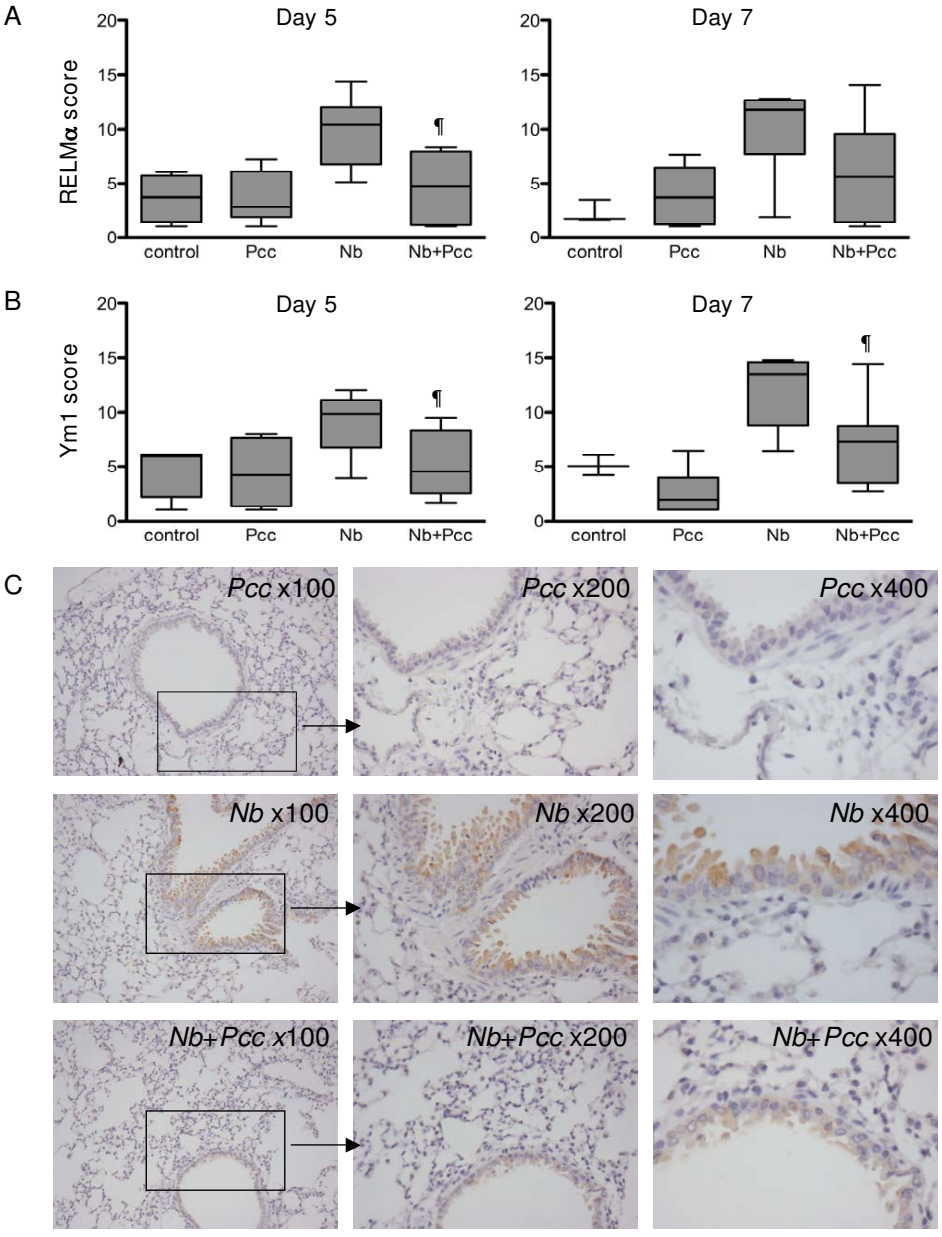

Figure 6

Immunohistochemical analysis of ChaFF protein expression in the lungs of *Nb, Pcc* and co-infected mice. Protein expression in lung sections corroborated BALF concentrations of ChaFF proteins observed around 7 days pi, as illustrated by days 5 and 7 pi staining intensity scores for RELMα (A) and Ym1 (B) as well as representative micrographs (at increasing magnification) of day 7 pi slides stained for RELMα (C). Down-regulation of ChaFFs was observed in co-infected mice (*Nb+Pcc*) compared to *Nb* mice; differences were significant for both RELMα and Ym1 at 5 days pi (RELMα $P = 0.0056$ and Ym1 $P = 0.0153$) and for Ym1 at 7 days pi ($P = 0.0171$). Scores were obtained by analyzing bronchial epithelium in ten fields per mouse using a scoring system described in Methods. The whisker-and-box graphs with minimum and maximum values depict results from a representative experiment with 4-9 mice per group. The symbol ¶ indicates significance of the difference between co-infected and *Nb* mice (*P* values included above and in Results text).

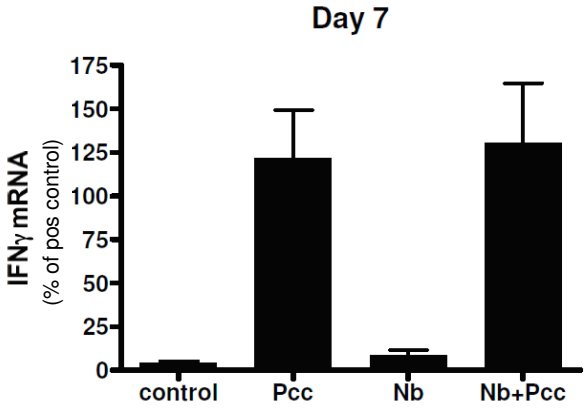

Figure 7
Lung mRNA for the Type 1 cytokine IFN-γ at 7 days pi. Real-time RT-PCR of mRNA from lung tissue harvested at 3, 5, 7 or 20 days pi using primers for Type 1 markers iNOS, IL-12p40, TNF-α, and IFN-γ were largely negative. For example, no iNOS nor IL-12p40 was detected in any mice, and although TNF-α was detected at low levels, its expression did not differ among groups of mice. At 7 days pi, however, IFN-γ was significantly upregulated in all mice with *Pcc* infection, with or without *Nb* co-infection, as depicted here. Data are expressed as percentage of a positive control sample (peritoneal macrophages of a thioglycolate-injected mouse [10]). Mean ± SEM from a representative experiment with 4-9 mice per group per timepoint are shown.

associated with chronic disease outcomes until longer term co-infection studies are performed.

We quantified the health of mice in our experiments using two measures that have proven informative during *Pcc* infection [49] and *Pcc*-nematode co-infection [50]: body mass and RBC density. To our knowledge, this is the first report demonstrating that murine *Nb* infection has a negative impact on both parameters, although reduced weight gain in young *Nb*-infected mice has previously been reported [40]. *Nb* caused a statistically significant, transient ∼3% loss of body mass from approximately 2-4 days pi, and in other experiments using a higher dose (500 L3s), mice lost closer to 10% of their starting body mass (unpublished data). Migration of *Nb* larvae through the lungs has previously been shown to cause two spells of inappetance and thus weight loss in rats, one associated with migration of larvae and the other with establishment of adults in the gut [41]. We detected only one period of loss of body mass; mice may be spared the second spell given the brief survival of adult *Nb* in mice, particularly for parasite strains, such as ours, that are not mouse-adapted. We also observed a transient loss of RBC density in *Nb*-infected mice. It was rather surprising that this effect - most likely caused by haemorrhaging of the lung following larval migration - was detectable at the systemic level. This suggests that the capillary damage and ingestion of RBCs by alveolar macrophages following lung migration of *Nb* [11,16] are associated with considerable blood loss.

A diverse range of outcomes is possible when helminths and malaria co-infect a host. Co-infected mice in our study experienced two periods of RBC loss in quick succession - first *Nb*-induced and then *Pcc*-induced. However, they had slightly higher RBC densities than *Pcc*-infected mice did, at the time of most severe malarial disease. This was associated with a small reduction in malaria parasitaemia in the blood. These results contrast with several studies of helminth-malaria co-infection in mice, in which malaria parasitemia was increased [51-54], and/or malarial symptoms exacerbated, in at least some groups of co-infected mice [50-55]. For example, in contrast to the lethal inflammatory liver disease recently described in mice simultaneously co-infected with *Heligmosomoides polygyrus* and *Pcc* [55], we observed subtle amelioration of malarial disease and no deaths. This disparity in the severity of co-infection could be due to the fact that we worked with a different mouse strain (BALB/c versus Helmby's C57BL/6) as well as a different helminth species that migrates differently through the host body. However, we detected an elevation in MSP-1_{19}-specific IL-6 due to co-infection, so it is possible that the emergent IL-17/IL-23 axis described by Helmby [55] may likewise be involved in our co-infection system, though not in organs that negatively impact short-term survival. Indeed, the mechanisms underlying the slightly protective effect of *Nb* observed here are not yet clear. We are investigating possible immunological causes of this protection, including innate mechanisms such as IFN-γ^+ NK cells [56] and adaptive mechanisms such as cytophilic antibody isotypes [33] that could promote malaria clearance; either might be altered by acute *Nb* co-infection. However, it is also possible that lower parasitaemia might be the consequence of the RBC density changes induced by *Nb*, as previous co-infection studies have shown that helminths can limit RBC availability to malarial parasites and thereby cap their replication (e.g., [57]). Control of microparasites by Th1 immunity and by RBC limitation are not mutually-exclusive possibilities [58] and both might be operating in our model system. Finally, it is possible that the sequestration habits of *Pcc* parasites [59] are altered by *Nb*. These mechanisms remain to be investigated.

Our results largely resemble those reported for other *Nb*-microparasite pairings. For example, during co-infection of mice with *Nb* and either *Toxoplasma gondii* [60] or *Chlamydophila abortus* [61], significantly reduced Th2 responses (compared to mice with *Nb* infection) have been observed, independent of the interval between infections [60,61]. These data suggest that *Pcc* is not the only

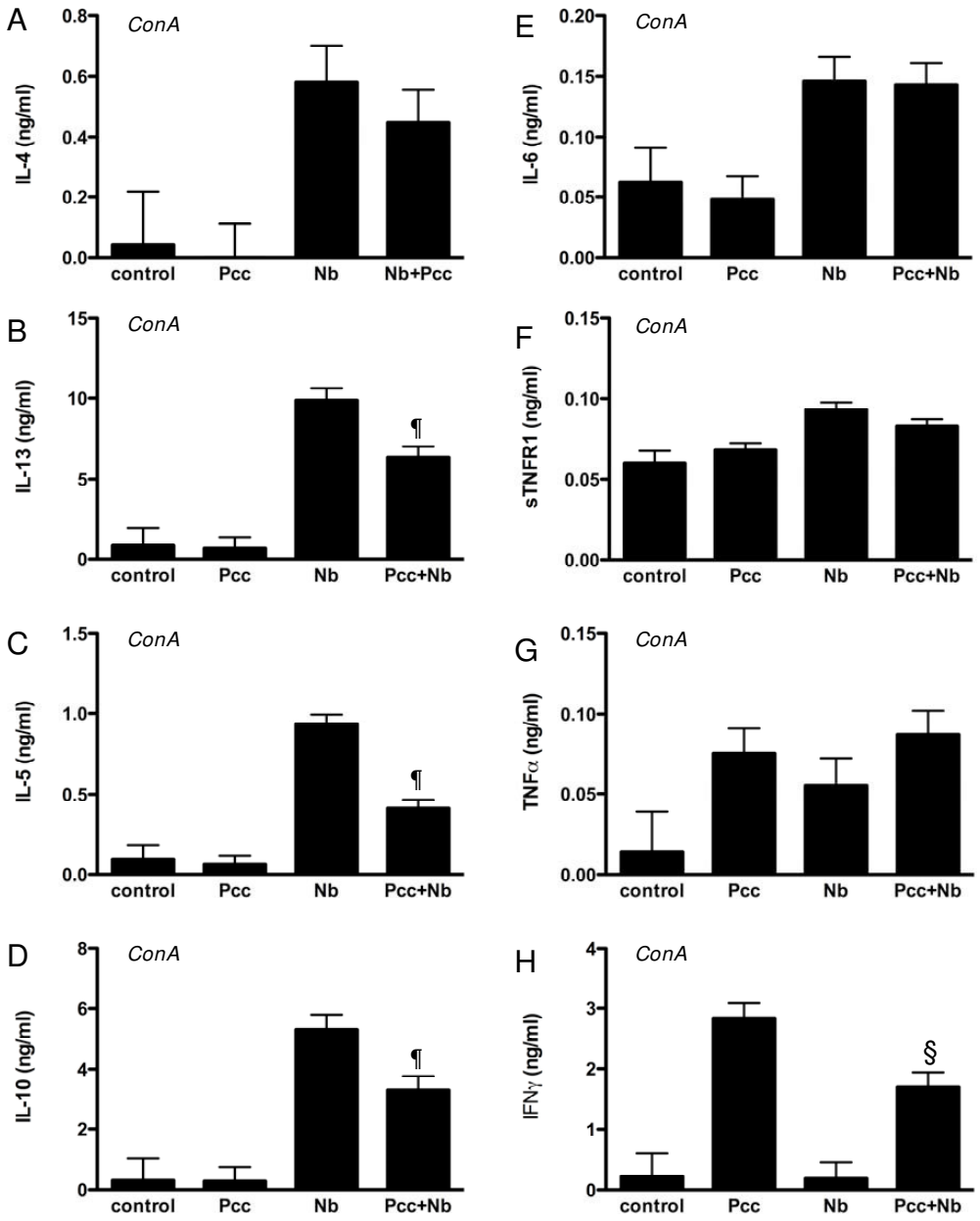

Figure 8

ConA-induced cytokine release in TLN lymphocyte recall assays. Cytokine concentrations in supernatants of ConA-stimulated TLN cells harvested at day 7 pi were measured with cytometric bead arrays. Groups did not differ in cytokine production at 3 or 5 days pi, and no antigen-specific responses were detected until 20 days pi. Data on day 7 pi IL-4 (A), IL-13 (B), IL-5 (C), IL-10 (D), IL-6 (E), sTNFR1 (F), TNF-α (G), and IFN-γ (H) production are shown. In these data, statistically significant down-regulatory effects of co-infection (*Nb+Pcc*) were found for IL-13 ($P = 0.011$), IL-5 ($P = 0.0001$), IL-10 ($P = 0.007$), and IFN-γ ($P = 0.006$), compared to relevant single-infection groups. Bars indicate mean ± SEM from 3 combined experiments, each with 4-9 mice per group. The symbol ¶ indicates significance of the difference between co-infected and *Nb* mice, while § indicates significance of the difference between co-infected and *Pcc* mice (*P* values included above and in Results text).

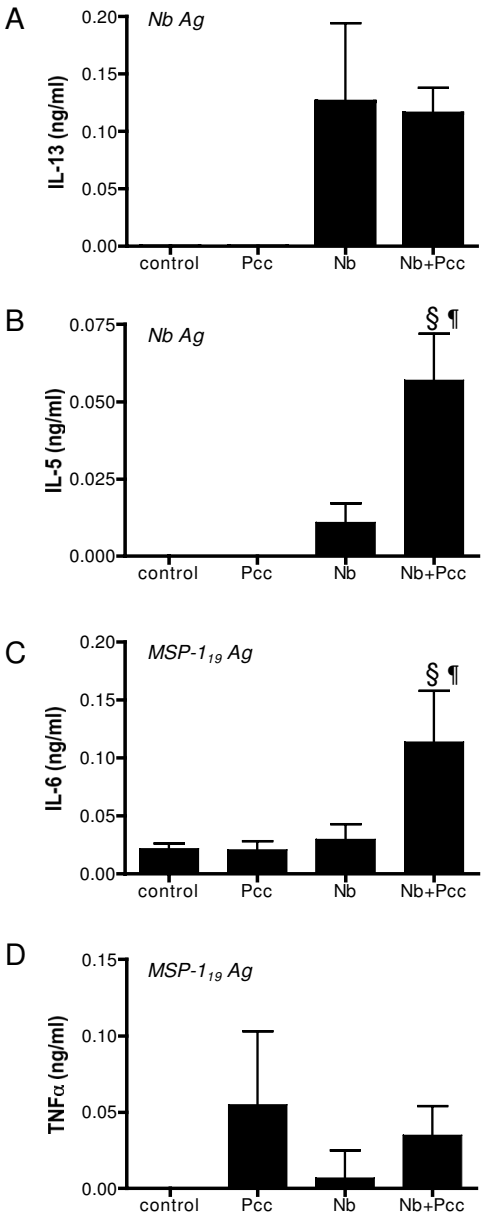

Figure 9

Antigen-specific responses in TLN lymphocyte recall assays. Cytokine concentrations in supernatants of TLN cells that were harvested at day 20 pi and then stimulated with antigen were measured with cytometric bead arrays. *Nb* Ag, a crude *Nb* adult lysate, and recombinant *Pcc* clone AS merozoite surface protein, MSP-1_{19}, were used as antigens *in vitro*. Although all cytokines shown in Figure 8 were measured, only IL-13 in response to *Nb* Ag (A), IL-5 in response to *Nb* Ag (B), IL-6 in response to MSP-1_{19} (C), and TNF-α in response to MSP-1_{19} (D) were above levels of spontaneous secretion. These figures thus depict antigen-specific cytokine (in ng/ml above background). Statistically significant differences among groups included increased NbAg-specific IL-13 in all *Nb* mice ($P < 0.0001$), increased *Nb* Ag-specific IL-5 in co-infected (*Nb+Pcc*) mice compared to both *Nb* ($P = 0.0025$) and *Pcc* mice ($P < 0.0001$), and increased MSP-1_{19}-specific IL-6 in *Nb+Pcc* compared to both *Nb* ($P = 0.0163$) and *Pcc* mice ($P = 0.0112$). Bars indicate mean ± SEM for 4-8 mice per group. The symbol ¶ indicates significance of the difference between co-infected and *Nb* mice, while § indicates significance of the difference between co-infected and *Pcc* mice (*P* values included above and in Results text).

microparasite that might reduce Th2 responses to *Nb* infection. *Mycobacterium bovis* BCG co-infection, however, does not significantly impact *Nb*-induced IL-4 in the mesenteric lymph nodes [62]; it would be of interest to know whether those lung-dwelling microparasites might have had similar effects to *Pcc* on Th2 responses, had they been measured in the TLN. Reported effects of *Nb* on the course of microparasite infections are likewise mixed: densities of *T. gondii* [60] and *M. bovis* BCG [62] are unaffected by the presence of the nematode, while *C. abortus* density increases dramatically [61]. Interestingly, the concurrent presence of influenza virus with migrating *Nb* larvae in the lung exacerbates the severity of lung disease compared to mice with influenza alone [40]. A two-week delay between *Nb* infection and influenza infection, or replacement of *Nb* with *H. polygyrus*, eliminates the added pathology, suggesting that the simultaneous presence of larvae and virus in the lungs is required [40]. Such may also be the case for *Pcc-Nb* co-infection. The *Nb*-influenza study did not include immunological measurements, so the role of the immune system in generating the observed pattern is not known. Indeed, this comparison illustrates that many details of anatomical location and parasite life cycles, as well as immunological interactions, must be taken into account to explain the diverse outcomes of helminth-microparasite co-infections [30,46].

Our most novel finding is that malaria infection has the capacity to modulate the host's pulmonary Type 2 response to nematode migration. However, the long-term impact of the altered Type 2 response is not possible to predict, because the function of Type 2 immunity in this setting is not yet fully understood. There are at least three potential outcomes of a helminth-induced Type 2 response in the lung. First, it may contribute to protection against incoming larvae [6]. Second, Type 2 responses are likely to be involved in repairing the damage that is inflicted by migrating parasites. Third, as recent studies have shown [5,16,18], lung migration and the associated Th2 responses have the potential to cause long-term lung pathology. Appropriate repair versus lung malfunction are likely to be flip sides of the same coin. Indeed, although ChaFFs and Arginase-1 are implicated in tissue repair, they are also associated with fibrosis, an overzealous repair process [19,24,43,63-65] (see also review by Wynn [66]). Predicting the effect of *Pcc* co-infection on long term *Nb*-induced lung disease is further complicated by recent data that suggest both Arginase-1 and RELMα can negatively regulate Th2-mediated pathology [27-29]. By this logic, inhibition of these molecules by malaria co-infection may ultimately exacerbate Th2-mediated lung damage.

However, our data suggest that the effect of malaria on ChaFF expression is not direct but rather via reduced Th2 cytokines. The effect of *Pcc* on *Nb*-induced ChaFFs was not apparent until 7 days pi, when the extent of the increase in ChaFF expression was inhibited by co-infection. This was correlated with differences in cytokine production in lymphocyte recall assays, suggesting that changes in ChaFF expression were driven by changes in the T lymphocyte populations after the onset of the adaptive Th2 immune response (around 5 days pi, as shown in *Nb*-infected IL-4 reporter mice [9]). A role for adaptive immunity is further supported by work showing that SCID mice are not able to sustain AAMϕ responses in the lung following *Nb* infection [11], and a demonstrated requirement for T cells to sustain the AAMϕ response in a mouse peritoneal infection model [24]. Remarkably, in SCID mice, in the absence of T cells and AAMϕ, the *Nb*-induced cellular infiltrate does not resolve [11]. The capacity of malaria to inhibit the transition to a full Th2 response by 7 days pi may likewise be detrimental to full resolution of the inflammatory response, a step necessary for appropriate tissue repair [67,68]. By day 20 pi, however, the residual Th2 responses in co-infected mice were as high as, or even higher than, in *Nb*-only mice. In support of this, day 20 antigen-specific IL-5 responses were particularly high in co-infected animals. Thus *Pcc* infection may protect against airway hyper-responsiveness through a reduction in peak Th2 activation, or else exacerbate it due to sustained Th2 activity. Transient passage of *Nb* larvae through the lung inflicts lasting damage [16,18]. Whether transient impairment of pulmonary Th2 responses by malaria co-infection also has lasting effects needs to be investigated experimentally.

A perhaps surprising finding in our study was the apparent absence of classical macrophage activation in the lung despite the clear presence of malaria parasites: we did not detect iNOS, IL-12p40 nor elevated TNF-α mRNA in lung tissue of *Pcc*-only or co-infected mice at any time point. One could argue that malaria parasites stay in the lung microvasculature and do not cross into tissue. However, this is unlikely to be the case, given the extensive lung damage due to *Nb* in co-infected mice, as well as evidence that malaria merozoites can be found dispersed in the lung [34]. Furthermore, IFN-γ mRNA was detectable in the lung of all *Pcc* mice regardless of co-infection, suggesting that lymphocytes were activated, perhaps by innate activation of NK or γδ T cells. The most likely explanation for the failure to detect classical macrophage activation may be that lung macrophages, which are exposed daily to inhaled microbes, have a remarkably high threshold for activation even in the presence of IFN-γ and microbial stimuli [69].

As with any laboratory model, it is important to acknowledge the potential disconnection between natural co-infections and the experimental systems and designs used

here, including the relative timing of the two infections, doses at which they were administered, and the fact that we have only studied primary and self-resolving (rather than secondary and/or chronic) infections. Permutation of any of these parameters is likely to quantitatively, if not qualitatively, alter outcomes. For example, repair processes might readily keep pace with lung damage when the rate of exposure to nematode larvae is low, unlike in most experimental models. We used a relatively low dose of L3 larvae (200 per mouse while others use ~500 [16,18] or as many as 750-1000 [60-62]) but still exceeded natural exposure levels. Furthermore, larval helminths and malaria parasites are unlikely to arrive in the lung within a few days of each other in nature, and it may be that pre-existing malaria would have had a different effect on pulmonary Type 2 responses to *Nb* migration, particularly if malaria parasites do not remain long in the lung. Indeed, the most likely natural exposure scenario may be chronic malaria infection into which helminth larvae are "trickled" [32], but experimental studies that mimic this scenario have yet to be carried out. Nonetheless, lung dysfunction is seen as a consequence of helminth migration [4,5] and both acute and persistent malaria infection [38] in people, so high-dose experimental *Nb* studies in which long-term lung pathology can be observed [16,18], combined with simultaneous malaria exposure, may provide useful models for disease states in people.

Conclusion

With the experiments reported here, we have established an acute laboratory model of helminth-malaria co-infection that will be suitable for future work exploring the details of how Type 1 inducing co-infections affect long-term, Type 2-mediated repair of the damage caused by migrating nematodes. Recently developed models of malaria-induced lung damage (e.g., [37]) might be analysed in animals co-infected with *Nb*. Corroborative studies in human populations may also be feasible. Like migratory helminthiases [3], severe falciparum malaria is associated with detectable lung injury, as measured by spirometry and clinical symptoms [38]. A study like Brooker *et al*'s analysis of whether the anaemia of hookworm and malaria are additive during co-infection [32] that used spirometry to assess the pulmonary health of malaria-infected, *A. lumbricoides*- or hookworm-infected, and co-infected people could assess whether co-infection exacerbates damage. Given the huge number of people with such co-infections, it is possible that clinical studies of malaria lung injury may gain insight from considering the presence, however transient, of helminths in the lung.

Methods

Mice, parasites, experimental design, and monitoring

Specific pathogen free, 8-10 week old female BALB/c mice (Harlan, UK) were maintained in individually ventilated cages on diet 41b *ad lib*. *Nippostrongylus brasiliensis* (*Nb*) was maintained by serial passage through Sprague-Dawley rats, as described previously [70]. Cryopreserved *Plasmodium chabaudi chabaudi* (*Pcc*) parasites of clone AS were passaged through two generations of donor BALB/c mice and inoculated into experimental mice as described previously [50]. The four co-infection experiments used a factorial design, with uninfected controls, *Pcc*-infected, *Nb*-infected, and co-infected mice. On day 0, 200 *Nb* L3 larvae and/or 10^5 *Pcc*-infected RBCs were injected subcutaneously and intraperitoneally, respectively. PBS and naïve mouse RBCs served as sham injections for *Nb* and *Pcc*, respectively; uninfected control animals received both sham injections. RBC density, body mass, and malaria parasites were then monitored daily, as described previously [50]. Briefly, RBC densities were measured by flow cytometry (Beckman Coulter), body mass was recorded to the nearest 0.1 g, and the proportion of RBCs parasitized was counted in Giemsa-stained thin blood films (at 1000× magnification). Mice were then culled 3, 5, 7, or 20 days pi (4-9 mice per infection type per timepoint). *Nb* parasite burden was assessed in the gastrointestinal tract of culled mice. Intestines were placed in PBS, slit lengthwise and the contents rinsed into muslin-lined funnels set over tubes containing PBS warmed to 37°C. Nematodes were left to filter through for >2 hours and counted via microscopy (at 40×). To elucidate the dynamics of *Nb*-induced alternative activation in the lung, a separate experiment was conducted for *Nb* only, with mice culled 3, 5, 7, 15, 20 or 26 days pi (4 *Nb*-infected mice per time point). All experiments were carried out in accordance with the animals (Scientific Procedures) Act 1986, and were approved by the UK Home Office inspectorate and institutional review committee.

Lung lavage and tissue sampling

Following terminal anaesthesia, tracheas were cannulated and lungs lavaged with 1 mL PBS. Cannulae were prepared from fine bore polythene tubing (Portex) and a 23 G needle. Following lavage, the left lung lobe was tied off, cut at the bronchus, and placed in RNAlater (Ambion) for mRNA extraction, while the right lobe was perfused in 4% formaldehyde and embedded in paraffin for immunohistochemistry. BAL cell concentrations were determined using a Scharf Instruments Casy Counter. BALF was centrifuged at 1,200 g for 5 mins and stored at -20°C for protein analysis by Western blot.

Immunohistochemistry

Expression of RELMα and Ym1 in lung sections was assessed by indirect immunoperoxidase techniques. Briefly, the paraffin embedded tissue sections were deparaffinised and rehydrated. After high temperature antigen unmasking (Vector Laboratories, UK), endogenous peroxidase was quenched with aqueous 2% H_2O_2 (Sigma

Aldrich, UK) for 15 minutes. Slides were then incubated 2 h with primary antibodies: rabbit anti-RELMα (0.25 μg/mL; Peprotech) or rabbit anti-Ym1 (1/100; StemCell Technologies) in antibody diluent (Dako Cytomation, Denmark) at RT, followed by the secondary antibody (goat anti-rabbit biotin, 1 mg/mL, Dako Cytomation, Denmark). Peroxidase-labelled ABC reagent and DAB substrate (Vector Laboratories, UK) were used for signal visualisation. Finally, the sections were counterstained with haematoxylin. RELMα and Ym1 staining intensities were scored by two researchers, blinded to experimental groupings, using a modification of a previously-published lung inflammation scoring system [71]. For each mouse, staining was assessed at 200× magnification for 10 fields. Each field included correctly inflated lung tissue and a complete transection of at least one bronchiole, blood vessel and alveolar airway. Cytoplasmic staining strength was scored in bronchial epithelial cells, infiltrating cells and alveolar macrophages on a scale of 1-4 (1 = no staining, 2 = weak, 3 = moderate, and 4 = strong staining, using a reference section of the same positive control sample (lung of an *Nb*-infected mouse at 7 days pi). The percentage of positive cells in each of these compartments was also scored on a scale of 1-4 (1 = none, 2<30%, 3 = 30-60%, 4>60% positive cells). Average cytoplasmic and cell positivity scores across the 10 fields were calculated. Finally, the overall staining score for each mouse was calculated by multiplying the average stain strength by average % positive cells. Control sections incubated with antibody diluent followed by secondary antibody only, or with normal rabbit serum alone, did not show any staining. Mouse lung pathology experts confirmed that stained cell types were correctly identified. Photomicrographs of representative sections were captured on a Zeiss Axioskop microscope with QCapture Pro Software.

RNA isolation and real-time RT-PCR

RNA isolation from lung tissue was carried out using TRIzol (Invitrogen). After DNase treatment (10 U/mL DNase1, Ambion), cDNA was synthesised using Moloney murine leukaemia virus reverse transcriptase (Stratagene). For quantification of Ym1, RELMα, Arginase 1, iNOS, IL-12p40, TNF-α, IFN-γ and IL-13 mRNA, real-time RT-PCR was performed using a LightCycler (Roche Diagnostics) and primers reported previously [63]. For each gene, five serial 1:4 dilutions of cDNA of a positive control sample (for RELMα, Ym1 and Arg1, peritoneal macrophages of a mouse implanted with *Brugia malayi* adult parasites for 3 weeks [72]; for IL-13, spleen of an MHV-68 infected IFNγR-KO mouse [63]; or for iNOS and other Type 1 markers, peritoneal macrophages of a thioglycolate-injected mouse [10]) were used in each reaction. Amplification was quantified and normalised using β-actin as a housekeeping gene. PCR reactions were carried out in 10 μl buffer containing 1 μl cDNA, 4 mM $MgCl_2$, 0.3 μM of each primer and the LightCycler-DNA SYBR Green I mix, under the following conditions: 30 s denaturation at 95°C, 5 s annealing of primers at 55°C or 63°C (Ym1), and 12 s elongation at 72°C, for 50 cycles. SYBR Green fluorescence was monitored after each cycle at 86°C (85°C for Ym1).

Real-time PCR to detect malaria in lung tissue

Real-time PCR for *Pcc* genomic DNA was carried out on DNA extracted with phenol/chloroform from homogenized lung tissue (stored in Trizol, Invitrogen; 75 mg tissue/mL). PCR was performed on an ABI Prism 7000 (Applied Biosystems), with primers for merozoite surface protein (MSP)-1 of clone AS, as described previously [73].

Western blot for Ym1 and RELM

20 μl BALF was mixed with sample buffer supplemented with denaturing buffer (NuPage, Invitrogen), heat denatured and resolved by SDS-PAGE using 4-12% gradient Bis-Tris gels (NuPage, Invitrogen) followed by transfer onto nitrocellulose membrane (Bio-Rad). Transfer and loading intensity were assessed with Ponceau Red staining (Sigma). After blocking with 0.05% Tween 20 in Starting Block (Pierce), membranes were incubated overnight at 4°C with polyclonal rabbit anti-Ym1 [10] (0.12 ng/mL) or rabbit anti-RELMα (0.2 μg/mL; Peprotech). After incubation with HRP-conjugated goat anti-rabbit IgG (heavy plus light chains; Bio-Rad; 1/2000), signal was detected with chemiluminescence (ECL kit, Amersham Pharmacia Biotech) and exposure to Hyper ECL film (Amersham). Control blots were incubated with secondary antibody only. Band intensity was determined with the FluorChem SP imager system and software (Alpha Innotech, USA) and expressed as percentage relative to a positive control (pooled lung lavages of three *Nb*-infected mice) on each blot.

Measurement of cytokine and cytokine receptor responses in local lymph nodes

Thoracic lymph node (TLN) cells were cultured at 5×10^5 cells per well, with 1 μg/mL Concanavalin A (ConA), 10 μg/mL adult *Nb* parasite extract, 1 μg/mL of recombinant *Pcc* Merozoite Surface Protein MSP-1_{19}, or medium alone at 37°C. After 72 h, supernatants were harvested. Concentrations of IL-4, IFN-γ, TNF-α, IL-5, IL-6, IL-10 and IL-13 were then measured using Cytometric Bead Array Flex Sets (BD Biosciences), with slight modifications from manufacturer's instructions: 50 μl samples/standards were incubated with capture beads (0.5 μl per sample per cytokine, plus diluent to 25 μl) in darkness with shaking for 1 h at RT. Plates were washed, spun at 200 g for 5 min, and then incubated with 25 μl of PE-conjugated anti-cytokine antibodies in darkness for 1 h. After washing and resuspension of beads, data were acquired on a FACSArray with FCAP software (BD Biosciences). Soluble TNF Receptor-1

(sTNFR1) concentrations were determined by sandwich ELISA, using 2 μg/mL capture antibody (clone MAB425), 200 ng/mL biotinylated detection antibody (BAF425), and recombinant mouse sTNFR1 as standard (425-R1; all from R&D Systems). Plates were blocked with 5% BSA in TBS for 2 h at RT and washed with TBS/0.05% Tween (TBST) before 50 μl samples were incubated overnight at 4°C. Plates were washed, incubated with detection reagent for 2 h at RT, washed again, and then incubated with streptavadin-HRP (Sigma Aldrich) at RT for 20 min. Plates were washed again and developed with TMB SureBlue substrate system (KPL 52-00-03). The reaction was stopped after 30 min with 1 M HCl and read on a spectrophotometer at 450 nm.

Statistical analysis

Most data were analysed with SAS System 9.1 mixed-model analyses of variance (ANOVA) or covariance (ANCOVA) [74] (see exceptions below). To meet the homogeneity-of-variance assumption of such analyses, data were logarithmically transformed. To account for slight differences among experiments in, for example, the magnitude of ConA-stimulated cytokine production, *experiment* was included as a random factor in all models. All significant effects of *infection* reported below have therefore remained significant after controlling for effects of *experiment*, if any. To account for differences among mice in starting body mass or RBC density, day 0 values were included as covariates. Pathology analyses focused on animals that experienced the full 20-day course of infection, and minimum body weight and RBC density were analysed in two time frames: the first week pi, and the entire experiment. In accord with the factorial design of the experiments, *Pcc* and *Nb* infection were fit as fixed factors. Wherever the interaction term was significant (indicating a potential effect of co-infection), post-hoc t-tests adjusted for multiple comparisons were run. A Tukey-Kramer-corrected $P < 0.05$ was used as the cut-off for significance. For relevant subsets of mice, an *Nb* fixed factor was used to test whether co-infection altered *Pcc* parasitaemia, while a *Pcc* fixed factor tested whether co-infection altered expression of ChaFFs.

Following logarithmic transformation to meet the assumptions of parametric statistical analysis, the ChaFF time courses, IHC scores, and lung *Pcc* data were analysed with unpaired *t*-tests in Prism 4 (Graph Pad Software, Berkeley, USA), with a two-tailed $P < 0.05$ designated as significant. Only two groups were compared per dataset, as specified in the Results section.

Abbreviations

AAMϕ: (alternatively activated macrophages); BALF: (bronchioalveolar lavage fluid); ChaFFs: (chitinase and Fizz/resistin family members); IHC: (immunohistochemistry); IL: (interleukin); *Nb*: (*Nippostrongylus brasiliensis*); *Pcc*: (*Plasmodium chabaudi chabaudi*); pi: (post-infection); RBC: (Red Blood Cell); RELMα: (resistin-like molecule α); Th: (T helper); TLN: (thoracic lymph node).

Authors' contributions

MAH conducted RT-PCR analysis of ChaFFs and PCR analysis of *Pcc* genomes, ran Western blots, statistically analysed some of the data, assisted with IHC scoring, and helped to draft the manuscript. KJM led the lung sampling, conducted cytokine RT-PCR reactions, assisted with Western blots, did IHC staining and scoring, and helped to draft the manuscript. Additionally, KJM collected all data for the *Nb* timecourse experiment. KJF-C, aided by ALG, set up all co-infection experiments and collected parasitemia, body mass and anaemia data. SM, aided by KJF-C, cultured lymph node cells and measured cytokines and cytokine receptors in the supernatants. JEA and ALG conceived of and designed the study, and drafted the manuscript. ALG performed most of the statistical analysis. All authors contributed to scientific discussions of the data, read and approved the final manuscript.

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