



**Loss and Recovery of Humoral Immunity to Influenza
Virus Following Malaria Infection**

Dorothy Hui Lin Ng

March 2012

Division of Parasitology and Division of Immunoregulation
MRC National Institute for Medical Research
The Ridgeway
Mill Hill, London
NW7 1AA

Division of Infection & Immunity
University College London

This thesis is submitted to University College London for the
degree of
Doctor of Philosophy

Declaration

I, Dorothy Hui Lin Ng, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Acknowledgements

I am indebted to my supervisors, Jean Langhorne and George Kassiotis. Their patience and kindness, as well as their academic experience, have been invaluable to me throughout my PhD and in the preparation of this thesis.

I am grateful to the UCL MB PhD Programme for giving me the opportunity to do research during my medical degree, and to A*STAR Graduate Academy and Medical Research Council for their generous financial support.

I am extremely grateful to my thesis committee, Andreas Wack and Benedict Seddon, for their support and advice; Bill Jarra, Anna Sponaas, Ana Paula Rosario, Robin Stephens, Phil Spence, Thibaut Brugat, Jenni Lawton, Rute Marques, Becki Pike and George Young for their perseverance in teaching me many protocols and techniques; John Skehel, who generously gave his time to prepare purified Influenza haemagglutinin; Radma Mahmood from Histology who patiently helped me figure out how to make bone marrow sections and stain for parasites; Claudia Berek and Van Trung Chu from Deutsches Rheuma Forschungszentrum Berlin for their hospitality and for teaching me how to make bone marrow cryosections; Isabel González Azcárate and Wiebke Nahrendorf for their help with much pipetting for ELIspots; Donald Bell from Confocal Microscopy for teaching me how to use the microscopes; Brian Trinnaman and Jackie Wilson for helping with large-scale hybridoma cultures; Donna Brown, Liz McMinn, Ula Eksmond, Tine Wagner, Tembi Huna, Prisca Levy and Sarah McLaughlin for their help in getting experimental reagents and for smoothing out many administrative processes; and especially to Graham Preece and the FACS facility staff, and Anna Sullivan, Rosemary Murphy, Jackie Holland and her team in LLY for their vigilance and for taking such excellent care of my mice.

Thanks to all the past and present members of the labs for the good friendships, their generosity, and for helping me to see the funny side in all situations. Thanks to the members of NIMDRAM and the Hillwalking group for many hours of laughter, and to Renee for humanizing me every morning with a cup of Costa coffee.

The support and encouragement of many friends have been indispensable. My parents and my brother have been a constant source of support, and this thesis would certainly not have existed without them.

Contents	
Declaration	2
Acknowledgements	3
Contents	4
Abstract	9
List of Tables	10
List of Figures	11
Abbreviations	14
Chapter 1: Introduction	18
1.1: Generation of memory B cells and long-lived plasma cells in the primary immune response.	21
1.1.1: Differentiation of naïve B cells, affinity maturation and isotype switching in the germinal centre response	21
1.1.2: Fate determination: Short-lived PC, LLPC or MBC?	25
1.2: Memory B cells – subsets and localisation	29
1.3: T cell-independent B cell memory	32
1.4: Long-lived plasma cells migrate to bone marrow niches	33
1.5: Maintenance of serum antibodies in the absence of antigen	36
1.5.1: Antibody maintenance by long-lived plasma cells	39
1.5.2: Antibody maintenance by memory B cells	40
1.6: Influenza overview	43
1.6.1: Innate immune response	43
1.6.2: Humoral immune response	44
1.7: Malaria overview	49
1.7.1: Immunity to malaria	50
1.7.2: Humoral immune responses to malaria	51
1.8: Reasons why humoral immunity to malaria may be short-lived	61
1.9: Aims of this study	67
Chapter 2: Methods and Materials	68

2.1: Mice	68
2.2: Influenza A infection	68
2.3: The mouse model of malaria	68
2.3.1: Plasmodium <i>chabaudi chabaudi</i> AS	68
2.3.2: Passage of <i>P. chabaudi</i> through BALB/c mice	69
2.3.3: Infection of experimental mice with <i>P. chabaudi</i>	69
2.3.4: Determination of parasitaemia using thin blood films	69
2.4: Chloroquine treatment	70
2.5: Preparing serum from whole blood	70
2.6: Tissue harvesting and making single cell suspensions	70
2.6.1: Splenocytes	70
2.6.2: Bone marrow cells	71
2.6.3: Peripheral blood mononuclear cells (PBMCs)	71
2.7. Immunoblotting using the LICOR/Odyssey system	71
2.8. ELISA	72
2.8.1: Measurement of HA-specific IgG	72
2.8.2: Measurement of <i>P. chabaudi</i> -specific IgG	73
2.9: Hybridoma culture and purification of HY1.2 mIgG2a and 2H7 mIgG2b	73
2.10: Immunodepletion with 2H7 mAb.	74
2.11: Determination of the half-life of serum Ab.	74
2.12: ELISpots	75
2.12.1: HA-specific antibody-secreting cell ELISpot	75
2.12.2: HA-specific memory B cells	76
2.12.2.1: Concanavalin A supernatant	76
2.12.2.2: CTLL-2 assay	76
2.12.2.3: HA-specific memory B cell ELISpot	77
2.13: Virus neutralisation assay	77
2.13.1: MDCK cells	78
2.14: Flow cytometry	78
2.15: Determining lung viral titres	79
2.15.1: RNA extraction and cDNA preparation	79

2.15.2: qRT-PCR.	79
2.16: Paraffin sections and H&E staining	80
2.17: Statistical analysis	80
Buffers, reagents, antibodies and mice (See List of Tables)	81
Chapter 3: The role of memory B cells and long-lived plasma cells in maintaining serum antibody titres after intranasal infection of BALB/c WT female mice with Influenza A/PR/8/34.	89
3.1: Introduction	89
3.1.1: Maintenance of serum antibodies by memory B cells or long-lived plasma cells	89
3.1.2: Correlative longitudinal studies on memory B cells and serum Ab in humans	91
3.1.3: Antibody maintenance after memory B cell depletion	95
3.1.4: Kinetics of the humoral immune response to Influenza A/PR/8/34 in mice	99
3.2: Aim	101
3.3: Objectives	101
3.4: Results	102
3.4.1: Development of long-lived specific systemic humoral memory after primary intranasal PR8 infection in BALB/c WT mice	102
3.4.2: Experimental protocol for depletion of MBCs from hCD20tg/BALB/c mice	103
3.4.3: The 2H7 mAb targets hCD20 on CD19+ B cells in spleens from hCD20tg mice but not in hCD20tg-negative littermates	104
3.4.4: Distribution of hCD20 on different cell lineages after PR8 infection	105
3.4.5: Depletion efficacy after 2H7 treatment	105
3.4.6: Depletion of HA-specific memory B cells and its effect on HA-specific long-lived plasma cells and HA-specific IgG	107
Chapter 3: Figures 1-17 and Figure Legends	109
3.5: Discussion	126

Chapter 4: Loss of previously established humoral immunity to Influenza A after sequential <i>Plasmodium chabaudi chabaudi</i> (AS) infection.	133
4.1: Introduction	133
4.1.1: Dislocation of pre-established long-lived plasma cells by new migratory plasmablasts	133
4.1.2: Clearance of pre-established long-lived plasma cells and memory B cells by causing apoptosis	136
4.2: Aim	139
4.3: Objectives	139
4.4: Results	140
4.4.1: Little serological cross-reactivity between Influenza A/PR/8/34 and <i>P. chabaudi</i> infections	140
4.4.2: Experimental protocol of sequential infection with PR8 and <i>P. chabaudi</i>	141
4.4.3: An infection with <i>P. chabaudi</i> in mice previously infected with PR8 reduces pre-established PR8-specific humoral immunity	142
4.4.4: An infection with <i>P. chabaudi</i> in mice previously infected with PR8 results in the loss of thymus-dependent serum antibody isotypes	143
4.4.5: An infection with <i>P. chabaudi</i> in mice previously infected with PR8 results in the loss of protective immunity to PR8	144
4.4.6: An infection with <i>P. chabaudi</i> in mice does not decrease the half-life of immunoglobulin	145
4.4.7: An infection with <i>P. chabaudi</i> in mice previously infected with PR8 results in the loss of HA-specific bone marrow plasma cells after <i>P. chabaudi</i> infection	146
4.4.8: Entrance of migratory plasmablasts into the bone marrow and loss of LLPCs during acute <i>P. chabaudi</i> infection (d8-12)	147
4.4.9: LLPCs and HA-specific plasma cells are not detected in PBMC during acute <i>P. chabaudi</i> infection (d8 and 10)	148
4.4.10: LLPCs undergo apoptosis during acute <i>P. chabaudi</i> infection (before d10)	149
4.4.11: <i>P. chabaudi</i> -induced bone marrow LLPC apoptosis is	

dependent on FcγRI,II,III	150
4.4.12: Sequestration of <i>P. chabaudi</i> parasites occurs during acute infection (d5 and d8, not d10 and d13)	152
Chapter 4: Figures 1-13 and Figure Legends	154
4.5: Discussion	167
Chapter 5: Final Perspectives	173
References	180

Abstract

Loss and Recovery of Humoral Immunity to Influenza Virus Following Malaria Infection

The mechanisms of maintenance of humoral immunity to infectious pathogens, particularly the contributions of memory B cells and long-lived plasma cells in maintaining specific serum antibody titres, are not well understood. Furthermore, it is not clear whether sequential heterologous humoral immune responses and disease pathology can result in the dysregulation and loss of previously acquired antibody-mediated immune responses to unrelated antigens. Here, depletion of memory B cells using anti-hCD20 monoclonal antibodies in hCD20 transgenic mice was used to dissect the role of memory B cells and long-lived plasma cells in maintaining long-term serum antibodies after intranasal Influenza A infection. Next, an experimental model of sequential infections with Influenza A/PR/8/34 and *Plasmodium chabaudi chabaudi* (AS) was set up, with a 15-20 week interval between the infections, in order to investigate whether sequential infection with *P. chabaudi* would affect pre-established humoral immunity to Influenza A.

This study demonstrates that memory B cells are essential for the maintenance of long-lived serum Ab titres to Influenza A, as depletion of memory B cells results in the eventual loss of long-lived plasma cells and serum antibodies. Sequential infection with *P. chabaudi* results in the loss of pre-established serum antibodies to Influenza A by inducing the loss of long-lived plasma cells in an Fc γ RI,II,III-dependent manner, and this renders mice susceptible to secondary infection with Influenza A. However, this loss of pre-established humoral immunity is temporary, as serum antibodies do eventually return to normal levels. These findings demonstrate a mechanism shared by memory B cells and long-lived plasma cells which ensures that serum antibodies are maintained for long periods of time in the face of continuous generation and incorporation of new specificities throughout the lifetime of the host. A more complete understanding of the parameters that affect the longevity of immunological memory and how heterologous infections influence this will be vital in our understanding of the effect of continuous exposure to infectious pathogens on the efficacy and longevity of previously established immune memory.

List of Tables

Chapter 1: Introduction

Table	Title	Page
1.1	Contributions to immunological memory by different cell types.	20
1.2	Factors which are required for the development of long-lived plasma cells and memory B cells.	26
1.3	Effector immune responses of various cell types during infection with <i>P. falciparum</i> or <i>P. chabaudi</i> .	54
1.4	Stage-specific mechanisms of immunity after various vaccination strategies.	56

Chapter 2: Methods and Materials

Table	Title	Page
2.1	Buffers	81
2.2	Primers	82
2.3	Hybridomas	82
2.4	Plasma cell panel	83
2.5	Memory B cell and Germinal Centre B cell panel	83
2.6	B cell panel	84
2.7	T cell, NK cell and NK T cell panel	85
2.8	Granulocyte panel	86
2.9	hCD20tg panel	86
2.10	ELISA and ELISpot Antibodies	87
2.11	Mouse Strains	88

List of Figures

Chapter 1: Introduction

Figure	Title	Page
1.1	Programming of differentiation from a naive B cell to MBC or LLPC.	23
1.2	The life cycle of the malaria parasite <i>P. falciparum</i> and acquisition of immunity in an area of endemic transmission.	55

Chapter 3: The role of memory B cells and long-lived plasma cells in maintaining serum antibody titres after intranasal infection of BALB/c WT female mice with Influenza A/PR/8/34
--

Figure	Title	Page
3.1	Development of long-lived specific humoral systemic memory after primary intranasal PR8 infection in BALB/c wild-type female mice.	109
3.2	Experimental protocol for depletion of memory B cells from hCD20tg/BALB/c mice.	110
3.3	Preservation of Ag epitopes and specificity of in-house made anti-hCD20 mIgG2b, 2H7 in hCD20tg CD19+ B cells.	111
3.4	Expression of hCD20 on different cell lineages.	112
3.5	Depletion efficacy of 2H7 treatment – Total spleen and bone marrow cellularity.	113
3.6	Depletion efficacy of 2H7 treatment – Neutrophils, monocytes, macrophages, dendritic cells, eosinophils and mast cells.	114
3.7	Depletion efficacy of 2H7 treatment – T cells and NK cells.	115
3.8	Depletion efficacy of 2H7 treatment – B cells.	116
3.9	Depletion efficacy of 2H7 treatment – B cells in peripheral organs.	117
3.10	Depletion efficacy of 2H7 treatment – Marginal zone, follicular and immature B cells.	118
3.11	Depletion efficacy of 2H7 treatment – Developing B cells in bone marrow.	119

3.12	Depletion efficacy of 2H7 treatment – Memory B cells analysed by flow cytometry.	120
3.13	Depletion efficacy of 2H7 treatment – Germinal centre B cells analysed by flow cytometry.	121
3.14	Depletion efficacy of 2H7 treatment – Long-lived plasma cells by flow cytometry.	122
3.15	Depletion efficacy of 2H7 treatment – HA-specific memory B cells analysed by ELISpot.	123
3.16	Depletion efficacy of 2H7 treatment – HA-specific ASC analysed by ELISpot.	124
3.17	Loss of serum HA-specific IgG after memory B cell depletion.	125

Chapter 4: Loss of previously established humoral immunity to Influenza A after sequential *Plasmodium chabaudi chabaudi* (AS) infection.

Figure	Title	Page
4.1	Little serological cross-reactivity between Influenza A/PR/8/34 and <i>P. chabaudi</i> infections.	154
4.2	Experimental protocol for sequential PR8 and <i>P. chabaudi</i> infection.	155
4.3	Loss of pre-existing HA-specific serum IgG after <i>P. chabaudi</i> infection.	156
4.4	No increase in rate of serum antibody clearance during acute or chronic <i>P. chabaudi</i> infection.	157
4.5	Loss of protective immunity PR8 infection after <i>P. chabaudi</i> infection.	158
4.6	Loss of HA-specific ASC from bone marrow at day 8 of <i>P. chabaudi</i> infection.	159
4.7	Increase in number of HA-specific MBC in spleens of PR8- <i>P. chabaudi</i> -infected mice on days 33 and 77 of <i>P. chabaudi</i> infection.	160
4.8	Entry of migratory plasmablasts on day 10 of <i>P. chabaudi</i> infection.	161
4.9	Migratory plasmablasts, but not long-lived plasma cells, are detected by multiparameter flow cytometric analysis in PBMC during acute <i>P. chabaudi</i> infection.	162
4.10	HA-specific ASC are not detected in PBMC during acute <i>P. chabaudi</i>	

	infection.	163
4.11	Long-lived plasma cells undergo apoptosis during acute <i>P. chabaudi</i> infection (before d10)	164
4.12	Loss of HA-specific ASC or HA-specific serum IgG after <i>P. chabaudi</i> infection is dependent on FcγRI,II,III.	165
4.13	Sequestration of <i>P. chabaudi</i> AS parasites in bone marrow during acute infection (d5 and 8).	166

Abbreviations and symbols

α	Alpha
Ab	Antibody
AID	Activation-induced cytidine deaminase
AMA1	Apical merozoite protein 1
APC	Antigen-presenting cell
APRIL	A proliferation inducing ligand
ASC	Antibody-secreting cell
AS	<i>Plamsodium chabaudi chabaudi</i> AS strain
β	Beta
BAFF	B-cell activating factor
BALB/c	<i>Mus musculus</i> strain
Bcl-6	B-cell lymphoma 6
BCR	B cell receptor
BM	Bone marrow
C57BL/6	<i>Mus musculus</i> strain
CD	Cluster of differentiation
CO ₂	Carbon dioxide
CpG ODN	CpG Oligodeoxynucleotide
CIITA	MHC class II transactivator
CXCR4	Chemokine receptor 4
CXCL12	Chemokine ligand 12
δ	Delta
d	Days
DC	Dendritic cell
DNA	Deoxyribonucleic acid
ε	Epsilon
ELISA	Enzyme-linked immunosorbant assay
ELIspot	Enzyme-linked immunosorbant spot
<i>et al.</i>	And others
Fc γ R	Fc-gamma receptors

FDC	Follicular dendritic cell
γ	Gamma
$\gamma\delta$ T cell	Gamma delta T cell
GC	Germinal centre
GFP	Green fluorescent protein
h	Hours
HA	Haemagglutinin
ICOS	Inducible T cell co-stimulator
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgG	Immunoglobulin G
IgH	Immunoglobulin heavy chain
IgM	Immunoglobulin M
IFN	Interferon
IL	Interleukin
IRF	Interferon regulatory factor
i.n.	Intranasal
i.p.	Intraperitoneal
i.v.	Intravenous
κ	Kappa
KO	Knock-out
L	Ligand
LCMV	Lymphocytic Choriomeningitis Virus
LFA-1	Lymphocyte function-associated antigen-1
LLPC	Long-lived plasma cell
μ	Mu
mAb	Monoclonal antibody
mg	Milligram
ml	Millilitre
mM	Millimolar
μ g	Microgram

μl	Microlitre
MBC	Memory B cell
MDCK	Madin-Darby canine kidney (cell line)
MHC	Major histocompatibility complex
MSP1	Merozoite surface protein 1
MSP1 ₁₉	19 kiloDalton sequence on the carboxyl terminal of MSP1
nAb	Neutralizing antibody
ng	nanogram
NIMR	National Institute for Medical Research
NK	Natural killer
NKT	Natural killer T cell
NP	4-hydroxy-3-nitrophenyl acetyl
ON	Overnight
OX40	T cell co-stimulatory molecule
R	Receptor
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Room temperature
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PC	Plasma cell
PE	R-. Phycoerythrin
PECAM	Platelet endothelial cell adhesion molecule
PRR	Pattern recognition receptor
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RBC	Red blood cell
RUNX	Runt-related transcription factor
SAP	SLAM-associated protein
SCID	Severe combined immunodeficiency
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SLAM	Signaling lymphocytic activation molecule

SMAD	Transcription factor
SRBC	Sheep red blood cell
T-bet	Th1-specific T box transcription factor
TACI	Transmembrane activator and calcium modulator and cyclophilin ligand interactor
TCR	T cell receptor
TD	Thymus-dependent
Tfh	T follicular helper cell
TGF β	Transforming growth factor beta
Th	T helper cell
TI	Thymus-independent
TLR	Toll-like receptor
TNF α	Tumour necrosis factor alpha
V	Volt
VCAM-1	Vascular cell adhesion protein 1
VLA	Very late antigen-alpha
w/v	Weight/volume percent (g/ml)
XBP-1	X box binding protein 1
-/-	Homozygous knockout
°	Degree Celsius
%	Percent

Chapter 1: Introduction

Immunological memory is the ability to ‘remember’ an antigen that a host has previously encountered (through natural infection or vaccination) and to mount a more rapid and robust secondary response (Gray 1993). This can result in attenuated symptoms, decreased pathogen burden or even sterile immunity to re-infection (Ahmed & Gray 1996). One of the theories to explain immunological memory is a greater precursor frequency of antigen-specific lymphocytes, which arise out of clonal expansion, and which are long-lived in the absence of antigen. These increased number of memory cells are also primed to subsequently give more specific, rapid and robust immune responses than naïve cells when re-encountering the antigen. Fine dissection of the qualitative and quantitative responses involved in mounting memory immune responses have been characterised extensively with model antigens (Nossal *et al.* 1968). Cells with these memory characteristics can differentiate from different types of naive lymphocytes, including innate cells (Bird 2010;Paust & von Andrian 2011), CD4⁺ and CD8⁺ T cells (Pepper & Jenkins 2011;Sallusto *et al.* 2004), and B cells (McHeyzer-Williams *et al.* 2011) (**Table 1.1**).

After vaccination, for example, with many viral vaccines such as the live attenuated Influenza A vaccines, or infections, for example, measles or smallpox or even malaria, protective immunity is usually correlated with antibody titres (Crotty & Ahmed 2004;Langhorne *et al.* 2008;Pulendran & Ahmed 2011;Welsh *et al.* 2004;Zinkernagel 2002). However, whether these high serum antibody titres are maintained by memory B cells, short-lived plasma cells or long-lived plasma cells is still unclear. In addition to antibodies, memory CD8⁺ T cells can also contribute to cellular immunity, for example, after Influenza A vaccination (Lalvani *et al.* 1997), but the correlation studies of memory T cells with either antibody levels or protective immunity is inconsistent (Zinkernagel *et al.* 1996) and possibly age-related (Forrest *et al.* 2008; Goronzy *et al.* 2001). Correlates to protective immunity can perhaps be complicated by the fact that protective immunity can be either towards disease or towards re-infection, which can be mediated through different mechanisms.

The memory niches and necessary survival resources which organise, maintain and even limit immunological memory cells have been a subject of intense study. The memory T cell niche was previously thought to be finite (Selin *et al.* 2004), perhaps restricted by the availability of cytokines such as Interleukin-15 and TNF-TNFR ligand interactions (reviewed by Sabbagh *et al.* 2004). Attrition of pre-existing memory CD8⁺ T cells in the spleen was demonstrated by either stochastic competition for survival resources (Chapdelaine *et al.* 2003) and also by active deletion as a result of infection (Kim & Walsh 2003). However, other studies have also demonstrated long-term maintenance of cell numbers particularly in the CD8⁺ T cell memory pool (Murali-Krishna *et al.* 1998; Odumade *et al.* 2012) or even an expandable CD8⁺ T cell memory pool (Vezyz *et al.* 2009). CD4⁺ and CD8⁺ memory T cells are able to undergo a slow homeostatic turnover which can enhance the longevity of antigen-specific populations (Murali-Krishna *et al.* 1999). In humans, memory B cells (MBCs) similarly are able to undergo homeostatic turnover (Macallan *et al.* 2005; Wirths & Lanzavecchia 2005), although it is not known whether memory B cells undergo homeostatic proliferation in mice, but the characteristics of their niche are not well understood. By contrast, long-lived plasma cells (LLPCs) have been found to predominantly reside in specialised bone marrow niches (Manz *et al.* 1997; Slifka *et al.* 1998; Tokoyoda *et al.* 2004) and require specific survival resources (Fairfax *et al.* 2008). It is currently believed that the niche for LLPCs is finite, and because they are terminally differentiated and are unable to replace themselves, LLPCs can be more susceptible to stochastically-determined attrition (Radbruch *et al.* 2006). It is important to understand the mechanisms that regulate the finite LLPC population in order to accommodate an increasing number of specificities throughout the host's lifetime.

The first aim of this thesis is to understand the relative contributions of the cells that mediate humoral memory – B-cell originated memory B cells (MBC), which can mount a heightened recall response to a secondary challenge, and long-lived plasma cells (LLPC), which secrete antibodies (Abs) of high affinity and specificity into the circulation and mucosa – towards maintaining persistently elevated serum Abs. The second aim of this to understand whether and how a sequential heterologous infection can affect pre-established

Table 1.1: Contributions to immunological memory by different cell types.

Cell name	Cell phenotype /characteristics*	Mechanism of action	Ref
T effector memory (T_{EM})	<p>CD8^{>}CD4 CCR7⁻ CD62L^{+/-**}</p> <p>Express homing receptors that facilitate migration to nonlymphoid sites of inflammation . Enriched in lung, liver and gut May require pMHCII stimulation for longevity Maintain stable Th1 or Th2 polarisation</p>	<p>Produce IFNγ, IL-4 and IL-5 within several hours of TCR stimulation. CD8 TEM carry large amounts of perforin Th1 or CTL: CCD5⁺ CXCR6⁺ CCR3⁺ Th2: CCR3⁺ CRTh2⁺ CCR4⁺ Selective chemokine expression enables homing into barrier sites – skin, gut, lymph nodes through HEV</p>	(Sallusto, Geginat, & Lanzavecchia 2004)
T central memory (T_{CM})	<p>CD4^{>}CD8 CD45RO⁺ CD62L⁺ and CCR7⁺ Enriched in lymph node and tonsils Higher sensitivity to TCR stimulation, require less costimulation signals, higher levels of CD40L Recirculate through secondary lymphoid organs High levels of phosphorylation of STAT5 and are capable of self-renewal; high levels of IL-15R Contains both uncommitted and polarised cells</p>	<p>Do not produce cytokines except IL-2. However can proliferate and differentiate into effector cells producing IFNγ and IL-4. CXCR5⁺ Tfh phenotype – produce IL-2 and IL-10 and provide spontaneous B cell help Can be induced to differentiate into Th1 or Th2 or CTL</p>	
NK	<p>Complex pattern of expression of the polygenic and polymorphic Ly49 family of innate receptors (and KIR in humans). Bind MHCI or MHCI-like molecules which are inhibitory or activating.</p>	<p>Like T cells. Population expansion, cytolytic activity/cytokine production.</p>	(Bird 2010;Paust & von Andrian 2011)
Memory B cell	<p>Undergoes homeostatic turnover Able to differentiate into daughter memory B cells or plasma cells rapidly Heterogenous population; Not all isotype switched, variable somatic hypermutation Can be T cell-dependent or T cell-independent Mainly reside in spleen, with a small proportion recirculating</p>	<p>Fewer activation requirements, may not need co-stimulation</p>	(McHeyzer-Williams, Okitsu, Wang, & McHeyzer-Williams 2011)
Long-lived plasma cell	<p>Terminally differentiated CD138⁺ intracellular Ig⁺ Not all isotype switched Highest levels of somatic hypermutation Can be T cell-dependent or T cell-independent Mainly reside in bone marrow niches, do not recirculate</p>	<p>Secretion of Ig</p>	(Shapiro-Shelef & Calame 2005)

*Defined in the human system

** Other markers, like CD45RA, CD27 and CD28 have been used to further divide TCM and TEM into subgroups

humoral memory. To this end, how MBC and LLPC are generated and maintained in their niches, various mechanisms of how they are thought to maintain serum Abs, and the humoral immune responses to Influenza A and *Plasmodium chabaudi*, which are the model infections used in the subsequent experiments, are outlined in this introduction.

1.1: Generation of memory B cells and long-lived plasma cells in the primary immune response.

1.1.1: Differentiation of naïve B cells, affinity maturation and isotype switching in the germinal centre response

During many viral infections, the appearance of neutralizing Abs coincides with the appearance of germinal centres (GCs) and marks the onset of recovery from the infection and clearance of virus from the circulation (Dörner & Radbruch 2007). GCs in the secondary lymphoid organs (spleen and regional lymph nodes) appear at day 4 of a primary immune response to protein immunisation, peak between days 12 and 14, and recede after three to four weeks (Allen *et al.* 2007; Pape *et al.* 2003). GCs are organised focal localisations consisting of foreign antigen, activated cells (B cells, T follicular helper (Tfh) cells, tangible body macrophages and follicular dendritic cells) and are the sites where B cells receive signals which are necessary for the differentiation into short-lived extrafollicular plasma cells (PCs), MBCs and LLPCs (**Figure 1.1**). The GC is also the site for somatic hypermutation and class-switch recombination of the genes encoding the B cell receptor (BCR), leading to affinity maturation as oligoclonal B cells are selected by antigen, as well as isotype switching and enhanced effector functions. In the GC, foreign antigen can be presented to B cells in different ways: 1) in follicles via fibroblastic reticular cells or small gaps in basement of lymph node sinus, where it is bound by antigen-reactive B cells in subcapsular sinus (SCS) (Roozendaal *et al.* 2009), 2) In the form of immune complexes or higher molecular weight by SCS macrophages (Batista & Harwood 2009), 3) Free diffusion of soluble antigen into lymphoid follicles (Pape *et al.* 2007), and 4). Dendritic cells (DC) migrating from peripheral organs can also transport antigens to

secondary lymphoid organs to access non-follicular B cells (Qi *et al.* 2006). Antigen-specific B cells process and present peptides complexed to MHC Class II to antigen-specific Tfh cells, which recognise these complexes. The co-stimulatory molecules provided by T cells, like CD40L, ICOSL and OX40L, engage the B cell during the formation of the immunological synapse and provide the necessary signals which activate the B cell and drive their differentiation into short-lived PCs, LLPCs or MBCs. This process is known as receiving cognate T cell help.

A recirculating naïve B cell repertoire produced by the bone marrow has, at any one time, 10^4 - 10^5 different specificities. The GC is a dynamic structure and naïve B cells can be seen moving through the GC to scan antigen presented on FDCs (Camacho *et al.* 1998). Signalling through the BCR as well as co-stimulation from Tfh cells activates antigen-specific B cells to proliferate intensely and undergo somatic hypermutation in the genes encoding the BCR, producing an oligoclonal population of B cells with different receptor affinities for the antigen. Somatic hypermutation is initiated by the enzyme Activation-Induced (Cytidine) Deaminase (AID), which deaminates cytosine into uracil in DNA, creating a uracil:guanine mismatch (Muramatsu *et al.* 2000). This mismatch is rapidly repaired by DNA mismatch repair enzymes like DNA polymerases (Casali *et al.* 2006). However, these DNA polymerases are error-prone and point mutations are introduced into the deaminated cytosine or the neighbouring base-pairs (Di Noia & Neuberger 2007). High levels of the anti-apoptotic transcription factor Bcl-6 in GC B cells suppress the cells' intrinsic response to DNA damage and allows gene mutations to occur in the variable region (Ranuncolo *et al.* 2007). Approximately one irreversible single-base pair mutation is introduced during each division cycle. Hence the final variable region of the BCR which is transcribed by the B cell is a variant within the oligoclonal population of rapidly proliferating B cells bearing mutated BCRs with varying affinities for the antigen (Allen *et al.* 2007). The highest affinity GC B cells have preferential survival rates, and over time the final affinity of the Ab produced by the LLPC or the BCR of the MBC evolves to becoming much higher than the affinity of the original BCR of the B cell which recognised the antigen. At the same time, negative selection against self-antigen occurs to remove the self-

reactive clones produced by somatic hypermutation. These proliferating GC B cells, which are known as centroblasts and centrocytes, form a distinct structure which can be seen as light zone and dark zones (MacLennan 1994). Follicular dendritic cells (FDCs) express the chemokines CXCL13 as well as integrins which attract centroblasts to the light zone. Movements between the light and dark zone are bidirectional, depending on the concentration gradient of the chemokines CXCL12 and CXCL13 and the corresponding expression of the chemokine receptors CXCR4 and CXCR5 on the differentiating centroblasts (Allen *et al.* 2004). This cycle of cellular movements, which is regulated by changes in expression of chemokine and chemokine receptors, and the accompanying molecular changes within the B cell, underpins the mechanisms that regulate antigen-specific clonal evolution during the development of B cell memory (Allen *et al.* 2007;Schwickert *et al.* 2007). Reiterative GC cycles of somatic hypermutation and affinity-based selection rapidly expands the highest-affinity variants of the original antigen-specific B cell compartment, whilst causing apoptosis of self-reactive and low-affinity clones (Takahashi *et al.* 1999), and this results in affinity maturation of the humoral immune response (Allen *et al.* 2007;Phan *et al.* 2009;Victora *et al.* 2010).

Isotype switching, which is the changing of the constant region of the Ab heavy chain, occurs via class switch recombination. This is a process which is stimulated by CD40 binding and switching from IgM and IgD to IgG, IgA or IgE is influenced by the local cytokine milieu (Cazac & Roes 2000;Snapper & Paul 1987) and the number of mitotic cycles the cell undergoes (Tangye *et al.* 2002). Unwanted constant region (C_H) exons are excised by the enzyme AID in the form of circular DNA by double-stranded breaks (DSB) at defined switch regions (Stavnezer *et al.* 2008). The gene segments surrounding the deleted portion are rejoined by non-homologous end joining (NHEJ) or microhomology joins to re-form a functional immunoglobulin gene that encodes a different heavy chain isotype, usually either a γ , α or ϵ exon. This process is mediated by several DNA repair enzymes including uracil DNA glycosylase and endonucleases. With the exception of the μ and δ genes, only one isotype is expressed by a B cell after leaving the GC. The IgG heavy-chain has a longer cytoplasmic tail and induces a unique signalling cascade that confers

to MBC a survival advantage over B cells with IgM and enhances the overall ability of the class-switched B cell to survive for longer periods of time (Horikawa *et al.* 2007). The activity of AID is controlled by transcription factors including paired box binding protein (PAX5) (Tran *et al.* 2009), homeobox C4 (HOXC4) (Park *et al.* 2009) and forkhead box O1 (FOXO1) (Dengler *et al.* 2008). Additionally, specific cytokines can induce the selective expression of transcription factors which enhance class-switch recombination to certain isotypes, for example, the cytokine IFN γ enhances the expression of the transcription factor T-bet, which promotes class switching of B cells to IgG2a (Mohr *et al.* 2010; Peng *et al.* 2002).

1.1.2: Fate determination: Short-lived PC, LLPC or MBC?

The combination of antigen recognition by the BCR and pro-survival signals from Tfh cells (Crotty 2011; Fazilleau *et al.* 2009) stimulate B cells to undergo genetic reprogramming to differentiate into extrafollicular short-lived PCs, LLPC or MBCs and exit the GC. Alternatively, these B cells can recycle through the dark zone to undergo further rounds of proliferation and affinity maturation (McHeyzer-Williams & McHeyzer-Williams 2005) (**Table 1.2**). Signals obtained outside of this, for example those provided by Toll-like receptors (TLR) ligands like bacterial lipopolysaccharide as well as the local cytokine milieu, can influence fate determination, affinity maturation and isotype class switching.

The activation and differentiation of naïve CD4⁺ T cells into a distinct transcriptional state of CXCR5^{hi} Tfh cells is important for providing cognate T cell help in terms of cross-linking of the BCR as well as co-stimulatory interaction such as CD40-CD40L, OX40-OX40L and ICOS-ICOSL (Crotty 2011; Fazilleau *et al.* 2009). Cognate T cell help is crucial for the generation of MBC and LLPC. In SLAM (Signaling lymphocytic activation molecule)-associated protein (SAP)-deficient mice, where activated CD4⁺ T cells fail to differentiate into Tfh cells and thus do not provide T cell help, long-lived humoral memory is severely impaired but not short-lived Ab responses (Qi *et al.* 2008). The complex factors which are required for the maintenance of cell contact between Tfh cells and B cells are

Table 1.2: Factors which are required for the development of long-lived plasma cells and memory B cells.

Cell type	Required for development humoral memory	External ligands	Transcription factor changes	Cytokine milieu	Ref
Plasma cell	<p>Recognition of pMHCII by the TCR of the cognate Th cell, i.e. “The Immunological Synapse”</p> <p>CD28-CD80/CD86</p> <p>B cell-Th cell interaction</p> <ul style="list-style-type: none"> • CD40-CD40L (CSR and GC formation) • OX40-OX40L (CSR) • ICOS-ICOSL <p>Appropriate spatio-temporal control of all molecular signals</p> <p>Apoptosis of low-affinity or self-reactive clones</p> <ul style="list-style-type: none"> • Bcl-2 ↓ • Bcl-XL ↓ <p>Positive selection of high-affinity clones</p> <ul style="list-style-type: none"> • T cell help 	<p>BCR – High avidity, may require several cycles through the GC</p> <p>GC dependent - extrafollicular tends to be short-lived</p> <p>ICOSL</p> <p>CD19, CD21/35</p> <p>CD40L, although CD40 overstimulation favours development of short-lived extrafollicular PCs</p> <p>TLR</p> <p>CD27-CD70 (in mice)</p>	<p>Retain EBL-2, which enables extrafollicular migration of LLPCs</p> <p>Blimp-1 ↑</p> <p>XBP-1 ↑</p> <p>Pax5 ↓</p> <p>CTH1A ↓</p> <p>Bcl-6 ↓⁺</p> <p>Blimp-1 ↑</p> <p>c-myc ↓</p> <p>Bcl-2 ↑</p> <p>SWAP-70 ↓</p>	<p>T cell derived cytokines – IL-2, IL-4, IL-21</p> <p>BAFF</p> <p>APRIL</p>	<p>(Shapiro-Shelef & Calame 2005)</p>
Memory B cells	<p>Positive selection of high-affinity clones</p> <ul style="list-style-type: none"> • T cell help 	<p>BCR – Moderate avidity, not as many mitotic cycles needed</p> <p>CD38 ligation</p> <p>Signalling through PLCγ2</p>	<p>Appears to be less stringent compared to LLPC</p> <p>**Bcl-6 ↓</p> <p>Bcl-2 ↑</p> <p>SWAP-70 ↑</p>	<p>IL-21 ↓</p>	<p>(McHeyzer-Williams & McHeyzer-Williams 2005; McHeyzer-Williams <i>et al.</i> 2011)</p>
Extrafollicular memory B cells					
GC B cells		<p>T cell interaction through SLAM-associated protein (SAP)</p>	<p>Lose EBL-2</p> <p>Bcl-6 ↑</p>	<p>IL-21</p> <p>IL-4</p>	

still being molecularly defined and include the cytokine IL-21 (Linterman *et al.* 2010; Ozaki *et al.* 2002; Zotos *et al.* 2010), dedicator to cytokinesis protein 8 (Dock8) (Randall *et al.* 2009), transcription factors like Bcl-6 (Kitano *et al.* 2011; Nurieva *et al.* 2009), and the micro-RNA miR-155 (Thai *et al.* 2007; Vigorito *et al.* 2007). Loss-of-function mutations in these components, which affect the interaction between B cells and Tfh cells, have a drastic effect on the development of either MBC or LLPC.

The formation of GCs *per se* does not appear to be strictly required for MBC formation but is required for LLPC formation. Bcl-6-deficient mice, which cannot make GCs nor have GC B cells, still can produce MBC which can persist in the quiescent state in spleen for up to 90 days, and can undergo isotype class-switching but not somatic hypermutation. However in these mice, there was a failure to generate LLPCs (Toyama *et al.* 2002). Similarly, mice deficient in Type 1 TNF receptor or TNF α also cannot form GCs in the spleen (Matsumoto *et al.* 1996), but these mice can still generate high affinity clones of MBC which are isotype switched and which are also very long lived, albeit at reduced numbers than in GC-competent mice. It is possible that these MBC are generated in GC-like structures in other secondary lymphoid tissue like lymph nodes (Fu *et al.* 1997). However there are markedly reduced LLPCs in TNF α -deficient mice and LLPCs also seem to decrease in number at a faster rate, suggesting a survival defect in these GC-independent PCs either due to an intrinsically truncated lifespan, or defects in homing or adapting to their long-term niche. Within the GC, antigen presentation and BCR-stimulated signalling through PLC γ 2, as well as ligation of CD38 (Ridderstad & Tarlinton 1998), are thought to influence the differentiation of B cells into MBC. It has been suggested that the reduction of Bcl-6 alone by default leads to the differentiation into MBC, whilst that reduction of Bcl-6 combined with upregulation of the transcription factor Blimp-1 leads to differentiation into PCs (Angelin-Duclos *et al.* 2000).

Quantitative differences in affinity for antigen amongst the positively selected centrocytes can control their fate to become MBC or LLPC. The highest affinity clones tend leave the GC early with few V-region mutations, to differentiate into short-lived extrafollicular PCs

first producing IgM and subsequently IgG (Dal Porto *et al.* 1998;Paus *et al.* 2006;Schwickert *et al.* 2011). By contrast, LLPC typically display the highest number of V-region mutations, and can take months to leave residual GCs, indicating that they have been through an extremely long-lived affinity maturation process (Phan *et al.* 2006;Smith *et al.* 1997;Takahashi *et al.* 1998). Moderate affinity clones typically differentiate into MBC and as a result, MBC can have more polyreactive BCRs than LLPC and can recognise viral escape mutants during a secondary response (Purtha *et al.* 2011). The introduction of a mouse with a selective defect in *Prdm1*, which encodes the ‘PC transcription factor’ Blimp-1, has allowed extensive analysis of the transcriptional changes that occur in developing pre-PCs. PC commitment occurs in GCs, where high-affinity BCR stimulation, in combination with other signals such as CD40L, may facilitate PC commitment by downregulation of the transcription factors Pax5 and Bcl-6, both of which maintain the naïve B cell state, and upregulation of X box-Binding Protein-1 (XBP-1), a transcription factor that regulates the unfolded protein response protecting the cell from damage induced by the continuous production and secretion of high amounts of Ab (Hu *et al.* 2009;Iwakoshi *et al.* 2003;Reimold *et al.* 2001;Todd *et al.* 2009). Although CD40 stimulation is required for development of PCs (Takahashi *et al.* 1998), overstimulation of CD40 curtails the production of LLPC and leads to differentiation into short-lived PCs (Erickson *et al.* 2002). Full commitment to PC differentiation occurs via induction of the transcription factor Blimp-1, the ‘master switch’ of PCs (Angelin-Duclos *et al.* 2000). Blimp-1 exerts a positive feedback loop that induces the expression of XBP-1 and represses Pax5, Bcl-6 and other genes important for B cell identity such as MHC class II transactivator (CIITA) and c-myc, allowing genetic ‘freedom’ of development into PCs (Calame *et al.* 2003;Shapiro-Shelef & Calame 2005). CD40 and TLR stimulation help to repress Bcl-6 through NF- κ B and IRF4-mediated pathways. Blimp-1 also represses the chemokine receptors CXCR5 and induces the expression of CXCR4 (Shaffer *et al.* 2002), which allows the PC to exit the GC, which expresses the chemokine CXCL13 and home to peripheral bone marrow niches which express CXCL12.

1.2: Memory B cells – subsets and localisation

MBCs derived from GC B cells have the hallmark features of having an increased frequency to a particular antigen, constitutive TLR expression, and having the ability to differentiate more rapidly into PCs which secrete affinity matured and isotype switched Abs (Good, Avery, & Tangye 2009). However it has become increasingly evident that MBCs have a variety of surface phenotypes, and not all MBCs have undergone somatic hypermutation or class switch recombination (McHeyzer-Williams & McHeyzer-Williams 2005; Sanz *et al.* 2008; Tangye & Good 2007; Yoshida *et al.* 2010). The MBC population is a heterogeneous one, but is still a functionally distinct population from other antigen-experienced B cell populations, such as GC B cells (PNA⁺ GL7⁺), or PCs (CD138⁺, Blimp-1^{hi}) and have discrete effector functions which are important in maintaining humoral memory.

Human MBCs can be distinguished by the downregulation of IgD, an identifier of a B cell that has undergone class-switch recombination. Based on this definition, IgD⁻ MBCs can be subdivided into IgM⁺ and IgG⁺ MBCs, both of which have undergone affinity maturation and have a more differentiated transcriptome than naïve IgD⁺ B cells. IgM⁺ MBC have been reported to be important in both thymus-dependent and thymus-independent immune responses and in cross-protective immunity during co-infection; for example they play a protective role during *Cryptococcus* co-infection of HIV patients (Subramaniam *et al.* 2009). Human MBCs can also be identified using the ‘pan MBC marker’ CD27 (Agematsu *et al.* 2000). Following this criteria, CD27⁺ MBCs are seen to comprise nearly half (40-50%) of total peripheral B cells from adult blood or secondary lymphoid organs. Surprisingly, using CD27 to define MBCs revealed that a large proportion of CD27⁺ MBCs had not undergone class-switch recombination and were still IgD⁺, with 40% of CD27⁺ MBC being IgM⁺ IgD⁺ compared with 20% of CD27⁺ MBC being IgM⁺ IgD⁻. However, these CD27⁺ IgM⁺ IgD⁺ peripheral blood B cells had undergone somatic hypermutation, indicating that they were *bona fide* antigen-experienced cells which had been through the GC reaction (Klein *et al.* 1998). Recently, in the light of the identification of the ATP-

binding cassette (ABC)B1 transporter being expressed only on human mature naïve B cells, while being absent on memory and transitional B cells, it was demonstrated that a small population of (ABC)B1- IgG⁺ MBCs were CD27⁻ (Wirhth & Lanzavecchia 2005). In summary, the MBC population in humans is a very heterogeneous one and bears a variety of surface markers and isotypes.

Due to the low frequency of Ag-specific MBC in mice, it is more difficult to characterise the MBC population in mice. Some groups have used transgenic mice and knock-in mice to increase the frequency of Ag-specific MBC in order to overcome this handicap. In one study using mice in which 2% of MBC were NP-specific after immunization, Anderson and colleagues (Anderson *et al.* 2007) found that NP-binding MBCs could be separated into subpopulations based on relative expression of CD80 and CD35. Surprisingly, the CD35⁺ CD80⁺ fraction, which comprised the majority (70%) of NP-specific MBC, had unmutated V genes, whereas only the remaining minority CD35⁻ CD80⁺ NP-specific MBC fraction had undergone somatic hypermutation. However the unmutated MBC were as long-lived as their mutated counterparts and maintained their distinct surface markers for the length of the study. In another study, Pape and colleagues used mice in which the BCR-specificity had been knocked into the endogenous H chain locus, which meant that the monoclonal B cell population was capable of class switching. They identified CD38 as a good marker to distinguish class-switched memory (CD38⁺) from GC B cells (CD38⁻), and using this, they demonstrated that IgM⁺ and IgG⁺ MBC have different sites of generation and kinetics after primary immunization (Pape *et al.* 2003).

One of the drawbacks of genetic manipulation to increase the frequency of MBC is that it may introduce confounding effects on the emerging MBC populations. Therefore a further study was done using a novel flow-cytometry based technique to characterise the phenotype of the endogenous population of R-Phycoerythrin (PE)-specific MBC formed after immunisation with PE. Confirming previous data, this study demonstrated that there were significant populations of both PE-specific IgD⁻ IgM⁺ and IgD⁻ IgG⁺ MBC (Pape *et al.* 2011). Furthermore, after adoptive transfer and re-exposure to antigen, most transferred

IgD⁻ IgG⁺ MBC differentiated into IgG-secreting PCs, whilst IgD⁻ IgM⁺ MBC predominantly gave rise to IgM⁺ and IgG⁺ GC B cells rather than differentiating into PCs, indicating that they had discrete effector functions (Pape *et al.* 2011). It is possible that the ability of IgG⁺ MBC to directly differentiate into PCs was due to their ability to be activated in the presence of pre-existing high-affinity neutralizing serum Ig, unlike their IgM⁺ counterparts which are inhibited from differentiating into PCs by pre-existing serum Ig (Pape *et al.* 2011).

Another study made use of a Rosa26-loxP-EYFP reporter mouse crossed with a transgenic knock-in mouse with Cre inserted into the *Acida* locus which encodes AID, which genetic labelling of AID-expressing cells with yellow fluorescent protein (YFP), i.e. antigen-experienced GC B cells and MBC. (Dogan *et al.* 2009). This study not only confirmed the existence of both IgM⁺ and IgG1⁺ YFP⁺ (i.e. Ag-experienced) MBCs after secondary challenge with SRBC, but also that IgM and IgG MBCs had distinct differentiation pathways after re-exposure to antigen. Furthermore, this study demonstrated that there were distinct localisations of MBC, with clusters of IgM⁺ and IgG1⁺ MBC persisting for up to 8 months within GC-like structures, and others residing in extrafollicular locations, and a minority being CD62L⁺ recirculating MBC (Dogan *et al.* 2009). The MBC which localised and proliferated in GC-like structures were in close contact with FDCs and CD4⁺ T cells where antigen could be maintained for long periods of time. These MBC were probably dependent on antigen for long-term maintenance, and these could be a distinct mechanism of survival from the population of recirculating quiescent MBC. By contrast, after immunisation with the NP-CGG in alum, these authors did not observe GC-like structures after 3 months, but instead NP-specific MBC were observed mainly outside the B cell follicles and in the circulation, indicating that the nature of the MBC response was highly influenced by the type of antigen. Whether it is an intrinsic difference in their genetic programming by the nature of the antigen, or their microenvironment that determine the separate functions and localisations of MBC remains to be studied.

1.3: T cell-independent B cell memory

Some MBC and LLPC can be generated in a thymus-independent (TI) manner outside of the GC (Defrance *et al.* 2011). These have been termed TI-MBC and TI-LLPC, and they are typically generated by TI-antigens such as pneumococcal capsular polysaccharides. Human studies have demonstrated that TI-MBC can be long-lived and can render protective humoral immunity in adults for at least 9 years after the initial dose (O'Brien *et al.* 2007). However their 'memory' features are a subject of controversy – some studies report that TI-MBC differ phenotypically and functionally from thymus dependent (TD)-'canonical' MBC, in that there is no enhanced sensitivity to Ag re-stimulation and no extended lifespan – in fact, phenotypically and functionally they are very similar to naïve B cells, except that there is an expansion of Ag-specific B cells, giving them a competitive edge in recognising Ag because of the increased pre-existing numbers during re-infection (Alugupalli *et al.* 2004). Indeed, because polysaccharide antigens tend to be retained in the host for longer periods of time, giving rise to continual effector responses, TI-MBC may not have developed these TD characteristics, and instead are subject to strict negative feedback regulation by pre-existing Ab (Obukhanych & Nussenzweig 2006). Indeed, TI-MBC are unresponsive to a secondary challenge with polysaccharide vaccine when secondary immunisation was performed 5 years after primary immunisation, possibly due to the inability of TI-MBC to differentiate into PCs in the presence of pre-existing specific Ab. In one study, in order to 'unmask' their effector memory function, TI-MBC were adoptively transferred into immunodeficient SCID mice, and this showed that TI-MBC were able to mount typically 'anamnestic' responses towards both TI and TD antigens, and they underwent isotype class switching and differentiation into IgG producing PCs (Moens *et al.* 2008).

TI-LLPCs have been shown to have a lower secretion capacity than TD LLPC, but they do not undergo a contraction phase like short-lived PCs, and so are maintained at higher numbers. CD5⁺ B-1b cells are thought to be the main precursors for TI-MBC (Alugupalli *et al.* 2004), but the precursors for TI-LLPC are unknown. How LLPC are generated outside

the GC is unknown as it has been shown that GCs are necessary for LLPC development, but it is possible that extensive cross-linking of the BCR can be induced by TI-Ag, which tend to have expansive regions of repetitive epitopes, while the necessary T cell help may be overcome by TLR agonists or inflammatory cytokines, particularly IL-1.

1.4: Long-lived plasma cells migrate to bone marrow niches

LLPCs can develop from any type of activated B cell: naive B-2, marginal zone or follicular, GC and MBCs. After a secondary immunisation of mice with ovalbumin, about 80-95% of the resulting PCs are short-lived PCs (SLPC) and contract after resolution of GCs, whilst the remaining can develop into LLPC (Manz *et al.* 1997). LLPC can have different IgG subclasses and tend to have more V-gene mutations than SLPC and MBCs (Smith *et al.* 1997). As they develop, dendritic cells and monocytes/macrophages secrete IL-6 and APRIL, providing survival factors to the PC as it migrates from the perivascular areas to the medullary cords (Moisini & Davidson 2009). Distinct changes in phenotype occur in LLPC intermediaries as they appear in the circulation on their way to the bone marrow. Recently, the Blimp^{GFP} reporter strain was used to define developmental stages of PC differentiation by characterising the phenotypic features of PCs at different stages of increasing BLIMP-1 expression (i.e. at different stages of maturity into terminally differentiated LLPC) (Kallies *et al.* 2004). An increase in GFP expression from low to high was associated with an increasing Ig secretion and a decreasing proliferative capacity (both downstream effects of BLIMP-1), confirming their identity as terminally differentiated PCs. As plasma cells accrued BLIMP-1 expression, they also downregulated B220, CD19 and MHCII, and all GFP^{high} cells in the bone marrow were CD19⁻ B220⁻, MHCII^{low}. All GFP⁺ cells expressed highly heterogeneous levels of CD138 (syndecan-1), a commonly used marker for ASC, as well as CD38, CD62L and CD43. Both GFP^{int} cells in the spleen and GFP^{high} cells in the bone marrow were CXCR5^{low} CXCR4^{high}, indicating that the transcriptional programmes mediating LLPC trafficking to the bone marrow occurred early in LLPC development.

The phenotype of LLPC precursors was also investigated using a Ig transgenic B cell adoptive transfer system by O'Connor and colleagues (O'Connor *et al.* 2002), where the transferred cells could be tracked with congenic and allotypic markers as they progressed through the primary immune response. Two weeks post-immunisation, Tg post-GC LLPC precursors in the bone marrow could be distinguished by their downregulation of B220, MHCII and surface Ig, and upregulation of LFA-1, VLA4, CD80, CD86, CD126 and CD127, when compared to Tg naive splenic B cells. Interestingly, irradiation of the mice at 7 and 14 days after immunisation caused >70% loss in BM ASC, whereas irradiating at Day 28 and 49 after immunisation did not result in this decay, indicating that PCs that enter the BM 2-3 weeks after immunisation are the true LLPCs.

These phenotypic changes have functional consequences. First, by downregulating surface Ig, MHCII and co-stimulatory molecules, the PC becomes unable to take, process and present antigen and unresponsive to further antigen stimulation. Second, they enable PCs to leave the GC and migrate to the bone marrow as LLPC precursors or terminally differentiated LLPC. Third, surface adhesion molecules like integrin $\alpha_4\beta_7$, E- and P-selectin, the integrins LFA and VLA, CD44, CD11a and CD18, or other molecules like B cell maturation antigen (BCMA) (O'Connor *et al.* 2004), CD28 and CD93 (the function of which is unknown) (Chevrier *et al.* 2009) are essential for LLPC to remain in the bone marrow niches, or increase their 'stickiness' in bone marrow niches in the face of competition from migratory plasmablasts, as deficiency or loss of these markers by loss-of-function genetic manipulation or injection of blocking antibodies results in normal LLPC development but inefficient retention in the bone marrow.

Trafficking from the spleen to bone marrow survival niches is regulated by selective expression of chemokine receptors and adhesion molecules. Developing LLPCs in the GC downregulate surface expression of the chemokines receptors CXCR5 and CCR7 (Ellyard *et al.* 2005), which control the trafficking of activated B cells into and within the GC; this enables the LLPC precursors to disengage from the original site of activation. Most plasmablasts, whether destined to become SLPC or LLPC, upregulate surface expression of

CXCR4 after activation, and this causes them to migrate towards its ligand CXCL12, which is expressed in by reticular cells in the red pulp of the spleen, medullary cords of the lymph nodes as well as stromal cells in the bone marrow (Hargreaves *et al.* 2001;Wols *et al.* 2002), clearly marking a pathway of egress from GCs to the bone marrow. LLPCs and CXCL12-secreting bone marrow stromal cells are very closely apposed (Tokoyoda *et al.* 2004). The specific LLPC stromal cells are VCAM-1⁺ PECAM⁻ IL-7⁻ which comprise approximately 17% of bone marrow mesenchymal stromal cells (Tokoyoda *et al.* 2004), and are believed to be responsible for limiting the estimated capacity in mouse BM for LLPCs at approximately 10⁶ PCs at any one time (Radbruch *et al.* 2006). These stromal niches could play an important role in maintaining LLPC ‘stickiness’, which makes them less susceptible to dislocation by the influx of new plasmablasts. Long-term PC survival in the spleen also depends on colonisation of limited niches, initially in close association to CD11chi mature DCs but later dispersing the red pulp. However the responsiveness of plasmablasts generated by a secondary immune response to CXCR3 and CXCR4 ligands only lasts for 1-2 weeks as measured in transwell migratory assays (Hauser *et al.* 2002). Therefore the role of CXCL12 expressed by stromal cells may be limited to attracting newly produced LLPC precursors to the bone marrow, rather than be a LLPC survival factor. After two weeks, LLPC precursors and LLPC lose all recirculatory potential and die if not contained within survival niches. Competition of LLPC precursors for limited survival niches is mediated by phenotypic changes that occur during development (Radbruch *et al.* 2006).

Expression of CXCR3 appears to be conditionally expressed on plasmablasts after exposure to interferon- γ (IFN γ) (Muehlinghaus *et al.* 2005), which is present in inflamed tissue. This attracts plasmablasts to migrate to sites of original infection and then, as IFN γ levels drop after resolution of inflammation, to be released. This is an important homeostatic regulatory system to prevent the accumulation of potentially autoimmune Ab-secreting cells in peripheral tissues. The persistence of inflammation is probably a precondition for the generation of pathogenic ectopic LLPC niches, which are found in many autoimmune diseases such as rheumatoid arthritis (Tsubaki *et al.* 2005).

Ex vivo bone marrow LLPC die within 24 hours, but can be rescued by *in vitro* culture with cytokines like interleukin (IL)-5, IL-6 (in synergy with CD44), tumour necrosis factor (TNF)- α , (Cassese *et al.* 2003) and a proliferation-inducing ligand (APRIL), which is a ligand for CD138 (syndecan-1) (Ingold *et al.* 2005). Although the cytokines IL-6, IL-10 and IL-5, are able to support LLPC survival in *in vitro* culture, mice deficient in these cytokines are still able to generate and maintain normal numbers of LLPC, suggesting that they are possibly redundant survival resources. IL-21 is required by B cells in order to differentiate into LLPC but whether it is required for the survival of LLPC is also unclear. Mice injected with blocking antibodies for APRIL (Benson *et al.* 2008) or B cell activating factor (BAFF) (Avery *et al.* 2003) have reduced numbers of LLPC. In APRIL-deficient mice, there is delayed but eventually normal accumulation of LLPC in BM, although class switching to IgA and survival of isotype switched MBC is impaired. Co-blockade of APRIL and BAFF has a significant impact on B cells, depleting naïve B cells, GC B cells and PC compartments, whilst leaving only the MBC compartment untouched, demonstrating that LLPC and MBC are discretely regulated compartments with independent survival needs. The cognate receptors of BAFF and APRIL, which are BCMA and TACI are important for survival of PCs in the bone marrow (Benson *et al.* 2008). APRIL has been shown to be secreted by a number of different bone marrow-resident cells, including eosinophils (Fröhlich *et al.* 2011), bone marrow macrophages (Belnoue *et al.* 2008), and in the periphery, inflammatory neutrophils can secrete APRIL and create ectopic PCs niches in the mucosa (Huard *et al.* 2008).

1.5: Maintenance of serum antibodies in the absence of antigen

After the clearance of infection and resolution of GCs, there is no or very little antigen remaining in the host. The half-life of serum Ig has been estimated to be about 3 weeks in humans, and about 1 week in mice (Vieira & Rajewsky 1988). Despite this, serum Ab can be maintained at persistently elevated titres for very long time, for up to 90 years in humans (Yu *et al.* 2008b) and for at least 250 days in the mouse, in the absence of re-infection.

MBC and LLPC both contribute to this maintenance of high Ab titres, however how they do this is still unknown.

The necessity for persisting antigen for the maintenance of long-lived serum is difficult to determine, because it is difficult to ascertain whether there are minute amounts of residual or cross-reactive antigens in both humans and laboratory animals. It has been argued that persisting antigen is necessary for the maintenance of high titres of Abs and it is dependent not on memory T and B cells nor on LLPCs, but on continuous antigen-driven maturation of naïve B cells to PCs (Gray 2002; Tarlinton 2006; Traggiai *et al.* 2003; Zinkernagel *et al.* 1996). The prolonged generation of LLPC for weeks to months after the original stimuli, with PC continuing to leave the secondary lymphoid organs from residual GCs, suggests this may be true and continuous replenishment of short-lived PCs may be necessary to replenish serum Ab titres. As described earlier, the finding that significant numbers of MBC are localised in persistent GCs in the spleen for up to 8 months after primary immunisation, and that the artificial programming of persistent Abs to Influenza requires the generation of persistent GCs in lymph nodes for up to 1.5 years, is evidence that antigen persistence and continuous differentiation of B cells and MBCs into PCs is important for the maintenance of long-term Ab titres.

In order to overcome the difficulties in obtaining an antigen-free system, one group made use of a mouse strain where the BCR specificity could be genetically switched following immunisation, so that the MBC no longer recognised the antigen that induced them (Maruyama *et al.* 2000). This showed that MBC could persist for long periods of time in the absence of cognate BCR stimulation. However, in a conditional knock-out mouse model where the enzyme phospholipase C γ 2 (PLC γ 2), which is a downstream component of BCR signalling, was excised with recombinant Cre under the C γ 1 promoter, the secondary humoral response was impaired due to a drastic reduction in the number of GCs and MBCs, demonstrating that BCR-mediated signals are necessary for the survival of MBC. The role of antigen sequestration on FDCs for the maintenance of MBCs has also been investigated but has given contradictory results. One group used mice where B cells

were unable to secrete Ig and therefore could not make immune complexes which could be bound by FDCs, and showed that immune complexes sequestration on FDCs was not necessary to maintain a long-lived MBC population (Hannum *et al.* 2000). By contrast, in a bone marrow chimera system where FDCs were deficient in complement receptor 2 (CR2) and therefore could not bind immune complexes, while B cells were wild-type, there was poor maintenance of serum IgG titres as well as an impaired secondary response due to a reduction in GC and MBC (Fang *et al.* 1998).

Nevertheless, immune complexes sequestered on FDCs has a ‘natural’ half-life and their decrease may be hastened by internalisation by centrocytes, meaning that, if Ab titres were dependent on continuous antigen-driven differentiation of B cells into ASC, then Ab levels would decay at the same rate as the decay of GCs or immune complexes (Kesmir & De Boer 1999). Mathematical modelling showed that the size and duration of GC reactions and indeed, the generation and rescue of high-affinity centrocytes as they recycle through the light and dark zones of the GC, is directly correlated with the availability (half-life and dose) of Ag, (Kesmir & De Boer 1999). The half-life of a protein Ag on FDCs seems to be of the order of 30 days (Oprea & Perelson 1997), but MBC and serum Ab can last for up to a lifetime of a mouse, and for up to 90 years in humans. Therefore the longevity of Ab outlasts the longevity of antigen sequestered and brings into question whether antigen alone is capable of maintaining serum Ab titres for very long-term time points.

It is not known whether both MBCs and LLPCs are equally generated by the immune response and can equally survive over the years. Certainly the inflammatory context of the infection or vaccination can influence the differentiation of B cells towards either MBC or LLPC. Their lifespans are probably genetically imprinted and MBC and LLPC appear to rely on mutually independent mixtures of intrinsic and extrinsic resources for survival. MBCs undergo a slow homeostatic turnover rate of less than 1 division per month and, unlike all other B-2 cell subsets and LLPC, are independent of the cytokines BAFF and APRIL for survival. By contrast, LLPC are terminally differentiated, do not undergo mitosis, and can maintain a non-proliferating, senescent phenotype *in vivo* for as long as 90

days, as demonstrated by bromo-deoxyuridine pulse-chase labelling of splenic LLPC after immunisation of mice with 4-hydroxy-3-nitrophenyl acetyl (NP)(Sze *et al.* 2000). They are crucially reliant on a specific transcriptome (Blimp-1, *Aiolos*, XBP-1, Ets-1, anti-apoptotic factors like BCL-2 family protein A1, A20 and IAP-2) and compete for external survival signals like BAFF and APRIL for survival against the stresses of continuously secreting large amounts of immunoglobulin (reviewed by (Chu *et al.* 2011;Radbruch *et al.* 2006;Shapiro-Shelef & Calame 2005)). The anatomic distribution of MBC and LLPC are also different – 80-90% of PCs reside in the bone marrow (Manz *et al.* 1997), while the anatomic distribution of IgM⁺ and IgG⁺ MBCs appears to be largely influenced by the context of different infections and immunisation strategies, as described previously (Doherty 1995).

Generally, models of how serum antigen-specific Ab responses are maintained are divided into MBC-dependent or LLPC-dependent mechanisms. In the first, Abs are maintained either by repeated re-stimulation of MBCs into short-lived PCs or LLPCs. Re-stimulation can occur through the BCR by re-infection, scheduled boosters, sequestered antigen or cross-reactive antigens, or in any inflammatory context with TLR ligands and bystander T cell help. If levels of serum Abs are a direct result of MBC, there would be peaks and troughs of Ab levels, where Ab levels correlate with numbers of circulating specific MBCs. In the second LLPC-dependent model, Abs are secreted autonomously by LLPC, where Ab levels are stable and correlate with numbers of LLPCs, do not correlate with MBC numbers and are independent of antigen stimulation (Amanna *et al.* 2007). These models are outlined briefly below, but longitudinal correlation studies in humans as well as the findings of various MBC or LLPC depletion studies will be discussed in greater detail in **Chapter 3**.

1.5.1: Antibody maintenance by long-lived plasma cells

The ability of LLPC to maintain serum Ab titres independently of MBC was demonstrated by two groups in the late 1990s. In the study by Manz *et al.*, irradiation of LCMV-immune

mice, which depleted all subsets of B cells including MBC and short-lived PC but not radiation-insensitive LLPC, demonstrated that in the absence of MBC, a substantial number of LLPC were still detected up to 250 days later, showing that LLPC can survive for long periods of time in the absence of replenishment by MBC (Manz *et al.* 1997). In the other study by Slifka *et al.*, adoptive transfer of OVA-specific LLPCs from bone marrow, but not MBCs, conferred specific and long-lasting Ab titres to antigen-free IgH syngeneic recipients, showing that LLPCs survived and were capable of continuously producing Ab in the absence of antigen or MBC (Slifka *et al.* 1998). More recently, two MBC depletion studies by Amana *et al.* and DiLillo *et al.*, where MBCs were depleted using anti-CD20 mAbs showed that, in spite of rapid and drastic depletion of the MBC compartment, LLPC numbers in spleen and bone marrow as well as pre-established serum Ab titres remained constant (Ahuja *et al.* 2008; DiLillo *et al.* 2008).

Some investigators argue that the Ab-secreting population in the bone marrow are not all uniformly terminally differentiated LLPC, but contains of a group of pre-LLPC precursors, which are able to undergo a final differentiation step to becoming a ‘true’ LLPC (O’Connor *et al.* 2002). They can theoretically do this when specific ‘true’ LLPCs diminish in number; hence they are the most likely precursor cell to replenish the LLPC niche. The exact transcriptional mechanisms which ‘halt’ development at the pre-LLPC stage, and what signals trigger the final differentiation step into LLPC, are not clear. Little is known about whether they are capable of undergoing homeostatic turnover, which would make them a self-replenishing pool of cells capable of replenishing the LLPC niche.

1.5.2: Antibody maintenance by memory B cells

LLPCs have a finite lifespan with a half-life of about 138 days in mice, and over time, LLPCs are believed to turnover, or undergo attrition from their finite niches by competition from new migrating plasmablasts generated by heterologous infections. This has led some investigators to argue that in these situations, long-lived serum Ab could be replenished by MBC (or, as described above, by ‘LLPC precursors’). MBC have a low turnover rate just

sufficient to maintain its own frequency and repertoire as a group. MBCs differentiate into Ab-secreting cells (ASCs) after stimulation through the BCR, or through signals from cytokine receptors and TLRs (Traggiai *et al.* 2003). In humans, MBCs constitutively and exclusively express high levels of Toll-like receptors, e.g. TLR9 (which binds unmethylated CpG) and TLR4 (which binds bacterial lipopolysaccharides) (Bernasconi *et al.* 2003). This allows only MBCs, and not naïve B cells, to respond to innate signals and differentiate into plasmablasts even in the absence of antigen-specific BCR signalling. Bystander CD4⁺ T cell help *in vitro* also stimulates non-specific MBCs to differentiate into PCs, possibly because of increased availability and upregulation of co-stimulatory molecules and production of Th2 cytokines (Bernasconi *et al.* 2003; Bernasconi *et al.* 2002). *In vitro*, addition of TLR9 agonists effectively elicited polyclonal MBC differentiation into PCs, whilst non-follicular naive B cells required TLR9 agonist as well as BCR and CD40 stimulation to promote a similar response. In human subjects receiving a booster for tetanus toxoid (TT) vaccination, besides the increase in TT-specific Ab, there was a polyclonal boost of lower magnitude for Ab titres for unrelated antigens, suggesting that during an antigen encounter, bystander help and the inflammatory context could stimulate some unrelated MBC to differentiate into PCs. However, this has not been subsequently confirmed in other studies in mice (Benson *et al.* 2009) or humans (Amanna & Slifka 2010), there are now alternative hypotheses proposed to explain the boost in non-specific Ab, for example, the residual Ab generated by a very prolonged primary immune response triggered by residual antigen sequestered on FDCs, or that the spike in unrelated serum Ab is a consequence of dislodgement of pre-existing LLPC from the BM niches by newly formed plasmablasts. MBC may ‘sense’ deficiencies in ‘old’ PCs or reduction in specific nAb titres, either by purely stochastic means or a negative feedback mechanism, and react by differentiating into PCs, which migrate to the bone marrow to re-enhance levels of specific nAb in serum.

The second aim of this thesis is to investigate what happens to pre-established humoral immunity during sequential heterologous infections. In this introduction, the humoral

immune responses to Influenza A and malaria infection will be described, as these are the two infectious models used in my experiments.

1.6: Influenza overview

Influenza is an infection caused by the RNA virus of the family Orthomyxoviridae. It is transmitted through the air in aerosols containing virus, or by direct contact with bird droppings or nasal secretions. In this thesis, the virus that is used is one of the five genera of the family, the Influenza A virus. Following infection of mice with a replication-competent recombinant influenza virus carrying a GFP reporter gene in the NS segment (NS1-GFP virus), which allowed visualisation of the early events of Influenza A infection, it was recently shown that Influenza A viruses grow rapidly in the respiratory mucosa, with respiratory epithelial cells being the primary target, where they replicate to produce large amounts of virus that then infects alveolar macrophages and local dendritic cell populations, B cells and NK cells. Infection with Influenza A viruses begins near the trachea and main stem bronchi, spreading with time into bronchioles (Manicassamy *et al.* 2010). Inoculation of influenza virus by the intranasal route results in a highly localised pulmonary infection because of the dependence of viral replication on a trypsin-like enzyme that is largely restricted to respiratory epithelial cells (Steinhauer 1999).

1.6.1: Innate immune response

The Influenza A virus single-stranded RNA triggers all three major families of the innate pattern recognition receptors (PRRs), including Toll-like receptors (TLR), Nod-like receptors (NLR), and the host cell cytoplasmic RNA helicases called retinoic acid-inducible gene I (RIG-I) like receptors (RLR) (Pang & Iwasaki 2010; Sanders *et al.* 2011). Activation of downstream pathways following triggering of these PRRs leads to production of Type 1 interferons (IFN α and IFN β), IL-1, IL-18 and IL-6, which creates an ‘anti-viral’ state, inhibiting protein synthesis in host cells and limiting virus replication (Alexopoulou *et al.* 2001). Influenza single-stranded RNA activates the NLR, NOD-like receptor family, pyrin domain containing 3 (NLRP3), which induces the formation of the inflammasome, generating active caspase-1, which cleaves pro-IL-1 β , IL-18, etc., resulting in the release of these cytokines, which are involved in the induction of Th17 and CD4⁺ T cell responses

(Allen *et al.* 2009). Activation of RIG-I is important for early cytokine production (Pichlmair *et al.* 2006). Activation of these early viral recognition signals is essential for protecting mice from death.

A number of resident innate lymphocytes like alveolar macrophages (Kumagai *et al.* 2007), DCs (Piqueras *et al.* 2006) and NK cells (He *et al.* 2004;Mandelboim *et al.* 2001), as well as respiratory epithelial cells (Herold *et al.* 2006), are activated through these PRRs (Tumpey *et al.* 2005). In addition, infiltrating cell populations like inflammatory monocytes, DCs migrating from lung and blood (Belz *et al.* 2004), natural killer (NK) cells and neutrophils are attracted by changes in chemokine expression on the respiratory epithelium (Herold, von Wulffen *et al.* 2006). The increase in innate cells in the lung results in inflammation, production of nitric oxide synthase (NOS) and direct killing of infected epithelial cells by the triggering of apoptosis through a TNF-related apoptosis-inducing ligand (TRAIL)-dependent manner. Furthermore, antigen presentation (GeurtsvanKessel *et al.* 2009a) initiation and maintenance of the GC reaction (GeurtsvanKessel *et al.* 2009b) leads to the activation and regulation of specific adaptive immune responses, primarily consisting of B cell and CD8⁺ T cell proliferation and survival (Wijburg *et al.* 1997).

1.6.2: Humoral immune response

Despite intranasal Influenza A infection being localised to the respiratory epithelium, it generates both a local and systemic humoral response, a phenomenon which is similar to the humoral immune response towards most respiratory infections. The humoral response is both essential for clearance of virus during primary infection (Gerhard *et al.* 1997) and for long-term protective immunity (Waffarn & Baumgarth 2011). High titres of serum neutralising Ab specific for the virus variable surface glycoprotein haemagglutinin (HA) and, to a lesser extent, against neuraminidase (NA), correlate very well with protective immunity after both natural infection and intramuscular vaccination. A single natural infection with any strain of influenza virus is capable of eliciting long-lived Ab-mediated

protection against the homologous infection, as evidenced by protective titres of nAb found in donors infected with the 1918 ‘Spanish’ influenza virus 90 years previously (Yu *et al.* 2008a), as well as after experimental Influenza A infections in mice (Hyland *et al.* 1994; Jones & Ada 1987).

Humoral responses towards respiratory viruses can be generated locally in the nasal-associated lymphoid tissue (NALT) and bronchus-associated lymphoid tissue (BALT) (Rangel-Moreno *et al.* 2005; Wijburg *et al.* 1997; Zuercher *et al.* 2002). B cells in the mediastinal lymph node encounter viral antigens transported from the site of infection by DCs by day 3 of infection, or directly capture viral antigens in the mediastinal lymph nodes (MLN) or at site of infection for transport to the lymph nodes (Manicassamy *et al.* 2010). GC-like structures, with distinct B and T cell zones appear in the BALT, and are sites of B cell proliferation. Furthermore, in the absence of peripheral lymphoid organs like spleen, lymph nodes and Peyer’s patches, the isolated immune response in the BALT was able to clear influenza infection and result in a small measure of protective immunity (Moyron-Quiroz *et al.* 2004). Both GC and extrafollicular B cell responses can be induced by Influenza A infection, although MBC and LLPC are predominantly produced by the GC reaction.

The mucosal humoral immune response is distinct from the systemic humoral immune response, in terms of having its own long-term niches for MBC and LLPC, and mucosal Abs have a different profile from serum Abs (e.g. IgA > IgG). The NALT is a site of residence for MBC and LLPC and long-term virus-specific Ab production (Liang *et al.* 2001). NALT is composed of a pair of **organised** (O-NALT) lymphoid aggregates located on the entrance to the nasopharyngeal duct and the less well-organised **diffuse** (D-NALT) lymphoid tissue lining the nasal passages. Local Ab-secreting cell (ASC) generation in NALT of BALB/c mice parallels detection of anti-HA Abs in nasal wash and correlates with viral clearance from the nose (Tamura *et al.* 1998). The frequency of ASCs is much greater in D-NALT than O-NALT over the course of a primary infection, with a higher number of ASCs continuing to secrete Ab for a longer time. Long-term virus-specific Ab is

detectable in the D-NALT 18 months after primary infection, whereas no ASCs are detectable in O-NALT after approximately 5 months. This appears to be a feature of respiratory infections, as intranasal immunisation with inactivated respiratory syncytial virus (RSV), in combination with bacterial outer membrane vesicles as adjuvant, also generates a population of NALT-resident RSV-specific ASCs and local long-term Ab production (Etchart *et al.* 2006).

Joo and colleagues (Joo *et al.* 2008) have demonstrated that MBC induced by influenza become broadly dispersed and localized to the lung mucosal tissue, particularly O-NALT, and do not undergo a contraction phase, unlike recirculating MBC, indicating that the O-NALT may be a unique depository for MBC after mucosal infection. The O-NALT and MLN, but not D-NALT, have measurable HA-specific IgA MBC after 8 weeks of infection. The expression of the integrin $\alpha_4\beta_7$ on IgA-expressing MBC leads to their preferential entry into O-NALT, where its ligand, MadCAM-1, is highly expressed. Given the preferential localisation of MBC to O-NALT, and PCs to D-NALT, the O-NALT could represent the 'central memory' niche, while D-NALT could support 'effector memory' PCs. Another study showed that a small subset of lung MBC bore similar surface markers to that of splenic MBC and were $CD73^+$ $CD80^+$ $CD273^+$ $CD69^+$ $CXCR3^+$, and persisted for at least 5 months, and mounted localised protective IgG and IgA responses to secondary challenge (Onodera *et al.* 2012). Recently, an effort to 'programme' long-lived Ab responses after vaccination of mice with synthetic nanoparticles containing Influenza antigens with adjuvant TLR-ligands demonstrated that long-lived Ab titres correlated with enhanced persistence of GCs and of PC responses, which persisted in the lymph nodes for >1.5 years (Kasturi *et al.* 2011), consistent with the idea that MBC play a significant role in maintaining protective immunity against secondary infection through continuous generation of PCs.

Ab-mediated protection from live-virus challenge is a combination of local neutralising Ab, secretory IgA and serum neutralising Ab; however, the relative importance of each is not clear. It is thought that mucosal Ab is correlated to protective immunity after natural

infection whilst serum Ab is correlated to protective immunity after intramuscular vaccination, and that serum Ab is particularly important during infection with highly pathogenic virus, viral pneumonia and immunity in the longer-term (Palladino *et al.* 1995). After primary influenza infection, PCs leave the GCs and appreciable numbers of PCs are seen in accumulating in bone marrow, spleen, mesenteric lymph nodes and lung, where they maintain high titres of nAb (Fazekas *et al.* 1994). Haemagglutinin (HA) is a lectin on the surface of the viral particle which mediates viral binding to sialic acid on the surface of cells, and enables fusion of the virus to the endosomal membrane and facilitates the release of virus RNA into the host cell cytosol (Markwell & Paulson 1980). It is the predominant critical target structure for the immune response mechanisms which eliminate Influenza virus. Preformed virus-specific Ab directed against HA, and to a lesser extent, NA or the matrix 2 (M2) proteins, in serum or in the airway mucosal surface can block virus entry and subsequent establishment of infection, which is important because, once established in respiratory epithelium, the virus is capable of extremely rapid replication. *In vivo*, only passive transfer of anti-HA Abs are able to neutralise the virus and prevent disease (i.e. offer sterilising immunity), but not Abs directed against other surface glycoproteins like NA or non-structural proteins like nucleoprotein (NP). NA-specific Ab do not block the initiation of infection but inhibit virus release, therefore they do not confer sterilising immunity but do protect from morbidity and mortality. Anti-M2 Abs provide only partial protection and are mainly studied as a vaccine target, as it is very poorly generated by a natural infection (Feng *et al.* 2006; Jegerlehner *et al.* 2004). Most of the antigenic structures recognised by neutralising anti-HA Abs are condensed within the HA1 polypeptide, and are highly specific for one of five defined recognition sites on the surface of the HA molecule. Likewise, the majority of the PC and MBC clones derived from mouse and human donors are specific for these few sites in HA1 (Caton *et al.* 1982; Wiley *et al.* 1981). NAbs are so specific that minor changes in the HA1 structure (antigenic drift) in its structure can lead to escape mutants, whilst major changes (antigenic drifts) leads to serologically distinct sub-strains with little sub-heterotypic cross-reactivity between anti-sera (Horimoto & Kawaoka 2005).

Another important feature of Influenza A immunity, particularly as a vaccine design strategy, is pre-existing antigen-specific CD8⁺ T cells directed towards conserved viral regions. In mice, in the absence of CD8⁺ T cells, virus clearance is delayed and pathology is augmented (Eichelberger *et al.* 1991). An expansion and trafficking of large numbers of virus-specific CD4⁺ and CD8⁺ T effector cells into the lung occurs within 1 week of infection (Flynn *et al.* 1998; Lawrence & Braciale 2004; Novak *et al.* 1999; Román *et al.* 2002). Memory CD8⁺ T cells are able to rapidly reduce the viral load through various mechanisms, e.g. production of pro-inflammatory cytokines like IFN γ , TNF α and IL-2 (Carding *et al.* 1993; Xu *et al.* 2004) and granule exocytosis or FasL-mediated killing of infected epithelial cells (Hou & Doherty 1995; Topham *et al.* 1997). However, an over-exuberant CD8⁺ T cell response, for example in the case of the highly pathogenic avian Influenza, is thought to mediate pathology which can be fatal (Mauad *et al.* 2010). CD4⁺ T cell help is important for CD8⁺ T cell primary and recall responses, as the size and function of the CD8⁺ T cell pool is smaller and clearance of virus is delayed in CD4⁺ T cell-deficient mice (Belz *et al.* 2002; Bennett *et al.* 1997). CD4⁺ T cells are also essential for providing help for the developing virus-specific B cell response and humoral immunity (Brown *et al.* 2006; Topham & Doherty 1998). Primed HA-specific TCR transgenic CD4⁺ T cells which are adoptively transferred and challenged can also have direct anti-viral activity through perforin-mediated cytolytic activity and induction of pro-inflammatory cytokine production (Brown *et al.* 2006).

1.7: Malaria overview

Malaria is caused by the single-cell protozoan parasite from the genus *Plasmodium*. There are five *Plasmodium* species known to infect humans – *Plasmodium falciparum*, *P. vivax*, *P. ovale* and *P. malariae*, which are transmitted to humans through the mosquito vector, and *P. knowlesi*, which is zoonotic and is transmitted by mosquito from its other host, the long-tailed macaque (Lee *et al.* 2011). Due to the limitations of obtaining and analysing human samples, animal models are an important resource to delineate many of the protective mechanisms against malaria (Craig *et al.* 2012; Lamb *et al.* 2006; Langhorne *et al.* 2002; Sanni *et al.* 2002), but there are important caveats to consider when translating the data in the context of human infection, for example, the fact that rodent parasites are not natural mouse pathogens and that initiation of experimental infection can occur by unnatural routes which bypass the natural intradermal route of inoculation via the mosquito bite. Mosquito-transmitted infections have often been done in mouse models, particularly with *P. berghei*. However there are still very few studies focusing on the cellular immune responses that occur after this infection route. Nevertheless, it is one of the best experimental systems for delineating the immune responses that can occur in the field. There are several mouse models of malaria, and the one used in this thesis is blood-stage infection with *P. chabaudi*, which has a synchronous 24h erythrocytic life-cycle and has a well-characterised acute phase and a chronic relapsing and remitting phase which lasts for up to 2-3 months. *P. chabaudi* has a variety of cloned lines and causes a spectrum of disease patterns, which are determined by both the parasite strain and host background. One of them, *P. chabaudi chabaudi* (AS), is a well-characterised model for immunological studies and is the one used in this study. A summary of the main mechanisms of protective immunity to *P. chabaudi* is in **Table 1.3** and are also reviewed by (Good *et al.* 2005; Lamb *et al.* 2006; Langhorne *et al.* 2002; Sanni *et al.* 2002; Stephens *et al.* 2011).

1.7.1: Immunity to malaria

There are two distinct forms of immunity to malaria – clinical immunity, which refers to protection against disease severity, and parasitological immunity, which refers to a reduction in parasitaemia. Anti-disease clinical immunity is acquired rapidly in endemic places and can be observed in young children and results in reduced mortality and severe clinical disease (Gupta *et al.* 1999) (**Figure 1.2a**). This is probably due to a ‘learned’ regulation of the inflammatory cytokine response to the pathogen. Conversely, anti-parasite immunity is more slowly acquired even in endemic areas, and results in reduction of high density parasitaemia which can be mostly seen in older age groups. However, in almost all cases, sterilising immunity is never achieved and asymptomatic carrier status is the norm in adults. Furthermore, there is strong evidence that the parasite can evade the host immune response through antigenic variation (Brown & Brown 1965). The relationship between clinical symptoms of malaria, parasitaemia and the host immune response are extremely complex and can be affected by a combination of host, parasite and environmental factors, for example, the stage of life when malaria is first encountered (infants, children, adolescence or adulthood), pregnancy and parity, co-morbidity or co-infection and medical/drug history, nutrition status, endemicity of the area, transmission intensity, and host genetic background (Doolan *et al.* 2009). There is a fine balance in the host immune response which determines whether the immune response leads to disease resolution or leads to severe malaria syndromes, namely severe malarial anaemia, cerebral anaemia and respiratory distress. Studies in Mali, Papua New Guinea and India have shown a correlation between increased levels of both pro-inflammatory cytokines like TNF α , IL-1B, IL-6 and IFN γ (Lyke *et al.* 2004;Prakash *et al.* 2006;Robinson *et al.* 2009) as well as the anti-inflammatory cytokine IL-10 (Kurtzhals *et al.* 1998;Othoro *et al.* 1999) with severe disease syndromes. In addition, host polymorphisms are associated with greater host susceptibility to immune pathology in response to malaria, particularly in genes encoding IFN γ , IRF, TNF, IL-10 and IL-4 (Carpenter *et al.* 2007).

Most of the information of protective immune responses in humans to erythrocytic stage parasites has been provided by longitudinal studies in endemic populations and determined by passive-transfer experiments. Naturally acquired immunity to malaria is gradual and requires uninterrupted heavy exposure, and protective Abs are rapidly lost upon cessation of exposure or after receiving medical treatment (Cavanagh *et al.* 1998; Fonjungo *et al.* 1999; Giha *et al.* 1999; Jennings *et al.* 2006; Kinyanjui *et al.* 2007); however, this may not be a complete loss as some people exposed to malaria infection even 30 years earlier are more resistant to clinical disease than completely naïve subjects (Lim *et al.* 2004). There is little documented heterologous immunity, and immunity to a specific strain is also somewhat stage-specific, for example, naturally-acquired immunity to *P. falciparum* appears to be mainly driven against the erythrocytic stages (Owusu-Agyei *et al.* 2001). Repeated infections brings about a broadening of specificity that helps with immunity against different stages, and an accumulation of a sufficiently diverse repertoire to recognise most variants encountered in the field, and at this stage there may be some heterologous immunity (Crompton *et al.* 2010; Weiss *et al.* 2010). Those exposed since birth have a very high degree of protection that is broader in scope. Interestingly, in vaccination strategies where the erythrocytic stages of the parasite life cycle are curtailed, for example by irradiation (Nussenzweig *et al.* 1969; Silvie *et al.* 2002) or genetic modification of the sporozoite (Mueller *et al.* 2004; Mueller *et al.* 2007), or by curative drug treatment (Belnoue *et al.* 2004; Friesen *et al.* 2010; Putrianti *et al.* 2009; Roestenberg *et al.* 2009), long-term sterile protection is established even after a single infection, making it the basis of the most advanced malaria vaccine strategy at present. Stage-specific immune mechanisms can be finely dissected through analysis of patients or animal models immunisation directed against the intrinsic mechanisms of immunity to pre-erythrocytic and erythrocytic stages (**Table 1.4** and **Figure 1.2b**).

1.7.2: Humoral immune responses to malaria

In humans, transfer of sera from ‘asymptomatic carrier’ adults in hyperendemic areas to children or naïve individuals is able to drastically reduce parasitaemia, particularly when γ -

globulin therapy was started during high density parasitaemia (Cohen *et al.* 1961). Similarly, passive transfer of hyperimmune sera from *P. yoelli*-infected mice can protect against re-infection or reduce parasitaemia in recipient mice (Jayawardena *et al.* 1978; White *et al.* 1991). Furthermore, mice which have a targeted disruption of the transmembrane exon of the Ig mu-chain gene (mu-MT mice), and hence are B cell-deficient, are not able to clear a blood-stage *P. chabaudi* infection, but have a fluctuating chronic parasitaemia, which can then be eliminated by the adoptive transfer of B cells (Der Weid *et al.* 1996), highlighting the importance of B cells and Ab for clearance of parasite. Malaria-specific Abs can mediate a number of anti-parasitic and anti-toxic functions, including:

- Inhibition of merozoite invasion of erythrocytes and intra-erythrocytic growth (Blackman *et al.* 1990)
- Enhancement of parasite clearance by preventing resetting and sequestration in small vessels (Carlson *et al.* 1990)
- Enhancing phagocytosis opsonisation (Bull *et al.* 1998)
- Ab-mediated cellular cytotoxicity (Bouharoun-Tayoun *et al.* 1995b)
- Providing help to developing T cell effector mechanisms (Langhorne *et al.* 1998)
- Inhibition of parasite-induced pro-inflammatory response, e.g. glycosylphosphatidylinositol (GPI)-specific Abs can neutralise GPI-induced TNF production by macrophages, which contributes to severe disease (de Souza *et al.* 2010)

The exact target antigen(s) on the surface of free merozoites or infected erythrocytes inducing protective immunity are not known and this is hampered by the fact that the majority of antigens encoded by the parasites are completely unknown. However the parasite's ability to evade the immune system suggests that it is unlikely for there to be a single effective target antigen. Indeed, vaccines directed against single antigens have limited success. In order to define the specificities of the Abs in malaria immune adults, Crompton and colleagues designed a protein microarray containing approximately 23% of

the *P. falciparum* 5,400-protein proteome and assessed antigen-specificity of plasma Ab taken from 220 individuals between the ages of 2-10 years and 18-25 years in Mali before and after the 6-month malaria transmission season. Although Ab increases were short-lived, there were modest incremental polyclonal increases in Ab reactivity with age, indicating that protective immunity is more likely to be a result of an accumulation of polyclonal Abs specific for a variety of antigens, and this eventually confers the increasing anti-parasite immunity in older age groups (Crompton *et al.* 2010). However, Abs in adults from hyperendemic areas are mainly directed towards the erythrocytic stages, particularly against the variant surface antigens (VSA) displayed on the surface of the infected erythrocyte.

Candidate antigens like the merozoite surface protein 1 (MSP1) and apical membrane antigen 1 (AMA1) have been identified by injection of monoclonal Abs which can block invasion by the merozoite, or show protective efficacy after vaccination with recombinant or native affinity-purified proteins (Holder & Freeman 1981). Several studies in mice showed that immunisation with the 19 kiloDalton sequence on the carboxyl terminal of MSP1, MSP1₁₉, elicits Ab-mediated protection particularly against a lethal challenge with *P. yoelii*, and this protection correlates well with MSP1₁₉-specific Abs (Daly & Long 1995; Ling *et al.* 1994; Tian *et al.* 1996). The homologous protein has been identified in several species of parasite including *P. falciparum* (Holder *et al.* 1985), and indeed, Abs against MSP1₁₉ can inhibit parasite growth *in vitro* (Egan *et al.* 1999). Moreover, MSP1 specific Abs form part of the endogenous Ab response against *P. falciparum* infection (Guevara Patiño *et al.* 1997). However, monoclonal Abs to MSP1 or MSP2 have not shown much inhibition of *P. falciparum* merozoite growth. More recently, it has been discovered that a blood-stage surface erythrocyte-binding protein on *P. falciparum*, the reticulocyte-binding protein homologue 5 (PfRh5), which forms a single receptor-ligand pair with the blood group antigen, Basigin (Crosnier *et al.* 2011), is essential for erythrocyte invasion by most strains of *P. falciparum*. PfRh5 is unique because it cannot be deleted in any *P. falciparum* strain and is therefore apparently absolutely essential for parasite growth as shown in blood stage culture (Baum *et al.* 2009). Importantly, parasite growth across several laboratory *P. falciparum* strains is inhibited by very low concentrations of anti-

Chapter 1: Introduction

Table 1.3: Effector immune responses of various cell types during infection with *P. falciparum* or *P. chabaudi*. Adapted from Stephens *et al.* 2012.

	Cell type	<i>P. falciparum</i>	<i>P. chabaudi</i>
Innate immune responses			
PRRs Byproducts of parasite metabolism like GPI, haemozoin, DNA, etc can activate various innate pathways: <ul style="list-style-type: none"> • TLR/MyD88 • NLR • C-type lectin receptors (CLR) 	Dendritic cells	<i>P. falciparum</i> : unclear; suppressive at times, but activate at low doses <i>P. vivax</i> : sporozoites activate DCs to kill hepatic stages. Produce cytokines IL-2, IL-10	Activation of CD11c ⁺ CD8 ⁺ DCs (Th1), and later post-peak of infection CD11c ⁺ CD8 ⁻ DCs (IL-4, IL-10) Antigen is processed within a 3-4 h timeframe to T cells Produce IL-2, IL-6, IL-10 and TNF
	Macrophages/monocytes	Monocytes phagocytose iRBCs. Macrophages sense parasite products such as GPI anchors and parasite DNA trapped in haemozoin, and produce inflammatory cytokines TNF, IL-6 and IL-12p40.	Produce IL-12 – associated with resistance Phagocytosis of iRBC and antigen presentation to T cells Produce iNOS.
	Inflammatory monocytes		Derived from atypical progenitors in the bone marrow – IFN γ -dependent Direct killing of iRBC by phagocytosis and ROS production
	NK cells		Produce IFN γ Cytotoxic effects on iRBC
	NK T cells		Act during the pre-erythrocytic stage by producing IFN γ and during the chronic phase by producing IL-4 to enhance the Ab response
	$\gamma\delta$ T cells		Contribute to protection during both acute and chronic phase as a source of inflammatory cytokines May be a back up when $\alpha\beta$ T cells are depleted
Adaptive immune response			
	CD4⁺ T cells	Mix of Th1 cells producing IFN γ with lower levels of Th2 and Th17 cells Th2 cells producing IL-4 Balance of IL-10 and TNF crucial Tregs correlated with susceptibility to infection	Th1 cells produce IFN γ ; Tregs and IL-10 ⁺ T cells regulate pathogenesis; TNF and IFN γ are crucial for clearance and host survival but cause pathology, IL-10 ⁺ T cells are crucial for ameliorating immunopathology Prime B cells Multi-functional CD4 ⁺ Th1 cells are associated with protection, e.g. TNF ⁺ IL-2 ⁺ IFN γ ⁺ while single producers are associated with immunopathology, e.g. IFN γ alone.
	CD8⁺ T cells		Produce IFN γ , IL-12, iNOS (in BALB/c) Lytic factors like perforin, granzyme B, FasL (in C57BL/6)
	B-1 cells		IgM-deficient mice are less able to control pathology and parasitaemia
	B-2 cells	Cytophillic antibody (IgG1) correlates with decreased parasitaemia Antibody to parasite variants correlates to exposure, protection Passive transfer of serum from previously infected donors or maternal Ab can offer protection Disorganisation of splenic architecture	Produce cytophillic antibody (IgG2a in mice) and IgG1. Passive transfer of Ab offers protection, where the number of infections given to donor mice correlates with greater protection Ab are necessary for parasite clearance particularly in the chronic phase Ab also aid the development of T cell help Large short-lived component to the Ab response and temporary disorganization of splenic architecture
	Memory cells	Specific Th1 cells shown to correlate with protection Memory B cells accumulate with repeated infections and can be long-lived	Effector memory Th1 cells develop, not exhausted Memory CD4 ⁺ T cells help to maintain long-lived Ab Functional and long-lived B cells generated

Table 1.4: Stage-specific mechanisms of immunity after various vaccination strategies.

Stage	Method of immunization	Primary immune response	Protective immunity against re-infection
Pre-erythrocytic stage	<p>Immunisation with whole irradiated sporozoites/radiation-attenuated sporozoites (RAS).</p> <p>Sporozoites take only seconds to reach the liver. They transmigrate into hepatocytes by traversing Kupffer cells, migrate through several hepatocytes before infecting a final one, with invagination of the cell plasma membrane to form a parasitophorous vacuole (Mota <i>et al.</i> 2001). Inside the vacuole, multiple rounds of nuclear divisions followed by schizogony results in release of thousands of erythrocyte-invasive merozoites. Merozoites are not delivered directly to the blood circulation but bud off from hepatocytes as merozoites (Sturm <i>et al.</i> 2006). Some can be trapped in mouse lung microvasculature, where they disintegrate and release merozoites in the lung capillaries (Baer <i>et al.</i> 2007).</p>	<p><i>In vitro</i>, sporozoites can interact directly with MOs and DCs. <i>In vivo</i>, sporozoites can encounter specialised DCs in skin, and liver (Plebanski <i>et al.</i> 2005).</p> <p><i>In vitro</i>, activated DCs incubated with <i>P. berghei</i> parasites can induce CSP-specific CD8⁺ T cells to produce IFNγ (Amino <i>et al.</i> 2006).</p> <p>CD8⁺ T cells are activated after DC priming in skin-draining lymph nodes and CD4⁺ T cell help</p> <p>Infiltrating neutrophils and macrophages create small granulomas in the liver and phagocytose infected hepatocytes and parasite material after merozoite formation, but <i>P. yoelii</i> and <i>P. berghei</i> parasites can escape phagocytosis (Sturm <i>et al.</i> 2006).</p>	<p>Experimental immunization of rodents, non-human primates and humans with multiple doses of RAS can induce sterile protective immunity (Nussenzweig <i>et al.</i> 1969). However immunity induced by RAS tends to be short-lived (Hoffman <i>et al.</i> 2002). RAS must remain capable of invading hepatocytes. Heat-inactivated sporozoites cannot induce protection.</p> <p>Neutralising Ab that inhibit sporozoite invasion and conventional $\alpha\beta$ IFNγ-secreting T cells that target the infected hepatocyte. The main target is likely to be CSP, which binds to liver HSPGs and allows them to rapidly sequester in the liver (Frevort <i>et al.</i> 1993). However further studies have cast doubt on the protective efficacy of vaccines directed against CSP (Gr⁺ner <i>et al.</i> 2007).</p> <p>IFNγ-producing CD8⁺ effector T cells are able to kill infected hepatocytes (Schofield <i>et al.</i> 1987). $\gamma\delta$ T cells (Lee <i>et al.</i> 2011) NK cells, NK T cells (Roland <i>et al.</i> 2006; Tsuji <i>et al.</i> 1994)</p>
	<p>Controlled WT-sporozoite inoculation under prophylactic cover with chloroquine or primaquine (Belnoue <i>et al.</i> 2004; Putrianti <i>et al.</i> 2009; Roestenberg <i>et al.</i> 2009) or antibiotic treatment (Friesen <i>et al.</i> 2010).</p>		<p>Sterile protective immunity (Belnoue <i>et al.</i> 2004; Putrianti <i>et al.</i> 2009; Roestenberg <i>et al.</i> 2009)</p> <p>Induces cross-stage immunity to pre-erythrocytic and erythrocytic stages (Belnoue <i>et al.</i> 2004; Roestenberg <i>et al.</i> 2009).</p> <p>CD4⁺ and CD8⁺ T cells and IFNγ and NO production (Belnoue <i>et al.</i> 2004). Role of B cells and Ab is still unclear.</p>
	<p>Genetically attenuated parasite (GAP) malaria vaccine.</p> <p>Genetic manipulation of rodent parasites which lack genes necessary for liver stage development, therefore generate sporozoites which can infect hepatocytes but are unable to mature (Mueller <i>et al.</i> 2004).</p>		<p>Sterile protective immunity</p> <p>CD8⁺ T cells and IFNγ production (Kumar <i>et al.</i> 2009; Mueller <i>et al.</i> 2007; Tarun <i>et al.</i> 2007).</p>
Erythrocytic stage	<p>Direct injection of large numbers of blood-stage parasites, bypassing the pre-erythrocytic cycle</p>	<p>Macrophages and the innate system Activated T cells</p> <p>Humoral immune responses directed against free merozoites or parasitized erythrocytes (Bull <i>et al.</i> 1998; Cohen <i>et al.</i> 1961b).</p> <p>Fine balance of inflammatory and anti-inflammatory cytokines required for parasite clearance without severe immunopathology.</p>	<p>Naturally-acquired clinical immunity develops after repeated exposure and is mainly directed against erythrocytic stages (Crompton <i>et al.</i> 2010; Marsh & Kinyanjui 2006).</p> <p>Humoral immune responses directed against free merozoites or parasitized erythrocytes (Bull <i>et al.</i> 1998; Cohen <i>et al.</i> 1961b). Immunisation with MSP1₁₉ induces protection in mice (Holder & Freeman 1981).</p>
Dermal stage	<p>Small numbers of sporozoites inoculated into the dermis and not directly into the blood circulation (as few as ten per mosquito bite).</p> <p>A significant proportion of these parasites remain in the dermis at the bite, while others are drained by lymphatic circulation and trapped in the proximal lymph node in close approximation with CD11c⁺ DCs. Some escape degradation and develop into exo-erythrocytic forms (Amino <i>et al.</i> 2008; Sidjanski & Vanderberg 1997; Yamauchi <i>et al.</i> 2007).</p>		<p>Tregs in the skin</p>

PfRh5 antibodies (Douglas *et al.* 2011). Furthermore, erythrocyte invasion by six fresh Senegalese field parasite isolates is inhibited by blocking of PfRh5-Basigin binding (Crosnier *et al.* 2011), making both Basigin and PfRh5 very promising vaccine targets. Nevertheless, at present there is no study in humans that definitively demonstrates that any monoclonal Ab response is protective in the field.

Longitudinal studies in humans show that the cryophilic Abs of the IgG1 and IgG3 isotype correlate highly with protection from *P. falciparum* (Groux & Gysin 1990) and are most efficient in neutralising parasite *in vitro*, while amounts of malaria-specific IgG4 and IgG2 Ab tend to be lower in immune adults. Analysis of sera from people from hyperendemic regions demonstrated that IgG1 and IgG3 did not inhibit parasite invasion of erythrocytes *in vitro*, but instead worked by Ab-dependent cellular inhibition (ADCI), by inducing TNF α production from monocytes in an Fc receptor-dependent manner (Bouharoun-Tayoun *et al.* 1990; Bouharoun-Tayoun *et al.* 1995a). In mice, infection with *P. yoelii* and *P. berghei* elicits protective Abs of mainly the IgG2a and IgG3 isotype (Waki *et al.* 1995; White *et al.* 1991). In *P. chabaudi* infection, IgG Ab plays an important role in protective immunity; however IgM has also been shown to be important in a primary *P. chabaudi* infection, as IgM-deficient mice appear more susceptible to pathology and blood-stage parasitaemia. These mice also appear less able to control recrudescence suggesting a role for IgM in the chronic stage (Couper *et al.* 2005). MSP1₁₉-specific IgG Ab titres are stably maintained after a primary infection with *P. chabaudi*, but at low levels, for several months following the decay of the acute peak of Ab response (Achtman *et al.* 2007).

In both humans and mice, Ab-mediated immunity to malaria may operate by an Fc receptor-mediated event like receptor-mediated phagocytosis or Ab-dependent cellular killing (ADCC). In humans, polymorphisms in Fc γ RIIIa Fc γ RIIb and Fc γ RIIIb have been implicated in susceptibility to severe malaria pathology (Cooke *et al.* 2003; Omi *et al.* 2002) and also protection (Shi *et al.* 2001). The systemic lupus erythematosus (SLE)-associated loss-of-function mutation in the human Fc γ RIIIb gene is commonly found in Africa,

suggesting that they are preferentially selected for (Clatworthy *et al.* 2007). One of the roles of Fc γ RIIb is to inhibit the phagocytosis of infected cells by macrophages and monocytes (Bouharoun-Tayoun *et al.* 1995b; Jafarshad *et al.* 2007), therefore losing the inhibitory signal from the Fc γ RIIb in people with SLE may result in enhanced phagocytosis of malaria parasites, thus conferring on them a survival advantage.

Studies delineating the downstream roles of Fc γ receptors by malaria-specific IgG have given contradictory results. Although protection against *P. yoelii* infection after the passive transfer of merozoite-specific monoclonal IgG1, IgG2a and IgG3 Abs has been demonstrated previously, transfer of IgG3 Abs alone is able to offer protection from lethal *P. yoelii* infection. However, IgG3 does not bind to Fc γ Rs and may not require a functional Fc γ -chain to exert its effector functions, instead possibly acting by steric hindrance of the interaction of the merozoite with the erythrocyte and preventing invasion (Freeman *et al.* 1980). Although monomeric IgG3 does not bind to Fc γ R, IgG3 immune complexes have been found to be able to bind to Fc γ R1 *in vitro* (Gavin *et al.* 1998). However in mice deficient in the γ -chain of Fc γ R1 and which therefore cannot signal through this receptor, passive transfer of MSP1₁₉-specific IgG3 Abs is still able to protect mice from *P. yoelii* infection (Vukovic *et al.* 2000). Furthermore, in this same study, MSP1₁₉-specific single-chain fragments (scFv) could also show dose-dependent protective anti-parasite effects despite lacking the Fc portion, suggesting that the effector functions mediated through the Fc portion are not required.

There is some evidence that complement activation through binding of Fc γ R is important for protection. Complement activation occurs by the binding of C1q to the Fc domain of immune complexes. Studies using transfer of human-mouse chimeric Abs composed of a murine V region specific for MSP1₁₉ and human IgG2b Fc regions, which can bind mouse Fc γ Rs and trigger *in vitro* phagocytosis of merozoites, but not activate mouse complement, failed to protect against lethal *P. yoelii* challenge (Pleass *et al.* 2003). By contrast, a further study using a similar chimeric Ab with human IgG1 Fc region recognising *P. falciparum* MSP1₁₉ was able to suppress parasitaemia after infection with a *P. berghei* transgenic for *P.*

falciparum MSP1₁₉, and this was critically dependent on the presence of transgenic human FcγR1 in the host mouse (McIntosh *et al.* 2007).

In *P. chabaudi* infection, although B cells are required for the control of chronic parasitaemia (Der Weid *et al.* 1996), and are important in helping anti-parasitic CD4⁺ T cell development and function (der Weld & Langhorne 1993;Langhorne *et al.* 1998), the role of Fc receptors in Ab-mediated protection during *P. chabaudi* infection is still unclear. In FcRγ-chain deficient mice, which lack surface expression of FcεE1 and FcγRIII, and lack binding to FcγRI, and have defects in phagocytosis and NK cell-mediated ADCC (Takai *et al.* 1994), there is no effect of the loss of FcRγ-chain on the course of *P. chabaudi* infection (P. Gardner, unpublished observations).

Plasmodium infection induces both thymus-independent and thymus-dependent B cell responses. Both IgM⁺ and IgM⁻ MBC were generated after a primary *P. chabaudi* infection in mice but, whilst their specificity was not known, only the IgM⁻ MBC and marginal zone (MZ) B cells expanded upon re-infection (Stephens *et al.* 2009). Upon re-infection, a faster kinetic and higher avidity of the IgG Ab response was observed, more bone marrow LLPC are generated, and splenomegaly and bone marrow hypocellularity were reduced compared to that seen in the primary immune response, indicating that functional parasite-specific humoral memory does develop in the primary infection (Stephens *et al.* 2009). In mice, MSP1₁₉-specific IgG MBC are detected for up to 8 months post-infection, even after chronic infection was terminated by chloroquine treatment, and can produce functional anamnestic responses for at least 3-4 months post infection (Ndungu *et al.* 2009). The percentage of peripheral blood MBC which was observed in mice (less than 5% of total B cells) during primary and secondary infection is lower than that typically found in humans. During primary *P. chabaudi* infection, there is a large short-lived extrafollicular CD138⁺ PC response that peaks on around day 10 and is reduced to background levels by day 21. However, functional MSP1-specific ASC can survive at a stable mean frequency for at least two months after infection irrespective of the presence of chronic parasitaemia. Interestingly, at this time point, approximately 40% of mice after *P. chabaudi* infection

actually did not have detectable numbers of MSP1₁₉-specific ASC, whilst others had high numbers of MSP1₁₉-specific ASC (Ndungu *et al.* 2009), which is either because there was no generation of MSP1₁₉-specific ASC in some of these mice, or that the MSP1₁₉-specific ASC are extremely short-lived (disappearing in less than two months).

In humans, there are a handful of studies that have looked at the longevity and characteristics of malaria-specific MBC in the peripheral blood of exposed individuals. One study in Madagascar, an area of very low transmission, reported that, 8 years after a single exposure to *P. falciparum*, parasite-specific MBC could still be detected in peripheral blood in low numbers (Migot *et al.* 1995). A similar study in northern Thailand, another area of extremely low malaria transmission, reported that both Ab and MBC responses to *P. falciparum* and/or *P. vivax* antigens were stably maintained over time in the absence of reinfection (Wipasa *et al.* 2010). The results from vaccination studies were similarly encouraging, demonstrating that long-lived MBC and Ab responses in the blood could be generated by immunisation of naïve people with two candidate malaria subunit protein vaccines and the adjuvant TLR9 ligand CpG (Crompton *et al.* 2009), although a follow up report showed this mechanism failed to have the same effect in malaria semi-immune adults (Traore *et al.* 2009). Therefore long-lived MBC are generated after infection with *P. falciparum* or *P. vivax* in areas of low transmission intensity. However, in these studies, the correlation of parasite-specific MBC and Ab is inconsistent, as subjects who have persistent titres of malaria-specific Ab can lack detectable recirculating malaria-specific MBC, whilst others who lack Ab do have detectable specific MBC.

In areas of high transmission intensity, it has been observed that the *P. falciparum*-specific MBC compartment increases with age in children and adults, indicating that acquisition of natural immunity to malaria in humans may require an increasingly polyreactive profile of MBC and serum Ab, that builds up with age after repeated infections (Weiss *et al.* 2010). The specificity of protective MBCs appears to be somewhat stage-specific, as there were no, or very low and intermittent, MBCs specific for some *Plasmodium* antigens (Dorfman *et al.* 2005; Weiss *et al.* 2010), and these are often undetectable outside of the transmission

season. In addition, a recent study reported increased numbers of the inhibitory receptor FCRL4 (Fc receptor-like protein 4)-expressing hyporesponsive MBC in malaria-exposed individuals, which possessed an atypical phenotype similar to the HIV-specific exhausted MBC reported previously, comprising higher expression of homing receptors, lower proliferative potential and having undergone few mitotic divisions and less somatic hypermutation (Weiss *et al.* 2009). This longitudinal study in Mali demonstrated that 20-60% of circulating B cell pool were exhausted B cells, even in children as young as 2 years of age, whereas they comprise only 1-2% of the pool in people from non-endemic countries. A follow up study showed that the degree of atypical MBC expansion increased with the intensity of *P. falciparum* transmission (Weiss *et al.* 2011).

1.8: Reasons why humoral immunity to malaria may be short-lived

The short-lived nature of humoral immunity to malaria in humans is well-documented (Crompton *et al.* 2010; Dorfman *et al.* 2005; Marsh & Kinyanjui 2006), although Ab responses against some antigens may be more persistent than others (Drakeley *et al.* 2005). In *P. chabaudi* infection of mice, malaria-specific Abs decrease significantly relatively early after a primary infection and are not as persistent as the Abs induced by protein immunisations or viral infections (Achtman *et al.* 2005; Achtman *et al.* 2007). If malaria-induced PC responses are intrinsically short-lived compared to those induced by protein immunisations or viral infections, it is currently unclear what the key differences are in these contexts that determines the life-span of the PC response. Alternatively, the reason for rapidly waning Abs could be due to the failure of generating PC and MBC with optimal functional qualities or with adequately protective specificities, allowing the parasite to evade the immune response. Alternatively, the PC and MBC generated are intrinsically long-lived, but their lifespan can be curtailed through a parasite-specific mechanism. There are various ways in which this can occur. The chronic parasitaemia and constant exposure to novel antigens could result in chronic simulation of naïve B cells into short-lived PCs, masking or attenuating the MBC response. Indeed there appears to be a constant turnover of MBC and LLPC during chronic infection, with LLPC still being generated 12 weeks

after primary *P. chabaudi* infection (Ndungu *et al.* 2009), indicating a constant exposure to novel antigens.

The formation of ‘optimum’ humoral immunity to infection requires appropriate signals from the antigen-presenting cell and T cell help and it is possible that parasite-specific MBC or LLPC are not ‘optimally’ generated (Kasturi *et al.* 2011; Reis e Sousa, Sher, & Kaye 1999). The modulation of the host immune response by the parasite is well documented (Coban *et al.* 2007; Stevenson & Riley 2004) and this could have adverse effects on the quality of parasite-specific MBC and LLPC generated during the infection. Indeed, innate immune responses are very sensitive to modulation by the by-products of the parasite metabolism, parasite growth rates and antigen loads, or ligands that can activate PRRs such as Toll-like receptors (TLR), and these factors can influence them to exert either activatory and inhibitory effector responses (Langhorne *et al.* 2004). While it has been shown in some experimental systems with various *Plasmodium* strains that DCs can be directly activated by the parasite (Seixas *et al.* 2001), others have demonstrated that DC maturation and ability to present antigen and stimulate T cells can be suppressed during infection by modulation from infected erythrocytes (Couper *et al.* 2007; Sponaas *et al.* 2006; Urban *et al.* 1999b). The DCs which encounter the pre-erythrocytic forms of the parasite are also subject to modulation, e.g. sporozoites can reduce the sensitivity of Kupffer cells to pro-inflammatory signals and induce apoptosis of KCs (Klotz & Frevort 2008), whilst merozoites can downregulate surface signals of infected erythrocytes that induce phagocytosis, e.g. phosphatidyl serine, which may enable them to escape phagocytosis, leading to decreased antigen presentation (Sturm & Heussler 2007). High antigen loads can cause apoptosis of DCs and hence reduce the numbers of DCs available to activate T cells (Elliott *et al.* 2007; Sponaas *et al.* 2006; Urban *et al.* 1999a). Haemozoin, a malarial byproduct, has been shown to be a key mechanism which suppresses DC function not only to itself (Urban & Todryk 2006) but also to heterologous antigens (Millington *et al.* 2006). However, haemozoin has also been shown to activate plasmacytoid DCs through TLR9 and constitute an important mechanism of parasite resistance (Coban *et al.* 2005), but which at the same time can inhibit cross-priming and

cross-presentation to a heterologous viral antigen (Wilson *et al.* 2006). Hemin, another parasite degradation product, has been shown to cause premature mobilization of granulocytes from bone marrow with a quantitative defect in the oxidative burst, which is advantageous for parasite survival but impaired the immune response to *S. typhimurium* co-infection (Cunnington *et al.* 2011).

The complex disulphide bonded structure of parasite proteins such as MSP1₁₉ may inhibit antigen processing and presentation by APCs and B cells, and this may induce poor provision of T cell help to GC B cells (Egan *et al.* 1997). Furthermore, a large proportion of parasite-specific and non-specific T cells undergo apoptosis and are reduced in number during infection by various lethal and non-lethal *Plasmodium* strains (Xu *et al.* 2002). It is also likely that, similar to the abnormally large proportion of atypical or exhausted MBC found in people living in malaria-endemic countries (Weiss *et al.* 2009), a large proportion of CD4⁺ T cells could be exhausted, indicating that they undergo abnormal differentiation and may only provide suboptimal help to B cells. Indeed, there appears to be overexpression of negative regulators of T cell activation, CTLA-4 and PD-1, on T cells during *P. berghei* infection, which correlates with the downregulation of pro-inflammatory responses and increased susceptibility to lethal infection (Hafalla *et al.* 2012). Dysfunctional and short-lived CD4⁺ T cell responses may lead to failure to maintain long-lived protective immunity to *P. chabaudi* infection (Freitas do Rosário *et al.* 2008). CD4⁺ T cell activation can be inhibited by IL-10 produced by infected erythrocyte-modulated DC and macrophages. It is possible that too many regulatory T cells are formed instead of effector T cell responses, leading to overproduction of anti-inflammatory cytokines IL-10 and TGFβ, which inhibits the anti-parasitic inflammatory response (Couper *et al.* 2008; Omer, de Souza, & Riley 2003), possibly due to the high antigen load (Finney *et al.* 2010). Therefore, the various ways that the parasite can modulate the innate immune response and development of CD4⁺ T cell help could result in the rapid loss of Abs if the resulting PC and MBC were not optimally ‘programmed’ or ‘imprinted’ with a long lifespan.

In addition to the attenuation of immune cell activation by a direct effect of parasite or an imbalance in regulatory mechanisms, there is also a massive disruption to the splenic microarchitecture in both humans (Looareesuwan *et al.* 1987) and mice during acute malaria. In mice, there is a striking and temporary changes in the leukocyte distribution during acute *P. chabaudi* infection, occurring maximally just after the peak of parasitaemia (Achtman *et al.* 2003; Cadman *et al.* 2008). This is in contrast to the immune response that occurs after immunisation with soluble antigen, which is highly organised and take place in very well defined cellular structures (Allen *et al.* 2007), but has been observed to be similar to that induced by administration of the TLR4 ligand, lipopolysaccharide (LPS) and other parasitic diseases such as leishmaniasis (Yurdakul *et al.* 2011). The remodelling of stromal tissue can affect antigen presentation, alter cellular movements and restrict access to appropriate or adequate cytokines and co-stimulatory signals which are required for the generation of a protective immune response. Therefore remodelling of stromal tissue of haematopoietic microenvironments like the bone marrow and spleen could be a mechanism of immune evasion by the parasite (Svensson & Kaye 2006).

During malaria infection, massive splenomegaly occurs, often increasing to several times the cellular size of a naïve spleen, and many changes occur in the splenic cellular microarchitecture and circulation (del Portillo *et al.* 2011). During infection with *P. chabaudi* in mice, there is severe but reversible disruption of the splenic white pulp architecture, with transient loss of follicular mantle and T zone integrity, atypical localisation of PCs, and loss of marginal metallophilic macrophages, marginal zone (MZ) B cells and macrophages from the splenic marginal zones (Cadman *et al.* 2008). Some MZ B cells are found in the red pulp, where they can contribute to the large extrafollicular short-lived PC response seen in acute *P. chabaudi* infection. Surprisingly, despite these drastic splenic alterations, large and persistent GCs still form during *P. chabaudi* infection. However, these GCs are atypical, as normal dark and light zones, where centrocytes cycle between and undergo proliferation, class-switch recombination and somatic hypermutation, are absent throughout acute *P. chabaudi* infection (Achtman *et al.* 2003; Kumararatne *et al.* 1987). There is a reduction in the avidity of the Ab response to a third-party antigen given

at the time of acute *P. chabaudi* infection, indicating that this is not restricted to the parasite-specific response (Achtman *et al.* 2003).

In humans, not only is the Ab response short lived, the existing B cell and Ab response to malaria is highly dysregulated, with over-exuberant non-specific polyclonal B cell activation (Banic *et al.* 1991;Freeman & Parish 1978;Rosenberg 1978), hypergammaglobulinaemia (Greenwood 1974), formation of large amounts of low-affinity immune complexes (June *et al.* 1979), skewing of malaria-specific Abs to less favourable isotypes, and also production of autoantibodies (Greenwood 1974;Rosenberg 1978) and frequent occurrence of B cell tumours in malaria endemic areas (Kafuko & Burkitt 1970). Atypical GCs have also been reported in *P. falciparum* infection (Zingman & Viner 1993) and the Ab response to vaccinations given at or around the time of malaria is impaired (Greenwood *et al.* 1972;Williamson & Greenwood 1978).

Another mechanism for the short-lived humoral immune response is that established memory is actively and rapidly curtailed by the parasite. The large numbers of B cells and PCs found in the spleen during acute *P. chabaudi* infection disappear from the spleen and blood, and are not subsequently recovered in the bone marrow, indicating that the majority have not matured into bone marrow LLPC (Achtman *et al.* 2003). Indeed, the large quantities of soluble antigen that are shed by the parasite during merozoite invasion could induce early apoptosis of any MBC and LLPC which are leaving the GC and therefore they are not detected in circulation. In one study, infection of mice with *P. yoelii* resulted in significant caspase-3-dependent apoptosis of pre-existing vaccine-induced MSP1₁₉-specific and unrelated MBCs and LLPC, hence contributing to the loss of vaccine-induced Ab (Wykes *et al.* 2005). Similar observations have been made in *Trypanosoma brucei* infection of C57BL/6 and BALB/c mice, which also resulted in reduction of not only pre-established anti-parasite MBC, but also unrelated hapten-protein conjugate- and vaccine-induced MBCs and LLPC, resulting in an increased susceptibility to a heterologous infection from which the host should have been protected from by the previous vaccination (Radwanska *et al.* 2008).

No mechanism has been described to explain parasite-induced abrogation of previously established humoral immunity, but this could be one explanation for the short-lived nature of malaria-induced MBC and LLPC. Furthermore, abrogation of previously established immune cells by parasitic infections has obvious implications on the longevity of vaccination-induced humoral immunity in areas where parasitic infections are endemic. One possible mechanism is that the circulation of large amounts of low affinity immune complexes produced by polyclonal B cell activation during parasitic infections can induce the apoptotic clearance of resident LLPC via the cross-linking of the Fc γ RIIb on the surface of resident LLPC, a recently described novel mechanism for regulating of the LLPC niche (Xiang *et al.* 2007). Alternatively, the maintenance of higher-than-average BAFF-R expression on malaria-induced MBC may be necessary for their longevity, as it was observed that there was a positive correlation with highest level of BAFF-R expression on B cells and the maintenance of schizont-specific IgG for period of more than 4 months in children (Nduati *et al.* 2011).

1.9: Aims of this study

The first aim of this study is to determine the cellular basis of long-lived serum Ab titres after a natural infection. In this study, the humoral immune response to an intranasal infection with Influenza A in BALB/c mice will be characterised. Next, hCD20 transgenic mice will be used to provide a system whereby memory B cells (MBCs) can be depleted, leaving long-lived plasma cells (LLPCs) intact. MBCs will be depleted using an anti-hCD20 monoclonal antibody after humoral memory to Influenza A is established. The selective depletion of MBC but not LLPC will allow discrimination of the contribution of the MBC and LLPC towards long-lived serum Ab titres.

The second aim of this study is to determine the effect of a heterologous infection on pre-established humoral immune responses. In this study, the first infection will be intranasal Influenza A infection. The second infection, which is blood-stage *P. chabaudi* infection, will be initiated after the resolution and establishment of humoral immunity to Influenza A. This will be the experimental model to investigate the effect of *P. chabaudi* infection on pre-established humoral memory.

Chapter 2: Methods and Materials**2.1: Mice**

All mice were bred in the specific pathogen free (SPF) unit at the National Institute for Medical Research (NIMR), London. hCD20tg BALB/c (Ahuja *et al.* 2007) and FcγRI,II,III^{-/-} (Van Lent *et al.* 2003) C57BL/6 mice were backcrossed for at least 7 generations (see **Table 2.11**). Experiments were carried out in a non-SPF unit in accordance to the Home Office Animal Act (1980). Experiments were initiated in 8 to 15 week old female mice of each strain.

2.2: Influenza A infection

For induction of non-lethal Influenza A infection, mice were infected with 250 hemagglutinin units (HAU) of the A/Puerto Rico/8/34 (PR8) strain of (H1N1) Influenza A virus by instillation into their nasal cavities without anaesthesia. For sub-lethal infection, mice were given light inhalation anaesthesia with isoflurane before intranasal instillation with 0.1, 1 or 10 HAU of PR8 and allowed to recover.

2.3: The mouse model of malaria**2.3.1: Plasmodium chabaudi chabaudi AS**

Plasmodium chabaudi chabaudi (*P. chabaudi*) was isolated from the African thicket rat (*Thamnomys rutilans*) and passaged in mice (Walliker *et al.* 1975). *P. chabaudi* parasites were cloned and maintained at the NIMR, London (Slade & Langhorne, 1989), with only four passages in C57BL/6 mice from the original isolate provided by Professor David Walliker (University of Edinburgh). Cryo-preserved parasite stabilates were tested negative for Mycoplasma and other common mouse pathogens by inoculation onto Mycoplasma Agar (Oxoid Ltd., CM0401) and a horse blood agar plate and examined under the light microscope at 50X magnification.

2.3.2: Passage of *P. chabaudi* through BALB/c mice

Cryopreserved parasite stabilates were used for initiating infections in the animals in the manner previously described (Langhorne J *et al*, 1989). Briefly, stabilates were thawed from liquid nitrogen, diluted 1:1 with 0.9% saline and injected i. p. into BALB/c mice. These parasites were passaged up to four times in mice by i. p. injection of 10^6 , 10^5 or 10^4 pRBC per mouse diluted in 100 μ l of Kreb's glucose saline (See **Table 2.1**) (Jarra and Brown 1985). The number of pRBC was calculated by determining the percentage of parasitaemia on thin blood films using 20% Giemsa stain (VWR) and assuming a RBC density of 2.5×10^9 /ml in peripheral venous blood.

2.3.3: Infection of experimental mice with *P. chabaudi*

Experimental mice were infected using pRBC taken from one of the passage mice before the peak of parasitaemia. Each experimental mouse received an i. p. injection of 10^5 pRBC diluted in 100 μ l of Kreb's glucose saline.

2.3.4: Determination of parasitaemia using thin blood films

Thin blood films were prepared on microscope slides from a drop of blood (approximately 5 μ l) obtained by tail nick. The blood films were air-dried and fixed with 100% methanol (BDH), stained for 25 minutes in 20% Giemsa stain (VWR), and then washed thoroughly in tap water and air-dried. Films were analysed under oil immersion on a Zeiss Axioskop light microscope with a 100X objective. For films with high parasitaemia, at least 2,000 RBC were counted for parasites and for those with low parasitaemia, at least 10,000 RBC were counted. Parasitaemia was quantified as a percentage of pRBC in total RBC counted.

Formula for obtaining % Parasitaemia = (total number of pRBC / total number of RBC) x 100%

2.4: Chloroquine treatment

Chloroquine for injection was prepared fresh from chloroquine diphosphate salt (Sigma) for each treatment. The treatment protocol was 10 daily i. p. injections of 40 mg/kg of chloroquine dissolved in sterile 0.9% saline.

2.5: Preparing serum from whole blood

Monitoring of serum antibody responses was performed by obtaining serum from venous blood, taken by incision across the lateral tail vein with a scalpel blade. No more than 15% of total blood volume was collected over any 28-day period. Blood was directly collected in clean Eppendorf tubes and incubated for approximately 2-5 hours (h) at room temperature to allow clotting to take place. Tubes were centrifuged at 900 xg for 10 minutes using the Heraeus Fresco 17 centrifuge (Thermo Scientific) and the serum removed from the clot into a clean Eppendorf tube. Some erythrocytes and insoluble material was usually transferred during this process, so the serum was re-centrifuged at 16,200 xg for 5 minutes to remove any transferred or remaining material. The serum was transferred again into clean Eppendorf tubes and heat-inactivated at 56 °C for 10 minutes. Heat-inactivated serum was stored at -20 °C until use.

2.6: Tissue harvesting and making single cell suspensions**2.6.1: Splenocytes**

Mice were sacrificed by cervical dislocation, stretched opened and pinned to a Styrofoam board. Spleens were dissected and transported from the animal housing unit to the sterile tissue culture room in 15 ml Falcon tubes containing 5 ml of complete IMDM medium (Gibco) on ice. Individual spleens were placed in separate sterile 60 mm Petri dishes containing 5 ml red blood cell lysing buffer (Sigma R7757). The plunger of a 5 ml syringe was used to mash and press the spleen gently through a 70 µm filter into the red blood lysing buffer, to release individual cells and lyse red blood cells at the same time. The cell suspension was transferred into a 15 ml Falcon tube. The Petri dish and 70 µm filter was rinsed with 5 ml of cold complete IMDM and added to the suspension to maximise yield and terminate the erythrolysis reaction. The cells were

washed by centrifugation (7 minutes, 425 $\times g$ 4°C, Heraeus Megafuge 1.0R using a swing bucket rotor) and re-suspended in complete IMDM or sterile FACS buffer.

2.6.2: Bone marrow cells

Femurs and tibias were dissected and cleaned by removing the attached muscle, fat and cartilage. The bones were gently opened at either end with surgical scissors and the marrow flushed out in red cell lysing buffer, using a 5 ml syringe and 27 G needle, into a sterile Petri dish. Marrow was mashed through a 70 μm filter using the plunger of a 5 ml syringe, to release individual cells and lyse red blood cells at the same time.

2.6.3: Peripheral blood mononuclear cells (PBMCs)

Mice were euthanised with terminal anaesthesia using i. p. injection of 50 μl pentobarbitone (source). The chest cavity was opened and flooded with 200 μl of heparinised KGS (see **Table 2.1**) The descending aortic arch was cut and blood was suctioned using a Pasteur pipette into Eppendorf tubes. Erythrocytes were lysed by incubating with 5 ml of red cell lysis buffer for 3 minutes, washing with 5 ml cold complete IMDM or FACS buffer and repeating the erythrolysis step for two more times in order to completely remove red blood cells.

2.7. Immunoblotting using the LICOR/Odyssey system

P. chabaudi parasite lysates were a kind gift from Jennifer Lawton, NIMR, London. Briefly, *P. chabaudi* infected blood was filtered through Plasmodipur (Euro Diagnostica) to remove mouse leukocytes and washed 3X in PBS at 490 $\times g$, 10 min each. Parasites were lysed in 0.1% saponin (Sigma) in PBS, and washed 3X in PBS at 3,0074.5 $\times g$ for 10 minute at 4 °C. Pelleted material was stored at -70 °C. The parasite lysate fraction contains the purified parasite and its parasitophorous vacuole membrane, but may also contain a small amount of contaminating pRBC membrane.

Bromelain-digested Influenza A/PR8/34 haemagglutinin was a kind gift from John Skehel, NIMR, London.

Proteins were resolved on NuPAGE 12% bis-Tris gels (Invitrogen) under non-reducing conditions in NuPAGE 1X morpholineethanesulfonic acid sodium dodecyl sulphate buffer (Invitrogen) according to the manufacturer's instructions. Proteins were run alongside 1X SeeBlue Plus2-prestained standard (Invitrogen) for 60-90 minutes at 200 V. The proteins were then electrophoretically transferred to Hybond C membrane (Amersham Biosciences) in buffer containing 10% methanol, 0.025 M Tris base (Sigma) and 0.2 M Glycine (BDH biosciences) for 3 h at 30 V. Membranes were blocked at 4 °C overnight in skimmed milk, then washed 6 x 5 minutes with PBS + 0.1% Tween20 on an agitating rocker. Specific proteins on the membranes were detected using a variety of anti-sera: Hyperimmune sera from mice multiply-infected with *P. chabaudi*; sera from PR8-infected mice, and sera from uninfected mice as a negative control. These were incubated at 1/100 dilution for 1 h at room temperature (RT) on an agitating rocker, wrapped in aluminium foil. The membranes were washed as before. The secondary Alexa 680-conjugated goat anti-mouse IgG (Licor Biosciences 926-32220) was used at a 1:15,000 dilution and incubated for 1 h at RT on an agitating rocker, wrapped in aluminium foil. Membranes were washed as before, then with PBS only for 30 minutes on an agitating rocker, wrapped in aluminium foil. The fluorochrome conjugated to the secondary antibody was revealed by scanning the membranes with the Odyssey scanner (Licor Biosciences) using 680EX nm/700EM nm filter settings

2.8. ELISA

2.8.1: Measurement of HA-specific IgG

96-well MaxiSorp™ plates (Nunc 439454) were coated with 0.25 µg/well of bromelain-released PR8 haemagglutinin (HA). Duplicate columns for the positive standard was included on each plate and coated with 0.25 µg/well of goat anti-mouse IgG (H+L) (Southern Biotech) and incubated overnight (ON) at 4 °C. Subsequently, the coat was removed and the residual surface of the wells were blocked with 200 µl/well of Blocking Buffer (PBS, 1% bovine serum albumin, 0.3% Tween20, 0.05% NaN₃) for 2 h at 37 °C to reduce non-specific binding. Wells were washed 4 times with PBS + 0.01% Tween20. Eight two-fold dilutions of serum samples were made with a dilution range of 1/50 – 1/128,000. Eight two-fold dilutions of purified immunoglobulin (Table 7) were

included on each plate starting with a pre-titrated known concentration. Dilutions of serum and standard were made in Blocking Buffer.

Serum and standard were incubated for 1-2 h at RT. Plates were washed 4 times with PBS + 0.01% Tween20. 100 µl/well of secondary alkaline phosphatase-conjugated detecting antibodies (Table 2) at a 1/1000 dilution was added and incubated for 1 h at RT. Plates were washed 4 times with PBS + 0.01% Tween20. 50 µl/well of a 1 mg/ml 4-Nitrophenyl phosphate disodium salt hexahydrate (Sigma S0942) in diethanolamine buffer pH 9.8 solution (see **Table 2.1**) was added and incubated in the dark until the colour reaction was completed. The optical density (O. D.) of each plate was measured at the reference wavelength of 450 nm using the Dynex MRX-TC Revelation Microtiter plate reader and analysed using Microsoft Excel (2003) software.

2.8.2: Measurement of *P. chabaudi*-specific IgG

Parasite lysate was prepared as described above. 250 µl of parasite lysate buffer was added to every 50 µl of parasite lysate and vortexed, followed by centrifugation at 16,200 $\times g$ for 2-5 minutes. The supernatant was diluted in PBS to give an OD₂₈₀ reading of 0.05 and 50 µl was used to coat PolySorp™ plates (Nunc 475094). Hyperimmune serum from mice multiply infected with *P. chabaudi* were used as a standard, starting from a pre-titrated dilution. The remaining steps of the ELISA were as described above for HA-specific IgG.

2.9: Hybridoma culture and purification of HY1.2 mIgG2a and 2H7 mIgG2b

The hybridoma cell line Hy1.2 was a gift from Professor Hans Ulrich Weltzein (Ortmann B 1992). The Hy1.2 hybridoma was grown up to 30 L by the NIMR large-scale facility in endotoxin-low 5% complete IMDM. Another hybridoma cell line, the 2H7 hybridoma was also grown up to 30 L in protein-free RPMI (**Table 2.3**). The concentrated hybridoma cell supernatants were sterilised by 0.2 µm filtration and antibodies were purified using a Protein G column according to the manufacturer's instructions (GE Healthcare Life Sciences). Briefly, Protein G sepharose (Hi-Trap, Pharmacia Biotech #17-0404-01) was used to fill a column, followed by the addition of 5 bed volumes of PBS. PBS pH 7.4 (Gibco) was added in a ratio of 1:1 to the culture supernatant and filtered through a 0.22 µm filter. The culture supernatant was then

added onto the column according to the manufacturer's protocol. The column was washed with PBS, and immunoglobulin was eluted using 3 bed volumes of 0.1 M Glycine pH 2.8 into tubes containing 1 M Tris (pH 2). After use, the column was regenerated with 2% ethanol and stored at 4 °C. Eluted fractions were pooled and dialyzed against 3 changes of endotoxin-free PBS (Gibco), using at least 100 times the sample volume using Slide-A-Lyzer Dialysis Cassettes, 10 K MWCO (Pierce cat: 66810). Dialyzed antibody was then run through polymixin B agarose (Sigma) to remove endotoxin and concentrated using Vivaspin columns (5,000 MWCO) (GE Healthcare Life Sciences), according to the manufacturer's instructions. The final antibody concentration was determined by spectrophotometry at OD₂₈₀ nm (Nanodrop ND-1000 Spectrophotometer, Labtech) and ELISA (as described above).

2.10: Immunodepletion with 2H7 mAb.

The mAb recognizing hCD20, 2H7, was used for B cell depletion. 2H7 is a mouse IgG2b that binds an epitope on hCD20 similar to that bound by rituximab and therefore, mimics rituximab treatment of humans (Ahuja *et al.* 2007). 2H7 was purified from culture supernatants as described above. Mice were injected i. p. with 2 mg/week of 2H7 in sterile 0.9% saline for 2 weeks.

2.11: Determination of the half-life of serum Ab.

The protocol for determination of the half-life of a non-specific serum Ab in mice was adapted from the ones used by Vieira and colleagues (Vieira & Rajewsky 1988). Briefly, 200 µg of anti-TNP mIgG2a (Hy1.2) in 200 µl PBS was injected i. p. into mice 24 hours or 60 days after infection with 10⁵ pRBC, and into uninfected controls. 25 µl of blood was taken by tail nick from all the mice 6, 24, 80, 150 and 193 h after injection. Serum was obtained from blood following the method described above. Concentrations of anti-TNP mIgG2a in serum were quantified using ELISA as described above, with the following exceptions: 96-well MaxiSorp™ plates (Nunc 439454) were coated with 25 ng/well of TNP-BSA (Biosearch Technologies T-5050; diluted in PBS) and duplicate standard columns were coated with 25 ng/well of goat anti-mouse Ig(H+L; Southern Biotech; diluted in PBS) and incubated overnight at 4 °C. Known concentrations of purified mouse IgG2a (Sigma M9144) was used as a standard control.

2.12: ELISpots**2.12.1: HA-specific antibody-secreting cell ELISpot**

Plasma cells specific to an antigen of choice were quantified by a direct *ex vivo* ELISpot, based on their ability to continuously secrete antibody. 96-well Multi-screen MCE filtration plates with mixed cellulose ester bases (Millipore MSHAN4550) were coated with 0.5 µg/well of PR8 haemagglutinin (HA) for HA-specific antibody-secreting cells (ASC) and 0.5 µg/well of purified goat anti-mouse IgG (Sigma M30100) for total IgG antibody-secreting cells. Plates were wrapped tightly in saran wrap and incubated at 4 °C overnight. Plates were then washed twice with PBS and blocked with 200 µl/well of sterile complete IMDM for 2 h at RT. Spleen and bone marrow cells were isolated and two-fold serial dilution suspensions made with a starting concentration of 5×10^5 cells per well. Complete IMDM was added to each well to make a final volume of 200 µl per well. Plates were incubated for 5 h at 7% CO₂ and 37 °C in a humidified incubator. After incubation, plates were washed twice with PBS and twice with PBS-0.025% Tween (PBS-T). 50 ng/well of biotinylated goat anti-mouse IgG (Sigma M30115) diluted in PBS-T+1% FCS was added and plates were incubated at 4 °C overnight. Plates were then washed 4 times with PBS-T and incubated with 50 µl/well pre-titrated 1/8000 dilution of streptavidin alkaline phosphatase (BD Pharmingen 554065) diluted in PBS-T+1% FCS for 1 h at RT. Plates were washed 4 times with PBS and 4 times with PBS-T. BCIP/NBT substrate (BioFX Laboratories BCIB-1000-01) was filtered through a 0.2 µm filter and pre-warmed to RT in the dark. 100 µl/well BCIP/NBT substrate was added and incubated in the dark for approximately 20-30 minutes until blue spots became sufficiently distinct, while making sure the background did not become too dark. The reaction was stopped by vigorously washing the plate 10 times with running cold water. The base of the plate was carefully removed and membranes air-dried overnight, shielded from direct sunlight. Plates were recorded using the ImmunoSpot[®] S5 UV Analyser and analysed using ImmunoSpot[®] Academic Software Version 4.0 (Cellular Technology Ltd) according to the manufacturer's guidelines. Experiments were done with at least 4 replicates.

2.12.2: HA-specific memory B cells

2.12.2.1: Concanavalin A supernatant

Single cell suspensions were made from spleen and erythrolysed. Cells were washed and re-suspended at a concentration of 1.25×10^6 /ml in fresh IMDM containing 5% FCS, penicillin/streptomycin (500 units/ml), L-glutamine (200 mM) and β -mercaptoethanol (50 μ M). Cells were cultured in a T-75 tissue culture flask in a 20 ml volume containing 2.5 μ g/ml of Concanavalin A (Pharmacia) and 20 ng/ml of PMA (Sigma), for 48 h at 7% CO₂ and 37 °C in a humidified incubator. After incubation, the supernatant was transferred to 50 ml Falcon tubes and centrifuged at 425 $\times g$ for 10 minutes to remove cellular debris. The supernatant was sterilised by filtering through a 0.2 μ m syringe filter. The supernatant was compared with previous batches using a CTLL-2 assay (see below). Aliquots of Concanavalin A supernatant were made and stored at -80 °C until use.

2.12.2.2: CTLL-2 assay

CTLL-2 is a IL-2 dependent cell line which grows in complete IMDM supplemented with 5% Concanavalin A supernatant and may therefore be used to test the efficacy of Concanavalin A supernatant. The CTLL-2 cell line was thawed from liquid nitrogen, grown and passaged at least twice before use for this assay. Cells were used when two-thirds confluent. Cells were washed 3 times with HBSS. In between the 2nd and 3rd wash, cells were left in HBSS for 30 minutes at RT to starve them. After the 3rd wash, cells were re-suspended in 5×10^4 /ml in complete IMDM. A serial dilution of Concanavalin A supernatant was made starting with a concentration of 50%. A positive control of a previous batch of Concanavalin A supernatant was also made. 100 μ l of CTLL-2 cell suspension (i.e. 5×10^3 cells) were added to each well. Cells were incubated for 24 h at 7% CO₂ and 37 °C in a humidified incubator. At this point, 0.25 uCi of 3H-Thymidine was added to each well and incubated for a further 12 h. Plates were then frozen at -20 °C, or cells were harvested and radioactivity determined using liquid scintillation (Wallac TriLux).

2.12.2.3: HA-specific memory B cell ELISpot

Memory B cells may be detected using an indirect *ex vivo* ELISpot assay by activating them *in vitro* to generate plasma cells and then quantifying the plasma cells. This is an ELISpot technique which has been adapted from the one reported by Crotty and colleagues (Crotty *et al.* 2004). Briefly, replicates of two-fold dilutions of cell suspensions of spleen were made on flat-bottomed 96-well plates (Costar) with 21 replicates and cultured for 6 days in 200 μ l/well complete Iscove's medium containing a 'stimulant mastermix' of 0.4 μ g R595 lipopolysaccharide (Alexis Biochemicals), 1×10^6 irradiated (1,200 rad) naive splenocytes and 20 μ l Concanavalin A supernatant, prepared as described above. After 6 days, cells were washed in complete IMDM+1% FCS, harvested and transferred to pre-coated 96-well Multi-screen MCE filtration plates. An *ex-vivo* ELISpot assay for HA-specific and total IgG plasma cell detection performed as described above. A graphical plot was made of the fraction of natural logarithm of the fraction of non-responding cultures as a function of the dose of cells placed in each culture and a straight line was fitted to cross 0 at the y- and x-axes. Assuming that one responding cell is sufficient to produce a positive culture, the cell dose yielding 37% negative cultures gives a frequency of cells in the population capable of responding to the antigen. Using the Microsoft Excel Trendline option, a straight line of best fit was plotted. The frequencies of HA-specific and total memory B cells were determined from the slope of the line. Values were accepted when r^2 values were greater than 0.7.

2.13: Virus neutralisation assay

Virus-neutralising Ab titres were determined using an assay as previously described (Kassiotis *et al.* 2006). Sera were collected at time points after PR8 infection, heat-inactivated for 10 minutes at 56 °C, and tested using a modified Madin-Darby canine kidney (MDCK)-based assay as previously described (Kassiotis, Gray, Kiafard, Zwirner, & Stockinger 2006). For this, 1:2 serial dilutions of test sera were made in 96-well flat-bottom plates with a dilution range of 1:100–1:12800 in IMDM containing 5% FCS. Wells containing 1:2 serum dilutions of a control serum with a dilution range of 1:100–1:12800 served as a control for inter-plate variation. These were incubated with a 95% tissue culture-infected dose of 0.3 HAU/1000 MDCK cells at 37 °C, 5% CO₂ for 30 minutes. Next, 1000/well of a fresh suspension of MDCK cells was added. Wells

containing virus and MDCK cells without serum served as background controls, and wells containing only MDCK cells without virus or serum were used to determine 50% proliferation. The plates were then incubated at 37 °C, 5% CO₂ for 3 days. Media was removed and wells gently washed with 100 µl IMDM containing 5% FCS. Cultures were pulsed with alamarBlue and incubated for 1-2 hours at 37 °C, 5% CO₂ to let the colour reaction develop. Resazurin, a non-fluorescent indicator dye, is converted to bright red–fluorescent resorufin via the reduction reactions of metabolically active cells (O'Brien *et al.* 2000). The amount of fluorescence produced is proportional to the number of living cells. Fluorescence was measured with SAFIRE II using 560EX nm/590EM nm filter settings.

Absorbance values correlated directly to the number of viable cells in the wells, which correlates with the neutralising activity of influenza-specific antibodies present in the serum. Absorbance values were used to calculate the neutralising antibody titres. The absorbance value was calculated after the mean value from the background control wells had been subtracted. Neutralising antibody titres were expressed as the reciprocal of the highest serum dilution that inhibited virus growth by $\geq 50\%$.

2.13.1: MDCK cells

MDCK cells were grown in IMDM containing 5% FCS and maintained at 37 °C, 5% CO₂ in a humidified atmosphere. The cells were sub-cultured at serial dilutions of 1:10 once a week. For the neutralisation test, cells were used when they formed an 80% monolayer.

2.14: Flow cytometry

For the surface staining procedure, 5×10^6 washed cells in 50 µl FACS buffer (PBS, 2% FCS, 0.05% NaN₃) were added to each well of a 96-well microtitre plate and mixed well with 50 µl of prepared multi-mixes of antibody panels (**Tables 2.4 – 2.9**). To prevent non-specific binding of monoclonal antibodies to the Fc receptors, 5 µl of the Fc receptor blocking antibody (clone 24G.2) was added with the multi-mix per 1×10^6 cells. Cells were incubated at RT in the dark for 20 minutes. Stained cells were centrifuged (425 xg, 1 minute), supernatant discarded by flicking the plate and washed with 200 µl of FACS buffer twice. For biotinylated antibodies, streptavidin-conjugated

fluorochromes were added at a dilution of 1/100 and further incubated for 10 minutes at RT and subsequently washed 3 times. Cells were acquired using the CyAn ADP flow cytometer (Beckman Coulter) within 2 hours. Data were analysed using FlowJo (Treestar). FlowJo was also used for graphical representation and statistical analysis.

For Annexin V staining, after surface staining was completed, cells were washed and re-suspended in Annexin V Binding Buffer (BioLegend) at a concentration of 1×10^6 cells/ml. 100 μ l of the cell suspension was filtered through a 0.2 μ m filter into polypropylene FACs tubes (BD 352002) and 5 μ l of Annexin V-Pacific Blue (BioLegend) was added. Cells were incubated for 15 minutes at RT in the dark. 400 μ l of Annexin V Binding Buffer was added to the tube just prior to analysis, as described above.

2.15: Determining lung viral titres

2.15.1: RNA extraction and cDNA preparation

RNA extraction was carried out according to the RNeasy mini kit protocol following the manufacturer's protocol (Qiagen cat: 74106). Total RNA was extracted from whole lung tissues using TRI reagent (Sigma-Aldrich) and subsequently was used for cDNA synthesis with the Omniscript reverse transcription (RT) kit (Qiagen, Hilden, Germany). 1 ng of RNA was used as the template, and cDNA synthesis was primed by a mixture of 1 μ M random hexamers and 1 μ M of a primer specific to a highly conserved region of the IAV *matrix* gene (5'-TCTAACCGAGGTCGAAACGTA-3'), as previously described (Ward *et al.* 2004). Reaction mixtures were incubated at 37 °C for 1 h and terminated by incubating the mixture at 90 °C for 5 minutes.

2.15.2: qRT-PCR

Expression of mRNA was determined by quantitative reverse transcription-PCR (qRT-PCR) using a DNA master SYBR green I kit (Roche, Mannheim, Germany) and the ABI Prism 7000 detection system (Applied Biosystems, Foster City, CA). The primers used for the amplification of target transcripts are in **Table 2.2** (Ward *et al.* 2004). Samples were analyzed in duplicate. The housekeeping gene *Hprt* was used to

normalize the critical threshold values for the genes of interest. Levels of IAV *matrix* mRNA are plotted as arbitrary units relative to *Hprt* mRNA levels.

2.16: Paraffin sections and H&E staining

Femurs were gently opened at either end and fixed in 10% neutral formalin buffer overnight, dehydrated through graded alcohols (Leica ASP300s), embedded in paraffin, and sectioned at 5 μm using a microtome. Sections were mounted on SuperFrost Plus Slides (Microm International) and stained by routine H&E (Harris haematoxylin and eosin, Leica Autostainer XL). Histological sections were examined and photographed under light microscopy using a 100X oil immersion objective lens.

2.17: Statistical analysis

Statistics were generated by Student's *t* test and Mann-Whitney *U* test performed using GraphPad Prism software.

Table 2.1: Buffers

Phosphate Buffered Saline (PBS)

5 mM KH_2PO_4
 5 mM K_2HPO_4
 0.1 M NaCl
 pH 7.5

FACS Buffer

PBS
 2% FCS
 5 mM EDTA
 0.05% NaN_3

ELISA/ELISPOT: Wash Buffer

PBS
 0.025% Tween20

ELISA: Blocking Buffer

PBS
 1% BSA
 0.03% Tween20
 0.05% NaN_3

ELISA: Substrate Buffer

48.5 ml Diethanolamine (source? Conc?)
 400 mg $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$
 0.05% NaN_3
 pH 9.8
 P nitrophenyl phosphate (PNPP) tablets (Sigma N-9389) were added just prior to use at a final concentration of 1 mg/ml

Giemsa Buffer

0.9% NaCl
 0.2 mM KH_2PO_4
 0.8 mM K_2HPO_4
 pH 7.0-7.2

Krebs' Glucose Solution

Buffer 222.2 ml
 Buffer contents:
 NaH_2PO_4 26 mM
 NH_4Cl 4.36 ml
 pH 7.4
 NaCl 55 mM
 KCl 4.6 mM
 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 2.4 mM
 Glucose 11 mM

For heparinised KGS, dilute heparin (LEO, 009876-04) 1/50 in KGS (100 IU/ml)

Parasite Lysate Buffer

50 mM Tris pH7.5
 1 mM EDTA
 0.05% SDS
 pH8

Complete Iscove's Modified Dulbecco's Medium (IMDM)

To IMDM medium 500 ml, the following were added:
 1 M HEPES 5 ml
 Penicillin G sodium/streptomycin sulfate (500 units/ml) 5 ml
 L-Glutamine (200 mM)
 B-mercaptoethanol (50 μM) 500 μl
 Heat-inactivated FCS 10% 50 ml

Reagents

Table 2.2: Primers

Primer	Sequence
<i>Hprt</i> forward	5'-TTGTATACCTAATCATTATGCCGAG-3'
<i>Hprt</i> reverse	5'-CATCTCGAGCAAGTCTTTCA-3'
<i>IAV matrix</i> forward	5'-AAGACCAATCCTGTCACCTCTGA-3'
<i>IAV matrix</i> reverse	5'-CAAAGCGTCTACGCTGCAGTCC-3'

Table 2.3: Hybridomas

Clone	Specificity	Isotype	Reactivity	Purified on
Hy1.2	2, 4, 6-trinitrophenyl	mIgG2a	Mouse	Protein G
2H7	Human CD20	mIgG2b	Human	Protein G

Antibody Tables

Table 2.4: Plasma cell panel

Company	Catalog #	Specificity	Fluorochrome	Channel	Stock concentration	Use at	Clone	Isotype
eBioscience	53-9991	CXCR4	Alexa Fluor® 488	FL-1	0.5 mg/ml	1/200	2B11	Rat IgG2b, κ
BD Pharmingen™	553714	CD138	PE	FL-2	0.2 mg/ml		281-2	Rat IgG2a, κ
BD Pharmingen™	560528	CXCR5	PerCP-Cy5.5	FL-4	0.2 mg/ml		2G8	Rat IgG2a, κ
eBioscience	25-0452	Human/Mouse CD45R (B220)	APC	FL-8	0.2 mg/ml		RA3-6B2	Rat IgG2a, κ
eBioscience	47-5321	MHC Class II (I-A/I-E)	APC-Cy7	FL-9	0.2 mg/ml		M5/114.15.2	Rat IgG2b, κ
BioLegend	640918	Annexin-V	Pacific Blue	FL-6		5 µl/100µl cell suspension containing 10 ⁵ cells		

Table 2.5: Memory B cell and Germinal Centre B cell panel

Company	Catalog #	Specificity	Fluorochrome	Channel	Stock concentration	Use at	Clone	Isotype
eBioscience	25-0452	Human/Mouse CD45R (B220)	PE-Cy7	FL-5	0.2 mg/ml	1/200	RA3-6B2	Rat IgG2a, κ
BioLegend	405712	IgD	Pacific Blue	FL-6	0.5 mg/ml		11-26c.2a	Rat IgG2a, κ
eBioscience	17-0381	CD38	APC	FL-8	0.2 mg/ml		90	Rat IgG2a, κ
BD Pharmingen™	562080	GL7 (B and T-Cell Activation Antigen)	FITC	FL-1	0.5 mg/ml		GL7	Rat IgM, κ

Table 2.6: B cell panel

Company	Catalog #	Specificity	Fluorochrome	Channel	Stock concentration	Use at	Clone	Isotype
eBioscience	25-0193	CD19	PE-Cy7	FL-5	0.2 mg/ml	1/200	eBio1D3 (1D3)	Rat IgG2a, κ
BD Pharmingen™	553818	CD21/CD35	FITC	FL-1	0.5 mg/ml		7G6	Rat IgG2b, κ
BD Pharmingen™	553137	CD23	Biotin		0.5 mg/ml		B3B4	Rat IgG2a, κ
BioLegend	406512	IgM	PerCP-Cy5.5	FL-3	0.2 mg/ml		RMM-1	Rat IgG2a, κ
BioLegend	405316	IgG (minimal x-reactivity)	APC-Cy7	FL-9	0.2 mg/ml		Poly4053	Goat Ig
BD Pharmingen™	561857	CD43	PE	FL-2	0.2 mg/ml	1/500	S7	Rat IgG2a, κ

Table 2.7: T cell, NK cell and NK T cell panel

Company	Catalog #	Specificity	Fluorochrome	Channel	Stock concentration	Use at	Clone	Isotype
eBioscience	12-5941	NK1.1	PE	FL-2	0.2 mg/ml	1/200	PK136	Mouse IgG2a, κ
eBioscience	25-5971	CD49b (Integrin alpha 2)	PE-Cy7	FL-5	0.2 mg/ml		DX5	Rat IgM, κ
eBioscience	11-0083	CD8b	FITC	FL-1	0.5 mg/ml		eBioH35-17.2 (H35-17.2)	Rat IgG2b, κ
eBioscience	45-0042	CD4	PerCP-Cy5.5	FL-3	0.2 mg/ml		RM4-5	Rat IgG2a, κ
BD Pharmingen™	559250	CD44	APC	FL-8	0.2 mg/ml		IM7	Rat IgG2b, κ
eBioscience	48-0621	CD62L (L-Selectin)	eFlour® 450	FL-6	0.2 mg/ml		MEL-14	Rat IgG2a, κ

Table 2.8: Granulocyte panel

Company	Catalog #	Specificity	Fluorochrome	Channel	Stock concentration	Use at	Clone	Isotype
BD Pharmingen™	552126	Siglec F	PE	FL-2	0.2 mg/ml	1/200	E50-2440	Rat IgG2a, κ
BioLegend	117324	CD11c	APC-Cy7	FL-9	0.2 mg/ml		N418	Armenian Hamster IgG
eBioscience	17-0112	CD11b	APC	FL-8	0.2 mg/ml		M1/70	Rat IgG2b, κ
BioLegend	128014	Ly6C	Pacific Blue	FL-6	0.5 mg/ml		HK1.4	Rat IgG2c, κ
BioLegend	127606	Ly6G	FITC	FL-1	0.5 mg/ml		1A8	Rat IgG2a, κ
BioLegend	123114	F4/80	PE-Cy7	FL-5	0.2 mg/ml		BM8	Rat IgG2a, κ
eBioscience	13-5898	FcεR1α (high affinity IgE receptor)	Biotin		0.5 mg/ml		MAR-1	Armenian Hamster IgG
BioLegend	406906	IgE	FITC	FL-1	0.5 mg/ml		RME-1	Rat IgG1, κ
eBioscience	12-1171	CD117 (c-kit)	PE	FL-2	0.2 mg/ml		2B8	Rat IgG2a, κ

Table 2.9: hCD20rg panel

Company	Catalog #	Specificity	Fluorochrome	Channel	Stock concentration	Use at	Clone	Isotype
eBioscience	12-0209	Human CD20	PE	FL-2	5 μL (0.06 μg)/test	1/20	2H7	Mouse IgG2b, κ
BioLegend	302314	Human CD20	APC-Cy7	FL-9		1/20	2H7	Mouse IgG2b, κ

Table 2.10: ELISA and ELISpot Antibodies

ELISA: Coating Antibodies			
Company	Catalog #	Name	Working dilution
Southern Biotech	1030-01	Goat Anti-Mouse IgG, Human ads-UNLB	1:1000; 50µl/well
Southern Biotech	1070-01	Goat Anti-Mouse IgG1, Human ads-UNLB	1:1000; 50µl/well
Southern Biotech	1080-01	Goat Anti-Mouse IgG2a, Human ads-UNLB	1:1000; 50µl/well
Southern Biotech	1090-01	Goat Anti-Mouse IgG2b, Human ads-UNLB	1:1000; 50µl/well
Southern Biotech	1100-01	Goat Anti-Mouse IgG3, Human ads-UNLB	1:1000; 50µl/well
Southern Biotech	1020-01	Goat Anti-Mouse IgM, Human ads-UNLB	1:1000; 50µl/well

ELISA: Secondary Antibodies			
Company	Catalog #	Name	Working dilution
Southern Biotech	1031-04	Goat anti-mouse IgG (H+L)-AP	1:1000; 50µl/well
Southern Biotech	1070-04	Goat anti-mouse IgG1-AP	1:1000; 50µl/well
Southern Biotech	1090-04	Goat anti-mouse IgG2a-AP	1:1000; 50µl/well
Southern Biotech	1080-04	Goat anti-mouse IgG2b-AP	1:1000; 50µl/well
Southern Biotech	1100-04	Goat anti-mouse IgG3-AP	1:1000; 50µl/well
Southern Biotech	1020-04	Goat anti-mouse IgM-AP	1:1000; 50µl/well

ELISA: Purified immunoglobulin used for standards, reconstituted to stock solutions of 1 mg/ml from ≥ 95% purified (SDS-PAGE) lyophilized powder.			
Company	Catalog #	Name	Clone
Sigma-Aldrich	I5381	IgG from mouse serum	
Sigma-Aldrich	M1398	IgG1, Kappa from murine myeloma	MOPC 31C
Sigma-Aldrich	M9144	IgG2a, Kappa from murine myeloma	UPC 10
Sigma-Aldrich	M8884	IgG2b, Kappa from murine myeloma	MOPC 141
Sigma-Aldrich	I3784	IgG3, Kappa from murine myeloma	DX
Sigma-Aldrich	M1520	IgM, Kappa from murine myeloma	TEPC 183

ELISPOT: Coating Antibodies			
Company	Catalog #	Name	Working dilution
Invitrogen	M30100	IgG (γ), Goat Anti-Mouse, (Purified)	1:1000; 50 μ l/well

ELISPOT: Secondary Antibodies and Streptavidin Alkaline Phosphatase			
Company	Catalog #	Name	Working dilution
Invitrogen	M30115	IgG (γ), Goat Anti-Mouse, (Biotin)	1:1000; 50 μ l/well
BD Pharmingen™	554065	Streptavidin-Alkaline Phosphatase	1:8000; 50 μ l/well

Table 2.11: Mouse Strains

Strain	Reference
BALB/c	NIMR
C57BL/6	NIMR
hCD20tg BALB/c	Ahuja, A., Shupe, J., Dunn, R., Kashgarian, M., Kehry, M. R., & Shlomchik, M. J. 2007, "Depletion of B cells in murine lupus: efficacy and resistance", <i>The Journal of Immunology</i> , vol. 179, no. 5, p. 3351.
Fc γ RI,II,III-/- C57BL/6	Van Lent, P., Nabbe, K. C., Boross, P., Blom, A. B., Roth, J., Holthuysen, A., Sloetjes, A., Verbeek, S., & Van Den Berg, W. 2003, "The inhibitory receptor Fc γ RII reduces joint inflammation and destruction in experimental immune complex-mediated arthritides not only by inhibition of Fc γ RI/III but also by efficient clearance and endocytosis of immune complexes", <i>The American journal of pathology</i> , vol. 163, no. 5, p. 1839.

Chapter 3: The role of memory B cells and long-lived plasma cells in maintaining serum antibody titres after intranasal infection of BALB/c WT female mice with Influenza A/PR/8/34.

3.1: Introduction

Longitudinal studies of antibody (Ab) responses in humans have shown that Abs appear to be maintained for long periods of time at detectable, and in many cases, protective, levels in the absence of re-exposure after infection with viruses such as measles (Panum *et al.* 1940), yellow fever virus (Sawyer 1931), polio (Paul *et al.* 1951) and Influenza A (Yu *et al.* 2008b). In addition, live attenuated vaccines for measles, yellow fever, polio and Influenza A, as well as vaccinia and rubella, are also able to induce successful life-long protective immunity (Amanna *et al.* 2007; Slifka & Ahmed 1996). The estimated half-life of Abs derived from these studies place vaccinia, rubella, mumps, measles and EBV as the vaccines that establish Ab titres with the longest serum half-lives, ranging from 92 to an infinite number of years (Amanna *et al.* 2007). However, how serum Abs are maintained for this long in the absence of antigenic stimulation is unknown (Ahmed & Gray 1996). In this chapter, I will investigate whether MBC is required for the maintenance of humoral immunity to Influenza.

3.1.1: Maintenance of serum antibodies by memory B cells or long-lived plasma cells

There have been a number of mechanisms proposed to explain how long-term Abs is maintained in the absence of antigen. Both memory B cells (MBCs) and long-lived plasma cells (LLPCs) can survive in the absence of antigen (Manz *et al.* 1998; Maruyama *et al.* 2000), summarized in (Amanna & Slifka 2010), but whether MBC or LLPC can maintain serum Abs independently of each other is unclear. MBC do not spontaneously differentiate into Ab-secreting plasma cells, and mechanisms which are dependent on MBC hinge upon antigenic stimulation or bystander stimulation and T cell help. MBC differentiate into plasma cells upon antigenic stimulation and they have the potential to react to a wider range of pathogenic epitopes than the Abs produced by LLPC, due to their lower-affinity, more polyreactive B cell receptors (BCRs) (Dal Porto *et al.* 2002; Tarlinton & Smith 2000), meaning that both homologous antigen and cross-reactive stimulation can stimulate the

MBC BCR. In addition, human and mouse MBC can differentiate *in vitro* into plasma cells upon non-BCR-mediated, non-specific Toll-like receptor (TLR) stimulation (Benson *et al.* 2009; Bernasconi *et al.* 2002). Therefore there are a number of ways for how MBC can maintain serum Abs. Over time, MBC can continually differentiate into Ab-secreting plasma cells whenever the host encounters homologous re-infection, cross-reactive heterologous infections, or in any inflammatory context with TLR ligands and bystander T cell help, and thus frequently boost serum Ab titres, and/or replenish the LLPC niche (Bernasconi *et al.* 2002).

LLPC have also been shown to have a significant role in maintaining serum Ab. Unlike mature naive and MBCs, LLPCs do not require, or respond to, antigenic or bystander stimulation and are terminally differentiated Ab-secreting cells (Tarlington *et al.* 2008). Instead, homeostatic regulation of LLPC occurs as they compete for space in finite survival niches where they continuously secrete large amounts of immunoglobulin, supported by intrinsic and extrinsic survival resources (Moser *et al.* 2006). Three papers have been published whereby MBC have been depleted either by irradiation or by Ab-mediated depletion, and these papers conclude that long-term Abs can be maintained exclusively by LLPC. Firstly, LCMV-induced LLPCs in mice continue to produce specific Abs for 250 days, despite irradiation to ablate dividing MBCs and inhibit differentiation of MBCs into new plasma cells (Slifka *et al.* 1998). In this study, LCMV-specific Ab levels showed a marked decrease directly after irradiation but thereafter remained stable, albeit at lower titres. Secondly, the B cell depleting mAb clone 2H7 directed against a human CD20 (hCD20) antigen was used by the Shlomchik and colleagues (Ahuja *et al.* 2008) in their hCD20 transgenic x B10 knock-in mice to show that the LLPCs generated by NP-CGG immunization were able to survive and maintain NP-specific serum Abs for up to 150 days *in vivo*, independently of naïve and MBCs, which had been depleted. This finding was consistent with another study, where murine anti-CD20 was directed against wild-type CD20 by the Tedder and colleagues (DiLillo *et al.* 2008), which demonstrated that the Ab responses generated by immunization with DNP-KLH was maintained for up to 77 days after CD20-mediated MBC depletion. Conversely, co-blockade of the adhesion receptors LFA-1 and VLA-4, which are components of the LLPC survival niche, caused the temporary loss of Ag-specific LLPC and the loss of specific serum Ab.

In summary, Ab titres can be maintained without Ag for long periods of time. During immune homeostasis, LLPCs generated by protein immunization have the ability to survive and maintain serum Abs in the absence of a MBC compartment for a long time *in vivo*. Interventions which result in the depletion of LLPC also result in the loss of serum Abs. However it has been suggested that in some contexts, stimulation of MBC may be required to maintain serum Ab titres beyond the finite lifespan of LLPC. In the context of LCMV infection, MBC may contribute to maintaining serum Abs at their optimum level, without which LLPCs are able to maintain Ab titres, albeit at a lower level.

3.1.2: Correlative longitudinal studies on memory B cells and serum Abs in humans

One way of determining the relative contribution of MBCs or LLPCs to long-lived serum Abs in humans is to correlate the concentrations of serum Abs to the abundance of specific MBCs in blood. This is especially informative when analyzing humoral immunity to vaccines with single/few protective antigens like those with protein antigens or single bacterial toxins (e.g. tetanus toxin) or invariant viruses (e.g. yellow fever virus), because in such cases high neutralizing Ab titres often are the best correlates with protective immunity and are used clinically to monitor vaccine efficacy (Amanna & Slifka 2010). Although Abs are the direct product of LLPCs, LLPCs are predominantly found in survival niches in the bone marrow and very rarely, if ever, detected in circulation, and are therefore very inaccessible for studies (Radbruch *et al.* 2006). On the other hand, the majority of MBCs are found in the spleen and lymph nodes, but a small number of specific MBCs recirculate and, in some contexts like NP immunization, are considered to be representative of the peripheral pool of specific MBCs within a month after immunization (Tarlinton & Smith 2000). It is possible that other infections and vaccinations can induce varying distributions of MBCs over different anatomic sites and recirculating MBCs may not necessarily reflect the total antigen-specific population in peripheral organs like spleen and lymph nodes (Doherty 1995).

Nevertheless, bearing these caveats in mind, longitudinal studies have been used to find correlative relationships between serum Abs and recirculating memory cells. If Ab kinetics matches that of a particular blood cell type, this suggests that Abs are maintained by that particular cell type. As mentioned above, some vaccines are able to induce Abs which have

a very long half-life. One of the best-studied examples in humans is the Ab response after immunization with vaccinia (DryVax), which generates serum Abs with an estimated half-life of 92 years (Amanna *et al.* 2007;Amanna *et al.* 2006). Correlative studies of long-term humoral immunity with MBCs after vaccinia vaccination are informative because: a) the tightest predictor of protective immunity to smallpox in the field, based on longitudinal studies (Slifka 2004), as well as in animal models (Davies *et al.* 2005;Shearer *et al.* 2005), is serum Abs. There is no consistent correlation between protective immunity and T cell memory, and neither is there a consistent correlation between T cell memory and serum Abs (Crotty *et al.* 2003;Hammarlund *et al.* 2003); b) since the cessation of vaccination with DryVax in 1972 and worldwide eradication of smallpox in 1980, there is unlikely to have homologous antigenic restimulation in humans for at least 30 years, with the exception of military personnel and people in specialized laboratories who still undergo smallpox vaccination. Smallpox is the only known virus in the orthopoxvirus family, which means that a cross-reactive by a closely related virus is unlikely, and it does not form chronic or latent infections.

DryVax is a live virus vaccine against smallpox derived from freeze-dried calf lymph, and was used from the 1940s to 2008. Immunization is via scarification of superficial epidermal layers. The establishment of several cohorts of human subjects immunized with DryVax in the 1940s has been followed by several very long-term studies examining the persistence of serum Abs to this vaccine. All these studies have shown that vaccinia-specific serum Abs and recirculating MBC responses are very long-lived. However, there has been no consistent correlation between MBC and serum Ab. Hammarlund and colleagues (Hammarlund *et al.* 2003) showed that there was detectable serum neutralizing activity and virus-specific re-circulating B and T memory cells present for up to 75 years after DryVax vaccination; however these cell populations displayed different decay kinetics over time and these authors concluded that they formed distinctly regulated compartments. There was no correlation between virus-specific T cell numbers and Ab titres at early or late time points. However, no direct correlations between MBCs and serum Abs were undertaken in this study. In another study, cross-sectional analysis of human vaccinees ranging from 4 weeks to more than 50 years post-immunization was done by Crotty and colleagues (Crotty *et al.* 2003), and this demonstrated a biphasic kinetic whereby the number of vaccine-specific MBCs increased and declined by about 90% during the first few

months/years post-vaccination and then appeared to stabilize, with vaccinia-specific IgG⁺ MBCs maintained for >50 years, and there was a positive correlation with virus-specific Ab titres, which was also maintained for more than 50 years.

Notably, another group found that, although the majority of smallpox virus-specific MBC are contained within the spleen, virus-specific Ab titres are not lower in splenectomised or B cell depleted individuals, where presumably the majority of virus-specific MBC would have been removed (Mamani-Matsuda *et al.* 2008). However, in this same study, several patients were identified who had virus-specific recirculating MBC but undetectable levels of virus-specific Abs, and conversely, some patients had high levels of virus-specific Abs but no detectable virus-specific recirculating MBC (Mamani-Matsuda *et al.* 2008), leading the authors to conclude that there was no clear correlation between virus-specific MBC and serum Abs, and that they belonged to distinctly regulated compartments. Finally, taking data from a recent study by Amana *et al.*, which followed the Ab responses to several childhood vaccine antigens in 45 adults for a period of 26 years (Amana *et al.* 2007), the same authors expanded their analysis to attempt to fit their data into 6 different models of MBC- or LLPC-dependent mechanisms (Amana & Slifka 2010). These authors did not observe a correlation between MBC-based models and Ab kinetics after vaccinia, varicella zoster virus, Epstein-barr virus, or tetanus-diphtheria vaccinations, but they reported a correlation after measles, mumps and rubella vaccinations (Amana & Slifka 2010). None of the MBC-dependent or LLPC-dependent models adequately fitted the kinetics of serum Ab loss following the complete course of vaccination (Amana & Slifka 2010), suggesting that the mechanisms that contribute to long-term serum Ab maintenance are probably more complex than the MBC or LLPC-dependent mechanisms proposed.

It is difficult to measure kinetics when there is little or no observable decline in serum Abs over time. In cases where vaccinations induce Abs with a shorter half-life, e.g. tetanus and/or diphtheria, it is possible to test a direct correlation with MBCs and serum Abs by following the response to booster immunization during later time points after immunization, when Ab levels decline, and immediately after booster immunization to see whether the rate of decrease in serum Abs matches the rate of decrease in MBC, and whether increases in Ag-specific MBC activation after booster immunization leads to concordant increases in Ab responses, which would then decline during the intervening

periods between outbreaks or vaccinations. These studies have also not shown a clear link between booster-induced elevation of serum Abs and numbers of recirculating MBC just after booster immunization (Leyendeckers *et al.* 1999;Nanan *et al.* 2001). One study reported a strong correlation between serum Abs and recirculating MBC years after the booster, with the additional observation that the number of specific recirculating MBC increased with the number of booster immunizations (Bernasconi *et al.* 2002), summarized by (Lanzavecchia *et al.* 2006).

Finally, vaccine failures, which are immunologically defined the rapid loss of serum titres below a protective threshold, have occurred despite the presence of effective immune priming, a robust early Ab response, and generation of high numbers of specific circulating MBCs, e.g. after *Bordetella pertussis* (Hendriks *et al.* 2010) or Hepatitis B vaccinations (Rosado *et al.* 2011). The latter study has shown that, although HBsAg-specific Abs soon fall below protective levels (<10 mIU/ml) within 5 years in 45 out of 99 of children tested, robust MBC recall responses can still be induced (Rosado *et al.* 2011). This non-correlation between waning Ab titres and the MBC recall response suggests that, in the absence of antigenic restimulation by booster immunization, MBCs do not in fact differentiate into plasma cells to maintain serum Ab. It could be that in the case of Hepatitis B, which is a virus which takes a few days to disperse from the site of inoculation, there is no need for MBCs to maintain serum Abs as there is enough time for them to differentiate into plasma cells and produce Abs to prevent the systemic spread of the virus.

In summary, although some infections and vaccines can induce long-lived serum Abs in the absence of antigen, and others induce Abs with a shorter half-life which requires periodic boosting, this does not consistently correlate with numbers of recirculating MBCs. The kinetics of serum Ab loss and numbers of recirculating MBCs appear to be different where there has not been any known antigenic re-stimulation, and *yet also* appear independent of homologous boosters. Therefore, from longitudinal studies following vaccination in humans, there is no clear resolution of whether MBCs are required for long-lived serum Abs after human infections or vaccinations.

3.1.3: Antibody maintenance after memory B cell depletion

A number of agents are able to selectively deplete MBCs or plasma cells (Clatworthy 2011;Hu *et al.* 2009). One of them, Rituximab™, which is a chimeric anti-human CD20 monoclonal Ab, is used as a biological treatment for diseases caused by abnormal B cell development, e.g. haematological neoplasms (Coiffier *et al.* 2002;Maloney *et al.* 1997;McLaughlin *et al.* 1998) or by pathogenic autoantibodies, e.g. autoimmune diseases such as rheumatoid arthritis (Rastetter *et al.* 2004;Silverman & Weisman 2003), and anti-rejection treatments for organ transplants by depleting B cells (Becker *et al.* 2004;Milpied *et al.* 2000;Vo *et al.* 2008). CD20 is a trans-membrane protein present on the surface of all B cells from pre-B cell stage onwards but not on terminally differentiated plasma cells (Gong *et al.* 2005). Depletion of B cells by Rituximab™ treatment is very specific and only depletes naïve, mature, MBCs and short-lived plasma cells. LLPCs, which have downregulated CD20, and other cell types like T cells and granulocytes, which do not express CD20, are not depleted by Rituximab™ (DiLillo *et al.* 2008;Gong *et al.* 2005). Thorough analysis of B cell depletion in the periphery in human patients is difficult; however it has resulted in a prolonged loss of peripheral blood CD19⁺ B cells (<0.0005 x 10⁶/ml) for more than 25 weeks in patients with rheumatoid arthritis (RA) (Cambridge *et al.* 2003;Edwards *et al.* 2004). Furthermore, Rituximab™ treatment results in the profound and rapid reduction of B cells in the peripheral blood (>99%), bone marrow (>90%) and lymph nodes (>80%) after a single treatment dosage in macaques (Reff *et al.* 1994). Although peripheral B cells recover within a year in patients treated for RA, the loss of MBCs after Rituximab™ treatment is prolonged, and there is a significant loss of recall humoral responses to vaccination boosters for up to 2 years post-treatment (Cambridge *et al.* 2003). Whether LLPC are truly not affected by Rituximab™ treatment has not been formally confirmed in human patients, but Rituximab™ therapy indirectly provides a system in humans to test the longevity of pre-established LLPC and their ability to maintain stable serum Ab titres, in the absence of a functional MBC compartment.

In most patients treated with Rituximab™, there is a significant reduction in auto-Abs for up to 1 year following treatment (Cambridge *et al.* 2003;Cambridge *et al.* 2006). By contrast, levels of pre-existing anti-microbial or anti-vaccine Abs, e.g. anti-pneumococcal or anti-tetanus Abs, have been found to remain stable for up to 1 year after Rituximab™

treatment (Cambridge *et al.* 2003;Cambridge *et al.* 2006), suggesting that pre-existing serum Abs can be maintained at normal levels by LLPCs in the absence of MBCs. However, risk of re-infection with Hepatitis B virus and a higher incidence of infection has been reported in patients (Cooper & Arnold 2010), indicating that mature and MBCs may be particularly important for protective immunity against those infections (Clatworthy;Cooper & Arnold 2010). Total serum immunoglobulin levels do not fall below normal levels (about 2 mg/ml) in the majority of patients treated with one course of Rituximab™ (Stasi *et al.* 2001). However, in relapsing and remitting cases of multiple sclerosis, where patients are given up to 5 cycles of Rituximab™ treatment, a progressive loss of the immunoglobulin subclasses IgM, IgG and IgG has been reported, in some cases falling below the normal range (Edwards *et al.* 2004). It has been noted that IgM levels tend to fall more than IgG and IgA levels (Keystone *et al.* 2007).

In contrast to CD20-targeted therapies like Rituximab™, which do not deplete LLPCs, CD19-targeted therapy has been found to also deplete 50% of murine bone marrow LLPCs, and is more efficacious at reducing circulating IgG Abs than CD20-targeted therapy. Depletion of plasma cells by Atacicept, which blocks the cytokines BAFF and APRIL, which are two survival resources crucial for LLPC survival in bone marrow, results in a significant reduction in serum IgG levels (Dall'Era *et al.* 2007). Patients who are treated by combination chemotherapy or bone marrow transplantation, whereby there is an almost complete loss of bone marrow plasma cells, there is a significant loss of serum immunoglobulin as well as loss of specific protective Ab titres against tetanus, diphtheria, measles and mumps in these patients (Lim *et al.* 2008;Nishio *et al.* 2006), possibly reflecting the contribution of LLPC to total serum immunoglobulin and Ab responses induced by these vaccinations. These findings are by no means universal: one study has reported that despite the use of Bortezimib (Everly *et al.* 2010;Wahrmann *et al.* 2010), a proteasome inhibitor which causes apoptosis of plasma cells, there is preservation of protective anti-microbial Ab titres and only very modest losses in some auto-Abs, indicating that LLPC may not be the exclusive source of anti-microbial serum Abs, and that they can contribute partially to the production of autoimmune Ab.

The chimeric monoclonal Ab 2H7, which recognizes epitopes similar to that recognized by Rituximab™, is used to deplete B cells in a hCD20 BAC transgenic mouse strain, where

the expression of hCD20 is similar to that of mouse CD20 (Ahuja *et al.* 2007;Gong *et al.* 2005). This transgenic model provides a clean system whereby the efficacy of B cell depletion can be extensively characterized. One study made use of genetically modified hCD20 transgenic mice crossed with B cell receptor B1-8 knock-in mice, in which approximately 2% of hCD20⁺ B cells were NP-specific and generated an abnormally large pool of MBC and LLPC after immunization (Ahuja *et al.* 2008). When these mice were depleted of MBCs after NP-CGG immunization, there was preservation of LLPC numbers and pre-existing NP-specific serum Ab, in the absence of MBCs for up to 16 weeks after B cell depletion (Ahuja *et al.* 2008). These results are consistent with another study where a mouse anti-CD20 Ab, 2B8, which binds to murine CD20 with similar affinity as the humanized 2H7, was used to deplete B cells in wild-type mouse strains, where pre-existing total serum immunoglobulin was also maintained for at least 77 days after depletion with 2B8 (DiLillo *et al.* 2008).

These hCD20 transgenic mice have also been crossed to autoimmune-prone mice and used to determine the role of autoimmune Abs to disease. In these studies, anti-hCD20 treatment or treatment with anti-mCD20 mAb has resulted in delayed symptoms or reversal of disease. However the mechanisms of amelioration of autoimmune symptoms do not always correlate with loss of autoimmune Abs, reflecting the role of multiple arms of immune responses to autoimmunity. In one study using the hCD20/Non-Obese Diabetic (NOD) model of Type 1 diabetes, (Hu *et al.* 2007) a 9-day cycle of 4 intravenous injections with a 3-day interval resulted very effective B cell depletion. B cell depletion resulted in prolonged prevention or delay of the onset of full-blown diabetes, even after insulinitis had begun, and was also able to reverse diabetes at a relatively advanced stage of the disease. The mechanism of recovery in this study, however, was found to be not due to reduction of autoantibodies, but due to the promotion of development of regulatory T and B cells and decrease in effector CD4 and CD8 T cells. Surprisingly, the role of autoantibodies was not examined in this study. In another study, using the K/BxN hCD20tg mouse model of inflammatory arthritis (Huang *et al.* 2010), which develop spontaneous arthritis in response to the auto-antigen glucose-6-phosphate isomerase (GPI), mice were treated with 1mg/wk anti-hCD20 mAb. Analysis of anti-GPI ASCs in bone marrow, spleen, lymph nodes, peripheral blood and peritoneal cavity by ELISpot revealed that the highest numbers of anti-GPI ASC were plasmablasts in spleen and lymph nodes, which expressed intermediate

levels of hCD20 and were susceptible to anti-hCD20 mAb-mediated depletion, leading to a reduction in serum titres of anti-GPI Abs over time, whereas total Ab titres were not affected. The main finding of this paper was that the reduction of anti-GPI Ab in serum was a result of depletion of GPI-specific short-lived plasma cells. By contrast, non-specific LLPC were not affected by anti-hCD20 mAb treatment.

Several papers have demonstrated that certain autoimmune contexts can pre-dispose hosts to ‘resistance’ to B cell depletion by anti-CD20 mAbs. In hCD20tg crossed to an lupus-prone MRL/MpJ-*Fas^{lpr}* background, B cells were very refractory to depletion compared with BALB/c mice across different anti-hCD20 clones (Ahuja *et al.* 2007), possibly due to impairment of IgG-mediated phagocytosis by macrophages and neutrophils exposed to lupus mouse serum (Ahuja *et al.* 2011). In these mice, significant, but not complete, depletion of B cells was only achieved with extremely high doses of anti-hCD20 mAb for prolonged periods of time (10mg/wk for 10 weeks) and similarly high doses were required when anti-mCD20 was used to deplete B cells in another autoimmune lupus-prone strain NZB/W. Using this high-dose regimen to deplete B cells, amelioration of lupus symptoms was found to be a combinatorial effect of a reduction in T cell activation, hypogammaglobulinaemia and lower titres of autoimmune Abs (Ahuja *et al.* 2007). The selective depletion efficacy of some B cell subsets was also shown in a murine model of spontaneous autoimmune thyroiditis in NOD.H-2h4 mice, where about 50-80% of splenic B cells were depleted by a single i.v. injection of 250µg anti-mCD20 mAb. In this system, splenic marginal zone B cells were resistant to depletion, whilst most follicular and immature B cells were effectively depleted. Despite the retention of marginal zone B cells, anti-mouse thyroglobulin autoantibody responses were significantly reduced and thyroiditis was reversed (Yu *et al.* 2008a). Therefore, in the autoimmune context, the depletion efficacy of anti-CD20 mAb treatment is reduced and may confound the interpretation of data from patients treated with Rituximab™ for autoimmune diseases such as SLE.

In summary, despite MBC depletion by Rituximab™ and prolonged suppression of MBC-mediated humoral recall responses in patients, the majority of treated patients maintain normal levels of some pre-existing serum anti-microbial immunoglobulin up to 1 year post-depletion. Autoantibodies tend to be preferentially reduced, and antimicrobial Abs tend to be maintained at pre-existing levels, in patients treated with anti-CD20 mAbs, but the

reason for this is not clear. It is difficult to assess the extent of depletion of peripheral B1, MBCs and short-lived inflammatory plasma cells from germinal centres in secondary lymphoid organs and ectopical germinal centres in patients treated with Rituximab™. LLPC are predominantly found in bone marrow niches and are also very inaccessible, and it is difficult to confirm whether LLPC are truly non-depleted. Therefore, as the depletion efficacy of peripheral B cells by Rituximab™ has not been extensively characterized in humans, it is difficult to draw conclusions as to which cell is the source of autoantibodies and anti-microbial Abs and whether LLPC and MBCs are independent sources of serum Ab. In mice, where anti-CD20 targeted B cell depletion has been extensively characterized, the LLPC and serum Abs induced by intraperitoneal protein immunization can be maintained for up to 77 – 150 days, in the absence of a MBC compartment and without any other immunological stimulus (Ahuja *et al.* 2008; DiLillo *et al.* 2008). However, the findings of anti-CD20 mAb mediated depletion in autoimmune-prone mice has revealed that depletion of B cells in an autoimmune context is relatively inefficient, suggesting that the same occurs in human patients. Therefore the conflicting data on serum Abs titres after MBC depletion may simply reflect the different contexts in which these experiments are done and the resulting variable depletion efficacies. It is possible that MBCs have a significant contribution to long-lived Abs titres in situations when there may be continuous Ag stimulation, e.g. autoimmune diseases or some infections, whilst LLPC play a predominant role in Ab maintenance in situations where Ag is more rapidly eliminated, e.g. protein immunization. It is still unknown whether LLPC generated by an infectious agent rather than immunization protocol also maintains serum Abs independently of MBCs.

3.1.4: Kinetics of the humoral immune response to Influenza A/PR/8/34 in mice

Influenza A/PR/8/34 (PR8) is a human-derived that is considered a prototype strain of the H1N1 subtype of Influenza A viruses. PR8 can affect mice and the mouse model for PR8 infection has been extensively used and characterized. The infection generates a predominantly Th1 response with the induction of virus-specific CD8 cytolytic T cells and a rapid Ab response comprising predominantly IgA and IgG isotypes. Elimination of CD8⁺ T cells does not abolish viral clearance and Abs to influenza virus haemagglutinin on their own are able to eliminate the virus from the lung, demonstrating the substantial contribution of the Ab response in anti-viral protection.

The outcome of experimental infection of mice with PR8 depends on many parameters, including the dose and route of administration. Infection via intranasal instillation results in either non-lethal respiratory disease (influenza model) or lethal viral pneumonia (viral pneumonia model) if light anesthesia is applied to allow viral inhalation directly into the lower respiratory tract. Regardless of route of administration, active infection with PR8, i.e. viral replication and generation of viable viral progeny, is typically confined to the airway epithelium although viral RNA can be detected in extrapulmonary tissues.

In the influenza model initiated by intranasal instillation, viral titres peak within 3-5 days and decline to undetectable levels by 14 days (Eichelberger *et al.* 1991). Intranasal PR8 infection initiates a systemic humoral immune response which comprises in an early appearance of IgM in the serum, followed by IgG2a and IgG1 which predominates from day 14 onwards (Jones & Ada 1986; Jones & Ada 1987). The titre of virus-specific IgA in the serum is usually very low. Virus-specific ASC can be detected in mesenteric lymph nodes, lung, spleen and bone marrow from day 7 to day 40 of infection, although the isotype distribution varies in each organ (Fazekas *et al.* 1994). Most studies of correlates of immune protection against influenza have focused on serum anti-HA1 Ab. The results of these studies generally agree that serum HA1-specific Abs are correlated with protection, whereas Abs specific for nucleoprotein (NP) and matrix protein 1 have less therapeutic potency, possibly because they are internal proteins not typically accessible on intact virions. HA1-specific serum IgG2a predominates in both quantity (Fazekas *et al.* 1994) and protective efficacy. The ability of serum IgG Abs to prevent re-infection has been documented by passive transfer of serum or HA-specific mAb into immunodeficient SCID mice, which is able to protect from infection (Gerhard *et al.* 1997; Palladino *et al.* 1995).

3.2: Aim

The intranasal route of infection with PR8 of wild-type mice is a well-characterized model and one that induces long-lived humoral immunity. However the cellular mechanisms for Ab maintenance are not known. In this chapter, I aim to determine whether LLPCs are able to survive and maintain serum PR8-specific Abs in the absence of a MBC compartment, by using the hCD20tg system of depleting MBCs.

3.3: Objectives

1. To determine whether intranasal infection with Influenza A/PR/8/34 of wild-type BALB/c female mice can induce long-term serum Abs, LLPCs and MBCs for up to 234 days post-infection.
2. To characterize hCD20tg mice and determine the extent of depletion using the anti-hCD20 mAb 2H7.
3. To determine the effect of 2H7-mediated depletion on pre-existing HA-specific serum IgG and LLPCs.

3.4: Results**3.4.1: Development of long-lived specific systemic humoral memory after primary intranasal PR8 infection in BALB/c WT mice.****Serum antibody**

Influenza A virus replicates in both the upper and lower respiratory tract. Viral replication peaks approximately 48 hours after inoculation into the nasopharynx and declines slowly, with little virus shed after about 6 days. The B cell response to influenza virus does not depend on extensive viral replication or viral persistence. No influenza virus genetic material can be demonstrated 14 days after infection by PCR (Eichelberger *et al.* 1991). To characterize the development of humoral immunity in BALB/c mice infected with intranasal instillation of PR8, specific serum Ab concentrations and specific plasma cells and MBCs were quantified at various time points after infection. The median value of the concentration of HA-specific serum IgG rose significantly from 116 µg/ml on d72 to 237.8 µg/ml on d91 (Mann-Whitney test; $P = 0.0317$), and to 401.4 µg/ml on day 190 but there was no statistical difference between d91 and d190 ($P = 0.2844$) (**Figure 3.1A**). The concentration of serum HA-specific IgM was analyzed from serum obtained on d14, 31, 72 and 190 post-infection. There was a significant increase (d14 vs. d31; $P = 0.0003$) in the median value of HA-specific IgM from 0.416 µg/ml on d14 to 2.03 µg/ml on d31. This then reached a plateau with no significant change to d72, but there was a significant decrease by d190 (d72 vs. d190; $P = 0.0286$) to comparable levels with d14 (d14 vs. d190; $P = 0.7641$). The results therefore demonstrated that a primary infection induced a slow accumulation of specific IgG by d91, thereafter remaining at a stable level for the rest of the period of observation until d190. This was accompanied by a faster but smaller increase in HA-specific IgM by d31, followed by a decrease by d190.

HA-specific antibody-secreting cells in spleen and bone marrow

The frequency and total number of HA-specific Ab secreting cells (ASC) in spleen and bone marrow from femur pairs were quantified using ELISpot at various time-points up to d234 after PR8 infection (**Figure 3.1B-C**). In spleen, there was no significant change in

either the frequency and number of HA-specific ASC between d150 and d234 (**Figure 3.1B**). The median frequency of HA-specific ASC in bone marrow increased significantly from $11.32/10^6$ cells on d28 and $67.75/10^6$ cells on d150; but did not significantly change after d150 (**Figure 3.1C**). However the total median number of HA-specific ASC in bone marrow reached a stable number by d63 and remained similar on d150, by which all of the mice had above background numbers of HA-specific ASCs, and up till d234 (**Figure 3.1C**). This data therefore demonstrates that HA-specific ASCs are detected in spleen and bone marrow, where they stabilize in number by d150 in the spleen and d63 in the bone marrow. LLPCs have been detected in the lymph nodes after intranasal influenza vaccination in association with certain adjuvants (Kasturi *et al.* 2011), but HA-specific LLPC in lymph nodes were not measured in these experiments.

HA-specific memory B cells in spleen

The frequency and total number of HA-specific MBCs (MBC) in spleens were quantified using limiting dilution ELISpot at various time-points up to d337 after PR8 infection (**Figure 3.1D**). Because the majority of MBCs are thought to reside in splenic reservoirs (Mamani-Matsuda *et al.* 2008), and due to the difficulty in doing limiting dilution ELISpot in more than one organ, I limited the quantification of HA-specific isotype-switched MBCs to cells in the spleen. Stable numbers of MBCs were established by a median value of 111.6 per spleen by d150 and were not statistically different from the median value of 168.6 per spleen on d337.

These data demonstrate that a primary intranasal infection with Influenza A/PR/8/34 induces stable concentrations of HA-specific Ab as well as stable numbers of HA-specific LLPCs and MBCs by 150 days post-infection.

3.4.2: Experimental protocol for depletion of MBCs from hCD20tg/BALB/c mice.

In order to determine whether MBCs or LLPCs were the source of long-lived Abs after intranasal Influenza A/PR/8/34 (PR8) infection, I designed a system whereby MBCs would be depleted 150 days after PR8 infection. If maintenance of Abs was dependent on the differentiation of MBCs into plasma cells, then this should result in the loss of Ab. In order

to do this, I used hCD20 transgenic mice and depleted mature and MBCs using the anti-hCD20 mAb, 2H7 (**Figure 3.2A**). By contrast, LLPCs have been documented to be resistant to depletion by 2H7, either because they have downregulated their surface hCD20 and/or due to their niche location in the bone marrow, and would therefore remain after 2H7 treatment. 6-10 wk old female hCD20 transgenic (hCD20tg) and hCD20tg-negative littermate control mice were intranasally infected with 250 haemagglutinin units of PR8. Serum was obtained 28, 56 and 84 days after infection to determine the Ab response to PR8 HA. After resting the mice for 20 weeks to allow both the MBC and ASC pools to become stably established, both groups of mice were given anti-hCD20 mAb for 2 weeks (8 x 0.5mg; 2mg/wk i.p.) to deplete B cells. Depletion was confirmed 24 hours after the last injection of 2H7 in a subset of mice. MBC, ASC and serum HA-specific IgG was quantified 42 and 150 days post-depletion.

In order to determine whether hCD20tg mice show similar responses as wild-type BALB/c mice, the concentration of HA-specific serum IgG after PR8 infection in hCD20tg and hCD20tg-negative mice and BALB/c WT mice was quantified by ELISA. There was no significant difference in the mean HA-specific IgG concentrations between the three groups (**Figure 3.2B**). Therefore, hCD20tg mice and their littermate controls mount similar Ab responses to PR8 HA compared to wild-type BALB/c mice.

3.4.3: The 2H7 mAb targets hCD20 on CD19⁺ B cells in spleens from hCD20tg mice but not in hCD20tg-negative littermates.

hCD20 transgenic mice were backcrossed for at least 7 generations with NIMR BALB/c mice. Spleen was obtained from 1 hCD20tg mouse and 1 hCD20tg-negative littermate and surface stained by flow cytometry using anti-hCD20 and anti-CD19 Abs. The anti-hCD20 mAb 2H7 was purified from a hybridoma supernatant as described in **Methods and Materials**. This 2H7 mAb recognized hCD20 on splenic CD19⁺ cells from the hCD20tg mouse but not in the hCD20tg-negative littermate control, when compared with a commercial 2H7-PE mAb and an isotype control (**Figure 3.3**). For all experiments, PBMCs were obtained from every hCD20tg and hCD20tg-negative mouse and screened with anti-hCD20 and anti-CD19 Abs before experiments were initiated.

3.4.4: Distribution of hCD20 on different cell lineages after PR8 infection

150 days after infection with PR8, hCD20 expression profile on various cell types in spleen and bone marrow was analyzed by flow cytometry in hCD20tg and hCD20tg-negative littermates (**Figure 3.4**). The lineage markers used and the cell types they delineated were CD19 (B cells), B220⁻ CD138⁺ (LLPCs), CD3 (T cells), DX5 (NK cells in BALB/c mice express DX5 but not NK1.1), CD11b (macrophages, monocytes, granulocytes, NK cells), CD11c (mainly dendritic cells, but also macrophages, monocytes, neutrophils, B cells) and F4/80 (macrophages). In agreement with previous studies (DiLillo *et al.* 2008), hCD20 is expressed by CD19⁺ B cells in spleen and bone marrow. In addition, hCD20 was expressed by B220⁻ CD138⁺ LLPCs in the spleen and at intermediate levels on approximately 40% of B220⁻ CD138⁺ LLPCs in the bone marrow. However, hCD20 is not expressed on any other cell type.

3.4.5: Depletion efficacy after 2H7 treatment

Having determined the expression profile of hCD20 on different cell types in hCD20tg and hCD20tg-negative mice, both groups were infected with PR8 and rested for 150 days. The short-term depletion efficacy of 2H7 treatment was tested in these PR8-immune mice using flow cytometric analysis before, and 1, 42 and 150 days post-treatment. After 2H7 administration, there was rapid loss of spleen cellularity (**Figure 3.5A**), but not in bone marrow cellularity (**Figure 3.5B**) when analyzed 1 day following the last dose of 2H7. In the spleen, there was recovery to approximately 50-60% of pre-depletion total cell numbers by days 42 and 150 post-depletion. The effect of 2H7 treatment on the frequencies of neutrophils, macrophages, dendritic cells, monocytes, eosinophils and mast cells was determined 1 day post-treatment using the gating strategy shown in the figure (**Figure 3.6A-B**). There was no change in the frequencies of neutrophils, macrophages, eosinophils, monocytes and mast cells in the bone marrow (**Figure 3.6C-G**). However, there was a significant decrease in dendritic cells in both the hCD20tg and hCD20tg-negative littermates (**Figure 3.6H**), suggesting that the slight depletion of dendritic cells was not a hCD20-specific effect but a bystander effect of the immunization. Similarly, there was also a depletion of both CD4 T cells (**Figure 3.7B**) and CD8 T cells (**Figure 3.7C**) in the bone marrow in hCD20tg but not in hCD20tg-negative littermates. There was no significant

change in DX5⁺ NK cells (**Figure 3.7D-E**). In summary, treatment with 2H7 resulted in a slight depletion of DCs, CD4⁺ and CD8⁺ T cells, but not neutrophils, macrophages, eosinophils, monocytes and mast cells.

By contrast, in the spleen, there was effective, rapid and specific B cell depletion. Surface markers CD19⁺, CD21⁺ and sIgD⁺ were independently used to quantify B cells (**Figure 3.8A**), and these markers were co-expressed on double plots (**Figure 3.8B**). In all three different analyses, B cells were depleted by 70-75% in the frequency and a greater than 95% reduction in the numbers 1 day post-depletion. Although frequencies were restored to pre-depletion levels by d150 post-treatment, the numbers of CD19⁺, CD21⁺ and sIgD⁺ cells were still significantly reduced at d150 (**Figure 3.8C-E**), perhaps indicating that there may have been a long-term disruption of particular splenic B cell niches which affected their ability to re-constitute naïve B cell numbers to pre-depletion levels. In contrast, splenic B cells before and after 2H7 treatment were very similar in hCD20tg-negative littermates, showing that depletion was 2H7 specific, rather than a bystander effect of the mAb treatment. CD19⁺ B cells were then quantified in spleen, peripheral blood mononuclear cells (PBMC), lymph nodes and bone marrow (**Figure 3.9A**). Similar to other studies, B cell depletion was not 100% complete and varied in spleen, lymph node, bone marrow and PBMC as assessed using CD19. Approximately 70-75% of CD19⁺ B cells were depleted in spleen, lymph node and PBMC and 50% depletion in bone marrow when analyzed 1 day post-depletion (**Figure 3.9B-E**).

Analysis of hCD20 expression on B cell subsets in spleen showed that hCD20 is expressed on immature, marginal zone and follicular B cells (**Figure 3.10A**). Despite all groups expressing hCD20, analysis of immature, marginal zone and follicular B cells 1 day post-depletion revealed very significant depletion of marginal zone (MZ) and follicular (FO) B cells, but no decrease in frequency of immature B cells (Imm) from the spleen (**Figure 3.10B-D**). In bone marrow, B cell depletion was highly specific and corresponded to the expression of hCD20 on pre-B cells (**Figure 3.11A**); only depleting pre-B cells and not pre-pro and pro-B cells (**Figure 3.11B**).

Splenic germinal centre B cells (B220⁺ IgD⁻ CD38⁻ GL7⁺) and MBCs (B220⁺ IgD⁻ CD38⁺ GL7⁻) both expressed hCD20 (**Figure 3.12A and 3.13A**). Assessment of short- and long-

term depletion of MBCs and germinal centre B cells was done on d1, 42 and 150 after 2H7 treatment. 2H7 treatment resulted in 85% reduction in frequency and number of splenic MBC (B220⁺ IgD⁻ CD38⁺ GL7⁻) even only 1 day after the last 2H7 administration, and the numbers of splenic MBC remained significantly decreased for up to 150 days post-treatment (**Figure 3.12B-C**). Surprisingly, splenic germinal centre B cells were found to be completely resistant to 2H7-mediated depletion. Instead there was rapid and drastic increase in both frequency and number of splenic germinal centre B cells, particularly in the hCD20tg-ve littermates, 1 day after the last 2H7 administration (**Figure 3.13B-C**).

Despite intermediate to high levels of expression of hCD20 on B220⁻ CD138⁺ plasma cells in spleen and bone marrow (**Figure 3.14A**), plasma cells were not depleted by 2H7 administration. In spleen, there was a large increase in the frequency and number of B220⁻ CD138⁺ plasma cells 1 day after the last 2H7 administration, but this had decreased to pre-depletion levels by day 42 (**Figure 3.14B-C**). In bone marrow, there was no significant change in the frequency and number of B220⁻ CD138⁺ plasma cells for up to 150 days after 2H7 treatment (**Figure 3.14D-E**).

3.4.6: Depletion of HA-specific memory B cells and its effect on HA-specific long-lived plasma cells and HA-specific IgG.

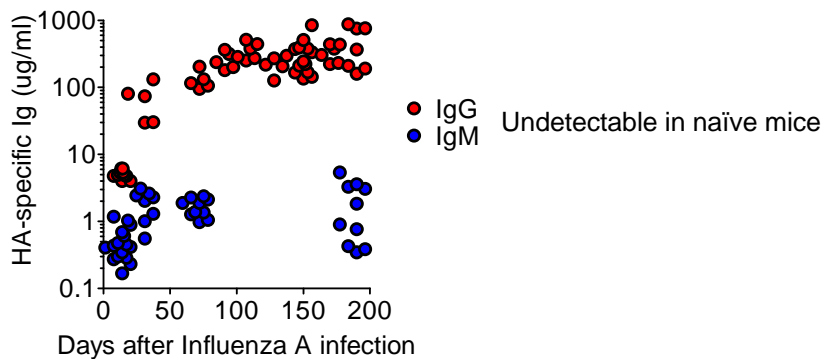
Having characterized the depletion efficacy and specificity by 2H7 treatment and established that treatment with 2H7 depletes CD19⁺ B cells but not B220⁻ CD138⁺ LLPC, the next task was to determine whether 2H7 treatment depleted pre-established HA-specific MBC from PR8-immune hCD20tg or hCD20tg-negative littermates. The numbers of functional HA-specific MBC in spleen was determined by limiting dilution ELISpot. There was a significant reduction of more than 80% of HA-specific IgG MBC from the spleen 1 day post-2H7 treatment, and this loss remained significant for up to 150 days post-treatment (**Figure 3.15A**). By contrast, although there was a greater than 95% reduction in total IgG MBC 1 day post-2H7 treatment, numbers of total IgG MBC returned to pre-depletion levels by day 42 and remained stable up to 150 days post-treatment (**Figure 3.15B**). There was no change in numbers of HA-specific MBC and total IgG MBC in the hCD20tg-negative littermate controls. Therefore, depletion of HA-specific MBCs was rapid but prolonged, remaining significantly depleted for up to 150 days after 2H7 treatment,

with 2 out of 3 hCD20tg mice at day 150 still having undetectable numbers of HA-specific MBC (individual data points not shown). Total IgG MBCs were reconstituted to pre-depletion levels by day 42, probably due to homeostatic division or stimulation of MBCs for other specificities by environmental antigens or other undetected infections throughout this time period.

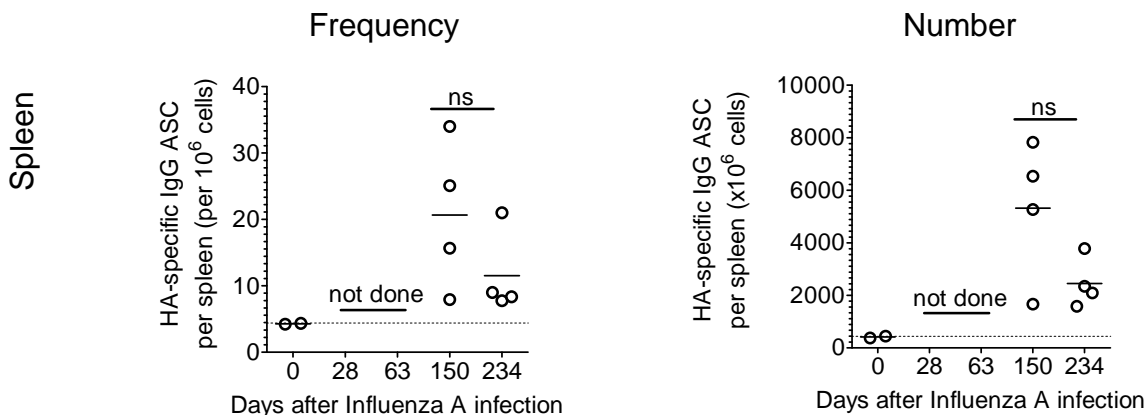
Having determined that 2H7 resulted in significant and prolonged depletion of HA-specific MBC, next I quantified HA-specific Ab-secreting cells (ASC) functionally by ELISpot. There was no reduction in HA-specific ASC in either spleen and bone marrow observed 1 day and 42 days post-2H7 treatment, but there was a significant decrease in HA-specific ASC in both spleen (**Figure 3.16A**) and bone marrow (**Figure 3.16C**) at day 150 post-treatment in hCD20tg mice. There was also a decrease in total IgG-secreting ASC in spleen (**Figure 3.16B**) but not in bone marrow (**Figure 3.16D**). Therefore, HA-specific ASC were not immediately depleted by the short two-week course of 2H7 treatment, consistent with the lower expression levels of hCD20 on plasma cells (**Figure 3.14A**). Instead, their loss at a late time point (at day 150 but not before day 42 post-2H7 treatment) suggests that the loss of HA-specific ASC was more likely to be a consequence of the long-term depletion of HA-specific MBC, possibly because of a lack of replenishment, than a direct effect of 2H7 treatment.

Finally, I quantified HA-specific serum IgG concentrations after 2H7 treatment and found that there was a significant decrease of HA-specific serum IgG only from day 90 up to day 150 post-treatment in hCD20tg mice, whereas HA-specific serum IgG remained stable in hCD20tg-negative littermates (**Figure 3.17A**). By contrast, there was no difference in total serum IgG concentrations either between hCD20tg or hCD20tg-negative littermates after 2H7 treatment, across the different time points (**Figure 3.17B**). These results suggested that MBCs contribute significantly to the long-term maintenance of HA-specific serum IgG.

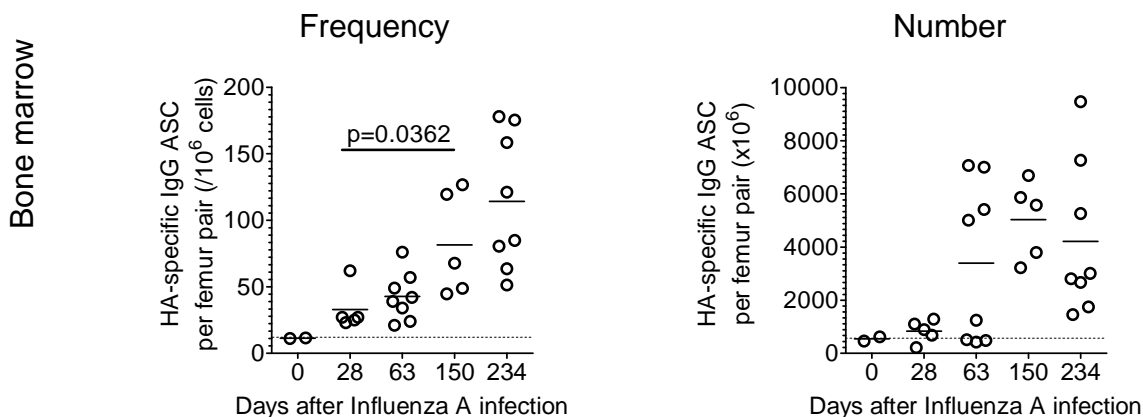
3.1A Serum antibody



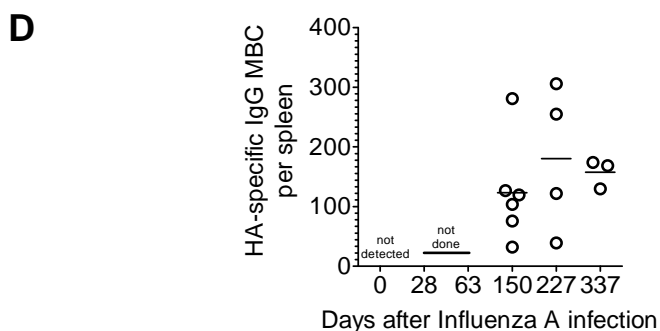
B ASC in spleen



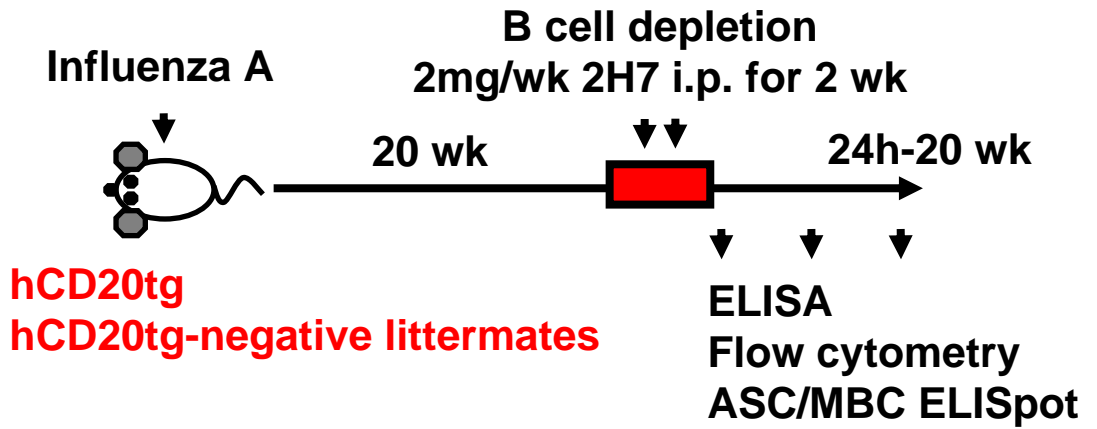
C ASC in bone marrow



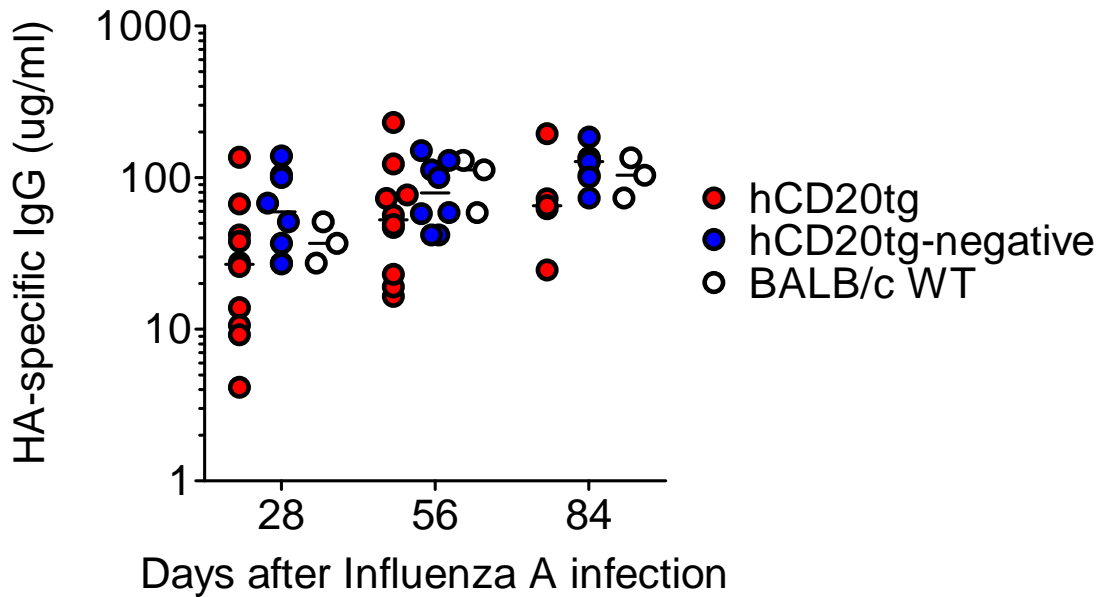
MBC in spleen



3.2A



B

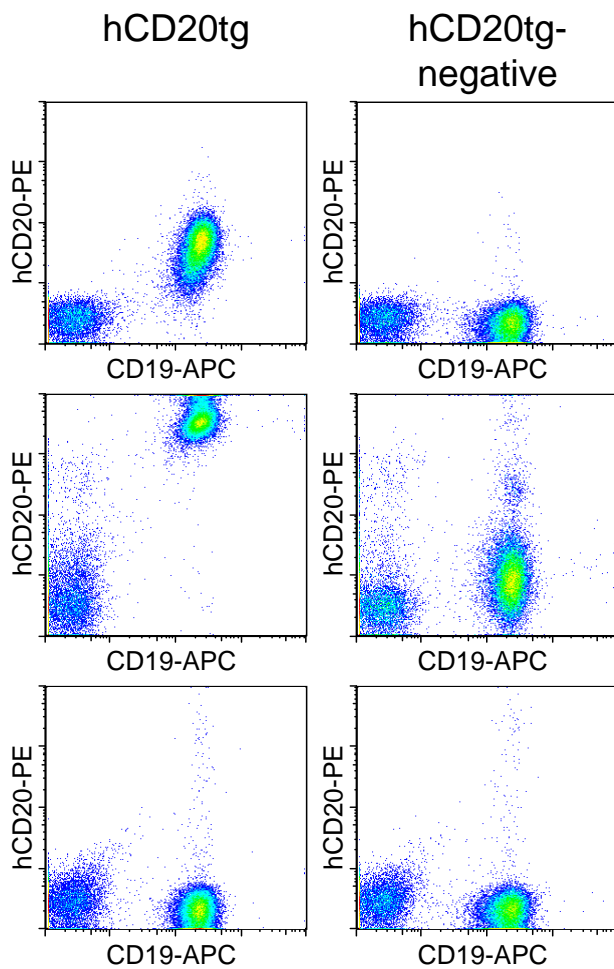


3.3

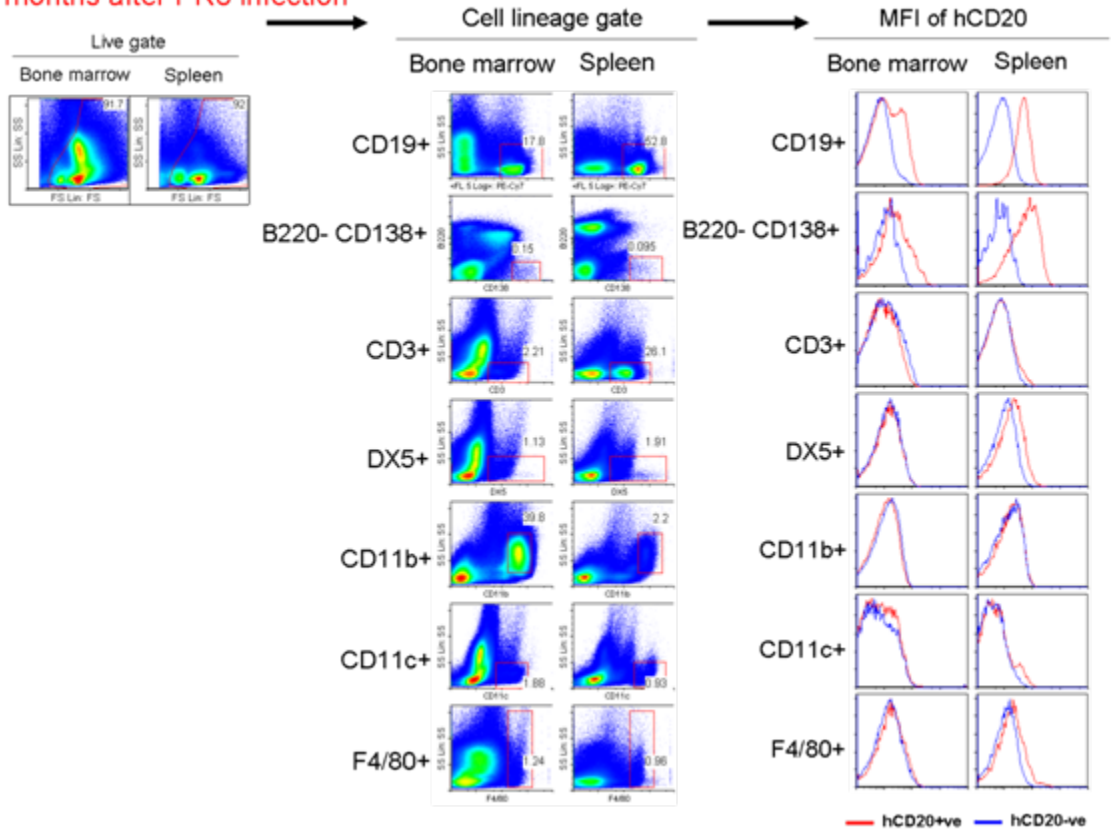
Commercial 2H7-PE antibody

Purified 2H7 antibody
+ anti-mouse IgG2b-biotin
+ streptavidin-PE

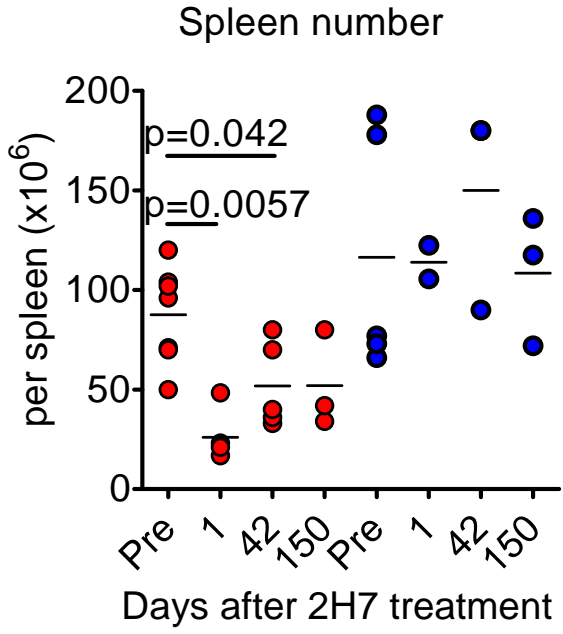
Mouse IgG2b-PE
isotype control



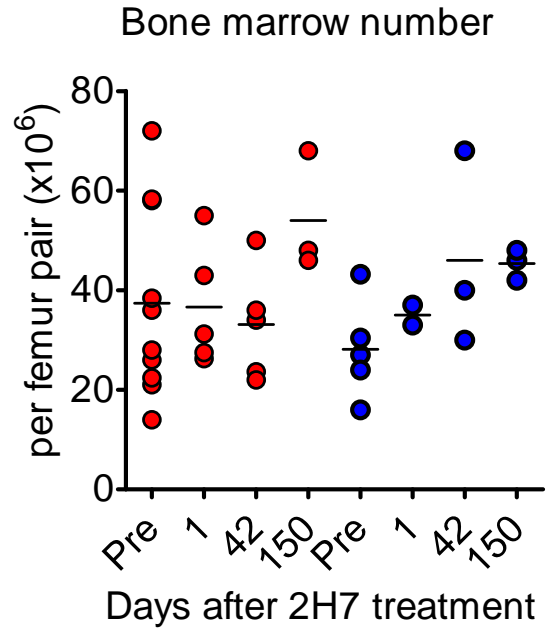
3.4 5 months after PR8 infection



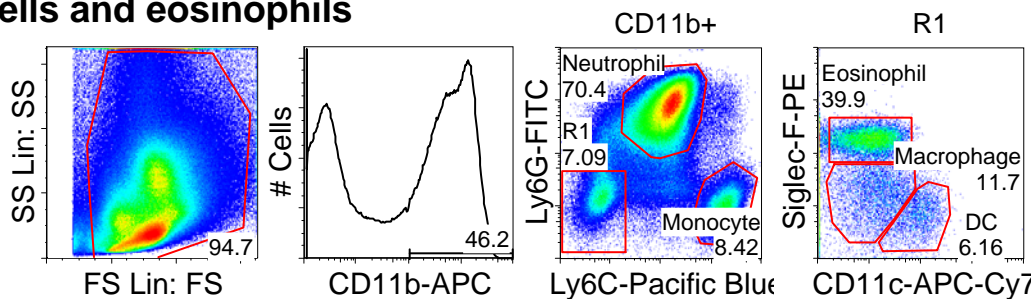
3.5A



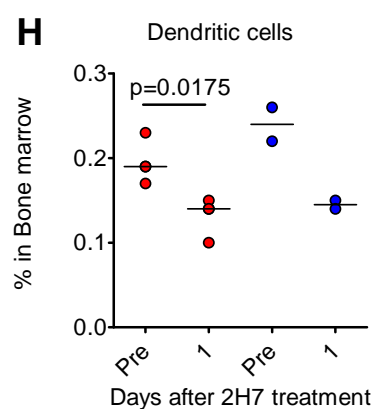
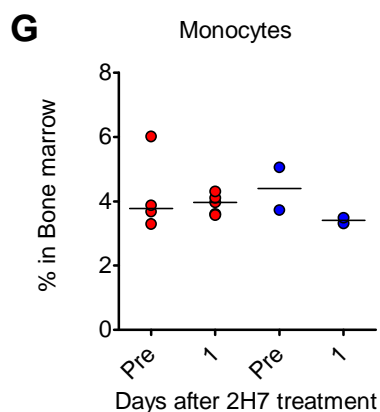
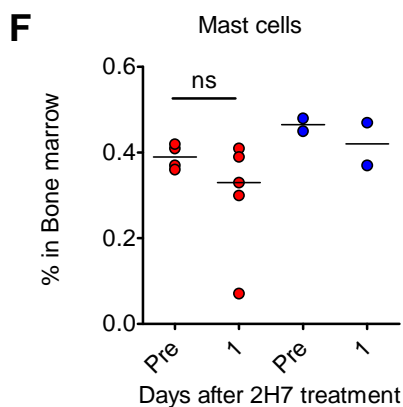
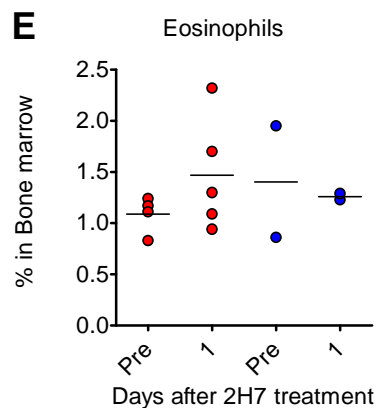
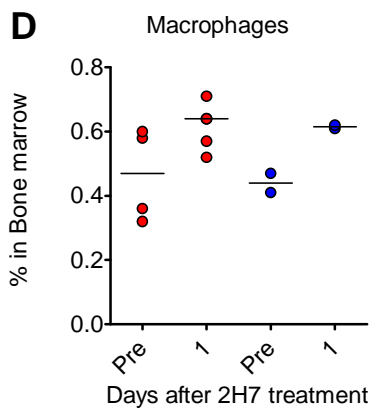
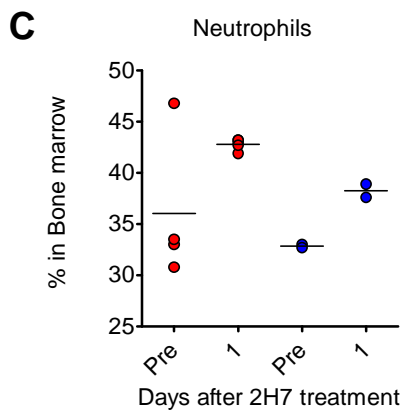
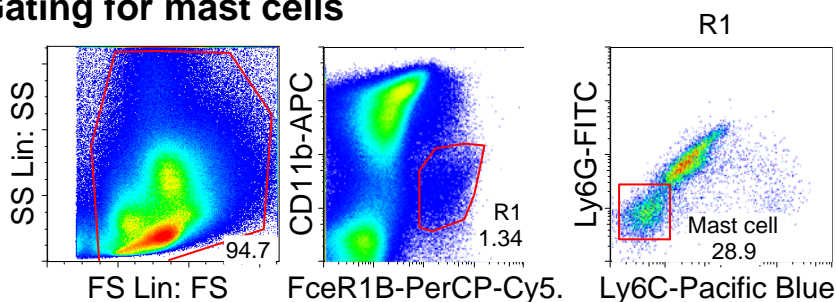
B



3.6A Gating for neutrophils, monocytes, macrophages, dendritic cells and eosinophils

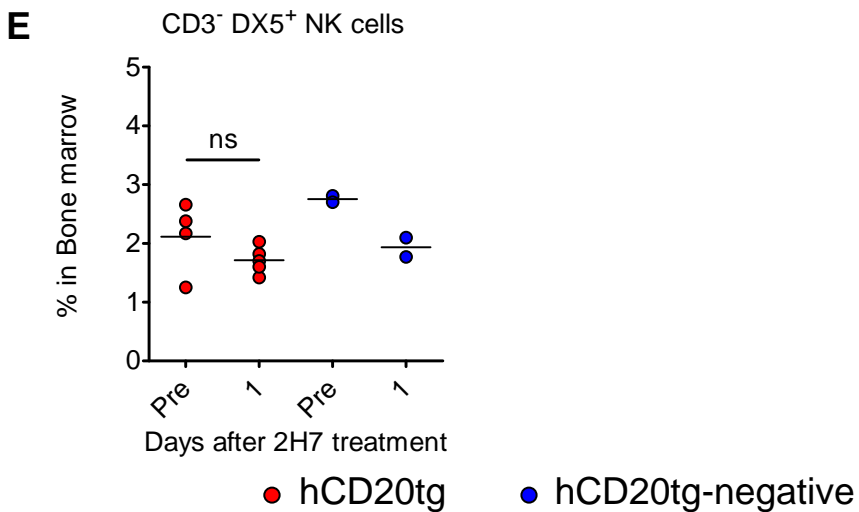
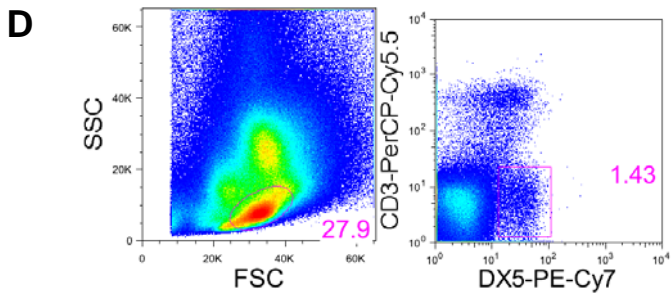
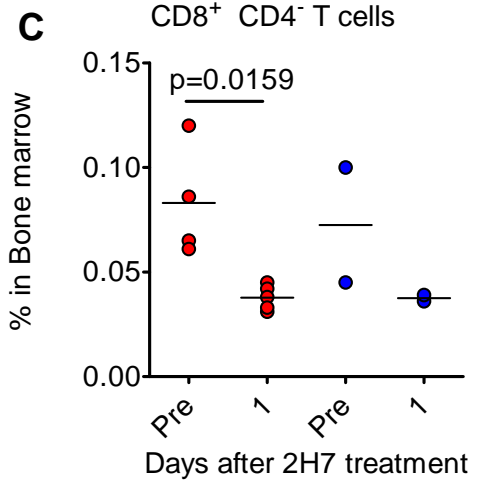
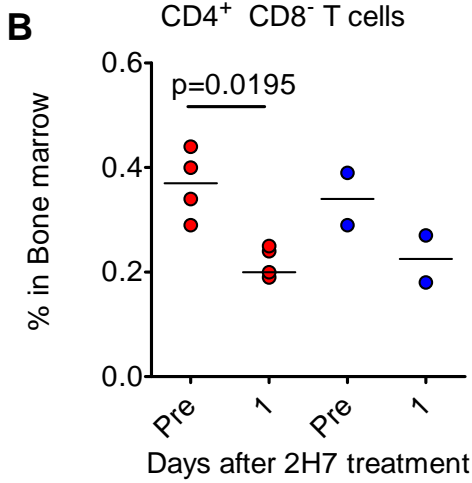
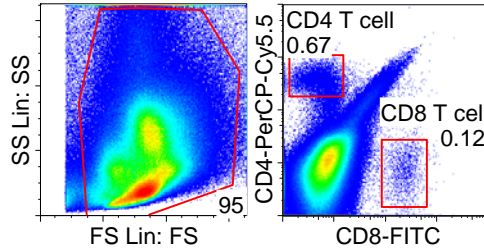


B Gating for mast cells

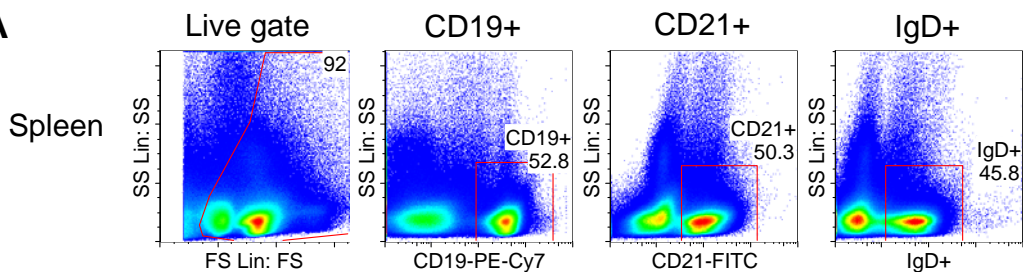


● hCD20tg ● hCD20tg-negative

3.7A

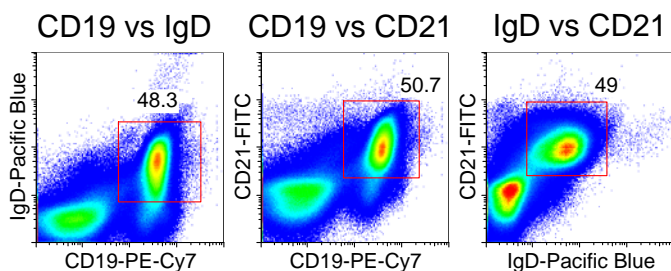


3.8A



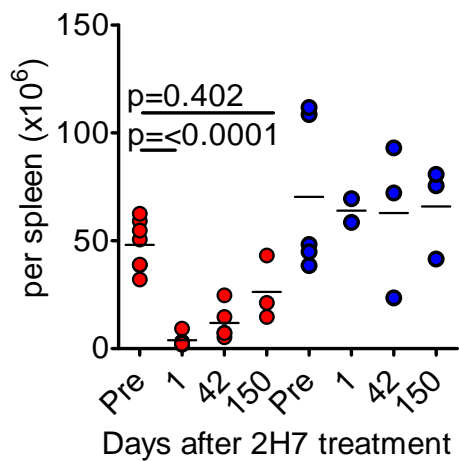
B

Double plots



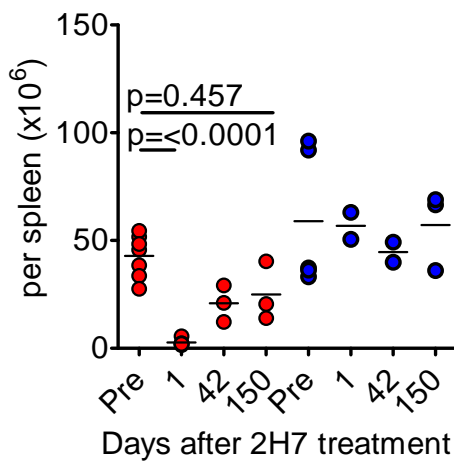
C

CD19



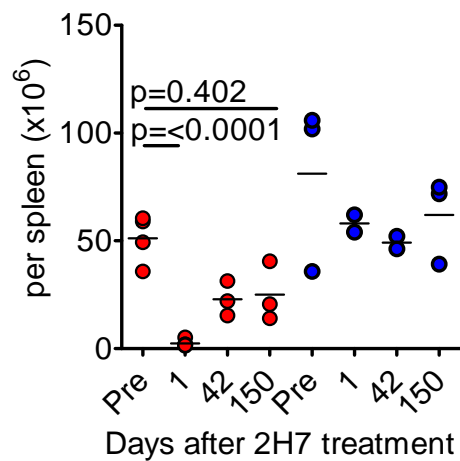
D

IgD



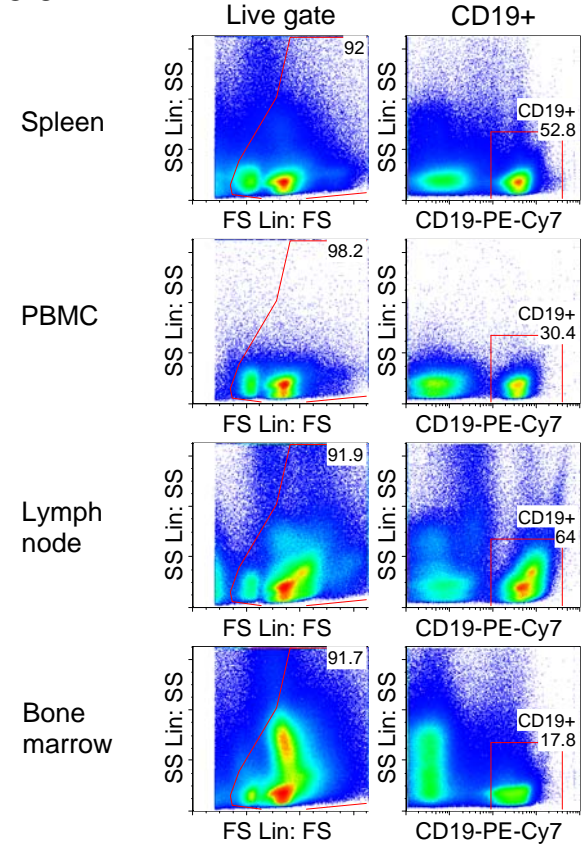
E

CD21

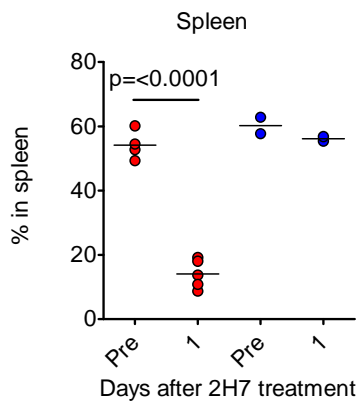


● hCD20tg ● hCD20tg-negative

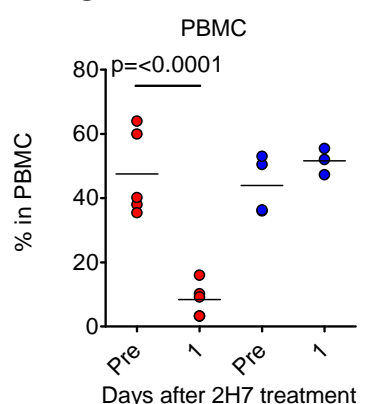
3.9A



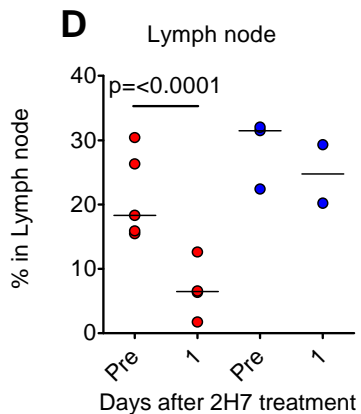
B



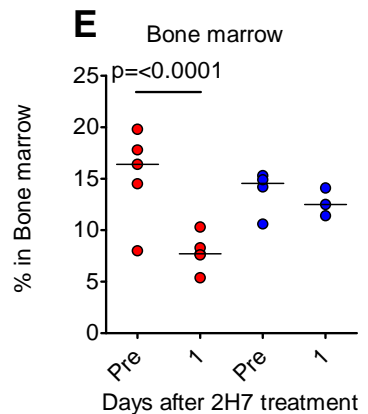
C



D

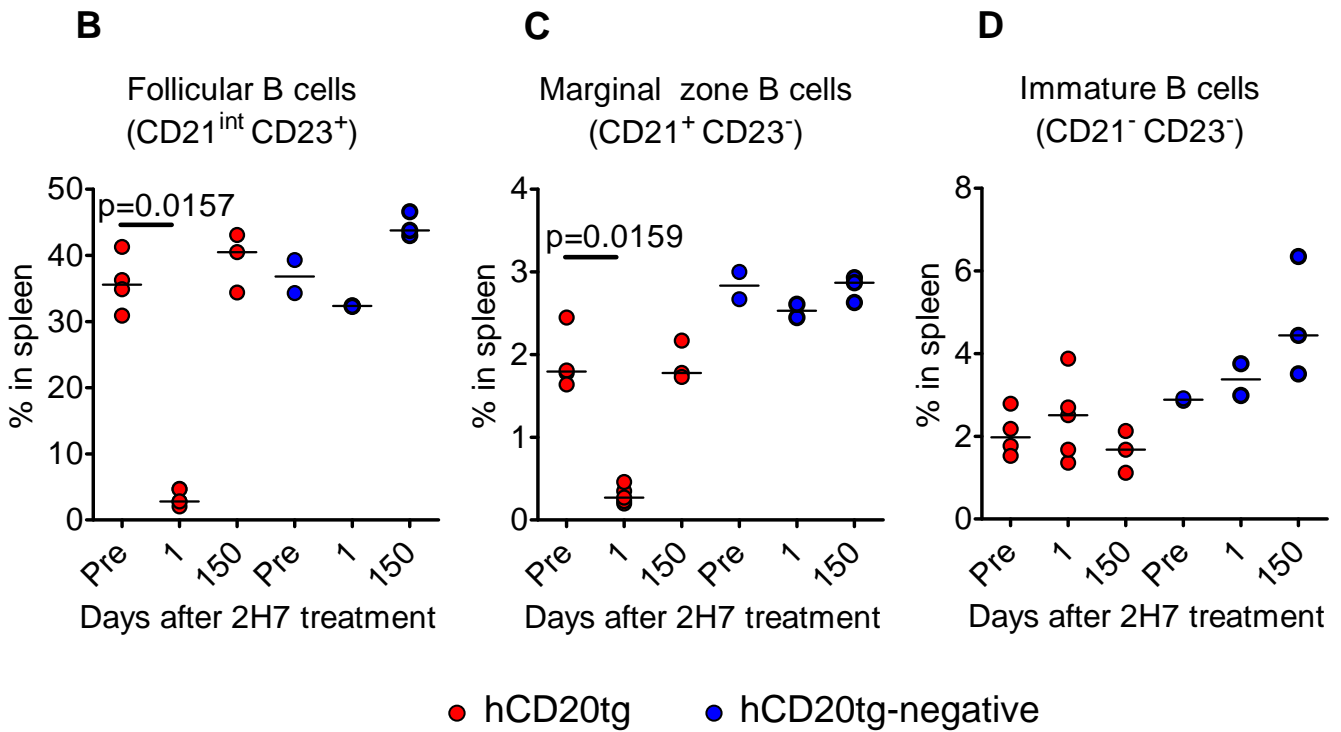
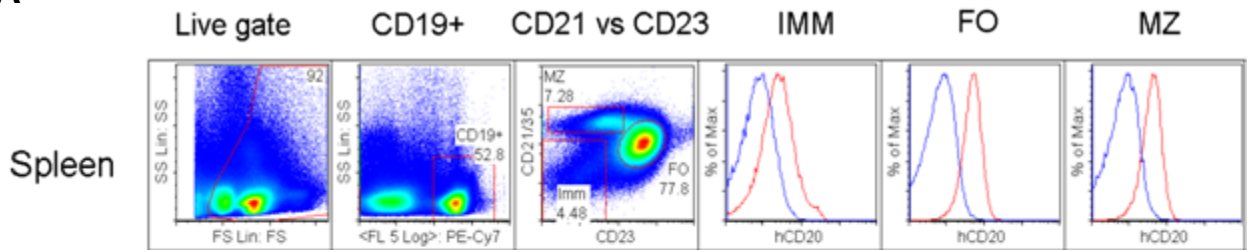


E

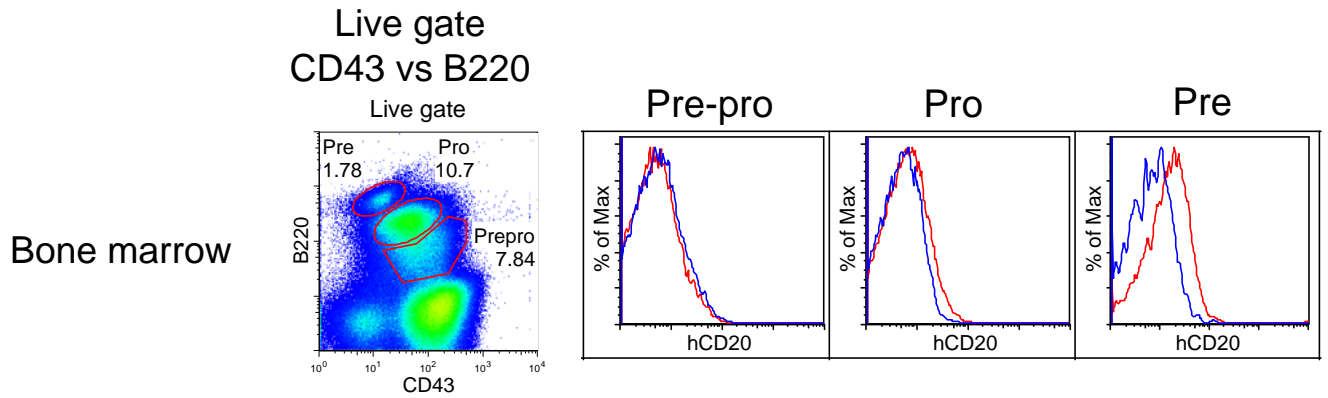


● hCD20tg ● hCD20tg-negative

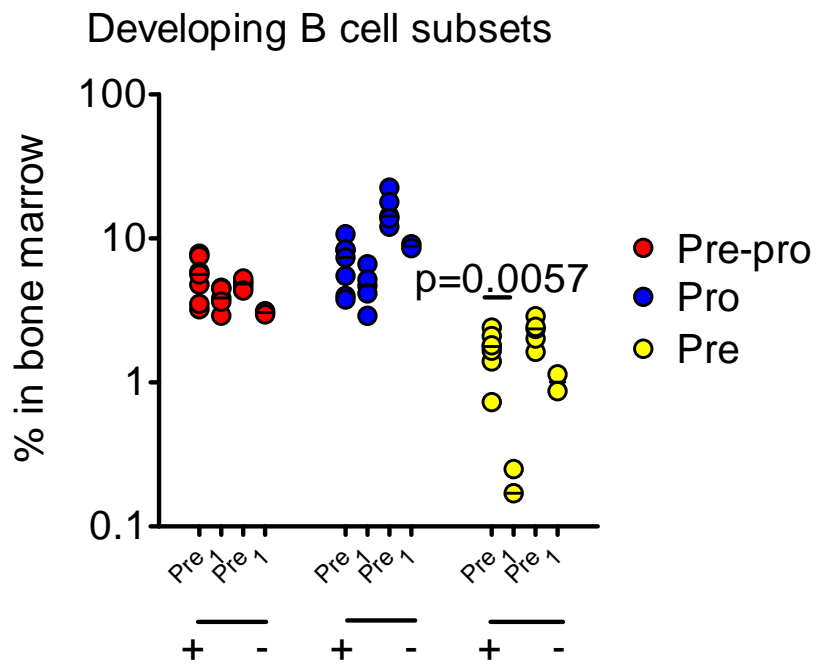
3.10A

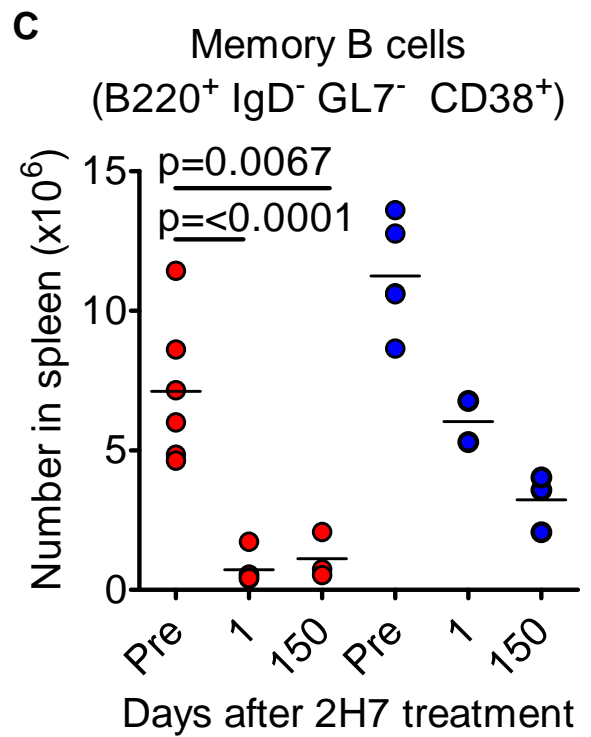
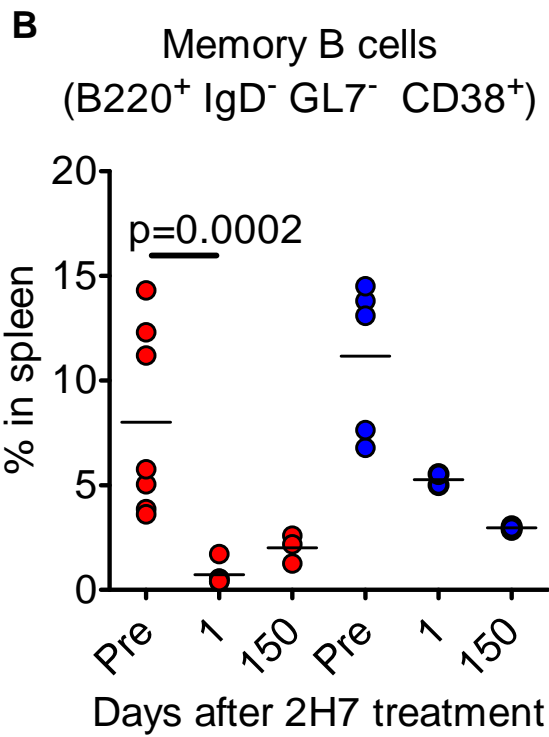
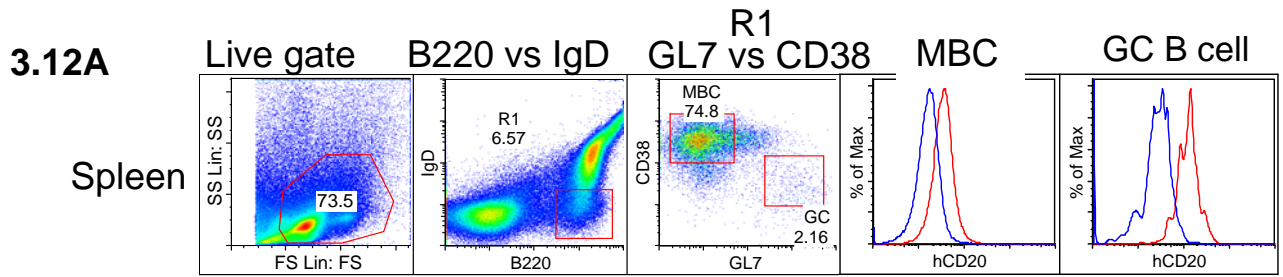


3.11A

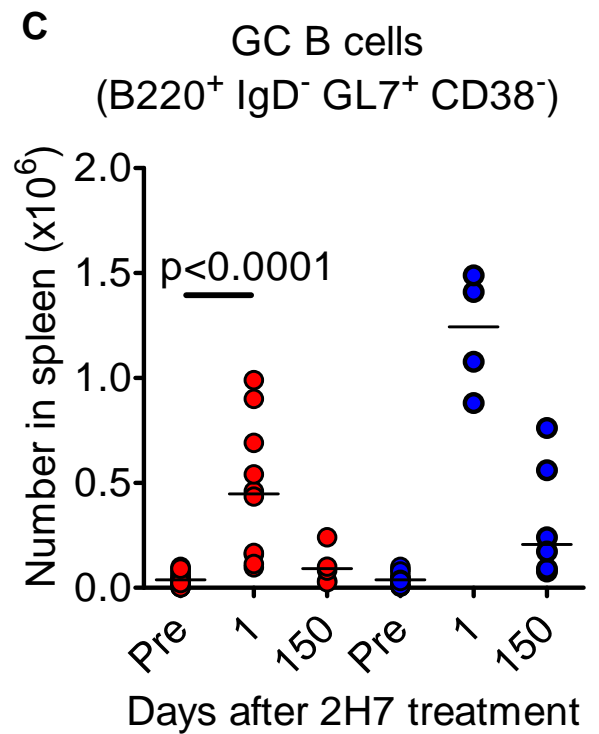
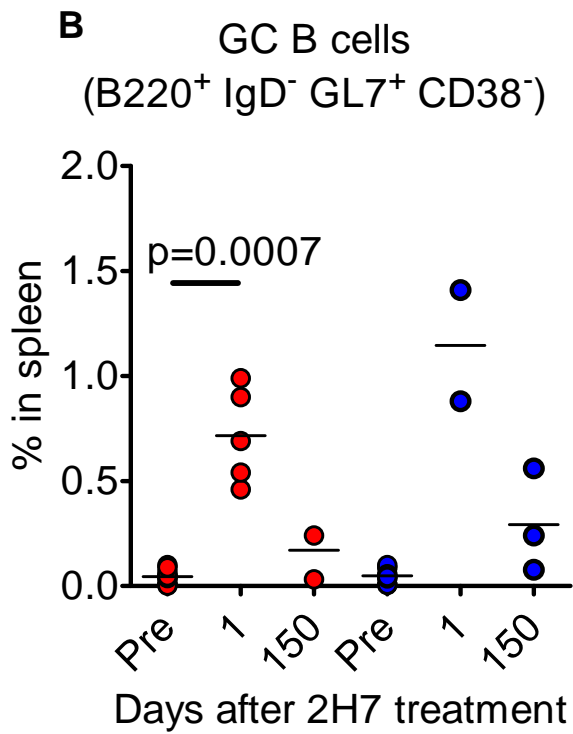
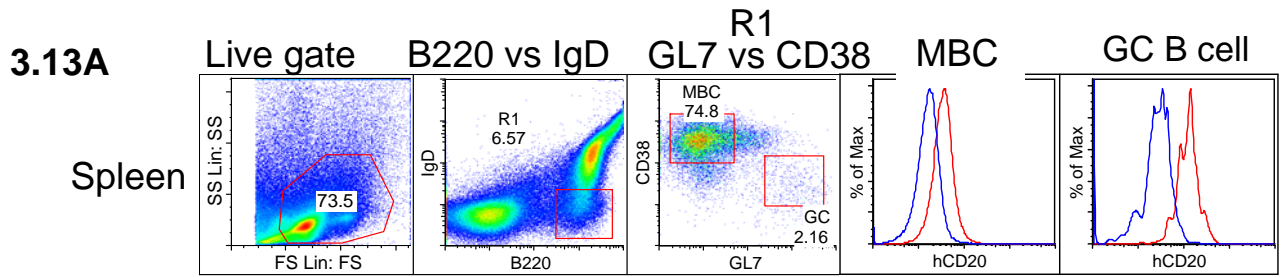


B



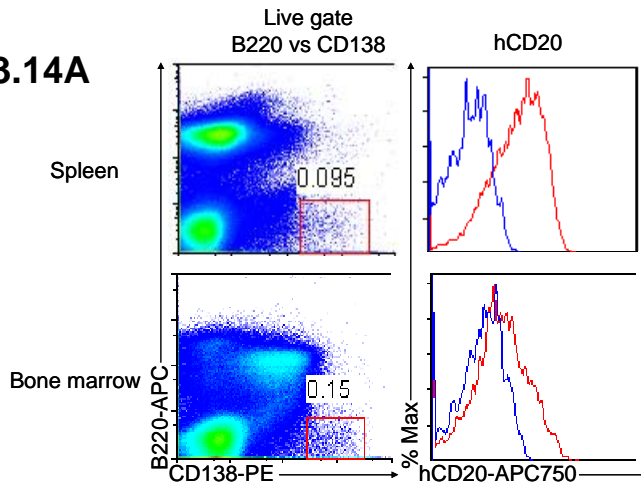


● hCD20tg ● hCD20tg-negative

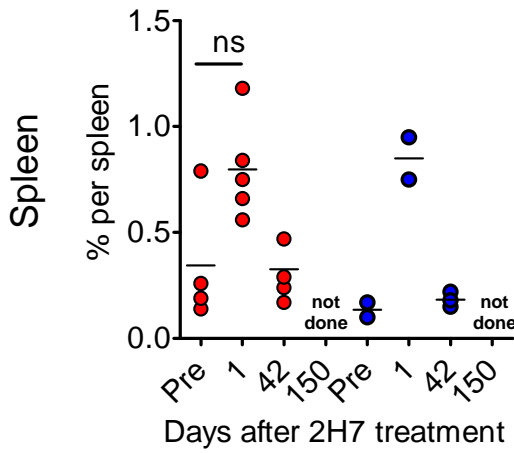


● hCD20tg ● hCD20tg-negative

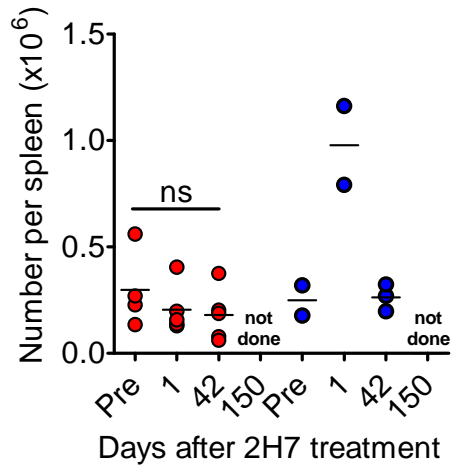
3.14A



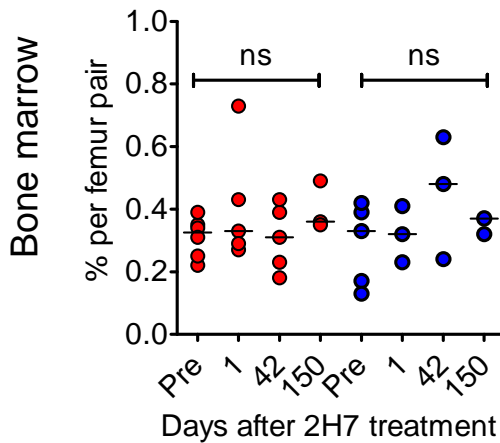
B Frequency Long-lived plasma cells



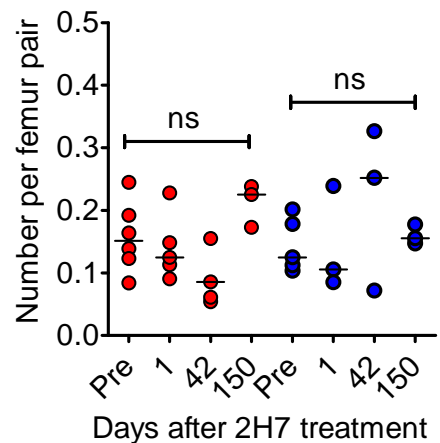
C Number Long-lived plasma cells



D Frequency Long-lived plasma cells



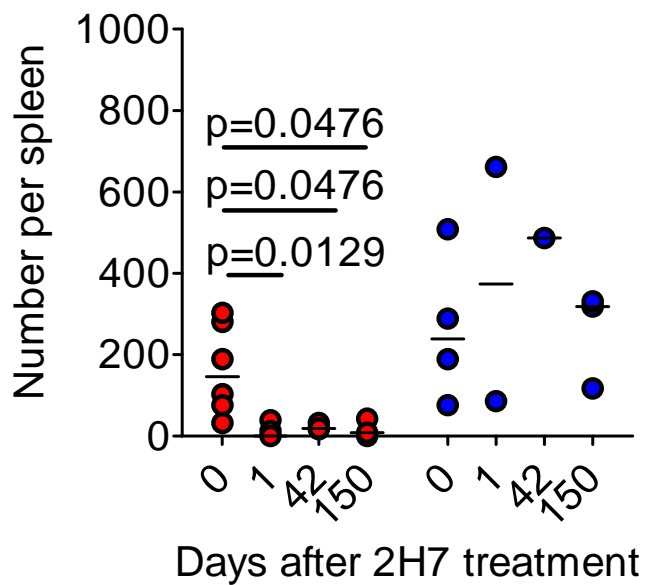
E Number Long-lived plasma cells



● hCD20tg ● hCD20tg-negative

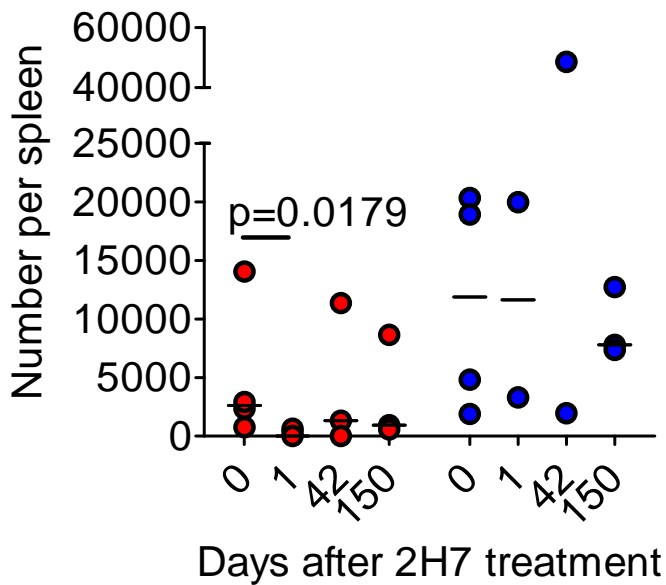
3.15A

HA-specific IgG MBC

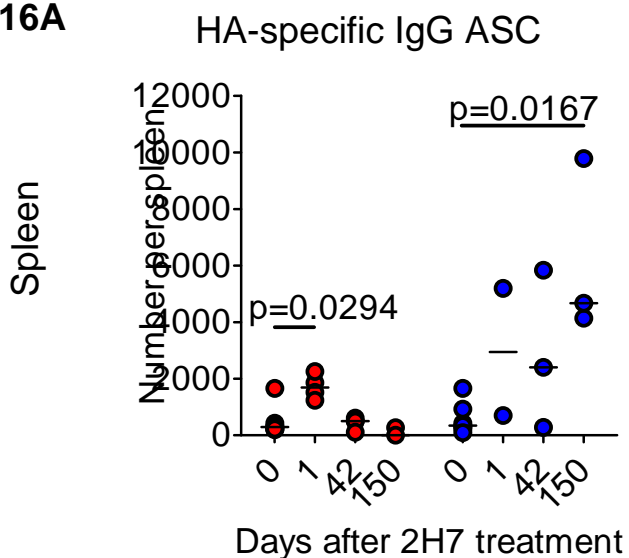


B

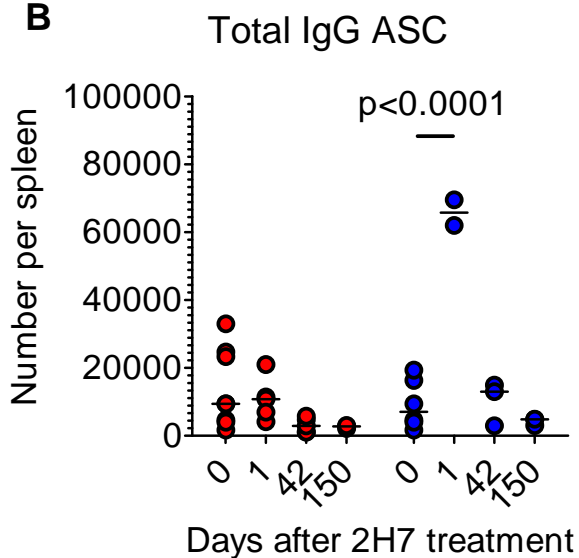
IgG MBC



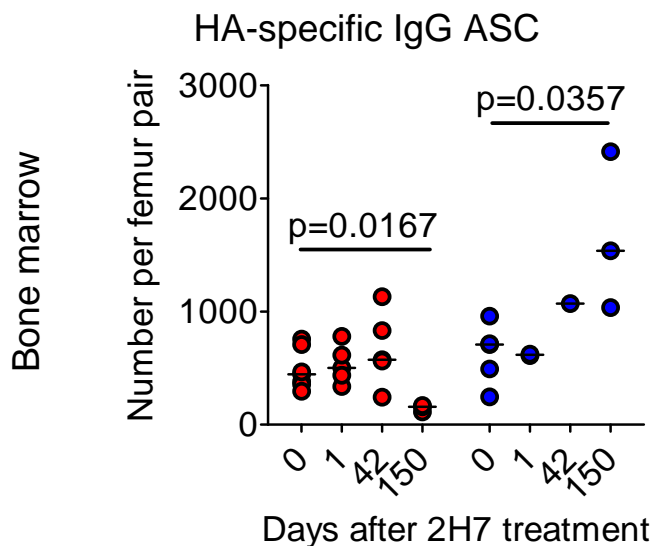
3.16A



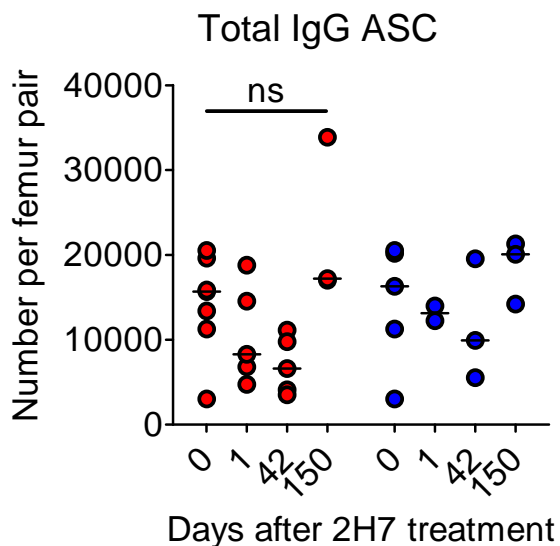
B



C



D



● hCD20tg ● hCD20tg-negative

Chapter 3: Figure legends

Figure 3.1: Development of long-lived specific humoral systemic memory after primary intranasal PR8 infection in BALB/c wild-type female mice.

8-10 wk old female BALB/c mice were infected by intranasal instillation of 250 HAU of Influenza A/PR/8/34. **A)** Venous blood was obtained at various time points up to 250 days post-infection and processed for serum. HA-specific IgG (●) ($n \geq 5$ per time point) and IgM ($n \geq 9$ per time point) (●) antibodies in serum were quantified by ELISA. **B)** Spleens and **C)** Bone marrow from femur pairs was obtained at various time points up to 234 days post-infection. HA-specific IgG ASCs were quantified by ELISpot. Graph shows frequencies and absolute numbers of HA-specific IgG ASCs **C)** per spleen [$n = 2(d0), 4(d150); 4(d234)$] or **C)** per femur pair [$n = 2(d0), 5(d28); 8(d63); 5(d150); 8(d234)$]. **D)** HA-specific MBCs were quantified by limiting dilution ELISpot. Graph shows total numbers of HA-specific IgG MBC per spleen [$n = 1(d0), 6(d150); 4(d227); 3(d337)$]. Line indicates the median value. Statistical values were calculated using the Mann-Whitney test using data pooled from 2-3 independent experiments. Background levels are indicated in dashed lines or were otherwise below the level of detection.

Chapter 3: Figure legends

Figure 3.2: Experimental protocol for depletion of memory B cells from hCD20tg/BALB/c mice.

A) 8-10 wk old female hCD20tg and hCD20tg-negative littermates and BALB/c WT mice were infected by intranasal instillation of 250 HAU of Influenza A/PR/8/34. 150 days post-infection, mice were treated with 0.5 mg/wk of 2H7/saline i.p every 48 hours for 2 weeks (2mg/wk). Analysis of efficacy of depletion was done 1 day post-depletion. Assessment of persistence of specific plasma cells and serum antibody was done 42, 84 and 112 days post-depletion by ELISA, flow cytometry and ASC and memory B cell ELISpot. **B)** 8-10 wk old female hCD20tg and hCD20tg-negative littermates and BALB/c WT mice were infected by intranasal instillation of 250 HAU of Influenza A/PR/8/34. Venous blood was obtained at various time points up to 84 days post-infection and processed for serum. The concentration of HA-specific IgG in serum from hCD20tg [●; n=9(d28), 9(d56) and 5(d84)], hCD20tg-negative littermates [●; n=8(d28), 8(d56) and 8(d84)] and BALB/c WT (○; n=3(d28), 3(d56) and 3(d84)] were quantified by ELISA. Line indicates median values.

Chapter 3: Figure legends

Figure 3.3: Preservation of Ag epitopes and specificity of in-house made anti-hCD20 mIgG2b, 2H7 in hCD20tg CD19⁺ B cells.

8-10 wk old female hCD20tg and hCD20tg-negative littermates were infected by intranasal instillation of 250 HAU of Influenza A/PR/8/34. Spleens were obtained 150 days post-infection and surface stained with CD19-APC in combination with either commercial 2H7-PE, in-house made 2H7 or an mIgG2b-PE isotype control. The figure shows the FACS plots derived from the splenic live gate and plotted with CD19 vs hCD20 from a pilot experiment with 1 hCD20tg and 1 hCD20tg-negative littermate control.

Chapter 3: Figure legends

Figure 3.4: Expression of hCD20 on different cell lineages.

8-10 wk old female hCD20tg and hCD20tg-negative littermates were infected by intranasal instillation of 250 HAU of Influenza A/PR/8/34. Spleens and bone marrow were obtained 150 days post-infection. hCD20 expression on cells of different lineages in the spleen and bone marrow was analysed by flow cytometry. The figure shows the live gate, lineage gate based on CD19, B220⁻ CD138⁺, CD3, DX5, CD11b, CD11c and F4/80, and relative mean fluorescence intensity (MFI) of hCD20 between hCD20tg (—) and hCD20tg-negative (—) littermates. These FACS plots are representative of 1 pilot experiment with 3 hCD20tg and 3 hCD20tg-negative mice.

Chapter 3: Figure legends

Figure 3.5: Depletion efficacy of 2H7 treatment – Total spleen and bone marrow cellularity.

8-10 wk old female hCD20tg and hCD20tg-negative littermates were infected by intranasal instillation of 250 HAU of Influenza A/PR/8/34. 150 days post-infection, mice were treated with 0.5 mg/wk of 2H7/saline i.p every 48 hours for 2 weeks (2mg/wk). Analysis of efficacy of depletion was done 1 day, 42 days and 150 days post-depletion. Single cell erythrolysed suspensions were obtained from spleens and bone marrow of hCD20tg and hCD20tg-negative littermates and counted using the haemocytometer with live/dead cell discrimination using Tryphan Blue. The graph shows the individual data points of **A)** spleen and **B)** bone marrow total cell counts just before treatment, 1 day, 42 days and 150 days post-treatment in hCD20tg [●; n=7(pre-treatment), 5(d1); 5(d42); 3(d150)] and hCD20tg-negative [●; n=5(pre-treatment), 2(d1); 3(d42); 3(d150)] mice. Line indicates the median value. Statistical values were calculated using the Mann-Whitney test using data pooled from 2 independent experiments.

Chapter 3: Figure legends

Figure 3.6: Depletion efficacy of 2H7 treatment – Neutrophils, monocytes, macrophages, dendritic cells, eosinophils and mast cells.

8-10 wk old female hCD20tg and hCD20tg-negative littermates were infected by intranasal instillation of 250 HAU of Influenza A/PR/8/34. 150 days post-infection, mice were treated with 0.5 mg/wk of 2H7/saline i.p every 48 hours for 2 weeks (2mg/wk). 1 day post-depletion, bone marrow was obtained and stained with surface markers as indicated for **A**) neutrophils (CD11b⁻ Ly6G⁺ Ly6C^{int}); monocytes (CD11b⁻ Ly6G⁻ Ly6C⁺); macrophages (CD11b⁻ Ly6G⁻ Ly6C⁻ Siglec-F⁻ CD11c⁻), dendritic cells (CD11b⁻ Ly6G⁻ Ly6C⁻ Siglec-F⁻ CD11c⁺), eosinophils (CD11b⁻ Ly6G⁻ Ly6C⁻ Siglec-F⁺ CD11c⁻) and **B**) mast cells (CD11b⁻ FcεR1B⁺ Ly6G⁻ Ly6C⁻). **C-H**) Frequencies of each cell type in bone marrow were determined before treatment and 1 day post-treatment from hCD20tg [●; n=4(pre-treatment), 5(d1)] and hCD20tg-negative [●; n=2(pre-treatment), 2(d1)] mice. Line indicates the median value. Statistical values were calculated using the Mann-Whitney test using data from 1 experiment. The *n* number of hCD20tg-negative mice was too small for Mann-Whitney test to be performed.

Chapter 3: Figure legends

Figure 3.7: Depletion efficacy of 2H7 treatment – T cells and NK cells.

8-10 wk old female hCD20tg and hCD20tg-negative littermates were infected by intranasal instillation of 250 HAU of Influenza A/PR/8/34. 150 days post-infection, mice were treated with 0.5 mg/wk of 2H7/saline i.p every 48 hours for 2 weeks (2mg/wk). 1 day post-depletion, bone marrow was obtained and stained with surface markers as indicated for **A**) CD4 T cells (CD4⁺ CD8⁻) and CD8 T cells (CD4⁻ CD8⁺) and **D**) NK cells (CD3⁻ DX5⁺). Frequencies of **B**) CD4 T cells, **C**) CD8 T cells and **E**) NK cells in bone marrow were determined before treatment and 1 day post-treatment in hCD20tg [●; n=4(pre-treatment), 5(d1)] and hCD20tg-negative [●; n=2(pre-treatment), 2(d1)] mice. Line indicates the median value. Statistical values were calculated using the Mann-Whitney test using data from 1 experiment. The *n* number of hCD20tg-negative mice was too small for Mann-Whitney test to be performed.

Chapter 3: Figure legends

Figure 3.8: Depletion efficacy of 2H7 treatment – B cells.

8-10 wk old female hCD20tg and hCD20tg-negative littermates were infected by intranasal instillation of 250 HAU of Influenza A/PR/8/34. 150 days post-infection, mice were treated with 0.5 mg/wk of 2H7/saline i.p every 48 hours for 2 weeks (2mg/wk). 1 day post-depletion, bone marrow was obtained and stained with surface markers as indicated for **A**) CD19, CD21 and sIgD B cells. **B**) These markers were co-expressed on the majority of B cells. Individual data points of the total number of **C**) CD19⁺, **D**) sIgD⁺ and **E**) CD21⁺ B cells in spleens were determined before treatment and 1 day post-treatment from hCD20tg [**●**; n=7(pre-treatment), 5(d1); 5(d42); 3(d150)] and hCD20tg-negative [**●**; n=5(pre-treatment), 2(d1); 3(d42); 3(d150)] mice. Line indicates the median value. Statistical values were calculated using the Mann-Whitney test. Statistical values were calculated using the Mann-Whitney test using data pooled from 2 independent experiments. The *n* number of hCD20tg-negative mice was too small for Mann-Whitney test to be performed.

Chapter 3: Figure legends

Figure 3.9: Depletion efficacy of 2H7 treatment – B cells in peripheral organs.

8-10 wk old female hCD20tg and hCD20tg-negative littermates were infected by intranasal instillation of 250 HAU of Influenza A/PR/8/34. 150 days post-infection, mice were treated with 0.5 mg/wk of 2H7/saline i.p every 48 hours for 2 weeks (2mg/wk). Analysis of efficacy of depletion of CD19⁺ B cells in spleen, PBMC, lymph node and bone marrow was done 1 day post-depletion by flow cytometry. **A)** CD19⁺ gating in spleen, PBMC, lymph node and bone marrow. **B-E)** Individual data points of the percentage of CD19⁺ cells in **B)** spleens, **C)** bone marrow, **D)** lymph node and **E)** PBMC were determined before treatment and 1 day post-treatment in hCD20tg [●; n=4-5(pre-treatment), 4-5(d1)] and hCD20tg-negative [●; n=2-4(pre-treatment), 2-3(d1)] mice. Line indicates the median value. Statistical values were calculated using the Mann-Whitney test using data from 1 experiment. The *n* number of hCD20tg-negative mice was too small for Mann-Whitney test to be performed.

Chapter 3: Figure legends

Figure 3.10: Depletion efficacy of 2H7 treatment – Marginal zone, follicular and immature B cells.

8-10 wk old female hCD20tg and hCD20tg-negative littermates were infected by intranasal instillation of 250 HAU of Influenza A/PR/8/34. 150 days post-infection, mice were treated with 0.5 mg/wk of 2H7/saline i.p every 48 hours for 2 weeks (2mg/wk). **A)** Spleens were obtained hCD20 expression on CD19⁺ cells from spleen, lymph node, PBMC and bone marrow 150 days post-infection. The figure shows the live gate, lineage gate based on CD19, gating of marginal zone (CD21⁺ CD23⁻), follicular (CD21^{int} CD23⁺) and immature (CD21⁻ CD23⁻) B cells, and relative mean fluorescence intensity (MFI) of hCD20 between hCD20tg (—) and hCD20tg-negative (—) littermates on each B cell subset. **B-D)** Individual data points of the percentage B) follicular, C) marginal zone and D) immature B cells in spleens were determined before treatment, 1 day and 150 days post-treatment in hCD20tg [●; n=4(pre-treatment); 5(d1); 3(d150)] and hCD20tg-negative [●; n=2(pre-treatment); 2(d1); 3(d150)] mice. Line indicates the median value. Statistical values were calculated using the Mann-Whitney test using data from 1 experiment. The *n* number of hCD20tg-negative mice was too small for Mann-Whitney test to be performed.

Chapter 3: Figure legends

Figure 3.11: Depletion efficacy of 2H7 treatment – Developing B cells in bone marrow.

8-10 wk old female hCD20tg and hCD20tg-negative littermates were infected by intranasal instillation of 250 HAU of Influenza A/PR/8/34. 150 days post-infection, mice were treated with 0.5 mg/wk of 2H7/saline i.p every 48 hours for 2 weeks (2mg/wk). **A)** The figure shows the live gate in total bone marrow cells and gating of pre-B cells (B220^{high} CD43^{low}), pro-B cells (B220^{int} CD43^{int}) and pre/pro-B cells (B220^{low} CD43^{high}) by FACS., and relative mean fluorescence intensity (MFI) of hCD20 between hCD20tg (—) and hCD20tg-negative (—) littermates on each B cell subset. **B)** Efficacy of depletion of developing B cells in bone marrow was determined before treatment and 1 day post-treatment in hCD20tg (+ on *x*-axis; n=7(pre-treatment); 5(d1)] and hCD20tg-negative (- on *x*-axis; n=5(pre-treatment); 2(d1)] mice. Line indicates the median value. Frequency of pre/pro-B cells (●), pro- (●) and pre- (●). Statistical values were calculated using the Mann-Whitney test using data from 1 experiment. The *n* number of hCD20tg-negative mice was too small for Mann-Whitney test to be performed.

Chapter 3: Figure legends

Figure 3.12: Depletion efficacy of 2H7 treatment – Memory B cells analyzed by flow cytometry.

8-10 wk old female hCD20tg and hCD20tg-negative littermates were infected by intranasal instillation of 250 HAU of Influenza A/PR/8/34. 5 months post-infection, mice were treated with 0.5 mg/wk of 2H7/saline i.p every 48 hours for 2 weeks (2mg/wk). Analysis of efficacy of depletion was done 1 day post-depletion by flow cytometry. **A)** The gating strategy for splenic memory B cells (B220⁺ IgD⁻ CD38⁺ GL7⁻) and GC B cells (B220⁺ IgD⁻ CD38⁻ GL7⁺) and relative mean fluorescence intensity (MFI) of hCD20 between hCD20tg (—) and hCD20tg-negative (—) littermates on MBC or GC B cells. Individual data points of the **B)** percentage and **C)** total number of splenic MBC were determined before treatment, 1 day and 150 days post-treatment in hCD20tg [●; n=7(pre-treatment); 5(d1); 2(d150)] and hCD20tg-negative [●; n=5(pre-treatment); 2(d1); 3(d150)] mice. Line indicates the median value. Statistical values were calculated using the Mann-Whitney test using data from 1 experiment. The *n* number of hCD20tg-negative mice was too small for Mann-Whitney test to be performed.

Chapter 3: Figure legends

Figure 3.13: Depletion efficacy of 2H7 treatment – Germinal centre B cells analysed by flow cytometry.

8-10 wk old female hCD20tg and hCD20tg-negative littermates were infected by intranasal instillation of 250 HAU of Influenza A/PR/8/34. 5 months post-infection, mice were treated with 0.5 mg/wk of 2H7/saline i.p every 48 hours for 2 weeks (2mg/wk). Analysis of efficacy of depletion was done 1 day post-depletion by flow cytometry. **A)** The gating strategy for splenic memory B cells (B220⁺ IgD⁻ CD38⁺ GL7⁻) and GC B cells (B220⁺ IgD⁻ CD38⁻ GL7⁺) and relative mean fluorescence intensity (MFI) of hCD20 between hCD20tg (—) and hCD20tg-negative (—) littermates on MBC or GC B cells. Individual data points of the **B)** percentage and **C)** total number of splenic GC B cells were determined before treatment, 1 day and 150 days post-treatment in hCD20tg [●; n=7(pre-treatment); 5(d1); 2(d150)] and hCD20tg-negative [●; n=5(pre-treatment); 2(d1); 3(d150)] mice. Line indicates the median value. Statistical values were calculated using the Mann-Whitney test using data from 1 experiment. The *n* number of hCD20tg-negative mice was too small for Mann-Whitney test to be performed.

Chapter 3: Figure legends

Figure 3.14: Depletion efficacy of 2H7 treatment – Long-lived plasma cells by flow cytometry.

8-10 wk old female hCD20tg and hCD20tg-negative littermates and BALB/c WT mice were infected by intranasal instillation of 250 HAU of Influenza A/PR/8/34. 5 months post-infection, mice were treated with 0.5 mg/wk of 2H7/saline i.p every 48 hours for 2 weeks (2mg/wk). Analysis of efficacy of depletion was done 1 day, 42 days and 150 days post-treatment by flow cytometry. **A)** The gating strategy for splenic or bone marrow long-lived plasma cells (B220- CD138⁺) and relative mean fluorescence intensity (MFI) of hCD20 between hCD20tg (—) and hCD20tg-negative (—) littermates on LLPC. The graphs show the mean and standard error of the **B)** percentage and **C)** total number of long-lived plasma cells in spleen and **D)** percentage and **E)** total number of long-lived plasma cells in femur pair were determined before treatment, 1 day, 42 days and 150 days post-treatment from hCD20tg [●; n=6(pre-treatment), 5(d1); 5(d42); 3(d150)] and hCD20tg-negative [●; n=5(pre-treatment), 3(d1); 3(d42); 3(d150)] mice. Line indicates the median value. Statistical values were calculated using the Mann-Whitney test using data pooled from 2 independent experiments. In **B)** and **C)**, the *n* number of hCD20tg-negative mice was too small for Mann-Whitney test to be performed.

Chapter 3: Figure legends

Figure 3.15: Depletion efficacy of 2H7 treatment – HA-specific memory B cells analysed by ELISpot.

8-10 wk old female hCD20tg and hCD20tg-negative littermates and BALB/c WT mice were infected by intranasal instillation of 250 HAU of Influenza A/PR/8/34. 5 months post-infection, mice were treated with 0.5 mg/wk of 2H7/saline i.p every 48 hours for 2 weeks (2mg/wk). Analysis of efficacy of depletion was done 1 day, 42 days and 150 days post-treatment by ELISpot. Splenic HA-specific memory B cells were quantified from mice using limiting dilution ELISpot. Individual data points of the total number of **A)** HA-specific IgG MBC and **B)** Total IgG MBC in spleens were determined before treatment, 1 day, 42 days and 150 days post-treatment from hCD20tg [●; n=6(pre-treatment), 5(d1); 5(d42); 3(d150)] and hCD20tg-negative [●; n=4(pre-treatment), 2(d1); 1(d42); 2(d150)] mice. Line indicates the median value. Statistical values were calculated using the Mann-Whitney test using data pooled from 2 independent experiments.

Chapter 3: Figure legends

Figure 3.16: Depletion efficacy of 2H7 treatment – HA-specific ASC analyzed by ELISpot.

8-10 wk old female hCD20tg and hCD20tg-negative littermates and BALB/c WT mice were infected by intranasal instillation of 250 HAU of Influenza A/PR/8/34. 5 months post-infection, mice were treated with 0.5 mg/wk of 2H7/saline i.p every 48 hours for 2 weeks (2mg/wk). Analysis of efficacy of depletion was done 1 day, 42 days and 150 days post-treatment by ELISpot. The graphs show the individual data points of the total number of splenic **A)** HA-specific IgG ASC and **B)** total IgG ASCs and bone marrow **C)** HA-specific IgG ASC and **D)** total IgG ASCs were determined before treatment, 1 day, 42 days and 150 days post-treatment from hCD20tg [●; n=7(pre-treatment), 4-5(d1); 5(d42); 3(d150)] and hCD20tg-negative [●; n=5-7(pre-treatment), 2(d1); 1(d42); 3(d150)] mice. Line indicates the median value. Statistical values were calculated using the Mann-Whitney test using data pooled from 2 independent experiments.

Chapter 3: Figure legends

Figure 3.17: Loss of serum HA-specific IgG after memory B cell depletion.

8-10 wk old female hCD20tg and hCD20tg-negative littermates and BALB/c WT mice were infected by intranasal instillation of 250 HAU of Influenza A/PR/8/34. 5 months post-infection, mice were treated with 0.5 mg/wk of 2H7/saline i.p every 48 hours for 2 weeks (2mg/wk). Serum was obtained from mice at various time points post-treatment. **A)** HA-specific serum IgG and **B)** total serum IgG concentrations was quantified using ELISA. The graphs show individual data points of **A)** HA-specific serum IgG and **B)** total serum IgG concentrations were determined before treatment, 1 day, 42 days and 150 days post-treatment from hCD20tg [●; n=13(pre-treatment), 4(d1); 8(d42); 8(d150)] and hCD20tg-negative [●; n=13(pre-treatment), 3(d1); 8(d42); 8(d150)] mice. Line indicates the median value. Statistical values were calculated using the Mann-Whitney test using data pooled from 2 independent experiments.

3.5: Discussion

The aim of this study was to determine whether the long-term Ab production after a primary PR8 infection was maintained by LLPCs independently of MBCs. For this I made use of the hCD20tg system of depleting MBCs, which allowed me to directly test whether LLPCs are long-lived in the absence of MBCs, and whether they can maintain serum Ab without reconstitution by MBCs differentiating into Ab-secreting plasma cells.

The key findings of this study are:

- A primary intranasal PR8 infection generates long-lived humoral immune responses (for up to 250 days) in terms of serum Ab, LLPCs and MBCs.
- hCD20 expression is restricted to CD19⁺ B cells and some B220⁻ CD138⁺ LLPC in spleen and bone marrow.
- 150 days after PR8 infection, depletion by 2H7 mAb is rapid, efficient and specific for CD19⁺ B cells. 2H7 treatment does not deplete B220⁻ CD138⁺ LLPC in spleen and bone marrow.
- Total B cells are reconstituted within 90 days of depletion.
- Depletion of HA-specific MBC is rapid but prolonged, remaining significantly depleted for up to 150 days after 2H7 treatment.
- Most importantly, in the absence of HA-specific MBC, there is an eventual loss of HA-specific ASC and loss of HA-specific serum Ab. This indicates that MBC are required for maintenance of long-term serum Ab and LLPC after intranasal PR8 infection.

The HA-specific Ab response induced by intranasal PR8 took at least 80 days to reach their peak value, but thereafter it stayed constant for up to 250 days post-infection. The majority of HA-specific Abs are of the IgG isotype, whilst HA-specific IgM decreases over time. The majority of HA-specific IgG ASCs were detected in the bone marrow and spleen, where total numbers peaked and remained stable from d63 onwards. If bone marrow taken from a femur pair contains 12.7% of bone marrow, and assuming that LLPC were only localised to spleen and bone marrow, it means that approximately 90% of total HA-specific IgG ASCs reside in the bone marrow and the remaining minority in spleen (approximately 3.2×10^4 in total bone marrow vs. 0.4×10^4 in spleen). This is consistent with the findings

in a study using OVA-immunisation where 90% of OVA-specific LLPC were localized to bone marrow after secondary OVA immunization (Manz *et al.* 1997). Therefore, the bone marrow is a major site for accumulation of HA-specific LLPC after primary Influenza A infection. A surprising finding in this study was that the number of HA-specific ASC in bone marrow and spleen only reached stable and peak numbers 150 days post-infection, indicating that, even though the PR8 is eliminated to undetectable levels by PCR within two weeks (Eichelberger *et al.* 1991), the primary immune response was prolonged. The continuous generation of LLPC and MBC could be a response to antigen sequestered by follicular dendritic cells in residual germinal centres. Further work would be to extend this study of the kinetics and tissue localisation of MBCs, LLPCs and germinal centre B cells to lymph nodes and NALT would provide a more complete description of the humoral response to intranasal PR8. Serum or bronchial IgA was not measured in this study, but I expect that a significant contribution to protective immunity comes from IgA-secreting ASC locally distributed in the nasal and bronchial mucosal tissue, particularly as this infection was via the intranasal route. Nevertheless, this study indicates that intranasal PR8 infection is able to generate long-lived systemic humoral memory, in terms of stable serum Ab concentrations, LLPC and MBC.

Using multi-parameter flow cytometry to characterise hCD20tg mice, I found that only CD19⁺ B cells and splenic B220⁻ CD138⁺ LLPCs expressed intermediate to high levels of surface hCD20. In this study, approximately 40% of bone marrow B220⁻ CD138⁺ LLPC expressed intermediate levels of hCD20 and all of spleen B220⁻ CD138⁺ LLPC expressed hCD20. Nevertheless, depletion by 2H7 treatment was highly specific towards B cells *in vivo*, depleting approximately 90% of CD19⁺, IgD⁺ and CD21⁺ B cells within 1 day of the last 2H7 injection. Amongst developing B cell subsets in bone marrow, hCD20 was expressed only on pre-B cells, and correspondingly only pre-B cells were depleted by 2H7 administration, a result that is consistent with previous studies (Ahuja *et al.* 2007;Gong *et al.* 2005). In spleen, naive, marginal zone and follicular B cells, as well as MBCs and germinal centre B cells expressed hCD20. However, follicular and marginal zone B cells were preferentially depleted, but naive B cells and germinal centre B cells appeared to be highly resistant to 2H7 depletion.

In summary, after 2H7 treatment in hCD20tg mice 5 months after PR8 infection, there is:

- Loss of spleen cellularity but not bone marrow cellularity.
- Loss of CD19⁺ B cells from spleen, lymph node, PBMC and bone marrow.
- Loss of pre-B cells from bone marrow.
- Loss of marginal zone and follicular B cells from spleen.
- Retention of naïve B cells and germinal centre B cells.

The reason for the resistance of immature B cells and germinal centre B cells cannot be explained by lack of expression of hCD20, as both express hCD20 as highly as other B cell subsets; or lack of accessibility to Ab, as other splenic B cell subsets are depleted efficiently. Immature B cells could have undergone renewal of naïve B cells from bone marrow although this is unlikely 1 day post-2H7 treatment. GC B cells increased 1 day post-2H7 treatment in both hCD20tg and hCD20tg-negative littermates and returned to pre-depletion levels by day 42. This transient increase in GC B cells is an important caveat in this study as MBCs can be generated by bystander T cell help induced by immune complexes formed by 2H7 treatment and sequestered on FDC. In another study, depletion by 2H7 used up to 16 mg/wk of mAb, in contrast to this study where a dose of 2 mg/wk was used; however reactivation of germinal centres as an effect of the high-dose mAb treatment was not examined. In this study, we observed that in both hCD20tg and hCD20tg-negative littermates, there was an increase in B220⁻ CD138⁺ plasma cells in the spleen 1 day after 2H7 treatment, and, similar to the numbers of GC B cells, numbers of B220⁻ CD138⁺ plasma cells returned to pre-depletion levels by d42 post-depletion. However, there was no increase in HA-specific IgG ASC in spleen or bone marrow either in hCD20tg or hCD20tg-negative littermates on day 1, 42 or 150 days post-2H7 treatment, suggesting that the transient increase in B220⁻ CD138⁺ plasma cells in the spleen was non-specific and could be a bystander effect from the repeated injections of exogenous Ab, causing regeneration of germinal centres via immune-complex-mediated idiotypic pathways or small amounts of contaminant TLR ligands, but not resulting in production of more HA-specific IgG ASC. Similarly, there was overall long-term depletion of HA-specific MBC and no increase in total IgG MBC.

Besides B cells, there was a small but significant depletion of dendritic cells in both hCD20tg and hCD20tg-negative littermates, suggesting that this was a bystander effect of

the immunization. It is known that dendritic cells are sensitive to levels of serum immunoglobulin and may have undergone apoptosis in response to increased clearance of opsonised and/or apoptosing B cells, or as a tolerogenic mechanism in response to the increased serum immunoglobulin (2 mg/wk of 2H7 mAb). In addition, there was a depletion of CD4⁺ and CD8⁺ T cells in hCD20tg mice but not in the hCD20tg-negative littermates, suggesting that this was 2H7-specific. However, the mechanism of depletion of CD4⁺ and CD8⁺ T cells is not known.

Long-term effects of memory B cell depletion on long-lived plasma cell numbers and serum antibody levels

HA-specific MBCs remained depleted for up to 150 days post-2H7 treatment, with 2 out of 3 mice at 150 days post-treatment still having completely undetectable HA-specific splenic MBCs by limiting dilution ELISpot. In contrast, total IgG-secreting MBCs were depleted by more than 95% but recovered to pre-depletion levels by 7 weeks post-depletion, probably via homeostatic turnover until the MBC ‘niche’ was filled. The efficacy of depletion of IgM, IgG and IgA MBCs in lymph nodes and NALT was not determined in these experiments but will be pertinent in accessing the contributions of mucosal MBC and LLPC to maintaining mucosal Ab of different isotypes after intranasal infection.

The purpose of characterizing this system was to determine the effect of MBC loss on numbers of pre-established LLPC and serum Ab. In hCD20tg mice, 2H7 treatment had no effect on HA-specific IgG ASC in either the spleen and bone marrow for up to at least 90 days after that, which is consistent with a previous finding that LLPCs are refractory to depletion by anti-CD20 mAb despite expressing low to intermediate levels of hCD20 (DiLillo *et al.* 2008). However on day 150, there was a significant loss of HA-specific IgG ASC from both spleen and bone marrow in hCD20tg mice but not in hCD20tg-negative littermates. As this was too late to be a direct depleting effect of the 2H7 mAb, it suggests that MBCs, which were depleted rapidly by 2H7 treatment, are required for the maintenance of HA-specific ASC in spleen and bone marrow, especially at very long-term time points. In the prolonged absence of 90% of HA-specific IgG MBCs, the concentration of serum HA-specific IgG remained stable for up to 42 days post-2H7 treatment but decreased by 90 days and remained significantly decreased for up to 150 days after MBC

depletion. In the spleen, there was also a decrease in the numbers of total IgG-secreting ASC, possibly because some of these were not 'true' LLPCs but rather plasmablasts which expressed hCD20 and were continually produced by non-specific stimulation of MBCs and therefore were absent after depletion of MBCs. The concurrent decrease in the number of bone marrow HA-specific IgG ASC and HA-specific serum Ab in hCD20tg mice indicates that the long-term maintenance of HA-specific serum Ab as well as the HA-specific LLPC pool was dependent on the presence of MBC.

MBCs do not spontaneously differentiate into Ab-secreting plasma cells but require a stimulus. The two main stimuli are antigen, or as proposed recently by Lanzevecchia and colleagues (Bernasconi *et al.* 2002) by non-specific TLR stimulation and bystander T cell help in the absence of antigen. *In vitro*, it is well established that MBC can be activated to differentiate by combinations of TLR agonists and cytokines in the absence of direct BCR stimulation (Bernasconi *et al.* 2002). However, when this was tested *in vivo*, there was little/no activation of pre-established PE-specific MBC or naïve follicular B cells observed in response to injection of LPS, CpG, or anti-CD3 Ab alone or anti-CD3 Ab and LPS or CpG (Benson *et al.* 2009), although 25-50% of both PE-specific MBC and naïve follicular B cells did proliferate in response to agonistic anti-CD40 Ab alone or anti-CD40 Ab coupled with LPS or CpG. Maximal MBC activation *in vivo* was observed by BCR activation by direct exposure to cognate antigen (Benson *et al.* 2009). In this study, there was no replenishment of HA-specific MBC, suggesting that there was little/no re-stimulation of any remaining HA-specific MBCs. Indeed, experimental mice were not intentionally exposed to homologous re-infection by PR8, and non-specific stimuli was minimized in a clean animal facility. The profound and prolonged loss of HA-specific MBC after 2H7 treatment was inadequate to restore serum Ab and replenish the LLPC niche. In the absence of HA-specific MBC, the half-life of HA-specific LLPC in these experiments approximately corresponds to that established for LCMV-specific LLPC in irradiated mice, which has been estimated to be about 138-141 days.

Data from longitudinal observational studies after vaccination and/or booster suggests that maintenance of serum Ab is complex. The recent mathematical modeling by Amanna and colleagues (Amanna *et al.* 2010) demonstrate that Ab kinetics is probably dependent on the context of immunization (route of administration, adjuvant, etc) or infection. Long-lived

serum Abs are most likely maintained by a combination of MBC-dependent and LLPC-dependent mechanisms. This study has shown that after intranasal PR8 infection, MBCs are required to maintain long-term serum Ab and bone marrow and splenic LLPCs. This finding is in contrast to the recent studies showing that MBCs are not required for the maintenance of serum Ab or the LLPC pool after systemic immunisation with protein (Ahuja *et al.* 2008; DiLillo *et al.* 2008). The difference in findings between our study and previous studies may simply reflect the heterogeneity of mechanisms that maintain long-lived humoral immunity under different immunisation or infection contexts. Perhaps when there is residual antigen, e.g. in chronic/latent infections or autoimmune disease, MBCs play a more important role in maintaining serum Ab, while when antigen is rapidly eliminated, e.g. in acute viral infections or after protein immunization, LLPCs are able to maintain serum Ab without MBCs. Furthermore, an additional level of homeostatic control exists, whereby pre-existing serum Ab concentrations affect whether MBCs respond to antigen by differentiating into Ab-secreting plasma cells or non-secreting daughter MBCs.

Like LLPCs, MBCs have the important role of maintaining serum Ab in the absence of antigenic re-stimulation. MBCs can be remarkably long-lived and are thought to represent an important second line of immune defence that is initiated especially if pre-existing Ab levels are inadequate to prevent infection or if the invading pathogen is able to circumvent the pre-existing Ab response (e.g. high dose exposure or antigenic mutation). These data demonstrate that pre-existing LLPCs can survive independently of the MBC compartment for at least 90 days but it requires MBCs to replenish the pool and maintain long-lived serum Ab.

Outstanding questions

There are several methods to reduce the number of LLPCs *in vivo* but leave the MBC compartment intact, which would enable us to test whether MBCs can maintain serum Ab in the absence of LLPCs. For example, using genetically-altered mice which fail to produce LLPCs but are able to produce MBCs and short-lived PCs after immunisation or infection [e.g. mice with deletions in homing receptor CXCR4, CD93 (Chevrier *et al.* 2009), CD28 (Rozanski *et al.* 2011)]. Other agents allow selective depletion of LLPCs but not MBCs, e.g. Bortezomib, as described previously, or by destruction of the LLPC survival niche e.g. by

depleting APRIL-producing eosinophils (Van Trung *et al.* 2011) or anti-BAFF/APRIL therapy (Benson *et al.* 2008). Whether by blocking differentiation of naïve B cells into LLPC, or by depleting pre-established LLPC, it would be very interesting to further this work by selectively depleting HA-specific LLPC after intranasal PR8 infection to test whether an intact MBC compartment can maintain serum Ab levels in the absence of HA-specific LLPC. Additionally, it would be important to determine whether mice depleted of MBC by treatment with 2H7 mAb would develop more severe infection after re-challenge with Influenza A virus.

Immunization with different vaccines, adjuvants or by different routes of administration has demonstrated that the longevity of serum Ab is dependent on, or in other words, 'imprinted' by, the context of immunization. Why some immunization strategies work better than others is not known, and it would be useful to find out whether the relative compartments of MBC or LLPC are altered by different immunization contexts, and the relative contributions of each compartment to long-lived serum Ab.

Chapter 4: Loss of previously established humoral immunity to Influenza A after sequential *Plasmodium chabaudi chabaudi* (AS) infection.

4.1: Introduction

Humoral immunity is defined as the antibody response and the accompanying cellular immune response that is initiated after vaccination or infection, and which has been correlated with protective immunity. High titres of specific serum and mucosal antibodies (Abs) are found only in hosts which have previously encountered the vaccine, infection or pathogen, and are therefore hallmarks and diagnostic indicators of the humoral immune memory repertoire (reviewed by Slifka & Ahmed 1996). Conversely, the absence or attrition of specific Abs, memory B cells (MBCs) or long-lived plasma cells (LLPCs) indicates that long-lived humoral memory was not established or that humoral memory has been established but was lost. Loss of humoral immunity can occur naturally with the waning of antibody levels after infection or failed vaccination. This could be due to inadequate priming or establishment of long-lived memory cells or attrition or apoptosis of pre-established LLPCs or MBCs.

In the previous chapter, I determined that persistent serum Abs after intranasal PR8 infection was partly maintained by both LLPCs and MBCs. The aim of this chapter is to investigate whether a subsequent infection with malaria could affect pre-established antibody persistence by affecting LLPCs or MBCs. The hypothesis is that infection with the malaria parasite could affect LLPCs through competitive dislocation by migratory plasmablasts, or by causing apoptosis of LLPCs; or affecting MBCs, which would then lead to inadequate replenishment of the LLPC pool and serum Abs.

4.1.1: Dislocation of pre-established long-lived plasma cells by new migratory plasmablasts

The size of the LLPC niche in the bone marrow is finite (Radbruch *et al.* 2006; Sze *et al.* 2000; Terstappen *et al.* 1990). However this finite niche has to accommodate LLPCs with specificities against different infections over time (Radbruch *et al.* 2006a). One of the

Chapter 4: Loss of previously established humoral immunity

mechanisms by which LLPC homeostasis is maintained is through occupation of and competition for the niche in a purely stoichiometric manner by migratory plasmablasts competing for survival resources, such as the chemokine CXCL12 or the cytokine APRIL, which are essential for homing into and survival in the bone marrow (Belnoue *et al.* 2008;Hargreaves *et al.* 2001). This means that in order to accommodate LLPC of new specificities in the bone marrow, some of the pre-established LLPC have to be ejected into the circulation, where they eventually die (Fairfax *et al.* 2008;Radbruch *et al.* 2006). The burden of migratory plasmablasts entering the bone marrow can be very large (Hofer *et al.* 2006). First, very little, if any, cross-reactivity exists between Abs against discrete pathogens or even closely-related pathogens. This means that, in order for serum Abs to persist against all pathogens, specific LLPC have to be generated and accommodated after each infection. Secondly, over time, hosts also have to cope with antigenic variation through genetic shifts and drifts in pathogens, each rendering previously established specific memory ineffective in preventing re-infection, and necessitating the generation of new LLPCs. Thirdly, these competitive stresses may even increase with age as the holding capacity of LLPC niches may be reduced with age (Han *et al.* 2003). If numbers eventually fall below the threshold required to sustain enough specific serum Abs to neutralise re-infection, the host effectively loses protective immunity to that pathogen. Therefore it is imperative that homeostatic regulation occurs to allow the host to accommodate new LLPC specificities without compromising pre-established LLPC.

Plasma cells appear in the blood during a narrow window of time after immunisation. To test whether attrition of pre-established LLPC from bone marrow into circulation can occur in humans, one group (Odendahl *et al.* 2005) tested human peripheral blood mononuclear cells (PBMCs) 7 days after secondary immunisation of healthy adults with recombinant tetanus toxoid, using a panel of specific Abs that could differentiate LLPC from newly generated migratory plasmablasts within the pool of PBMCs. In this study, LLPC and plasmablasts were differentiated by their relative surface expression of human leukocyte antigen (HLA-DR), the cell surface markers CD38 and CD20, chemotactic responsiveness to ligands for the chemokine receptors CXCR3 (CXCL9) and CXCR4 (CXCL12), and specificity of secreted antibody by ELISpot. A small number of HLA-DR^{low} LLPC appeared at the same time as HLA-DR^{high} TT-specific migratory plasmablasts on days 6

Chapter 4: Loss of previously established humoral immunity

and 7 after booster immunisation. Using ELISpot, they established that the HLA-DR^{low} LLPC did not secrete TT-specific Ab, nor did they respond to the chemokines CXCL9 and CXCL12, indicating that they were mature LLPC which had lost chemotactic responsiveness to those homing chemokines. This was the first indication that competitive dislocation was occurring at the level of the bone marrow niche.

Using these and other published data (Bernasconi *et al.* 2002;Manz *et al.* 1997;Slifka *et al.* 1998), Hofer and colleagues designed a mathematical model to determine whether homeostatic dislocation alone would be able to bring the number of pre-established plasma cells down below the threshold level of protection (Hofer *et al.* 2006). Assuming that a host encounters four novel pathogens each year, and, a) each infection generates an influx of a constant fraction of new cells into the bone marrow niche; b) competitive displacement is stoichiometrically determined; c) memory declines in an cell-autonomous fashion, d) a critical frequency of plasma cells is required to maintain protective antibody titres for a given pathogen; it is possible to predict the waning of humoral memory over time. Using the data from a previous study (Manz *et al.* 1997), it would take an ovalbumin-immune BALB/c mouse 367 subsequent antigenic challenges in order to lose serum antibody. Based on data obtained from another study (Odendahl *et al.* 2005), a human child vaccinated with tetanus toxoid would theoretically require 692 heterologous infections or immunogens generating a secondary response of similar magnitude, to be rendered non-immune to Tetanus. To encounter that number of disparate infections in their lifetime is unrealistic for both laboratory mice and humans, and if most infections generate a constant fraction of new cells into the bone marrow niche, then the majority of pre-established functional humoral immunity is unlikely to be eroded by competitive displacement alone, if the antigens encountered have one or few dominant epitopes.

However there may be situations where a pathogen can create an overwhelming migratory plasmablast load, which could affect the LLPC niche. In this laboratory, it has been observed that a primary blood-stage infection with the rodent malaria *P. chabaudi* generates approximately 6000 B220⁺ CD138⁺ migratory plasmablasts per femur pair entering the bone marrow per day from day 12 to 25 of primary blood-stage infection. Out of these, approximately 1000 MSP1-specific ASC are added to the LLPC pool from day 30

Chapter 4: Loss of previously established humoral immunity

and remain stable for up to 8 months after primary infection with *P. chabaudi* (Nduati *et al.* 2010;Ndungu *et al.* 2009). It is believed that a large proportion of the first wave of plasmablast entry into the bone marrow is not protective as it may be a low-affinity non-specific response generated by malarial mitogens or the very large antigenic load caused by the shedding of parasite material and/or antigenic variation (Achtman *et al.* 2007b;Greenwood 1974;McLean *et al.* 1982). Furthermore, parasites such as *Plasmodium* can continuously generate new migratory plasmablasts over a long period of time as they can induce chronic infection (Jarra 1982). This can occur through multiple possible mechanisms, including stimulation by malarial mitogens, bystander activation of helper T cells (Ho & Webster 1990), antigenic variation (McLean *et al.* 1982) or revelation of novel intracellular auto-epitopes after invasion (Rosenberg *et al.* 1973). In human bone marrow aspirates or smears taken during acute infection with the human parasites *P. falciparum* or *P. vivax*, an increase in bone marrow plasma cells has been observed (Wickramasinghe *et al.* 1987). Compared to a secondary OVA immunisation, which generates a total of approximately 3000 OVA-specific LLPC, the large number of migratory plasmablasts generated by a single *P. chabaudi* infection competing for space in the LLPC niche in the bone marrow may have the potential to dislocate LLPC from their niches (Nduati *et al.* 2010b;Ndungu *et al.* 2009a). Hence it is possible that malaria infection could exert a greater competitive stress on the limited bone marrow plasma cell niches than the comparatively fewer LLPC generated by other acute infections or sub-unit vaccinations, making it an ideal model to test the hypothesis of whether attrition of humoral immunity can occur in a purely stoichiometric manner of competitive dislocation based on finite survival resources.

4.1.2: Clearance of pre-established long-lived plasma cells and MBCs by causing apoptosis

Infection of mice with *P. yoelii* has been shown to result in significant apoptosis of both vaccine-induced MSP1₁₉-specific and unrelated MBCs and LLPC (Wykes *et al.* 2005). Similar observations have been made in *Trypanosoma brucei* infections of C57BL/6 and BALB/c mice, which also result in reduction of pre-established anti-parasite, hapten-protein conjugate- and vaccine-induced MBC and LLPC, leaving mice with increased susceptibility to previously-encountered infections (Radwanska *et al.* 2008).

Some pathogens, in particular parasitic infections such as those caused by *Plasmodium sp.*, can directly affect B cell memory niches irrespective of the immune responses they induce. Bone marrow hypocellularity is a feature of acute malaria (Wickramasinghe & Abdalla 2000) but the exact cause of this is unknown. However it is known that malaria can cause cellular dysregulation, development of atypical cell populations, cellular infiltration and extrusion, and disruption or acceleration of lymphatopoiesis and haematopoiesis (Belyaev *et al.* 2010; Kurtzhals *et al.* 1997; Palfreman *et al.* 1998; Silverman *et al.* 1987). A recent report demonstrated a decrease in CXCL12 mRNA in bone marrow which was proposed to result in the loss of developing B cells from their bone marrow niche (Viki & Nathalie 2011). This could also be a cause for the loss of LLPC, as they share overlapping survival niches on CXCL12⁺ VCAM-1⁺ stromal cells with developing B cells. In spleen, the niches for LLPC and MBCs are less well defined, but physical changes in splenic microarchitecture and modifications in B cell compartments (Castillo-Méndez *et al.* 2007), as well as their relation to the compartments containing T cells and innate cells could possibly reduce their access to survival resources. A novel mechanism for regulating of the LLPC niche involving FcγRIIB has been proposed whereby circulating low affinity Abs and the immune complexes produced by polyclonal B cell activation, which is a prominent feature of many parasitic infections, can induce the apoptotic clearance of resident LLPC via the cross-linking of the FcγRIIb on the surface of resident LLPC (Xiang *et al.* 2007). Therefore, it is not known whether the loss of LLPC from their bone marrow niche is merely a bystander effect of a general bone marrow hypocellularity or whether it is preferentially depleted in comparison to other cell types. It is important to note that bone marrow cellularity is a feature of or by induction and release of large amounts of inflammatory cytokines, which may cause dysregulation of the bone marrow niche.

Sequestration of *Plasmodium* parasite is the accumulation of erythrocytes parasitized with mature stages of parasites (trophozoites and schizonts) in particular organs and their adhesion to host endothelium. As the expression of endothelial cells surface receptors implicated in iRBC cytoadherence (such as CD36, ICAM, VCAM and P-selectin) is induced by inflammatory cytokines (such as TNFα, IFNγ and LTα) (Favaloro 1993; Mota *et al.* 2000), this phenomenon increases with inflammation. In humans, sequestration of *P.*

Chapter 4: Loss of previously established humoral immunity

falciparum-parasitized erythrocytes (pRBC) has been documented, and is thought to be linked to pathology such as cerebral malaria and respiratory distress. They have been found adhering to endothelium microvasculature in brain, heart, lung, liver, spleen, placenta and bone marrow (MacPherson *et al.* 1985) (reviewed by (Rowe *et al.* 2009)). In humans, sequestration of *P. falciparum* pRBC in bone marrow has been recorded but has only been shown to be by gametocyte-infected erythrocytes rather than asexual forms (Smalley, Abdalla, & Brown 1981). In culture, these gametocyte-infected erythrocytes adhere to bone marrow stromal and endothelial cells (Rogers *et al.* 2000). In mouse models, sequestration of *P. chabaudi*-parasitized erythrocytes has been reported in C57BL/6 mice (Gilks *et al.* 1990;McLean *et al.* 1982;Mota *et al.* 2000). *In vitro*, *P. chabaudi* parasitized red blood cells can bind to primary mouse endothelial cells (namely via CD36) and in an IFN γ R-dependent manner (Mota *et al.*2000). *In vivo*, sequestered *P. chabaudi* parasites have been found predominantly in the liver but also in the lung and spleen (D. Cunningham and T. Brugat, in press; and (Gilks *et al.* 1990)) but the mechanisms of sequestration *in vivo* are not known and how/whether sequestration causes pathology is unclear (Miller *et al.* 2002). Little is known whether sequestering parasites, in both humans and mice, can cause local immunomodulation or dysregulation of the LLPC niche and whether they can directly affect the survival of pre-established LLPC. It is important to note that perhaps the effect of parasite sequestration on the survival of bone marrow LLPC, if any, may be less relevant in non-sequestering parasite strains, e.g. *P. vivax* infection in humans and *P. yoelii* and *P. berghei* NK65 infections in mice.

4.2: Aim

The aim of this chapter is to establish whether *P. chabaudi* causes a reduction of pre-established Influenza-specific Abs.

4.3: Objectives

1. To determine whether the antibody response induced in *P. chabaudi* infection is cross-reactive with Influenza.
2. To determine whether *P. chabaudi* infection increases clearance of pre-established Influenza-specific Abs in serum.
3. To determine whether *P. chabaudi* infection causes reduction of pre-established Influenza-specific LLPC in bone marrow, and if so, to investigate the mechanism:
 - Whether the migratory plasmablasts induced by malaria causes competitive dislocation of pre-established bone marrow LLPC from their niches.
 - Whether infection with *P. chabaudi* causes apoptosis of LLPC through Fc γ RI,II,III-mediated mechanisms.
 - Whether *P. chabaudi*-induced apoptosis of LLPC can be related to the kinetics of parasite sequestration in the bone marrow.

4.4: Results

4.4.1: Little serological cross-reactivity between Influenza A/PR/8/34 and *P. chabaudi* infections.

A blood-stage infection with *P. chabaudi* generates a large polyclonal and non-specific B cell response. This results in hypergammaglobulinaemia which is characterised by IgG Ab with specificities for both plasmodial and non-plasmodial antigens (Achtman *et al.* 2003; Seixas and Ostler 2005). This is consistent with the hypergammaglobulinaemia observed after infection with *P. chabaudi adami* and *P. yoelli* (Langhorne *et al.* 1984). Hypergammaglobulinaemia including non-specific Ab has also been observed even after mosquito-transmission of *P. berghei* (Mori *et al.* 1987). This persistent hypergammaglobulinaemia may be a result of a large increase in B cells and plasma cells during early infection, which can persist for up to day 40 of infection (Castillo-Méndez *et al.* 2007).

To ensure that there were no Abs generated by a *P. chabaudi* infection that would bind to Influenza A/PR/8/34 (PR8) and thus confound interpretation of the results, I determined levels of cross-reactivity in serum Abs against PR8 and *P. chabaudi*.

First, three different types of sera were obtained from: 1) a pool of several sera from BALB/c mice infected previously with PR8; 2) a pool of immune sera from BALB/c mice recovered after 2-4 sequential infections with *P. chabaudi*, called “hyperimmune sera”; and 3) a pool of sera from naïve BALB/c mice. Second, a lysate of *P. chabaudi*-parasitized erythrocytes (“parasite lysate”) and bromelain-digested haemagglutinin cleavage product 1 (HA) from PR8 were used as antigens in three different assays to measure antibody cross-reactivity: 1) ELISA; 2) Western blot; and 3) *In vitro* virus neutralisation assay.

The binding of these three pools of sera to HA was first quantified by ELISA, using HA as the capture antigen. No cross-binding Abs to HA were detected in *P. chabaudi* hyperimmune sera or naïve sera (**Figure 4.1A**). Surprisingly, when parasite lysate was used as the capture antigen, a small amount of cross-binding of Abs in PR8 sera to parasite lysate was detected (**Figure 4.1B**).

Chapter 4: Loss of previously established humoral immunity

To confirm the ELISA data, three independent preparations of HA and parasite lysate were separated by SDS-PAGE and a Western blot was performed using these three sera as the primary Abs. The gel confirmed that there was little cross-reactivity between PR8 and *P. chabaudi* serum Abs, as HA1, an approximately 46 kDa product (Wang *et al.* 2006), was not recognised by serum Abs from hyperimmune sera and parasite lysate was not recognised by HA-specific Abs in sera obtained from PR8-infected mice in the Western blot. Surprisingly, there was also a doublet of approximately 20 kDa that was present and recognised by both PR8 sera and hyperimmune sera but not naïve sera (**Figure 4.1C**). The identity of that approximately 20 kDa doublet is at present unknown.

These three pools of sera were also tested in a viral neutralisation assay to determine their ability to neutralise PR8 virus *in vitro*. Hyperimmune sera and naive sera did not have any neutralising activity against PR8 *in vitro* (**Figure 4.1D**). BALB/c mice were infected with 10^5 *P. chabaudi*-parasitised erythrocytes intraperitoneally and 60 days later, infected with 250 haemagglutinating units (HAU) of PR8 intranasally. Sera obtained 0, 14 and 28 days after PR8-infection was tested using the neutralisation assay and showed that a previous infection with *P. chabaudi* did not alter the primary nAb response to PR8 (**Figure 4.1E-F**).

In summary, there was little cross-reactivity of serum Abs to parasite lysate and HA detected using ELISA, Western blot and neutralisation assay. Hyperimmune serum from mice multiply infected with *P. chabaudi* do not bind to HA in ELISA and Western blot and not neutralise PR8 virus *in vitro*. There is some cross-reactivity between *P. chabaudi*-specific sera and PR8 bromelain-digested HA; however a previous *P. chabaudi* infection and pre-existing *P. chabaudi*-specific Ab in BALB/c mice does not appear to change the development of PR8-specific nAb *in vivo* from that of naïve mice.

4.4.2: Experimental protocol of sequential infection with PR8 and *P. chabaudi*.

Having determined that there are little cross-reactive Abs induced by the individual PR8 and *P. chabaudi* infections, I set up a model of sequential infection model in BALB/c female mice, where the second infection with *P. chabaudi* was initiated 5 months after the first PR8 infection (**Figure 4.2A**). At 150 days after PR8 infection, the frequencies of

Chapter 4: Loss of previously established humoral immunity

germinal centre B cells in the spleen had returned to levels similar to naïve mice (**Figure 4.2B-C**), indicating that there was probably very few new HA-specific LLPC and MBC being generated at this late time-point. At this time point, a primary blood-stage *P. chabaudi* infection was initiated by intraperitoneal injection of 10^5 *P. chabaudi*-parasitized erythrocytes while a primary PR8 infection was initiated by intranasal instillation of 250 HAU of virus-containing fluid without anaesthesia. The course of parasitaemia after an intraperitoneal injection of 1×10^5 *P. chabaudi*-parasitised erythrocytes has been described previously in both BALB/c and C57BL/6 mice (Meding *et al.* 1990; Helmby *et al.* 2000; Ndungu *et al.* 2009), with acute high parasitaemia followed by a 2 to 3 month low-grade chronic parasitaemia. The course of parasitaemia in naïve BALB/c mice in my experiments is similar to that of C57BL/6 mice (**Figure 4.2A, graph in box**). This model of sequential PR8-*P.chabaudi* infections would enable me to investigate how pre-established humoral immunity was maintained after a heterologous infection at a cellular level. The impact of a sequential infection on pre-established humoral immunity to PR8 will be assessed by a) concentration of PR8 HA-specific Abs of different isotypes measured by ELISA; b) neutralizing anti-PR8 antibody titres measured by *in vitro* virus neutralization assay; c) numbers of antibody-secreting cells and MBCs quantified by ELISpot; d) numbers of LLPC and migratory plasmablasts quantified by multiparameter flow cytometric analysis using a panel of Abs specific for these cell types (**Figure 4.2A, text in box**).

4.4.3: An infection with *P. chabaudi* in mice previously infected with PR8 reduces pre-established PR8-specific humoral immunity

BALB/c mice were infected intranasally with 250 HAU of PR8 and rested for 105 or 150 days to ensure that the majority of HA-specific LLPC were stably established in the bone marrow (**Results Chapter 3; Figure 3.1**). Mice were then infected with *P. chabaudi* as described in **Figure 4.2A**. The concentration of HA-specific serum IgG Ab was measured by ELISA at various time points for up to 100 days after *P. chabaudi* infection. When mice were infected with *P. chabaudi* 105 days after PR8, there was a significant reduction in HA-specific IgG at 21, 42 and 63 days after *P. chabaudi* infection, when compared with serum from PR8 immune mice without *P. chabaudi* infection (**Figure 4.3A**). This was also the case when *P. chabaudi* was initiated 150 days after PR8 infection (**Figure 4.3B**). When

Chapter 4: Loss of previously established humoral immunity

there was an interval of 150 days between PR8 and *P. chabaudi* infection, HA-specific IgG started to return to the level of PR8 immune mice without *P. chabaudi* infection by 42 days after *P. chabaudi* infection (**Figure 4.3B**). Therefore a primary infection with *P. chabaudi* resulted in attrition of pre-established serum Ab 105 or 150 days after PR8 infection, but this effect appears to be transient, at least with a 150-day interval.

4.4.4: An infection with *P. chabaudi* in mice previously infected with PR8 results in the loss of thymus-dependent serum antibody isotypes.

Malaria infection induces a pro-inflammatory cytokine response with a rapid but regulated production of the cytokines interleukin-12 (IL-12), tumour necrosis factor- α (TNF- α), and interferon- γ (IFN- γ) (Langhorne *et al.* 2004; Su & Stevenson 2000). *P. chabaudi* has been shown to induce a large production of IL-12, TNF- α , and IL-6 (Langhorne *et al.* 2004; Stevenson *et al.* 1995), thus inducing a CD4 Th1 response (Langhorne *et al.* 1989). A role for specific isotypes, in particular IgG2a, in protective immunity to PR8 is inferred from the experimental models and immuno-epidemiological studies in which high antibody (Ab) titres and in some cases restricted immunoglobulin (Ig) isotypes to particular antigens (Ag) like HA correlate with immunity. Having found that an infection with *P. chabaudi* in mice previously infected with PR8 resulted in a global loss of HA-specific IgG, I hypothesized that *P. chabaudi* could affect some HA-specific Ig isotypes more than others and this may result in loss of protective immunity to PR8.

Four weeks post-PR8 infection, the predominant anti-HA serum Ab isotypes were IgG2a followed by IgG1. After *P. chabaudi* infection, the isotypes which were particularly reduced in the PR8-*P. chabaudi*-infected group were the thymus-dependent isotypes (Coffman *et al.* 1988; Reinhardt *et al.* 2009; Snapper & Paul 1987), IgG1, IgG2a and IgG2b. In contrast, the thymus-independent isotypes IgG3 and IgM were not significantly reduced in serum (**Figure 4.3C**). This suggests that *P. chabaudi* infection particularly affected the thymus-dependent isotypes of HA-specific serum antibody response.

4.4.5: An infection with *P. chabaudi* in mice previously infected with PR8 results in the loss of protective immunity to PR8.

Pre-established HA-specific Ab was transiently lost after infection with *P. chabaudi*, despite no decrease in serum Ab half-life. The loss of serum Abs was comprised mostly of IgG1, IgG2a and IgG2b isotypes. I then hypothesized that this loss was physiologically important in increasing susceptibility to re-infection with PR8. Therefore the neutralising antibody (nAb) response to PR8 was determined by a neutralising assay which has been previously described (Kassiotis *et al.* 2006) (**Figure 4.4A**). Briefly, the ability of serum from influenza-immune mice was quantified by its ability to inhibit killing of the cell line MDCK by PR8. The Influenza PR8 virus kills MDCK cells within 3 days of culture, but these cells are rescued by the addition of anti-sera. Sera were obtained at various time points after PR8-*P. chabaudi* infection and heated-inactivated for 10 min at 56°C to remove complement.

In agreement with the data obtained from ELISA showing a decrease in HA-specific serum IgG (**Figure 4.4A-B**), there was a highly significant reduction in serum nAb-mediated *in vitro* neutralisation of PR8 virus for up to 60 days post-*P. chabaudi* infection, compared with PR8 only control serum (**Figure 4.4A**).

To confirm that the loss of *in vitro* neutralisation indeed was an indication of a loss of *in vivo* protection, PR8-*P. chabaudi*-infected mice, as well as the PR8-only and naïve mice, were treated with chloroquine 21 days after the *P. chabaudi* infection to eliminate any residual parasites, rested for a further 21 days, and then re-infected with 10 HAU of PR8 intranasally under light anaesthesia. Three days later, viral titres in the lung were measured by qRT-PCR (**Figure 4.4B**). Whilst control naïve mice had high lung viral titres, the majority of PR8-immune mice had below detectable levels of viral titres in the lung. In contrast, mice which had been infected with PR8-*P. chabaudi* had intermediate viral titres in the lung (**Figure 4.4C**). This is in agreement with the loss of the neutralising activity of serum after *P. chabaudi* infection (**Figure 4.4A**), although correlation of viral loads with weight loss or lung pathology has not been determined. Therefore *P. chabaudi* infection initiated after establishment of protective immunity to a previous infection results in loss of

neutralising Ab and loss of resistance to the previous infection, regardless of the presence of parasite.

4.4.6: An infection with *P. chabaudi* in mice does not decrease the half-life of immunoglobulin.

Immunoglobulin (Ig) concentration in serum is under homeostatic regulation by balancing production by plasma cells and clearance by antibody, complement and Fc receptors (Baiu *et al.* 1999; Ward *et al.* 2003). Ig half-life is dependent on its plasma concentration (Fahey & Sell 1965). Malaria infection causes a dramatic and prolonged polyclonal hypergammaglobulinaemia as well as increase in spleen antibody-secreting cells (ASC) (Achtman *et al.* 2007; Cadman *et al.* 2008). Therefore it is possible that pre-established IgG is cleared faster in the PR8-*P. chabaudi* infection than in PR8 alone, in the host's attempt to maintain normal serum Ig concentrations.

Consistent with previous studies, PR8-immune BALB/c mice which were subsequently infected with *P. chabaudi* developed the peak of parasite-specific IgG on day 42 of *P. chabaudi* infection (**Figure 4.5A**). By contrast, there was a rapid increase in total serum IgG concentrations from the peak of parasitaemia (approximately day 10), which then remained elevated for up to day 60 (the last time-point observed) (**Figure 4.5B**).

Due to this rapid and prolonged hypergammaglobulinaemia, I investigated if the transient reduction in anti-PR8 Abs was due to a globally increased rate of antibody clearance caused by the hypergammaglobulinaemia. Age-matched BALB/c female mice were infected with *P. chabaudi* and then injected intraperitoneally with 200 µg of anti-2,4,6-trinitrophenyl (TNP) IgG2a 24h (acute infection) or 60 days (during the chronic infection) later. Serum was collected every 2 days and the concentration of anti-TNP IgG2a was monitored using TNP-BSA-coated ELISA plate (**Figure 4.5C**), in a similar protocol to one described in mice to determine the half-lives of serum antibody (Vieira & Rajewsky 1988). From this assay, the half-life of a non-specific mouse IgG2a could be determined during both the acute and chronic phases of primary *P. chabaudi* infection (**Figure 4.5D**). Antibody half-life was calculated by use of linear regression on log₂ transformed antibody concentrations

Chapter 4: Loss of previously established humoral immunity

in relation to hours after injection. Antibody half-life was expressed as the inverse reciprocal of the regression line slope (boxed in **Figure 4.5D**; expressed in hours). Despite hypergammaglobulinaemia generated by *P. chabaudi* infection occurring in three separate experiments, there was no difference in the Ab half-life in mice where *P. chabaudi* infection had been initiated 1 day previously, or in mice in late stages (d60) of *P. chabaudi* infection, compared to the Ab half-life in naïve control mice (**Figure 4.5D**).

4.4.7: An infection with *P. chabaudi* in mice previously infected with PR8 results in the loss of HA-specific bone marrow plasma cells after *P. chabaudi* infection.

The loss of HA-specific serum IgG could be the result of the loss of LLPCs (LLPC), therefore I quantified HA-specific antibody-secreting cells (ASC) in the bone marrow, where the majority of LLPC reside. First, BALB/c mice were infected with *P. chabaudi* and the absolute number of bone marrow cells from femur pairs were counted every 1-2 days for 80 days after *P. chabaudi* infection. In addition, parasitaemia was monitored by thin blood films throughout acute *P. chabaudi* infection. Bone marrow cellularity fell drastically from 4.85×10^7 in naive mice to 1.5×10^7 on day 8 post-infection, coinciding with the peak of parasitaemia, and then rising rapidly to 4.5×10^7 on day 20 as the parasitaemia dropped, and remaining stable for up to 80 days post-infection (the last time point observed) (**Figure 4.6A**).

Next, to investigate whether the effect of a subsequent *P. chabaudi* infection on HA-specific serum IgG was due to a loss of HA-specific plasma cells, an ELISpot assay was used to quantify HA-specific ASC in the bone marrow after PR8-*P. chabaudi* infection. BALB/c female mice were infected intranasally with PR8, rested for 150 days, and then infected with *P. chabaudi*. Bone marrow was obtained at the peak (d8) of *P. chabaudi* parasitaemia, where the greatest bone marrow loss was observed, and HA-specific ASC in the bone marrow were enumerated by ELISpot (**Figure 4.6B**). Mice infected with PR8-*P. chabaudi* had significantly reduced numbers of HA-specific IgG ASCs in the bone marrow when compared to mice only infected with PR8 (Median:) (**Figure 4.6C**). Therefore, infection with *P. chabaudi* reduced the frequency of HA-specific IgG ASC in the bone marrow. However, despite a loss of bone marrow cellularity and the loss of HA-specific

Chapter 4: Loss of previously established humoral immunity

IgG ASC, there was no loss of total ASC (**Figure 4.6D**), suggesting that size of the total LLPC niche was not compromised by the *P. chabaudi* infection. The preservation of total IgG ASC numbers in the face of bone marrow loss suggested that new migratory plasmablasts were entering the bone marrow during acute *P. chabaudi* infection, therefore keeping the total number of plasma cells the same (**Figure 4.6D**) in the face of the loss of HA-specific plasma cells (**Figure 4.6C**). By contrast, I observed a significant increase in the total number of HA-specific MBC in the spleen at d33 and d77 after *P. chabaudi* infection (**Figure 4.7**).

4.4.8: Entrance of migratory plasmablasts into the bone marrow and loss of LLPCs during acute *P. chabaudi* infection (d8-12).

The loss of HA-specific ASC could be due to attrition either via dislocation and mobilisation or apoptosis of pre-established ASC in the bone marrow by new migratory plasmablasts entering the bone marrow. To determine whether the loss of HA-specific ASC from bone marrow was due to competitive dislocation by migratory plasmablasts generated by *P. chabaudi* infection and entering the bone marrow, I investigated the kinetics of the entry of new migrating plasmablasts induced by *P. chabaudi* infection into the bone marrow during the acute and chronic phases of infection by flow cytometry. *P. chabaudi* was initiated 150 days after PR8 infection. Mice were sacrificed on day 0, 8, 10, 12, 25, 45 and 75 of *P. chabaudi* infection and bone marrow was stained with anti-B220 and anti-CD138 Abs to discriminate LLPC (B220⁻ CD138⁺) and migratory plasmablasts which have not downregulated B220 (B220⁺ LLPC⁺). A large percentage of B220⁺ CD138⁺ plasmablasts was observed to enter the bone marrow from day 10 onwards, and from d25 onwards, the B220⁺ CD138⁺ migratory plasmablast population appeared to downregulate B220, indicating their maturity into LLPC (Manz *et al.* 1998) (**Figure 4.8A**). This downregulation of B220 on migratory plasmablasts coincides to the time point when MSP1-specific IgG ASC start to appear in the bone marrow in a previous study in C57BL/6 mice (Nduati *et al.* 2010). In contrast, B220⁻ CD138⁺ LLPC decreased in percentage from day 10 onwards, only increasing to pre-*P. chabaudi* infection frequencies at the last time point observed (day 75) (**Figure 4.8A**).

Chapter 4: Loss of previously established humoral immunity

In terms of absolute numbers per femur pair, migratory plasmablasts peaked in number on day 25 with a mean of 108733 and dropped in number to 51600 on day 75, still very much increased from baseline (**Figure 4.8B**). LLPC decreased from a mean of 45533 pre-*P. chabaudi* infection to 10323 on day 8, and only recovered numbers by day 45 (**Figure 4.8B**). Interestingly, between days 45 and 75, total numbers of bone marrow LLPC increased from 66147 to 75500, which is significantly higher than day 0 of *P. chabaudi* infection (day 150 after PR8 infection) (**Figure 4.8B**).

4.4.9: LLPCs and HA-specific plasma cells are not detected in PBMC during acute *P. chabaudi* infection (d8 and 10).

With increased numbers of migratory plasmablasts entering the bone marrow during *P. chabaudi* infection, there could be a very large competitive stress placed on the finite LLPC niches in the bone marrow. It has been proposed that LLPC can be competitively dislocated from their niche into peripheral blood by homeostatic mechanisms (Odendahl *et al.* 2005). The movement of plasma cells from spleen, through peripheral blood, to bone marrow after a primary blood-stage *P. chabaudi* infection has been previously described (Nduati *et al.* 2010;Ndungu *et al.* 2009) and I investigated whether a similar kinetic occurred when *P. chabaudi* infection was initiated in mice previously infected with PR8. Spleen, peripheral blood mononuclear cells (PBMC) and bone marrow were obtained from PR8-*P. chabaudi*-infected mice at the peak of *P. chabaudi* infection and anti-B220 and anti-CD138 Abs were used to discriminate LLPC from migratory plasmablasts in the three organs. Compared against the negative controls which were spleen, PMBC and bone marrow obtained on day 0 of *P. chabaudi* infection or stained with the isotype control for anti-CD138 Ab, B220⁺ CD138⁺ migratory plasmablasts appeared in blood during a very transient window day 8-12, and in bone marrow from day 10-12 onwards (**Figure 4.9A**). However, B220⁻ CD138⁺ LLPC were not detected in blood during days 8-12 of *P. chabaudi* infection (**Figure 4.9A**), suggesting that, despite the large population of migratory plasmablasts induced during acute *P. chabaudi* infection, LLPC were not dislocated into peripheral blood in adequate frequencies for detection by flow cytometric analysis.

Chapter 4: Loss of previously established humoral immunity

To further confirm the identity of the B220⁺ CD138⁺ migratory plasmablasts that appeared in blood on days 8-12 of *P. chabaudi* infection, spleen, PMBC and bone marrow were obtained on day 10 of *P. chabaudi* infection and stained with anti-B220 and anti-CD138 Abs (**Figure 4.9B**). The B220⁺ CD138⁺ plasmablasts in the spleen, PMBC and bone marrow, as well as B220⁻ CD138⁺ LLPC in the bone marrow were further analysed using a panel of surface markers to distinguish migratory plasmablasts and LLPC by flow cytometry, similar to that described previously (Odendahl *et al.* 2005). Migratory plasmablasts are CD138⁺ and B200⁺, CD19⁺, MHC Class II⁺, and have downregulated CXCR5 and upregulated CXCR4. Conversely, LLPC are CD138⁺ but are B220⁻, CD19⁻, CXCR4⁺, CXCR5⁻ (**Figure 4.9B**). The relative expression of these surface markers on these cells demonstrated that migratory plasmablasts but not LLPC appeared in PMBC during the peak of *P. chabaudi* infection (**Figure 4.9B**), indicating that LLPC were not dislocated from bone marrow.

To confirm that pre-established LLPC were not expelled into peripheral blood, PBMC were obtained on days 8 and 10 of *P. chabaudi* infection and analysed for the presence of HA-specific IgG ASC. Although a significant increase in total IgG ASC in PBMC was detected on days 8 and 10 of *P. chabaudi* infection (**Figure 4.10B**), there were no HA-specific IgG ASCs detected in PBMC at these time points (**Figure 4.10A**).

In summary, there are changes in spleen, blood and bone marrow as migratory plasmablasts are generated and migrate to their bone marrow niche during acute *P. chabaudi* infection. A wave of migratory plasmablasts entering the bone marrow coincides with the loss of LLPC from the bone marrow. However, there is no evidence for the dislocation and mobilisation of LLPC into PMBC.

4.4.10: LLPCs undergo apoptosis during acute *P. chabaudi* infection (before d10).

As neither LLPC nor HA-specific IgG ASC could be detected in PBMC during acute *P. chabaudi* infection, the alternative hypothesis was that bone marrow LLPC were undergoing apoptosis during acute *P. chabaudi* infection. This was supported by the fact that B220⁻ CD138⁺ LLPC was already reduced on d8, before the entry of B220⁺ CD138⁺

Chapter 4: Loss of previously established humoral immunity

migratory plasmablasts into the bone marrow from d10 onwards (**Figure 4.6A-B**). BALB/c mice were infected with PR8-*P. chabaudi* and bone marrow was obtained on d0, 4-5, 6-8 and 10 of *P. chabaudi* infection. Apoptosis was determined by flow cytometric detection of Annexin V surface expression on total bone marrow cells and bone marrow LLPC (**Figure 4.11A**). There was a significant increase in the frequency of bone marrow cells expressing Annexin V on days 4-5 and 6-8 of *P. chabaudi* infection, going back to pre-*P. chabaudi* infection frequencies by day 10 (**Figure 4.11B**). Similarly, there was an increase in the frequency of B220⁻ CD138⁺ LLPCs expressing Annexin V during acute *P. chabaudi* infection, particularly on day 4-5, decreasing on day 6-8 and going back to pre-*P. chabaudi* infection frequencies by day 10 (**Figure 4.11C**). Therefore pre-established bone marrow cells and LLPC underwent increased apoptosis during *P. chabaudi* infection, and this occurred at very early time-points in infection. This result will be confirmed using other assays like Annexin-V and 7-AAD surface co-staining, as well as intracellular caspase-3 staining.

4.4.11: *P. chabaudi*-induced bone marrow LLPC apoptosis is dependent on FcγRI,II,III.

FcγRIIB is the only inhibitory FcR in humans and mice and facilitates negative feedback and dampening of humoral and cell-mediated responses (Ravetch & Bolland 2001). Although it is widely expressed on many cell types, including dendritic cells, macrophages, activated neutrophils, mast cells and basophils (Nimmerjahn & Ravetch 2008; Ravetch & Kinoshita 1991), it is the only IgG Fc receptor expressed by B cells (Amigorena *et al.* 1989). Mice deficient in FcγRIIB are susceptible to autoimmunity, and indeed can develop spontaneous autoimmune conditions, and they have elevated antibody titres to antigens with reduced cellular activation thresholds (Takai *et al.* 1996). FcγRIIB is a low affinity receptor that binds immune-complexed IgG, particularly of the subclasses IgG1, IgG4 and IgG2 in humans (Bruhns *et al.* 2009) and IgG1 in mice (Nimmerjahn *et al.* 2005). Cross-linking of FcγRIIB triggers the ITIM-mediated signalling cascade. Recently, ligation of FcγRIIB on LLPC by low-affinity immune complexes was found to lead to apoptosis and death of pre-established LLPC (Xiang *et al.* 2007). It has previously been demonstrated in this laboratory that an unusually high splenic B220⁺ CD138⁺ plasma cell response is

Chapter 4: Loss of previously established humoral immunity

produced early in *P. chabaudi* infection (Achtman *et al.* 2007), although the reason for this is not known. Possibly the exuberant splenic short-lived plasma cell generation is due to short-lived polyclonal B cell responses, which rapidly develop into extrafollicular plasmablasts which produce low-affinity Abs (Achtman *et al.* 2007; Stephens *et al.* 2009). At the same time, large quantities of soluble antigen are shed during merozoite invasion and can form immune complexes which can circulate and crosslink Fc γ RIIB on LLPC, causing death of LLPC and thereby creating newly vacant niches.

Since there was hypergammaglobulinaemia during *P. chabaudi* infection which could engage Fc γ RIIB on LLPC and cause apoptosis, I hypothesized that *P. chabaudi*-induced bone marrow LLPC apoptosis could be dependent on Fc γ RIIB. Five months after PR8 infection of wild-type BALB/c mice, bone marrow LLPC in these PR8-immune mice expressed Fc γ RIIB (**Figure 4.12A**). Fc γ RIIB KO mice were unavailable in the institute at the time; therefore I used Fc γ RI,II,III KO mice on a C57BL/6 background, which were available in the institute. 8-10 week old female Fc γ RI,II,III KO and wild-type C57BL/6 controls were infected with 250 HAU PR8 intranasally and their HA-specific IgG response was monitored by ELISA for up to 150 days (**Figure 4.12B**). Fc γ RI,II,III KO mice had higher HA-specific IgG concentrations on day 28 after intranasal PR8 infection compared to C57BL/6 controls (**Figure 4.12B**). This was probably due to an enhanced antibody response in the absence of Fc γ RIIB, which is consistent with the augmented humoral response in Fc γ RIIB KO mice to immunisation with T-dependent or T-independent antigens (Takai *et al.* 1996). This is unsurprising given the role of Fc γ RIIB in negative regulation of the B cell activation threshold (Amigorena *et al.* 1989). Nevertheless, HA-specific IgG concentrations in Fc γ RI,II,III KO mice returned to similar concentrations as C57BL/6 controls (approximately 75-77 μ g/ml) by day 56 for up to day 150 (**Figure 4.12B**). Indeed, Fc γ RI,II,III KO and C57BL/6 mice had similar concentrations of HA-specific IgG to BALB/c mice from day 56 post PR8-infection onwards (**Figure 4.12B**). 150 days after PR8 infection, Fc γ RI,II,III KO and C57BL/6 mice were infected with *P. chabaudi* and had similar parasitaemia curves throughout acute *P. chabaudi* infection (**Figure 4.12C**). Although peak % parasitaemia was similar between Fc γ RI,II,III KO and C57BL/6 mice, there was no change in bone marrow cellularity on day 8 of *P. chabaudi* infection Fc γ RI,II,III KO mice, whilst in comparison there was a significant loss of bone

Chapter 4: Loss of previously established humoral immunity

marrow cellularity in the C57BL/6 controls (**Figure 4.12D**). Similarly, when HA-specific IgG ASC was quantified by ELISpot, a significant decrease in bone marrow HA-specific IgG ASC was only observed in C57BL/6 controls at the peak of parasitaemia (**Figure 4.12E**), while there was no change in bone marrow HA-specific IgG ASC in FcγRI,II,III KO mice (**Figure 4.12E**). There was no significant difference in the total number of IgG ASC at the peak of *P. chabaudi* infection in both FcγRI,II,III KO and C57BL/6 controls (**Figure 4.12F**), although there appeared to be a non-significant increase in total number of IgG ASC in FcγRI,II,III KO at the peak of parasitaemia (**Figure 4.12F**). When the concentration of HA-specific serum IgG was monitored for 56 days following *P. chabaudi* infection, there was a subsequent loss of HA-specific serum IgG in C56BL/6 mice over the next 56 days after *P. chabaudi* infection, whereas there was no loss of HA-specific serum IgG in FcγRI,II,III KO mice (**Figure 4.12G**). Therefore FcγRI,II,III appears to be important in the loss of pre-established HA-specific bone marrow LLPC and pre-established HA-specific serum Ab during *P. chabaudi* infection. However, this does not formally exclude other mechanisms of apoptosis such as Fas-Fas ligand interactions or ligation of TNF receptors, which should be ruled out with the use of monoclonal blocking antibodies against Fas and TNF receptors.

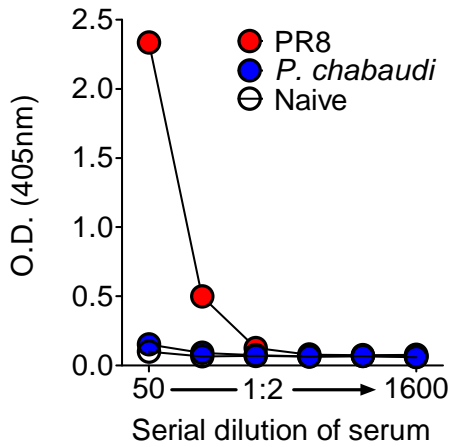
4.4.12: Sequestration of *P. chabaudi* parasites occurs during acute infection (d5 and d8, not d10 and d13).

In humans, *P. falciparum* parasitized-erythrocytes have been observed in bone marrow tissue sections (MacPherson *et al.* 1985), and sequestration of gametocytes has been observed in *in vitro* culture with bone marrow endothelium (Rogers *et al.* 2000a). In mice, *P. chabaudi*-parasitized erythrocytes have been observed sequestering to various tissues, particularly in the liver (Mota *et al.* 2000), and it is possible that they could also be sequestering in the bone marrow, and have direct effects on the local microenvironment which could affect pre-established LLPC. In order to examine whether parasite sequestration could have a role in LLPC loss, bone marrow was examined histologically after sequential infection. 8-10 week old female BALB/c mice were infected intranasally with 250 HAU PR8. 150 days after PR8 infection, mice were infected with *P. chabaudi* as previously described. Femurs were collected on d0, 5, 8, 10 and 13 after *P. chabaudi*

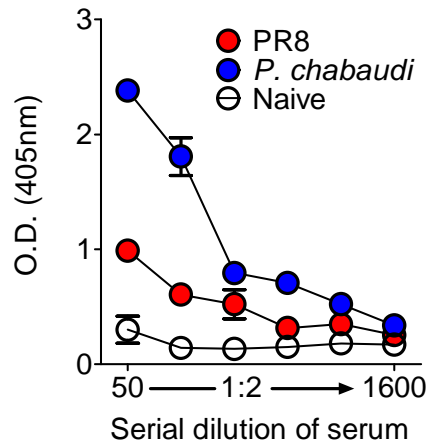
Chapter 4: Loss of previously established humoral immunity

infection for histology. Paraffin-embedded 5 μ m sections were prepared and stained with haematoxylin and eosin. By visual inspection under a light microscope using a x100 objective with oil immersion, parasitized erythrocytes adhering to endothelial cells of bone marrow sinusoids and blood vessels were observed at particularly on days 5 and 8, but not on days 10 and 13, of *P. chabaudi* infection (**Figure 4.13**). The kinetic of sequestration of parasitized-erythrocytes in the bone marrow particularly early time points (days 5 and 8) of infection correlates with the apoptosis of bone marrow LLPC (**Figure 4.11C**), therefore it is possible that sequestration has a causal effect on LLPC apoptosis, although confirmation of this hypothesis requires much more extensive investigation, such as analysis of sequestration in Fc γ RI,II,III KO mice, where is no loss of LLPCs. Furthermore, it would be interesting to examine whether apoptosis of bone marrow LLPC occurs during infection in mice where *P. chabaudi* parasites are thought not to sequester, such as in CD36 KO mice (Mota *et al.* 2000), or using non-sequestering parasite strains such as the recently described schizont membrane-associated cytoadherence protein (SMAC)-deficient *P. berghei* ANKA (Fonager *et al.* 2011) compared with infection with wild-type *P. berghei* ANKA.

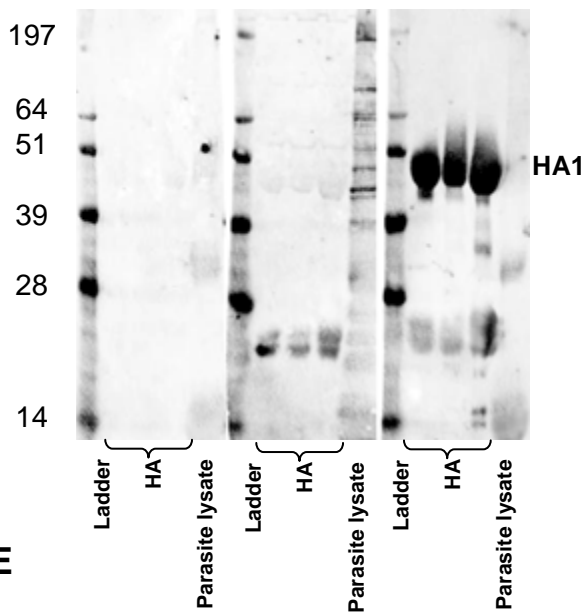
4.1A *P. chabaudi* hyperimmune sera do not bind to HA



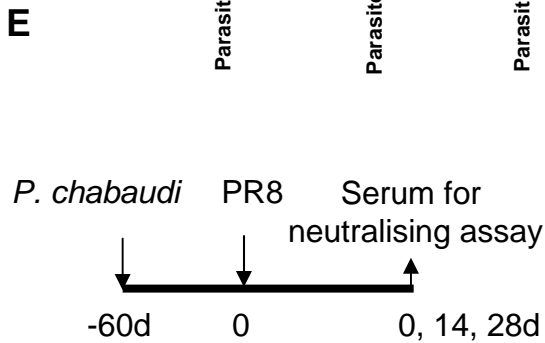
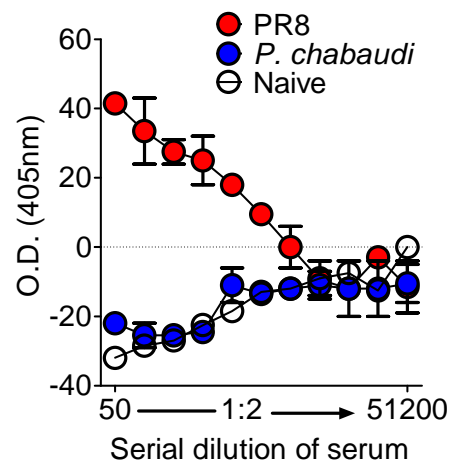
B PR8 immune sera binds weakly to parasite lysate



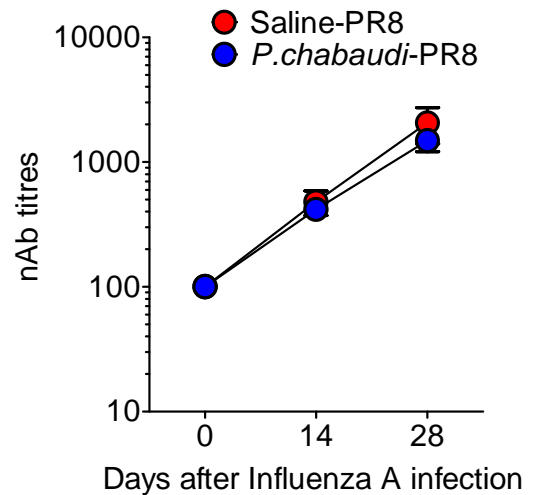
C Naive *P. chabaudi* PR8



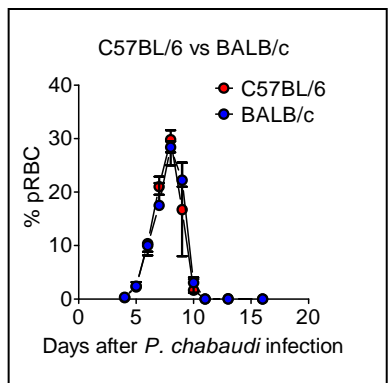
D *P. chabaudi* hyperimmune sera does not neutralise PR8 *in vitro*



F PR8-specific nAb is not affected by a previous *P. chabaudi* infection



4.2A



Influenza-specific serum IgG

- ELISA with virus haemagglutinin (HA)
- Virus neutralisation assay

Influenza-specific antibody-secreting cells

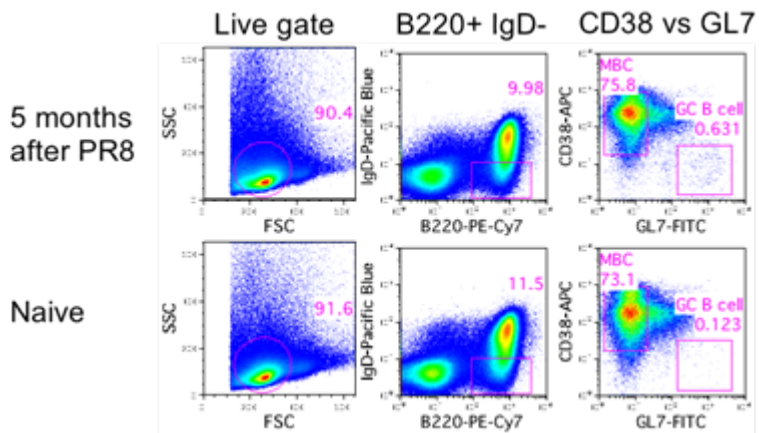
- ELISpot

Protective immunity against Influenza

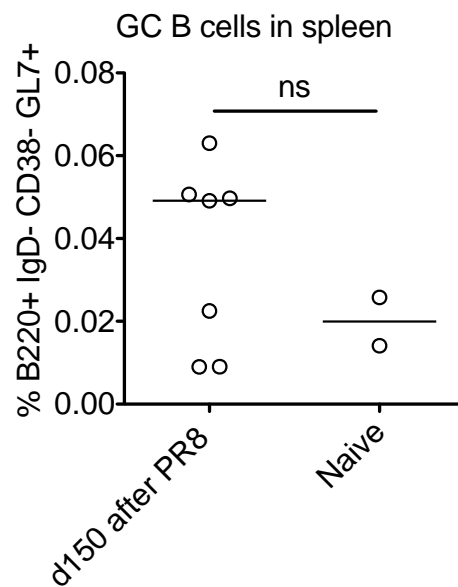
- Re-challenge
- qRT-PCR of lung viral RNA



B

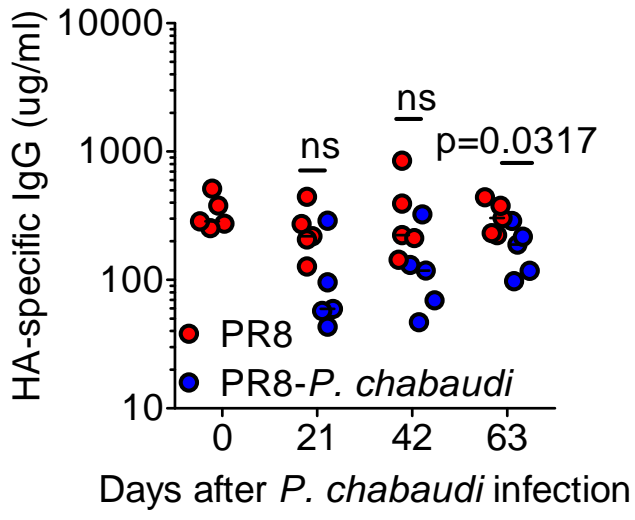


C



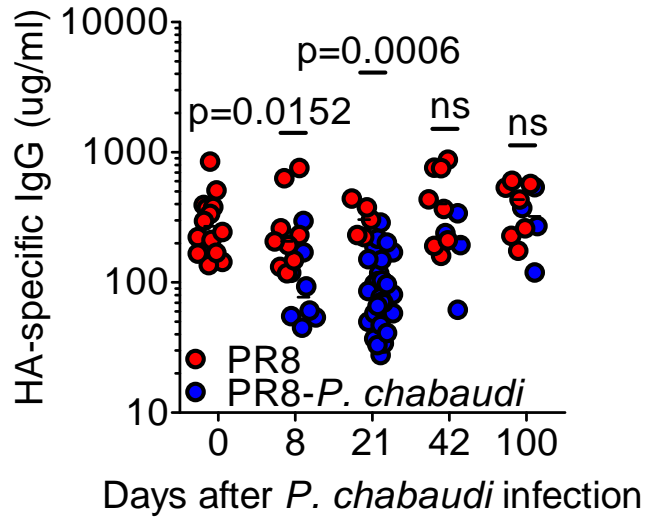
4.3A

105-day interval

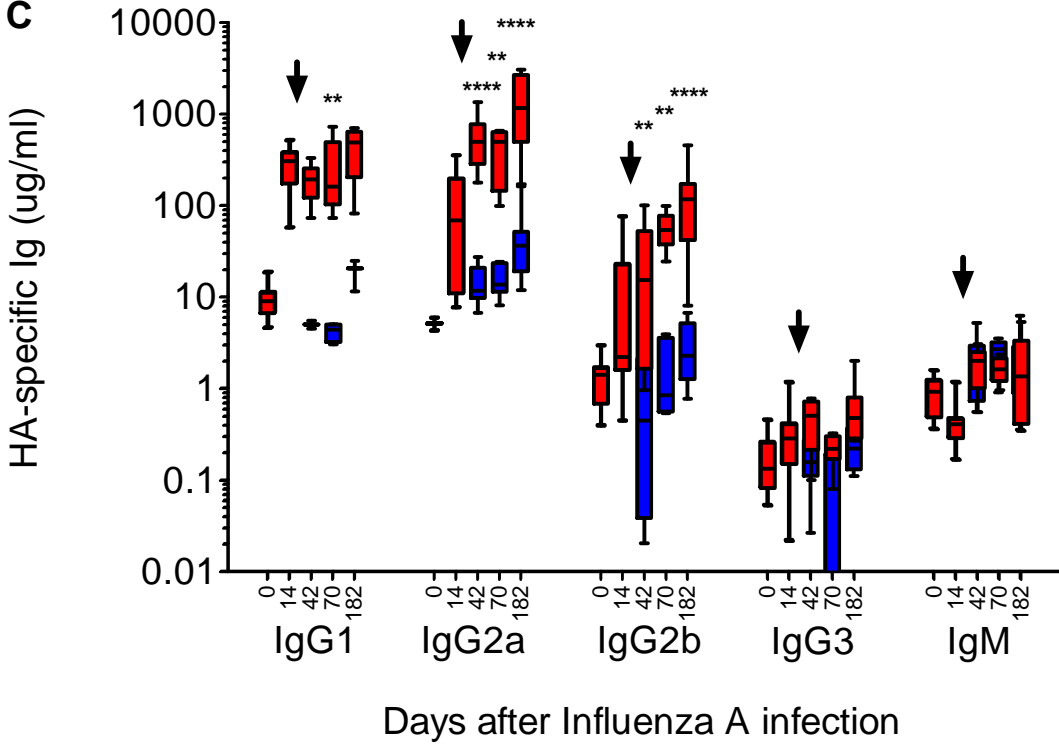


B

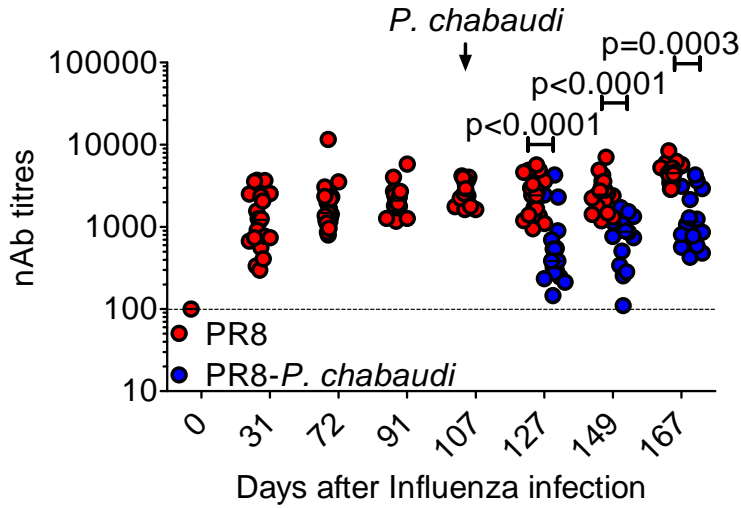
150-day interval



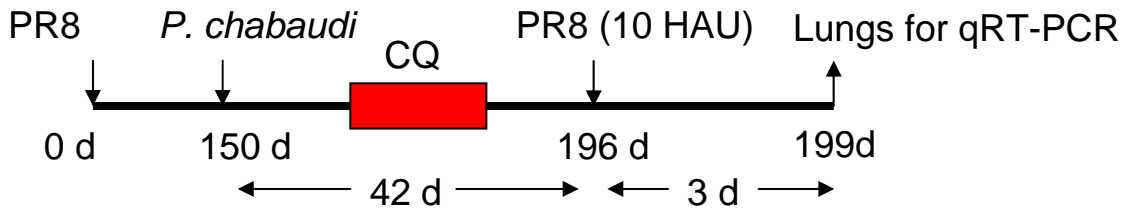
C



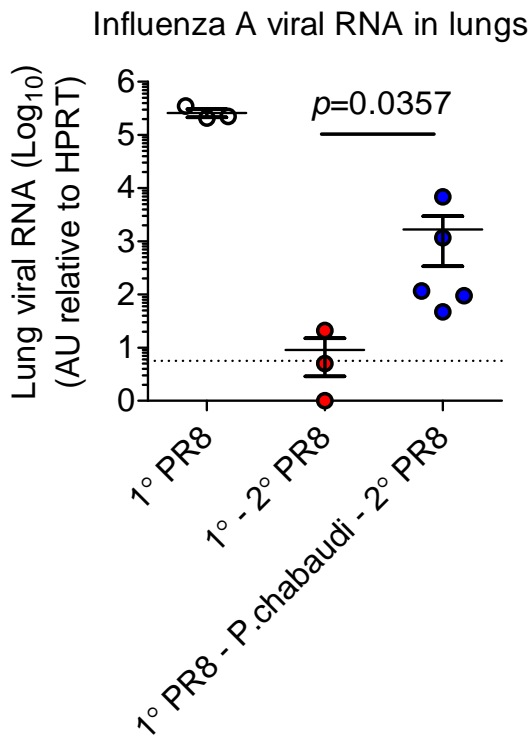
4.4A



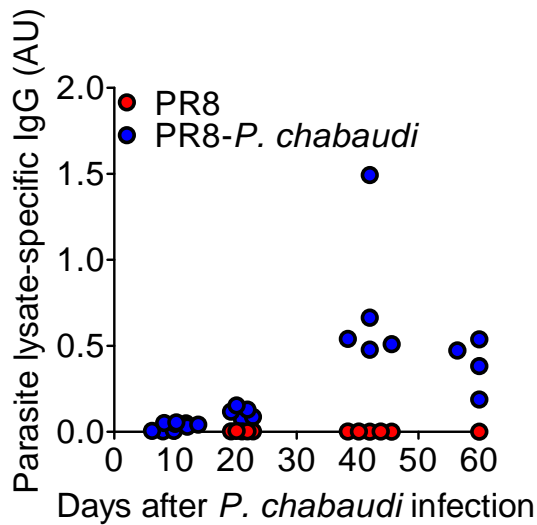
B



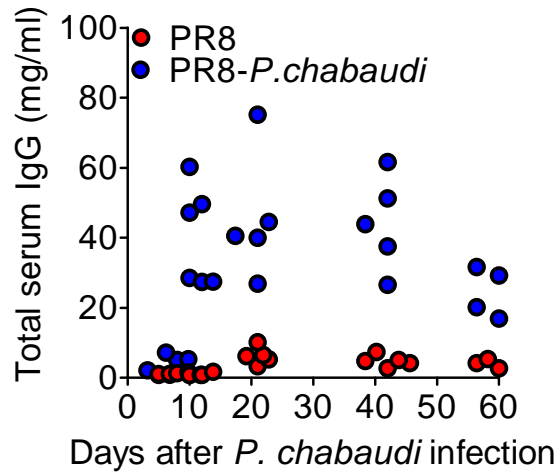
C



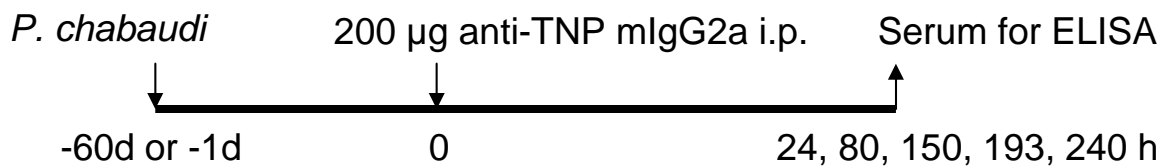
4.5A



B

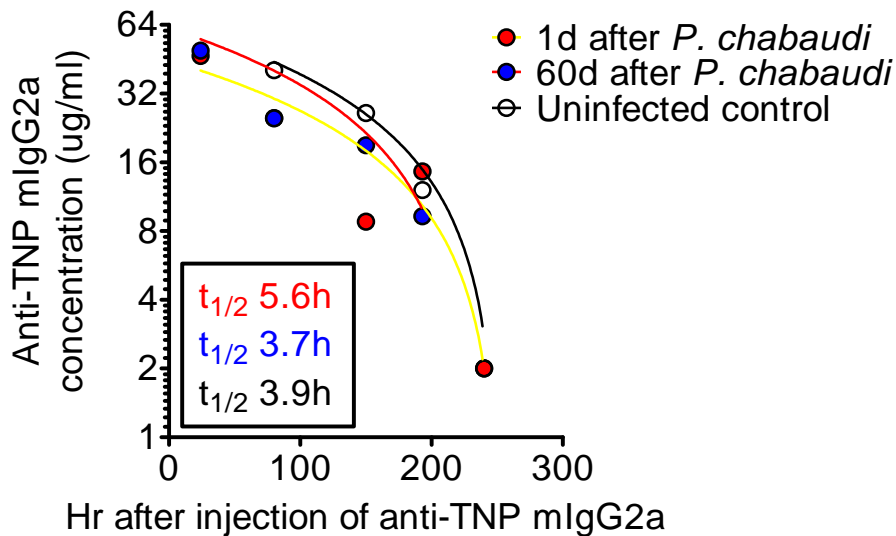


C



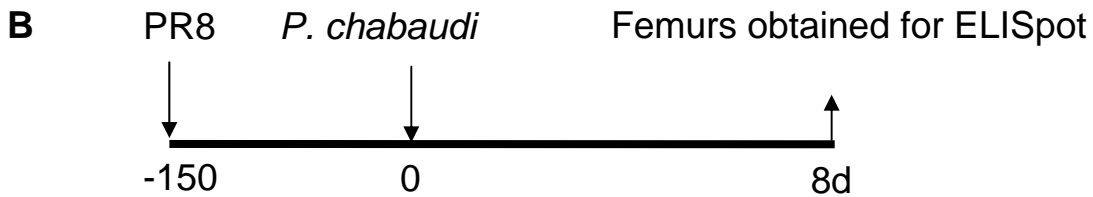
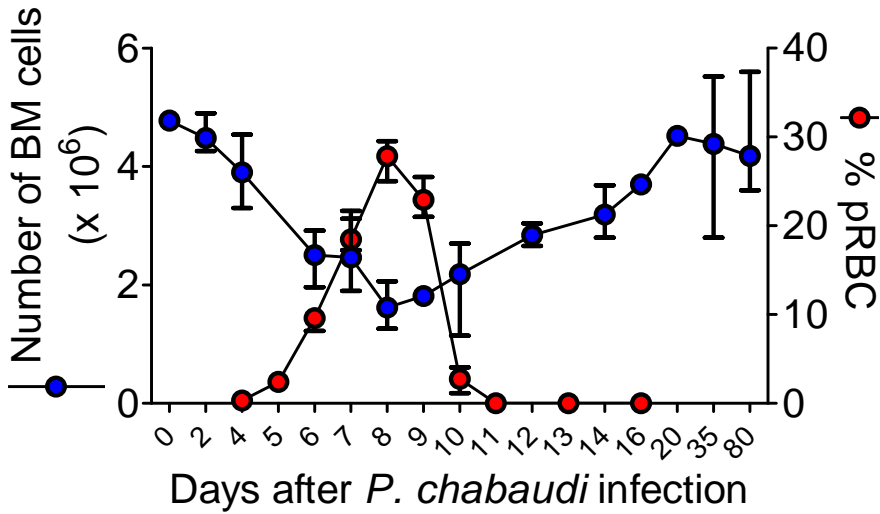
D

Half-life of serum Ig during acute or chronic *P. chabaudi* infection

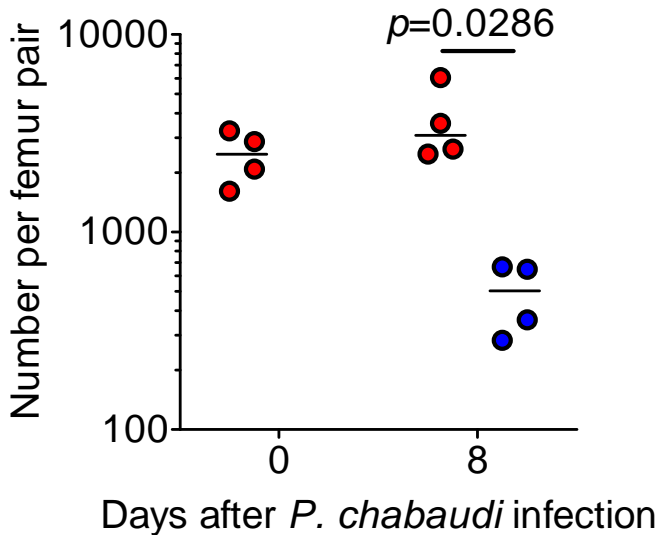


4.6A

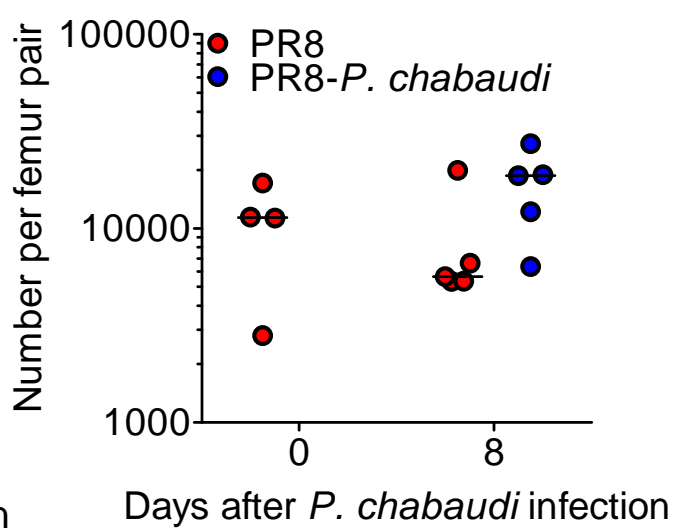
Parasitaemia vs bone marrow cells



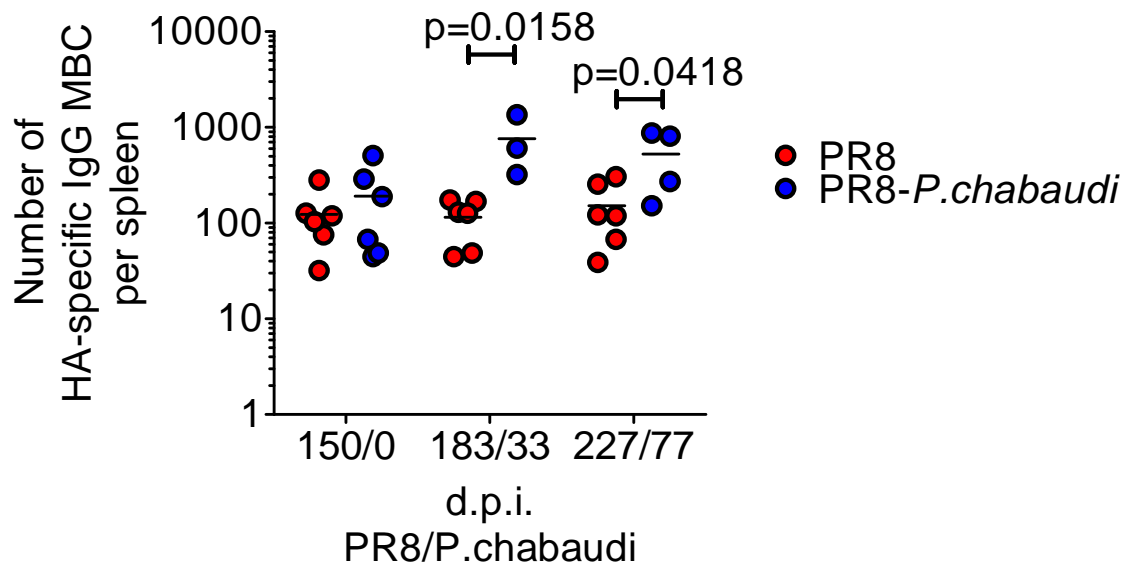
C HA-specific IgG ASC



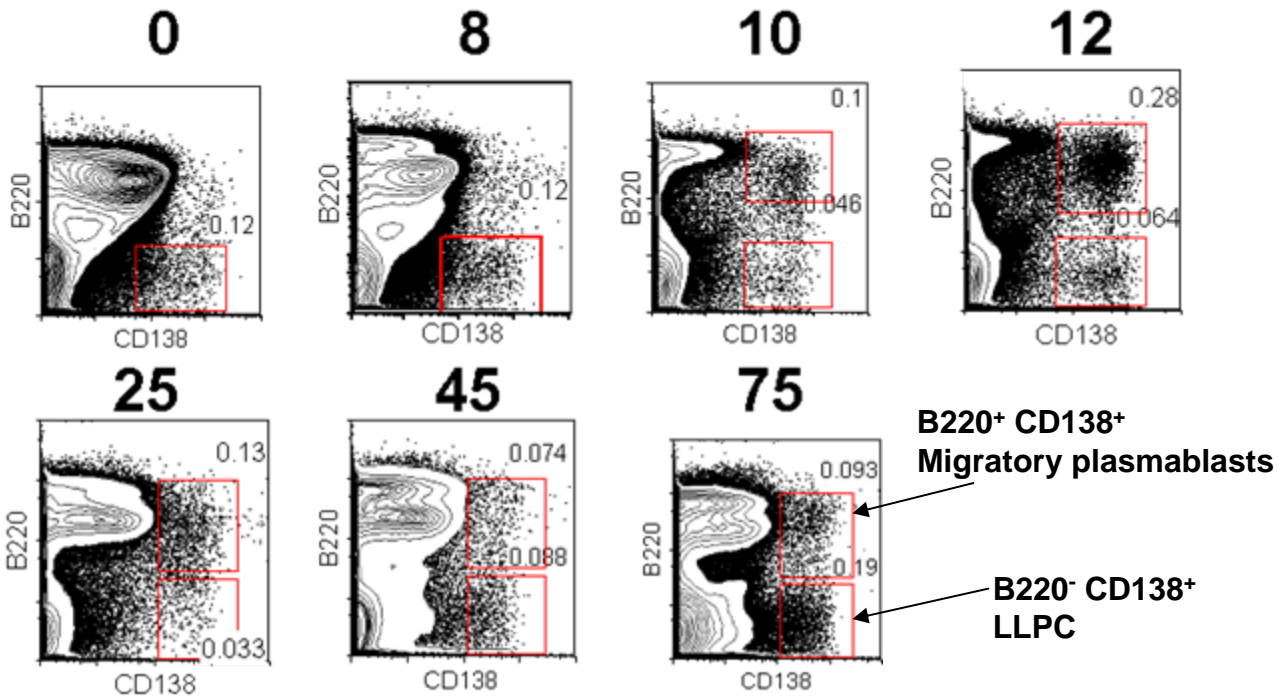
D Total IgG ASC



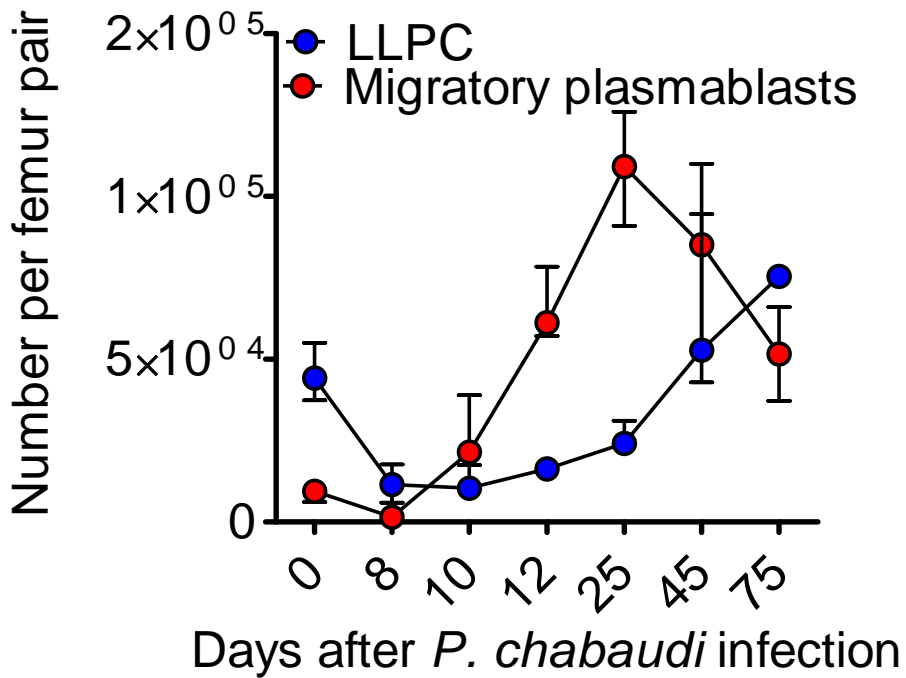
4.7



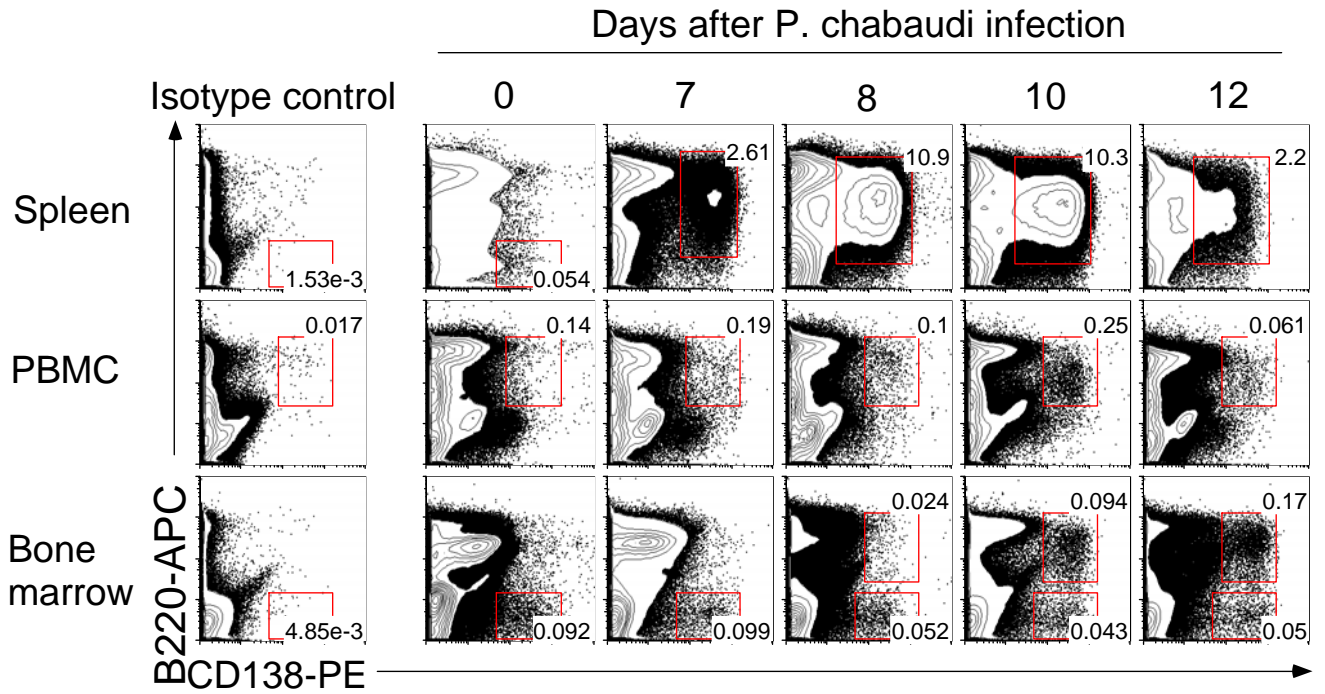
4.8A



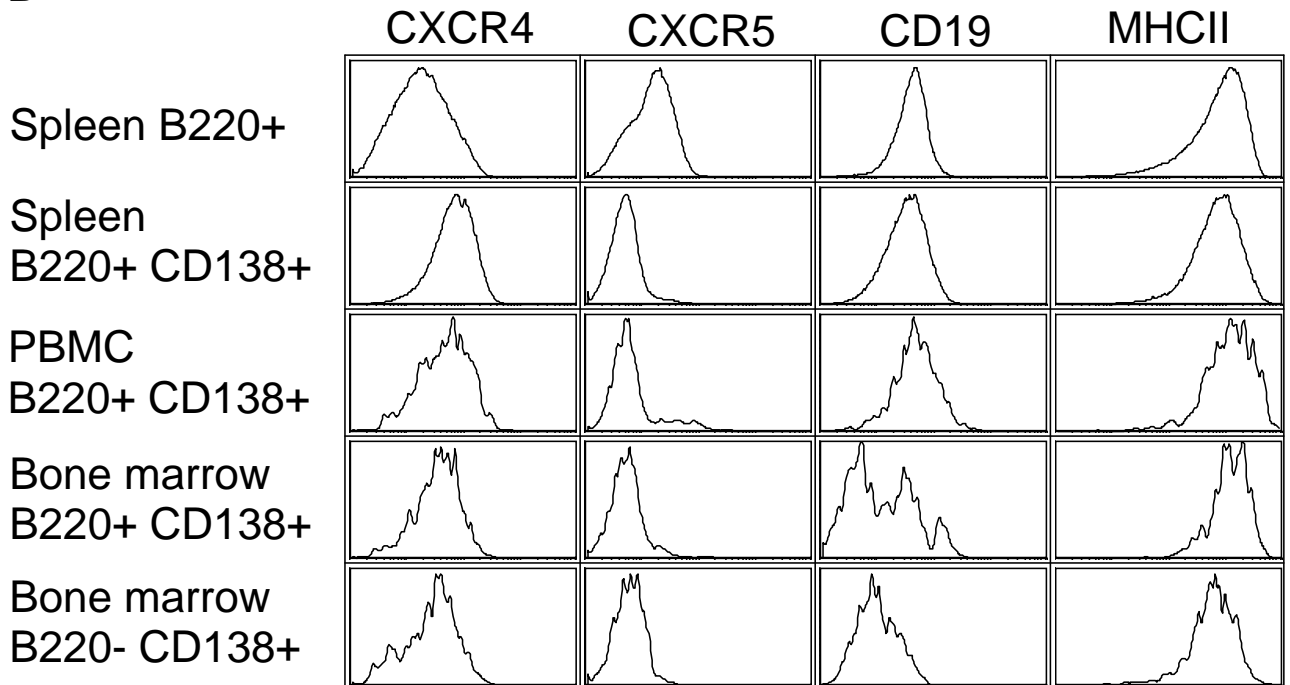
B



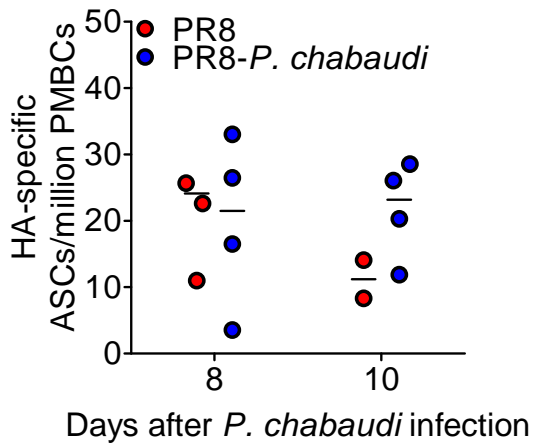
4.9A



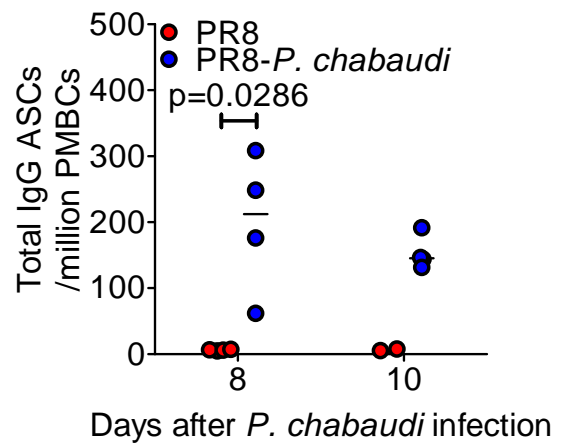
B



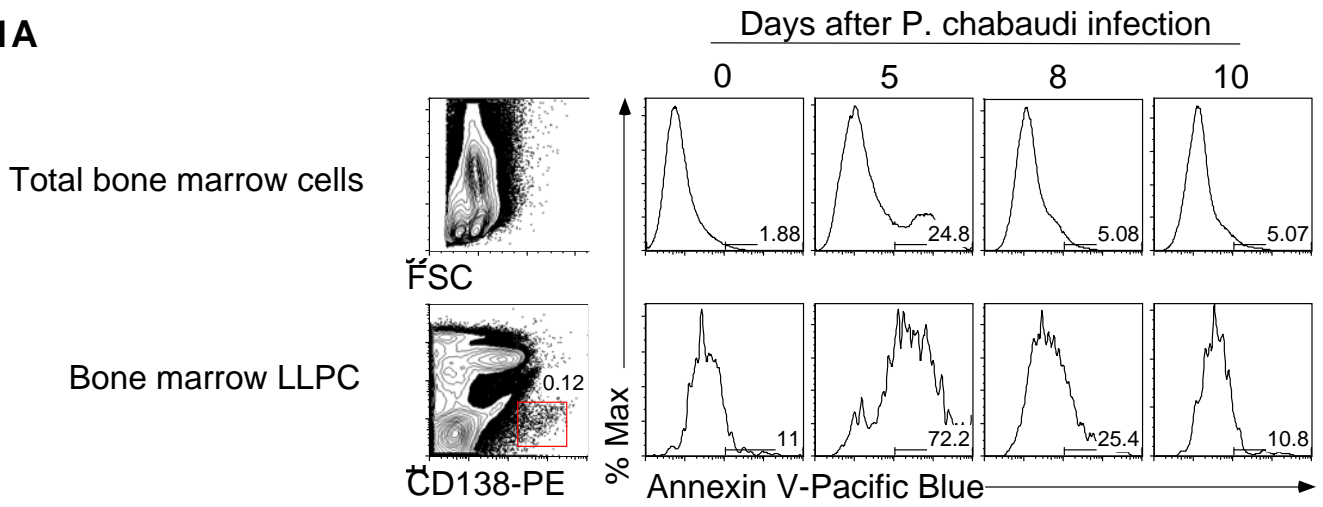
4.10A



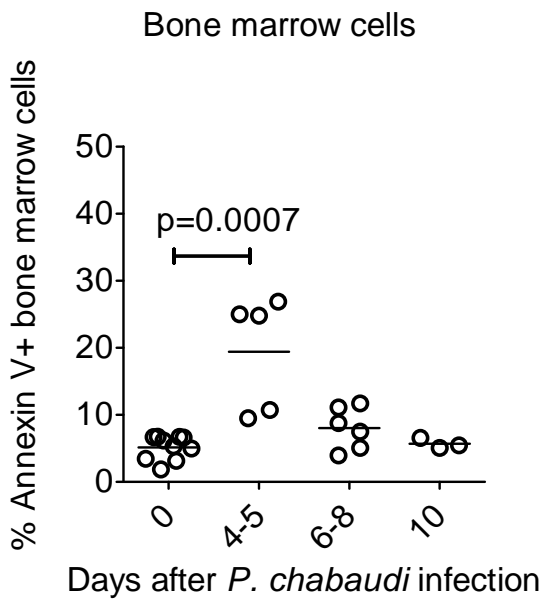
B



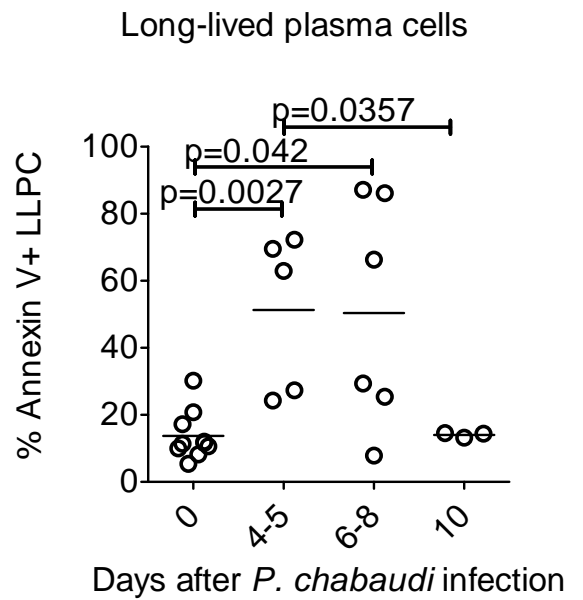
4.11A



B

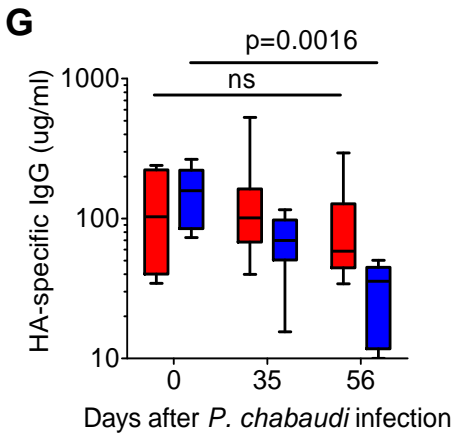
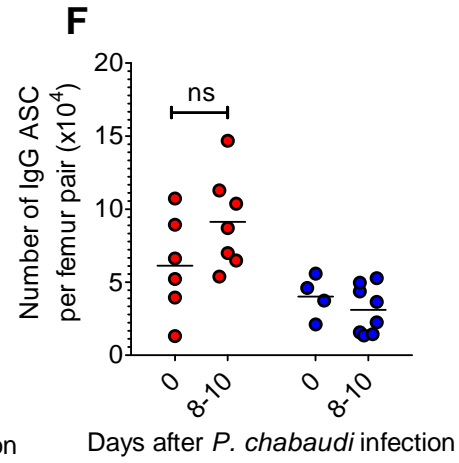
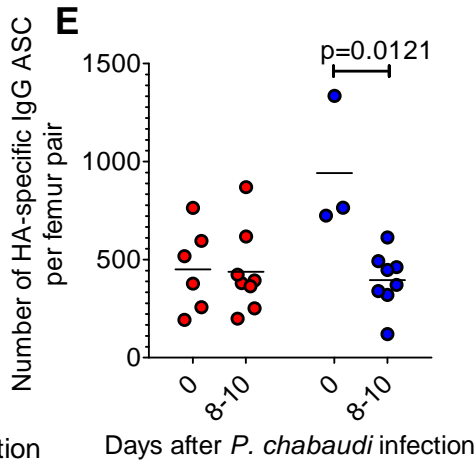
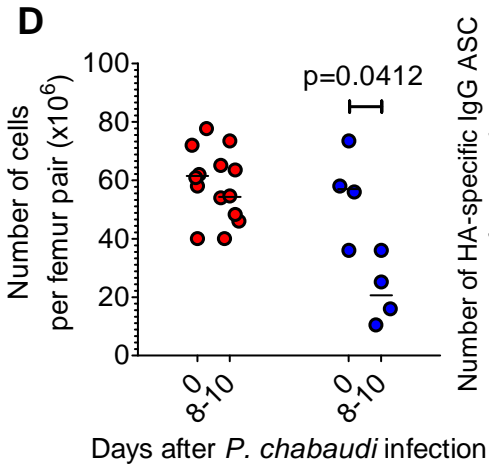
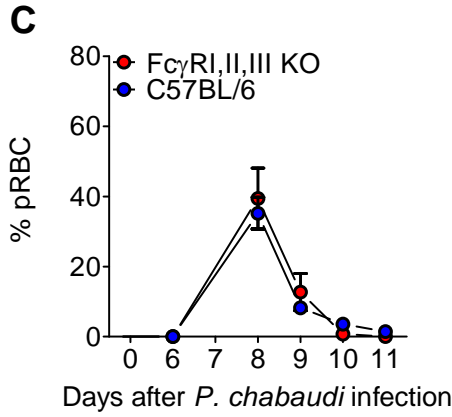
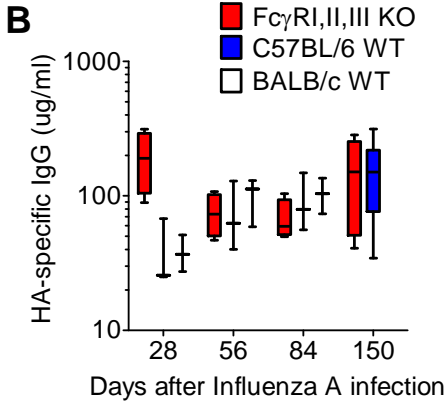
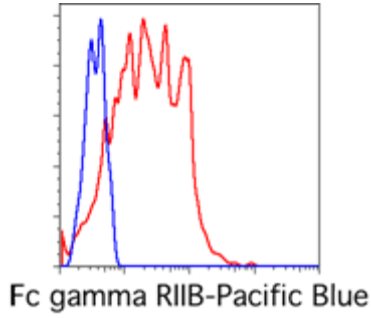


C



4.12A

— Isotype
— B220⁻ CD138⁺ LLPC



4.13

0

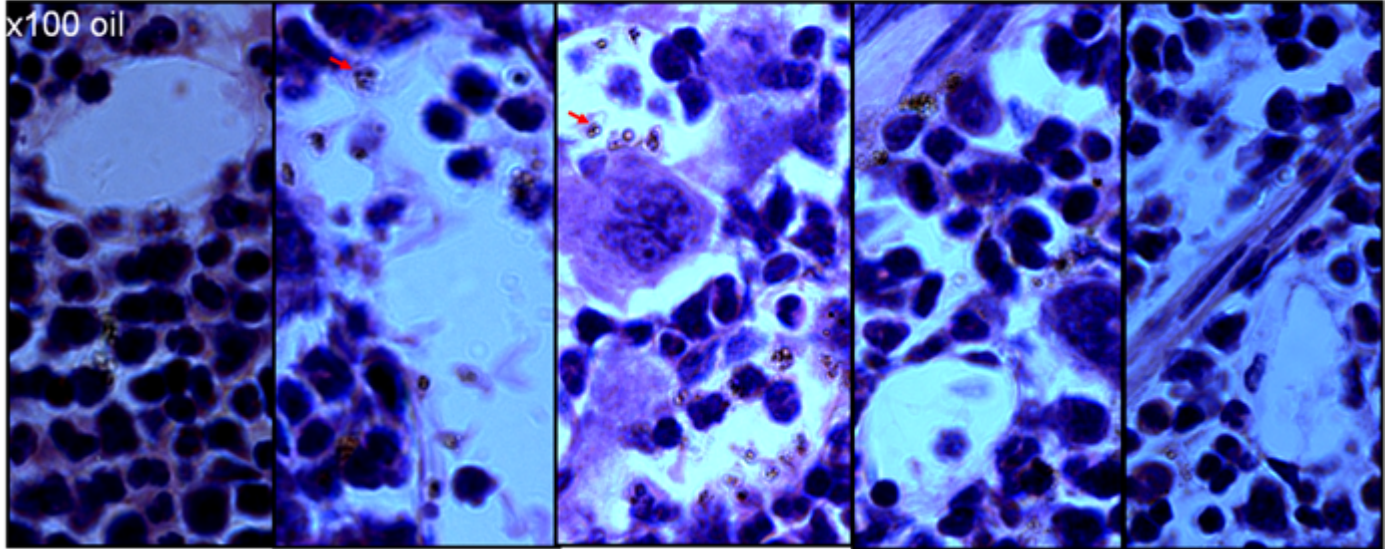
5

8

10

13

x100 oil



Chapter 4: Figure legends

Figure 4.1: Little serological cross-reactivity between Influenza A/PR/8/34 and *P. chabaudi* infections.

Graphs in **A)** and **B)** plot the O.D. (405nm) values obtained by ELISA after incubating normal mouse sera (○), hyperimmune anti-*P. chabaudi* sera (●), and anti-PR8 sera (●) with either HA or parasite lysate. Results were obtained from one experiment performed in duplicates with 6 two-fold dilutions starting from 1/50. In **A)**, only PR8 sera contained antibodies binding to PR8 HA. In **B)**, *P. chabaudi* sera and, to a lesser extent, PR8 sera, contained antibodies binding to parasite lysate. **C)** Three different preparations of bromelain-digested PR8 HA and *P. chabaudi* lysate were resolved on NuPAGE 12% bis-Tris gels alongside 1x SeeBlue Plus2-prestained standard, electrophorated on Hybond C membrane and probed with normal mouse sera (left panel), hyperimmune *P. chabaudi* sera (middle panel), and PR8 sera (right panel). Some cross-reactive antibodies were detected between *P. chabaudi* and PR8 sera. The 46 kDa, fragment of HA was only recognised by PR8 sera. **D)** Graph of the arbitrary neutralising Ab titres in normal mouse sera (○), hyperimmune anti-*P. chabaudi* sera (●), and anti-PR8 sera (●). Reactive serum titres of PR8-neutralising antibodies in the serum was measured using a virus neutralising assay. No neutralising antibodies were detected from 1:2 serial dilutions of 1:50 – 1:51200 in naïve BALB/c serum and anti-*P. chabaudi* serum. Only PR8 sera were able to neutralise PR8 *in vitro*. **E)** Schematic representation of experiment. BALB/c female mice were infected with *P. chabaudi*, rested for 60 days, then infected with an intranasal instillation of 250 HAU PR8. Neutralising assays were performed with sera taken 2 and 4 weeks after PR8 infection. **F)** Data shows the neutralising Ab response in mice infected with *P. chabaudi* and then PR8 (●); and age-matched control mice infected with saline and then PR8 (●). Results show no significant difference between the two groups. This represents one experiment with 5 mice per group. Error bars indicate standard error of the mean.

Chapter 4: Figure legends

Figure 4.2: Experimental protocol for sequential PR8 and *P. chabaudi* infection.

A) Schematic representation of sequential infection model in BALB/c female mice, where the second infection (blood-stage *P. chabaudi*) was initiated 105 – 150 days after the first (intranasal PR8). This would model a natural sequential heterologous infection in order to investigate whether attrition of humoral immunity occurs after sequential infection. Both these experimental infections generate LLPC, of which the kinetics have been previously characterized (Ndungu *et al.* 2009; Nduati *et al.* 2010). A comparison of parasitaemia curves was made by Giemsa-staining of thin blood films (inserted graph) obtained from 4 mice per group. After sequential infection, the antibody response to PR8 was determined by ELISA, neutralising assay and ELISpot at various time points post-infection. Protective immunity to PR8 was determined by qRT-PCR of lung viral titres after lethal secondary PR8 infection. **B)** Representative FACs plots and **C)** Frequencies of germinal centre B cells in spleens of BALB/c mice 5 months after PR8 infection (n=7) and in naïve BALB/c mice (n=2).

Chapter 4: Figure legends

Figure 4.3: Loss of pre-existing HA-specific serum IgG after *P. chabaudi* infection.

8-10 wk old female BALB/c mice were infected by intranasal instillation of 250 HAU of PR8. **A)** 105 or **B)** 150 days post-infection, mice were infected with *P. chabaudi* as previously described. **A)** and **B)** Concentration of serum HA-specific IgG in PR8-*P. chabaudi*-infected mice (●) and control PR8 only mice (●) after the two time intervals. The *x*-axis indicates day after *P. chabaudi* infection. Data were obtained from two experiments each with 5-10 mice per group. The line indicates the median value. Statistical values were calculated using the Mann-Whitney test. **C-D)** 8-10 wk old female BALB/c mice were infected by intranasal instillation of 250 HAU of PR8. 28 days later, mice were infected with *P. chabaudi* as previously described. **C)** Concentration of serum HA-specific IgG1, IgG2a, IgG2b, IgG3 and IgM in PR8-*P. chabaudi*-infected mice (blue bars) and age-matched control PR8-only mice (red bars) at various time points after PR8 infection. The black arrow indicates the time when *P. chabaudi* infection was initiated (day 28 of PR8 infection). Data were obtained from two experiments each with 5-10 mice per group. Error bars indicate 95% confidence intervals. P values were calculated using the Mann-Whitney test. P values in **C)** are indicated by: ** <0.01; *** < 0.001; **** <0.0001.

Chapter 4: Figure legends

Figure 4.4: Loss of protective immunity to PR8 infection after *P. chabaudi* infection.

A) 8-10 wk old female BALB/c mice were infected by intranasal instillation of 250 HAU of PR8. 105 days later, mice were infected with *P. chabaudi* as previously described. Serum was obtained from mice at various time points after PR8-*P. chabaudi* infection. Virus neutralising Ab titres in PR8-*P. chabaudi*-infected mice (●) and control PR8 only mice (●) were quantified using a neutralising assay as previously described (Kassiotis *et al.* 2006). The black arrow indicates the time when *P. chabaudi* infection was initiated (day 105 of PR8 infection). The *x*-axis indicates days after PR8 infection. Data were obtained from two experiments each with 5-10 mice per group. The line indicates the median value. Statistical values were calculated using the Mann-Whitney test. **B)** 8-10 wk old female BALB/c mice were infected by intranasal instillation of 250 HAU of PR8. 150 days later, some mice were infected with *P. chabaudi* as described previously and drug-cured with chloroquine. 6 weeks after *P. chabaudi* infection, mice were re-infected with PR8 by intranasal instillation of 10 HAU of PR8 under light anaesthesia and recovery. 3d after the secondary PR8 infection, mice were sacrificed and lungs processed for qRT-PCR of viral RNA loads as described in *Methods and Materials*. **C)** Graph shows data obtained from naïve BALB/c mice (○), PR8-immune mice (●) and PR8-immune mice infected with *P. chabaudi* (●). The dotted line indicates the limit of detection. P values were calculated using the Mann-Whitney test.

Chapter 4: Figure legends

Figure 4.5: No increase in rate of serum antibody clearance during acute or chronic *P. chabaudi* infection.

A-B) 8-10 wk old female BALB/c mice were infected by intranasal instillation of 250 HAU of PR8. 105 days later, mice were infected with *P. chabaudi* as previously described. Serum was obtained at various time-points after *P. chabaudi* infection. **A)** Titres of serum parasite lysate-specific IgG and **B)** Concentration of total serum IgG in PR8-*P. chabaudi*-infected mice (●) and control PR8 only mice (●) were determined by ELISA. Graphs show individual data points obtained from 3-5 mice per time point in one experiment. **C)** Schematic representation of experimental plan for determining antibody half-life during *P. chabaudi* infection. 8-10 week old naïve female BALB/c mice were infected with *P. chabaudi*. 24h or 60 days post-infection, mice were injected i.p. with 200ug of anti-TNP mIgG2a grown from the Hy1.2 hybridoma. Serum was obtained at various time points after injection. **D)** Concentration of anti-TNP mIgG2a in serum was quantified by ELISA throughout acute *P. chabaudi* infection (1d post-infection) (●) or chronic infection (60d post-infection) (●) and compared with uninfected age-matched controls (○). Graph showing the mean and standard error of data obtained from three independent experiments with 5 mice per group. Antibody half-life was calculated by use of linear regression on \log_2 transformed antibody concentrations in relation to hours after injection. Antibody half-life was expressed as the inverse reciprocal of the regression line slope (boxed in **Figure 4.5D**; expressed in hours).

Chapter 4: Figure legends

Figure 4.6: Loss of HA-specific ASC from bone marrow at day 8 of *P. chabaudi* infection.

A) Reduction in bone marrow cellularity during acute *P. chabaudi* infection. 8-10 wk old female BALB/c mice were infected with *P. chabaudi* as described previously. Bone marrow from femur pairs was obtained at various time points post-infection, erythrolysed and counted using a haemocytometer with live/dead cell discrimination made using Trypan Blue. Parasitaemia at these time points was determined through microscopic examination of thin blood films. Total bone marrow cellularity per femur pair (●; left y-axis) and parasitaemia (●; right y-axis) over the course of *P. chabaudi* infection was determined. Each point is the mean of data obtained from one experiment with 3-4 mice per time point. **B)** Schematic representation of experimental plan for determining whether HA-specific ASC was lost from bone marrow during *P. chabaudi* infection. 8-10 wk old female BALB/c mice were infected by intranasal instillation of 250 HAU of PR8. 5 months later, some mice were infected with *P. chabaudi* as described previously. 8 days after *P. chabaudi* infection, bone marrow from femur pairs were obtained and **C)** HA-specific and **D)** total IgG antibody-secreting cells (ASCs) in mice infected with PR8 (●) or PR8-*P. chabaudi* (●) were quantified using ELISpot. Data was obtained from one experiment with 4 mice per time point. Line indicates the median value. Statistical values were calculated using the Mann-Whitney test.

Chapter 4: Figure legends

Figure 4.7: Increase in number of HA-specific MBC in spleens of PR8-*P. chabaudi*-infected mice on days 33 and 77 of *P. chabaudi* infection.

8-10 wk old female BALB/c mice were infected by intranasal instillation of 250 HAU of PR8. 5 months later, mice were infected with *P. chabaudi* as previously described. Spleens were obtained 33 and 77 days after *P. chabaudi* infection. At these time points, HA-specific MBC in PR8-*P. chabaudi*-infected mice (●) and PR8 only control mice (●) were quantified using ELISpot. Graph shows individual data points obtained from one experiment with 3-5 mice per group. Statistical values were calculated using the Mann-Whitney test.

Chapter 4: Figure legends

Figure 4.8: Entry of migratory plasmablasts on day 10 of *P. chabaudi* infection.

8-10 wk old female BALB/c mice were infected by intranasal instillation of 250 HAU of PR8. 5 months later, mice were infected with *P. chabaudi*. Spleen, PBMC and bone marrow from femur pairs were obtained at various time points after *P. chabaudi* infection. Bone marrow from femur pairs were obtained at various time points after *P. chabaudi* infection and migratory plasmablasts (● B220⁺ CD138⁺) and long-lived plasma cells (● B220⁻ CD138⁺) were quantified using flow cytometry. **A)** Representative B220 vs CD138 FACs plots of live gated bone marrow cells. **B)** Median and error of the total number of migratory plasmablasts or LLPC at various time points after *P. chabaudi* infection. Data was obtained from one experiment with 3 mice per time point.

Chapter 4: Figure legends

Figure 4.9: Migratory plasmablasts, but not long-lived plasma cells, are detected by multiparameter flow cytometric analysis in PBMC during acute *P. chabaudi* infection.

A) Representative FACS plots of B220 vs CD138 on spleen, PBMC and bone marrow cells on days 0, 7, 8, 10 and 12 of *P. chabaudi* infection. **B)** Spleen, PBMC and bone marrow from femur pairs were obtained on day 10 after *P. chabaudi* infection, when the peak of the appearance of B220⁺ CD138⁺ migratory plasmablasts in PMBC was observed. The relative levels of CXCR4, CXCR5, CD19 and MHC Class II were determined by multi-parameter flow cytometry on B220⁺ splenic B cells; B220⁺ CD138⁺ splenic plasmablasts; B220⁺ CD138⁺ migratory plasmablasts in PMBC; B220⁺ CD138⁺ migratory plasmablasts in bone marrow; and B220⁻ CD138⁺ long-lived plasma cells in bone marrow on day 10 of *P. chabaudi* infection. FACS plots are representative of 3 mice in one experiment.

Chapter 4: Figure legends

Figure 4.10: HA-specific ASC are not detected in PBMC during acute *P. chabaudi* infection.

8-10 wk old female BALB/c mice were infected by intranasal instillation of 250 HAU of PR8. 5 months later, some mice were infected with *P. chabaudi* as previously described. PBMC were obtained on days 8 and 10 after *P. chabaudi* infection. **A)** HA-specific and **B)** total IgG antibody-secreting cells (ASCs) in PBMC in mice infected with PR8 (●) or PR8-*P. chabaudi* (●) were quantified using ELISpot. Data was obtained from one experiment with 4 mice per time point. The line indicates the median value. Statistical values were calculated using the Mann-Whitney test.

Chapter 4: Figure legends

Figure 4.11: Long-lived plasma cells undergo apoptosis during acute *P. chabaudi* infection (before d10).

8-10 wk old female BALB/c mice were infected by intranasal instillation of 250 HAU of PR8. 5 months later, mice were infected with *P. chabaudi* as previously described. Bone marrow was obtained from femur pairs of these mice at various time points after-*P. chabaudi* infection. Apoptosis of total bone marrow cells and CD138⁺ B220⁻ LLPCs was quantified by percentage surface Annexin V staining. **A)** Representative FACS plots of total bone marrow cells and bone marrow LLPC; and relative expression of Annexin V on days 0, 5, 8 and 10 of *P. chabaudi* infection. % Annexin V expression on **B)** total bone marrow cells and **C)** bone marrow LLPC. Data from days 4 and 5 of *P. chabaudi* infection, and days 6 and 8 were pooled from two independent experiments (see *x*-axis groupings). Data was obtained from 2 experiments with 3-10 mice per time point. Statistical values were calculated using the Mann-Whitney test.

Chapter 4: Figure legends

Figure 4.12: Loss of HA-specific ASC or HA-specific serum IgG after *P. chabaudi* infection is dependent on FcγRI,II,III.

8-10 week old female C57BL/6 (blue bars) and FcγRI,II,III KO (red bars) mice were infected by intranasal instillation of 250 HAU of PR8. 150 days later, mice were infected with *P. chabaudi* as previously described. 8 days after *P. chabaudi* infection. **A)** Representative FACS plot showing the mean fluorescence index of FcγRIIB (—) versus the isotype control (—) on B220⁻ CD138⁺ LLPC in the bone marrow. This plot is representative of 3 mice in one experiment. **B)** Concentration of HA-specific serum IgG in FcγRI,II,III KO (n=4-6) and C57BL/6 (n=3) and BALB/c (n=3) mice on days 28, 56, 84 and 150 after PR8 infection. Error bars indicate maximum range of values obtained from one experiment. **C)** % parasitaemia throughout acute *P. chabaudi* infection in FcγRI,II,III KO and C57BL/6 mice, when *P. chabaudi* infection was initiated 150 days after PR8 infection. Data was obtained from 2 mice per group in one experiment. **D)** Total bone marrow cellularity per femur pair at peak parasitaemia (d8) in FcγRI,II,III KO (n=6-8) and C57BL/6 mice (n=4) obtained from one experiment. **E)** HA-specific and **F)** total IgG ASC quantified using ELISpot in in FcγRI,II,III KO (n=6-8) and C57BL/6 mice (n=4-8) obtained from one experiment. **G)** Venous blood was obtained from FcγRI,II,III KO (n=6-10) and C57BL/6 mice (n=5-7) on days 0, 35 and 56 of *P. chabaudi* infection and processed for serum. HA-specific IgG antibodies were quantified by ELISA. Data was obtained from one experiment. Error bars indicate maximum range of values. Statistical values were calculated using the Mann-Whitney test.

Chapter 4: Figure legends

Figure 4.13: Sequestration of *P. chabaudi*-parasitized erythrocytes in bone marrow during acute infection (d5 and 8).

8-10 week old female BALB/c mice were infected by intranasal instillation of 250 HAU of PR8. 150 days later, mice were infected with *P. chabaudi* as previously described. Paraffin-embedded 5µm sections of femurs were made on days 0, 5, 8, 10 and 13 of *P. chabaudi* infection and stained with hematoxylin and eosin. Images were obtained using the Ziess Axioplan 2 imaging microscope using the 100X objective under oil immersion and analysed using the AxioVision 4.6 programme. Red arrows indicate parasitized erythrocytes. These images are representative of two independent experiments with 3 mice in each time point.

4.5: Discussion

In this chapter, I have found that infection with *P. chabaudi* results in a reduction of pre-established specific serum antibodies and bone marrow plasma cells to Influenza A. Furthermore, PR8-*P. chabaudi*-infected mice have increased lung viral loads after re-infection with PR8, indicating a significant loss of ability to clear virus as compared to PR8-immune control mice. However it is not possible at present to determine if protection against disease was also affected by *P. chabaudi* infection, as correlation of viral loads with visible signs of pathology such as weight loss or histological examination of lung pathology was not done. The transient loss of serum Abs is not due to decreased half-life, and it is likely to be a direct result of the reduction in bone marrow LLPC. It is possible that the recovery of serum Abs could be due to supplementation by the differentiation of HA-specific MBC into antibody-secreting plasma cells.

In these experiments, I have found little evidence that pre-established LLPC are being dislodged by new migratory plasmablasts generated by *P. chabaudi* infection; instead, apoptosis of LLPC *in situ* is more likely as demonstrated by the upregulation of Annexin V. In this sequential infection, apoptosis of LLPC during *P. chabaudi* infection is linked to FcγRI,II,III. I have also observed sequestration of *P. chabaudi* parasitized-erythrocytes in bone marrow during acute infection occurring at a similar kinetic to bone marrow LLPC apoptosis (before day 10) and this suggests that sequestration of parasitized-erythrocytes could potentially affect pre-established LLPC or their niches, resulting in the loss of LLPC, although extensive investigation is required to examine this hypothesis.

The lack of cross-reactivity between sera obtained from PR8- and *P. chabaudi*-infected BALB/c mice *in vitro* and *in vivo* validated our system using PR8 and *P. chabaudi* infections to investigate whether sequential infection with unrelated pathogens had any effect on the maintenance of humoral memory to the first infection. Each infection selects for non-cross-reactive B cell clones that generate distinct cohorts of LLPC, which produce non-cross-reactive Abs. Therefore any change that was observed in the HA-specific antibody profile or HA-specific ASC frequencies after subsequent malaria infection would not be confounded by cross-reactive B cell memory responses. Furthermore, this indicated

Chapter 4: Loss of previously established humoral immunity

that both neutralising assays and bromelain-digested PR8 HA-coated ELISAs were valid *in vitro* assays for following the HA-specific antibody response in sequentially-infected mice. When PR8 infection was initiated 60 days after *P. chabaudi* infection, there was no difference in the development of PR8-specific neutralising Ab titres compared to those of PR8 initiated in naive mice, indicating that the presence of *P. chabaudi*-induced immune response did not alter the development of neutralising Ab titres towards PR8.

The concentration of HA-specific serum IgG fell by more than 20% in mice infected with *P. chabaudi* 105 or 150 days after PR8 infection. There was a similar reduction in the concentration of HA-specific Ab titres, and an accompanying 2-3 fold loss of HA-specific IgG plasma cells in the bone marrow. HA-specific serum IgG concentrations remained subnormal for up to 63 days with the 105 day interval, and 42 days with the 150 day interval after *P. chabaudi* infection or drug-cured *P. chabaudi* infection. 42 days after *P. chabaudi* infection, antibody concentrations in PR8-*P. chabaudi*-infected mice were no longer different from those of the PR8 only controls. This result is strikingly similar to that obtained using pre-BSA immunised mice and *P. y. yoelii* and *P. berghei* (Strambachova-McBride & Micklem 1979) in that when *P. y. yoelii* or *P. berghei* was initiated 114 days after primary BSA immunisation, there was a similar 20-25% reduction in serum BSA-specific Ab titres, which returned to similar levels to the non-infected controls by day 53 of infection. However, when PR8-*P. chabaudi*-infected mice were challenged 42 days after *P. chabaudi* infection with PR8, they had higher lung viral titres compared to the PR8-only control group. It will be pertinent to determine whether *P. chabaudi* infection has caused a reduction of NALT HA-specific IgA and IgG and CD8⁺ T cells, and whether there has been long-term pathological damage to lung tissues which could also delay or prevent lung viral clearance.

P. chabaudi infection in mice previously infected with PR8 resulted in an overall reduction in HA-specific serum T cell-dependent isotypes IgG1, IgG2a and IgG2b, while concentrations of IgG3 and IgM, which are already very low, appear to be unaffected. It has been observed that most *Plasmodium spp.* skews the isotype distribution of non-specific serum Ig towards predominantly IgG2a (and IgG2b, in *P. chabaudi adami* infection) subclasses (Quin & Langhorne 2001;Langhorne *et al.* 1985), probably as a result of the Th1

Chapter 4: Loss of previously established humoral immunity

cytokine production induced during the early phase of infection (Langhorne *et al.* 2004), or a result of polyclonal and continuous B cell activation, which is observed in many parasitic infections (Langhorne *et al.* 1985;Minoprio *et al.* 1986). Isotype switching to IgG1, IgG2a and IgG2b is dependent on the persistence of splenic germinal centres and the disruption to splenic microarchitecture could disrupt pre-existing germinal centres.

It has been suggested that the malaria infection can cause transient loss of pre-established Abs by inducing increased clearance or catabolism of serum Abs due to hypergammaglobulinaemia, leading to a reduced half-life of non-specific serum Ab (Goumard *et al.* 1982;Strambachova-McBride & Micklem 1979). Indeed, malaria chemoprophylaxis taken before *P. falciparum* infection is associated with a slower rate of decay of Abs to a meningococcal vaccine among Kenyan children, suggesting that acute malaria globally accelerates the clearance of Abs, not just malaria-specific Abs (Kinyanjui *et al.* 2007). The exact mechanism of how the hypergammaglobulinaemia induced by malaria can cause a reduction in half-life of serum Ab is unknown, but the formation of low-avidity immune complexes during acute malaria may facilitate their clearance by macrophages (Langhorne *et al.* 2004). Alternatively, immune complex-induced nephritis can occur during malaria and can result in proteinuria and possibly accelerated clearance of serum Ab (Ward *et al.* 1969). In our sequentially infected mice, total serum IgG increased from approximately 1-2 mg/ml to approximately 45 mg/ml at 10 days of infection and remained at that level for up to 42 days, finally falling to approximately 23mg/ml only after 60 days of *P. chabaudi* infection, indicating that hypergammaglobulinaemia induced by *P. chabaudi* infection was very large and prolonged. Despite this, and contrary to previous studies (Strambachova-McBride & Micklem 1979), our experiments did not demonstrate a reduced half-life of serum IgG either during acute or chronic *P. chabaudi* infection. As the half-life of serum Ab did not appear to be affected by the presence or absence of parasite, it is unlikely that accelerated IgG clearance can fully explain the transient reduction in pre-established HA-specific serum Abs during *P. chabaudi* infection.

In line with previous studies (Radwanska *et al.* 2008;Wykes *et al.* 2005), I found that *P. chabaudi* infection can result in a loss of pre-established LLPC to unrelated specificities. One hypothesis as to how this happened was that pre-established LLPC were dislocated by

Chapter 4: Loss of previously established humoral immunity

newly generated migratory plasmablasts, a mechanism which is thought to take place during every new immune response in order to homeostatically accommodate new LLPC in finite survival niches (Radbruch *et al.* 2006). Previously, investigations of this were limited by the fact that models using protein-hapten immunisations elicited few plasmablasts, e.g. only 3000 after secondary immunisation with OVA (Manz *et al.* 1997). Here, I have shown that *P. chabaudi* infection induces very large numbers of migratory plasmablasts entering the bone marrow, which are likely to exert a stronger competitive stress on the finite niche. However, despite this, no dislodged LLPC from the bone marrow were detected in the blood during acute *P. chabaudi* infection, either by flow cytometry or by ELISpot. It is possible that the numbers of LLPC extruded by this huge migratory plasmablast population were too small to be picked up by these methods, which in that case would make their impact probably insignificant, or that they were undergoing apoptosis *in situ* and not expelled into the circulation at all.

Here, I found that *P. chabaudi* infection resulted in apoptosis of bone marrow LLPC at particularly early time points (d4-6) of infection. This result is consistent with an earlier study using *P. y. yoelii* where increased apoptosis of bone marrow LLPC occurred on days 3 and 7 of *P. yoelii* infection (Wykes *et al.* 2005). In these previous studies, no clear mechanisms for LLPC apoptosis during infection with parasites were elucidated, although (Wykes *et al.* 2005) suggested that it could occur in a caspase 3-dependent manner. An earlier study suggested that the increase in apoptotic cells in the spleen during *P. chabaudi* infection could be mediated by increased Fas ligand binding (Helmbly *et al.* 2000). My data suggests that Fc γ RI,II,III may play an important role in *P. chabaudi*-induced LLPC apoptosis. Ideally, Fc γ RIIB KO mice should have been used for these experiments but were not available at the time. Despite being lacking three Fc γ receptors, these Fc γ RI,II,III KO mice were able to generate comparable HA-specific serum IgG to that of C57BL/6 mice. Furthermore, Fc γ RI,II,III KO mice were able to control parasites and had similar parasitaemia curves to that of C57BL/6 mice. Importantly, Fc γ RI,II,III KO mice retain pre-infection numbers of HA-specific IgG ASCs in the bone marrow and maintain their pre-established Ab for up to 56 days after *P. chabaudi* infection, unlike the C57BL/6 controls, which have significantly reduced numbers of HA-specific ASC at peak parasitaemia and significantly reduced HA-specific serum IgG for up to 56 days of *P. chabaudi* infection.

Chapter 4: Loss of previously established humoral immunity

The fact that a sterile ‘cocktail’ of immunogens can also trigger LLPC apoptosis via FcγRIIB (Xiang *et al.* 2007) reinforces the implication that this mechanism is not specific to the *P. chabaudi* infection but is a global phenomenon that uses programmed cell death as a mechanism to homeostatically regulate the LLPC niche. FcγRIIB can induce LLPC apoptosis via two independent routes: 1) phosphorylation of ITIM sequence contained in its cytoplasmic domain, which leads to recruitment of SHIP or leads to activation of MAP kinases; 2) ITIM-independent clustering of FcγRIIB, which generates a pro-apoptotic signal through the transmembrane sequence, which acts via Btk. Nevertheless, because of the confounding effects of the absence of FcγRI and FcγRIII in FcγRI,II,III KO mice, it will be necessary to confirm these findings in FcγRIIB KO mice to allow me to dissect the mechanism of FcγRIIB-mediated apoptosis of LLPC in the context of sequential PR8-*P. chabaudi* infections. Other general mechanisms of apoptosis, such as Fas-Fas ligand interactions or ligation of TNF receptors, can be tested in this system with the use of monoclonal blocking antibodies against Fas and TNF receptors.

Outstanding questions

The direct affect of parasite on LLPC has not been previously studied. I have observed sequestration of *P. chabaudi*-parasitized erythrocytes in bone marrow endothelium at particularly early time points of infection (d5 and 8), which correlates very well with the kinetics of LLPC apoptosis and loss of HA-specific ASC in bone marrow. Therefore, it is pertinent to determine whether there is increased apoptosis of bone marrow LLPC during infection with non-sequestering parasites. Further work to investigate the immunomodulatory effects of *P. chabaudi* sequestration in bone marrow would be to investigate the time course of parasite sequestration in bone marrow over acute and chronic parasitaemia, which stage of the parasite life cycle sequesters (mature ring stages or gametocytes) and determine which cell type the sequestering parasite is residing in (mature erythrocytes, reticulocytes, or other cell types such as phagocytes). In addition, I would like to determine which cell type the parasite is sequestering to and investigate if there are histopathological changes caused by the sequestering parasite which could affect pre-established LLPC or their niche.

Chapter 4: Loss of previously established humoral immunity

The effect of *P. chabaudi* on pre-established heterologous memory B cells remains to be determined. However, from previous studies with *P. y. yoelii* and *Trypanosoma brucei* (Radwanska *et al.* 2008; Wykes *et al.* 2005), it is predictable that *P. chabaudi* would also cause a reduction in pre-established splenic memory B cells. In my previous chapter, a significant reduction in pre-established HA-specific serum IgG was observed 84-150 days after memory B cell depletion, indicating that memory B cells are important in the maintenance of HA-specific serum IgG. Therefore it is possible that the recovery of HA-specific serum IgG after *P. chabaudi* infection is due to the reconstitution of HA-specific LLPC and serum Ab by HA-specific memory B cells. Further work would therefore be to determine whether pre-established memory B cells were affected by *P. chabaudi* infection, and whether and how memory B cells replenish a depleted LLPC, as a mechanism for maintaining constant serum antibody concentrations over time.

Final perspectives**Memory B cells are required to maintain persistent serum Abs to influenza A in the absence of re-infection.**

In this study, the use of hCD20 transgenic mice revealed that memory B cells contribute to the maintenance of Influenza-specific long-lived serum Abs after an intranasal influenza infection. In agreement with previous studies (Ahuja *et al.* 2008; DiLillo *et al.* 2008; Gong *et al.* 2005), the anti-hCD20tg mAb had no observable effect on B220⁺ CD138⁺ LLPC frequencies and numbers immediately after the course of treatment, whereas it depleted peripheral mature B cell subsets rapidly and efficiently. This is probably because of the lower expression of hCD20 on LLPC compared to B cells and MBC. However it rapidly depleted the majority of B cells and MBC. In this study, frequencies of peripheral B cells returned to normal pre-treatment levels within 3 months after depletion. By contrast, the loss of Influenza haemagglutinin (HA)-specific MBC was sustained for up to 5 months. Interestingly, HA-specific plasma cells in the bone marrow, which did not change in number for up to d42 after 2H7 treatment, were subsequently observed to decrease at a particularly late time point of 150 days after 2H7 treatment. Pre-established serum Abs to Influenza also decreased significantly 90-150 days after depletion of MBCs. In this case, the kinetics of LLPC loss approximately matched the kinetics of serum Ab loss. These data therefore suggests that MBCs were required for maintaining the numbers of LLPCs in the bone marrow, as well as to maintain persistent Influenza-specific serum Abs after primary intranasal influenza infection.

The results of this depletion study is in accordance with previous studies, which point to the important contribution of MBC to maintaining Influenza-specific serum Abs. Influenza-specific MBC are very long-lived, as observed in human survivors of the 1918 Spanish influenza pandemic, who have detectable recirculating MBC which are able to mount recall responses even 90 years after (Yu *et al.* 2008b). Recently, Bali Pulendran's group used a novel vaccine strategy using synthetic nanoparticles containing Influenza antigens in combination with TLR ligands to programme the innate immune response to induce long-lived neutralising Ab titres (Kasturi *et al.* 2011). In this study, successful long-term serum

neutralising Ab titres against Influenza were established by generating long-lived germinal centres producing persistent MBC and ASC in lymph nodes for more than 1.5 years in mice, demonstrating that MBC can be an important contributor to maintaining long-term serum Ab.

However these findings are at odds with the notion that serum Abs are maintained only by LLPC, which was inferred by previous B cell depletion studies in mice (Ahuja *et al.* 2008; DiLillo *et al.* 2008), which demonstrated that the LLPC generated by protein-immunisation and acute infection with LCMV (Armstrong) (Slifka *et al.* 1998) are capable of surviving and maintaining pre-established serum Ab titres for long periods of time, despite MBC depletion by monoclonal Abs or irradiation. This difference between the findings in this study and in those previous studies could be due to the contexts of LLPC generation. Intraperitoneal protein immunisation or infection may ‘imprint’ a long intrinsic lifespan on LLPC, but in the context of intranasal Influenza infection, long-term serum Influenza-specific serum Abs may have a greater reliance on the MBC pool for their maintenance.

Indeed, MBCs have various properties which enable them to maintain serum Abs in the absence of re-infection. MBCs can survive independently of antigen stimulation and in the absence of mitosis (Maruyama *et al.* 2000), are intrinsically programmed for faster signalling and self-renewal (Tomayko *et al.* 2008), and have been documented to recirculate for up to 90 years after the last known infection (Crotty *et al.* 2003; Yu *et al.* 2008b). Furthermore, they are unique from other B cell subsets and LLPC in their independence of the cytokines BAFF and APRIL for their survival (Benson *et al.* 2008) and have their own specialised niches like the spleen (Mamani-Matsuda *et al.* 2008), although the properties of these niches are not well characterised. MBCs have a higher propensity to differentiate into PCs than naïve B cells upon activation (Benson *et al.* 2009; Bernasconi *et al.* 2003) and bear more polyreactive BCRs as compared to LLPCs (Dal Porto *et al.* 2002; Tarlinton & Smith 2000). Therefore serum Abs can be maintained by chronic reactivation of MBC both by persistent antigen and by cross-reactive antigen. In addition, MBC differentiation can also be driven by cytokines or TLR ligands inducing a bystander activation of MBC independently of antigen (Bernasconi *et al.* 2002). Therefore there is a

strong biological basis for the importance of MBC, not just in the anamnestic response, but also in the general maintenance of long-lived serum Abs in the absence of re-infection.

Given the importance of long-lived serum Abs for protective immunity after many vaccines and infections, it would be interesting to compare the relative importance of MBC and LLPC to long-term serum Abs across different infections, for example in other viral infections or parasitic infections, and indeed with different vaccination strategies and routes of administration. The information obtained from such studies would not only expand our understanding of the basic biological parameters of long-lived humoral immunity, but also enable better vaccine design. For example, this study suggests that strategies designed to induce persistent humoral immunity to Influenza A should induce long-lived MBC responses in order to generate the most robust and long-lasting serum Ab titres.

Transient loss of pre-established antibody-mediated immunity to Influenza A infection during acute *P. chabaudi* infection.

The second main finding is that pre-established Influenza HA-specific Abs are lost following *P. chabaudi* infection. Pre-established bone marrow HA-specific LLPCs are also lost during acute *P. chabaudi* infection. Moreover, PR8-*P. chabaudi* infected mice have much higher viral loads in the lungs after re-infection with Influenza A compared to the PR8-only controls, where viral loads are controlled to nearly below the detectable level. These findings are consistent with the very few previous studies using other rodent *Plasmodium* species such as *P. yoelii* and *P. berghei* (Strambachova-McBride & Micklem 1979; Wykes *et al.* 2005), as well as a study using the protozoan parasite, *Trypanosoma brucei* (Askonas *et al.* 1979; Radwanska *et al.* 2008), which all demonstrate a loss of serum Abs during acute infection, accompanied by apoptosis and reduction in number of pre-established non-specific MBCs and LLPCs. One hypothesis has been that pre-established LLPCs are competitively dislocated by newly generated migratory plasmablasts (Radbruch *et al.* 2006), but that hypothesis is not supported by the data obtained in this study. Although very large numbers of migratory plasmablasts are generated, and which enter the bone marrow, during acute *P. chabaudi* infection, I did not observe any pre-established LLPC dislocated into the circulation during the peak of the new migratory plasmablast

wave. Instead, apoptosis rather than homeostatic dysregulation of the finite LLPC niche is likely to be the cause of the loss of the loss of Influenza-specific ASC, as indicated by the increased Annexin V expression on bone marrow LLPC during acute *P. chabaudi* infection.

Several possible mechanisms have been put forward to explain the induction of apoptosis in ASC. An early study in the 1980s implicated the increased production of immune-suppressive low-density lipoproteins from *P. chabaudi*-infected mice (Goumard *et al.* 1982). During *Trypanosoma brucei* infection, there is a rapid loss of developing B cells from the bone marrow, and this has been attributed to the loss of CXCL12 expression (Viki & Nathalie 2011). Since CXCL12 is also required for retention of LLPCs (Tokoyoda *et al.* 2004), such a loss could also explain the loss of ASC but this was not investigated formally in this study. In intracellular *Trypanosoma cruzi* infection, apoptosis of IgG⁺ B cells was mediated by Fas/Fas ligand interactions resulting in B cell-B cell killing (Zuñiga *et al.* 2002). In the study with *P. yoelii*, apoptosis of MBCs and LLPCs was shown to be dependent on caspase-3 (Wykes *et al.* 2005), however what triggered the apoptosis of LLPCs was not determined. Apoptosis of LLPCs following injection of an immunogenic cocktail of antigens has been shown to be induced by binding of immune complexes to the inhibitory FcγRIIB expressed on B cells and plasma cells (Xiang *et al.* 2007). However this has not been demonstrated to take place following an infection. In this thesis, the apoptosis of Influenza-specific LLPCs is likely to be mediated through FcγRIIB. Five months after PR8 infection, the B220⁻ CD138⁺ LLPCs in the bone marrow express elevated levels of FcγRIIB. Mice lacking this receptor did not display either bone marrow hypocellularity or loss of pre-established LLPC following a *P. chabaudi* infection. Engagement of FcγRIIB on LLPCs is probably the result of the extensive hypergammaglobulinaemia and the generation of immune complexes during acute *P. chabaudi* infection (Achtman *et al.* 2007), which would have the ability to engage FcγRIIB and trigger apoptosis. This is particularly important as this a feature shared amongst many other viral and parasitic infections, and therefore may be a widespread phenomenon as well as an immune evasion mechanism of the parasite to prevent establishment of MBCs or LLPCs to its own variant antigens.

These data have implications for the longevity of protective efficacy of vaccinations in malaria-endemic countries. There is a lack of field data in humans on the impact of malaria

infection on pre-existing immunity. Vaccine efficacy amongst children tends not to be as good in malaria-endemic countries like Nigeria or the Gambia as compared to non-endemic areas (Abdurrahman *et al* 1982; Greenwood *et al* 1980), and there are documented outbreaks of infectious diseases like polio despite a high level of vaccination coverage (Hanlon *et al* 1987), and the reason for this is unclear. However, as the majority of vaccine efficacy studies are done in very young infants and children, the inefficacy of vaccine-induced immunity could be due to a number of factors, such as the young infants' immature immune system (Galindo *et al* 2000) or high pre-existing titres of maternal Abs which inhibit the development of MBC and LLPC, making data obtained from this age group difficult to interpret. It has been documented that a co-infection with *P. falciparum* (i.e. detectable parasitaemia) suppresses the development of vaccine-induced immune responses (Greenwood *et al* 1980; Williamson and Greenwood 1978), although there are also reports that overall vaccine-induced immunity has not been affected in malaria-endemic countries (Temple *et al* 1991; Campbell *et al* 1990; McLennan *et al* 2001; Cutts *et al* 2005). Similar studies in farm animals have shown that infection with African trypanosomes significantly reduced the efficacy of several commercial vaccines (Holland *et al.* 2003; Mwangi, Munyua, & Nyaga 1990; Rurangirwa *et al.* 1983; Sharpe *et al.* 1982; Whitelaw *et al.* 1979); however almost all these studies were done with vaccinations given during the time of Trypanosome infection and so do not provide answers to whether a parasite infection caused a loss of pre-established immunity. There is a dearth of investigations into the longevity of pre-established immunity in older age groups living in or moving into malaria-endemic countries where the parasite may have the ability to abrogate pre-established immunity and render the host susceptible to secondary infection. This point is extremely relevant in the context of multiple vaccination programmes in malaria-endemic countries. A longitudinal study of serum Abs in pre-immune adults travelling to malaria-endemic regions would provide a clue to whether attrition of pre-established immunity occurs in the field.

Sequestration of parasitized-erythrocytes in the bone marrow.

This study is the first to document the sequestration of parasitized erythrocytes in the bone marrow during acute *P. chabaudi* infection. Further investigation is required to

characterise the kinetics of parasite sequestration during acute and chronic parasitaemia, and to determine the type of red cell the sequestering parasite is residing in (e.g. mature erythrocytes, normoblasts or reticulocytes), or other cell types, e.g. phagocytes. Determining which cell type the parasite is sequestering to and if there are histopathological changes caused by the sequestering parasite may shed some light as to whether there are direct affects on LLPC or their niches.

Replenishment of serum antibodies.

Finally, unlike previous studies, this study also has shown that the loss of pre-established serum Abs is transient, as Influenza-specific serum Abs does eventually return to pre-established levels. It was previously suggested that the transient loss of pre-established serum Abs during infection of CBA mice with non-lethal *P. yoelii* 17X or *P. berghei* NK65 was due to increased clearance of serum Abs as a consequence of the hypergammaglobulinaemia generated (Strambachova-McBride & Micklem 1979). The rate of clearance of non-specific serum Ab during *P. chabaudi* infection of BALB/c mice was examined in this thesis; however I did not observe any impact on the clearance of non-specific serum Ab during either the acute or chronic phase of infection with *P. chabaudi*.

It is possible that HA-specific MBC are being reactivated, differentiating into PCs and repopulating the HA-specific LLPC niche. From the observations of previous investigators, it is likely that MBC will also be decreased during *P. chabaudi* infection (Radwanska *et al.* 2008; Wykes *et al.* 2005), although that has not yet been formally determined in this study. However, unlike the hCD20 system, which permanently depletes the majority of MBC, sometimes to undetectable levels, it is unlikely that *P. chabaudi* will deplete all pre-established MBC, and the remaining MBC will have the ability to undergo homeostatic turnover and differentiate into LLPC and replenish serum Ab. The inflammatory cytokine milieu induced during acute *P. chabaudi* infection and the ensuing chronic parasitaemia can potentially stimulate the regeneration of MBC and supplementation of the reduced LLPC niche.

Therefore it now remains to be determined whether bystander inflammation and re-generation of germinal centres in re-stimulation is the main mechanism the supplementation of Influenza-specific serum Abs and LLPCs after *P. chabaudi* infection. It would also be interesting to investigate whether chronic parasitaemia is necessary for re-stimulating pre-established Influenza-specific MBCs. This can be determined using a curative drug regimen with chloroquine to compare the effect of the presence or absence of chronic parasitaemia. If this is so, then elimination of the parasite by curative drug treatments would also abrogate the bystander stimulation of pre-established MBC.

In summary, this study demonstrates that HA-specific Abs can be maintained for very long periods of time in the absence of re-infection and that memory B cells are necessary for maintenance of long-lived serum Abs after an intranasal Influenza A infection. However, immediately after a sequential infection with *P. chabaudi*, there is a temporary dysregulation in the MBC and LLPC niches, causing apoptosis of pre-established LLPC and the loss of serum Abs. Importantly, there is also an increased susceptibility to opportunistic secondary infections with Influenza during this period of time. However, serum Abs are eventually replenished over time, possibly by differentiation of MBCs into plasma cells. This is a way of ensuring that pre-established serum Abs can be maintained for long periods of time, in the face of sequential heterologous infections and continuous generation and incorporation of new specificities throughout the lifetime of the host.

References

1. Abdurrahman,M.B., Greenwood,B.M., Olafimihan,O. & Whittle,H.C. Measles antibody levels from birth to 9 months of age in Nigerian infants. *African Journal of Medicine and Medical Sciences* **11**, 113 (1982).
2. Achtman,A.H., Khan,M., MacLennan,I. & Langhorne,J. *Plasmodium chabaudi chabaudi* infection in mice induces strong B cell responses and striking but temporary changes in splenic cell distribution. *The Journal of Immunology* **171**, 317 (2003).
3. Achtman,A.H., Bull,P.C., Stephens,R. & Langhorne,J. Longevity of the immune response and memory to blood-stage malaria infection. *Immunology and Immunopathogenesis of Malaria* 71-102 (2005).
4. Achtman,A.H., Stephens,R., Cadman,E.T., Harrison,V. & Langhorne,J. Malaria-specific antibody responses and parasite persistence after infection of mice with *Plasmodium chabaudi chabaudi*. *Parasite Immunology* **29**, 435-444 (2007).
5. Agematsu,K., Hokibara,S., Nagumo,H. & Komiyama,A. CD27: a memory B-cell marker. *Immunology Today* **21**, 204-206 (2000).
6. Ahmed,R. & Gray,D. Immunological memory and protective immunity: Understanding their relation. *Science* **272**, 54-60 (1996).
7. Ahuja,A. *et al.* Depletion of B cells in murine lupus: efficacy and resistance. *The Journal of Immunology* **179**, 3351 (2007).
8. Ahuja,A., Anderson,S.M., Khalil,A. & Shlomchik,M.J. Maintenance of the plasma cell pool is independent of memory B cells. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 4802-4807 (2008).
9. Ahuja,A. *et al.* An Acquired Defect in IgG-Dependent Phagocytosis Explains the Impairment in Antibody-Mediated Cellular Depletion in Lupus. *The Journal of Immunology* **187**, 3888-3894 (2011).
10. Alexopoulou,L., Holt,A.C., Medzhitov,R. & Flavell,R.A. Recognition of double-stranded RNA and activation of NF-kappa B by Toll-like receptor 3. *Nature* **413**, 732-738 (2001).
11. Allen,C.D.C. *et al.* Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. *Nature Immunology* **5**, 943-952 (2004).
12. Allen,C.D.C., Okada,T., Tang,H.L. & Cyster,J.G. Imaging of germinal center selection events during affinity maturation. *Science* **315**, 528 (2007).
13. Allen,C.D.C., Okada,T. & Cyster,J.G. Germinal-center organization and cellular dynamics. *Immunity* **27**, 190-202 (2007).

14. Allen, I.C. *et al.* The NLRP3 inflammasome mediates in vivo innate immunity to influenza A virus through recognition of viral RNA. *Immunity* **30**, 556-565 (2009).
15. Alugupalli, K.R. *et al.* B1b lymphocytes confer T cell-independent long-lasting immunity. *Immunity* **21**, 379-390 (2004).
16. Amanna, I.J., Slifka, M.K. & Crotty, S. Immunity and immunological memory following smallpox vaccination. *Immunological Reviews* **211**, 320-337 (2006).
17. Amanna, I.J., Carlson, N.E. & Slifka, M.K. Duration of humoral immunity to common viral and vaccine antigens. *New England Journal of Medicine* **357**, 1903-1915 (2007).
18. Amanna, I.J. & Slifka, M.K. Mechanisms that determine plasma cell lifespan and the duration of humoral immunity. *Immunological Reviews* **236**, 125-138 (2010).
19. Amigorena, S., Bonnerot, C., Choquet, D., Fridman, W.H. & Teillaud, J.L. FcγRII expression in resting and activated B lymphocytes. *European Journal of Immunology* **19**, 1379-1385 (1989).
20. Amino, R. *et al.* Quantitative imaging of *Plasmodium* transmission from mosquito to mammal. *Nature Medicine* **12**, 220-224 (2006).
21. Amino, R. *et al.* Host cell traversal is important for progression of the malaria parasite through the dermis to the liver. *Cell host & microbe* **3**, 88-96 (2008).
22. Anderson, S.M., Tomayko, M.M., Ahuja, A., Haberman, A.M. & Shlomchik, M.J. New markers for murine memory B cells that define mutated and unmutated subsets. *The Journal of Experimental Medicine* **204**, 2103 (2007).
23. Andrewes, C.H., Laidlaw, P.P. & Smith, W. The susceptibility of mice to the viruses of human and swine influenza. *The Lancet* **224**, 859-862 (1934).
24. Angelin-Duclos, C., Cattoretti, G., Lin, K.I. & Calame, K. Commitment of B lymphocytes to a plasma cell fate is associated with Blimp-1 expression in vivo. *The Journal of Immunology* **165**, 5462 (2000).
25. Askonas, B.A., Corsini, A.C., Clayton, C.E. & Ogilvie, B.M. Functional depletion of T- and B-memory cells and other lymphoid cell subpopulations during trypanosomiasis. *Immunology* **36**, 313 (1979).
26. Avery, D.T. *et al.* BAFF selectively enhances the survival of plasmablasts generated from human memory B cells. *Journal of Clinical Investigation* **112**, 286-297 (2003).
27. Böttiger, M., Gustavsson, O. & Svensson, A. Immunity to tetanus, diphtheria and poliomyelitis in the adult population of Sweden in 1991. *International Journal of Epidemiology* **27**, 916 (1998).

28. Baer,K., Klotz,C., Kappe,S.H.I., Schnieder,T. & Frevert,U. Release of hepatic *Plasmodium yoelii* merozoites into the pulmonary microvasculature. *Plos Pathogens* **3**, e171 (2007).
29. Baiu,D.C. *et al.* Modulation of the humoral immune response by antibody-mediated antigen targeting to complement receptors and Fc receptors. *The Journal of Immunology* **162**, 3125 (1999).
30. Banic,D.M., Viana-Martins,F.S., De Souza,J.M., Peixoto,T.D. & Daniel-Ribeiro,C. Polyclonal B-lymphocyte stimulation in human malaria and its association with ongoing parasitemia. *The American Journal of Tropical Medicine and Hygiene* **44**, 571 (1991).
31. Batista,F.D. & Harwood,N.E. The who, how and where of antigen presentation to B cells. *Nature Reviews Immunology* **9**, 15-27 (2009).
32. Baum,J. *et al.* Reticulocyte-binding protein homologue 5-an essential adhesin involved in invasion of human erythrocytes by *Plasmodium falciparum*. *International Journal for Parasitology* **39**, 371-380 (2009).
33. Becker,Y.T., Becker,B.N., Pirsch,J.D. & Sollinger,H.W. Rituximab as treatment for refractory kidney transplant rejection. *American Journal of Transplantation* **4**, 996-1001 (2004).
34. Belnoue,E. *et al.* Protective T cell immunity against malaria liver stage after vaccination with live sporozoites under chloroquine treatment. *The Journal of Immunology* **172**, 2487 (2004).
35. Belnoue,E. *et al.* APRIL is critical for plasmablast survival in the bone marrow and poorly expressed by early-life bone marrow stromal cells. *Blood* **111**, 2755 (2008).
36. Belshe,R.B. *et al.* Correlates of immune protection induced by live, attenuated, cold-adapted, trivalent, intranasal influenza virus vaccine. *Journal of Infectious Diseases* **181**, 1133 (2000).
37. Belyaev,N.N. *et al.* Induction of an IL7-R(+)-Kit(hi) myelolymphoid progenitor critically dependent on IFN-gamma signaling during acute malaria. *Nature Immunology* **11**, 477-U41 (2010).
38. Belz,G.T., Wodarz,D., Diaz,G., Nowak,M.A. & Doherty,P.C. Compromised influenza virus-specific CD8+-T-cell memory in CD4+-T-cell-deficient mice. *Journal of Virology* **76**, 12388 (2002).
39. Belz,G.T. *et al.* Distinct migrating and nonmigrating dendritic cell populations are involved in MHC class I-restricted antigen presentation after lung infection with virus. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 8670 (2004).

40. Bennett,S.R.M., Carbone,F.R., Karamalis,F., Miller,J.F.A.P. & Heath,W.R. Induction of a CD8+ cytotoxic T lymphocyte response by cross-priming requires cognate CD4+ T cell help. *The Journal of Experimental Medicine* **186**, 65 (1997).
41. Benson,M.J. *et al.* Cutting edge: the dependence of plasma cells and independence of memory B cells on BAFF and APRIL. *The Journal of Immunology* **180**, 3655 (2008).
42. Benson,M.J. *et al.* Distinction of the memory B cell response to cognate antigen versus bystander inflammatory signals. *The Journal of Experimental Medicine* **206**, 2013 (2009).
43. Bernasconi,N.L., Traggiai,E. & Lanzavecchia,A. Maintenance of serological memory by polyclonal activation of human memory B cells. *Science* **298**, 2199 (2002).
44. Bernasconi,N.L., Onai,N. & Lanzavecchia,A. A role for Toll-like receptors in acquired immunity: up-regulation of TLR9 by BCR triggering in naive B cells and constitutive expression in memory B cells. *Blood* **101**, 4500-4504 (2003).
45. Bird,L. Natural Killer cells: Making memories. *Nature Reviews Immunology* **10**, 811 (2010).
46. Blackman,M.J., Heidrich,H.G., Donachie,S., McBride,J.S. & Holder,A.A. A single fragment of a malaria merozoite surface protein remains on the parasite during red cell invasion and is the target of invasion-inhibiting antibodies. *The Journal of Experimental Medicine* **172**, 379-382 (1990).
47. Blink,E.J. *et al.* Early appearance of germinal center–derived memory B cells and plasma cells in blood after primary immunization. *The Journal of Experimental Medicine* **201**, 545-554 (2005).
48. Bockstal,V. *et al.* *T. brucei* Infection Reduces B Lymphopoiesis in Bone Marrow and Truncates Compensatory Splenic Lymphopoiesis through Transitional B-Cell Apoptosis. *Plos Pathogens* **7**, e1002089 (2011).
49. Borrmann,S. & Matuschewski,K. Protective immunity against malaria by ‘natural immunization’: a question of dose, parasite diversity, or both? *Current Opinion in Immunology* (2011).
50. Borrmann,S. & Matuschewski,K. Targeting *Plasmodium* liver stages: better late than never. *Trends in Molecular Medicine* (2011).
51. Bouharoun-Tayoun,H., Attanath,P., Sabchareon,A., Chongsuphajaisiddhi,T. & Druilhe,P. Antibodies that protect humans against *Plasmodium falciparum* blood stages do not on their own inhibit parasite growth and invasion in vitro, but act in cooperation with monocytes. *The Journal of Experimental Medicine* **172**, 1633-1641 (1990).

52. Bouharoun-Tayoun,H., Oeuvray,C., Lunel,F. & Druilhe,P. Mechanisms underlying the monocyte-mediated antibody-dependent killing of *Plasmodium falciparum* asexual blood stages. *The Journal of Experimental Medicine* **182**, 409-418 (1995).
53. Brown,D.M., Dilzer,A.M., Meents,D.L. & Swain,S.L. CD4 T cell-mediated protection from lethal influenza: perforin and antibody-mediated mechanisms give a one-two punch. *The Journal of Immunology* **177**, 2888 (2006).
54. Brown,K.N. & Brown,I.N. Immunity to malaria: antigenic variation in chronic infections of *Plasmodium knowlesi*. *Nature* **208**, 1286 (1965).
55. Buisman,A.M., De Rond,C.G.H., Ozturk,K., Ten Hulscher,H.I. & Van Binnendijk,R.S. Long-term presence of memory B-cells specific for different vaccine components. *Vaccine* **28**, 179-186 (2009).
56. Bull,P.C. *et al.* Parasite antigens on the infected red cell surface are targets for naturally acquired immunity to malaria. *Nature Medicine* **4**, 358-360 (1998).
57. Cadman,E.T. *et al.* Alterations of splenic architecture in malaria are induced independently of Toll-like receptors 2, 4, and 9 or MyD88 and may affect antibody affinity. *Infection and Immunity* **76**, 3924 (2008).
58. Calame,K.L., Lin,K.I. & Tunyaplin,C. Regulatory mechanisms that determine the development and function of plasma cells. *Annual Review of Immunology* **21**, 205-230 (2003).
59. Camacho,S.A., Kosco-Vilbois,M.H. & Berek,C. The dynamic structure of the germinal center. *Immunology Today* **19**, 511-514 (1998).
60. Cambridge,G. *et al.* Serologic changes following B lymphocyte depletion therapy for rheumatoid arthritis. *Arthritis & Rheumatism* **48**, 2146-2154 (2003).
61. Cambridge,G. *et al.* B cell depletion therapy in systemic lupus erythematosus: effect on autoantibody and antimicrobial antibody profiles. *Arthritis & Rheumatism* **54**, 3612-3622 (2006).
62. Cancro,M.P. The persistence of memory: A unique niche for IgG memory B cells. *Proceedings of the National Academy of Sciences* **107**, 12737 (2010).
63. Caraux,A. *et al.* Mobilization of plasma cells in healthy individuals treated with granulocyte colony stimulating factor for haematopoietic stem cell collection. *Immunology* **132**, 266-72(2010).
64. Carding,S.R., Allan,W., McMickle,A. & Doherty,P.C. Activation of cytokine genes in T cells during primary and secondary murine influenza pneumonia. *The Journal of Experimental Medicine* **177**, 475-482 (1993).
65. Carlson,J. *et al.* Human cerebral malaria: association with erythrocyte rosetting and lack of anti-rosetting antibodies. *The Lancet* **336**, 1457-1460 (1990).

66. Carpenter,D. *et al.* Immunogenetic control of antibody responsiveness in a malaria endemic area. *Human Immunology* **68**, 165-169 (2007).
67. Casali,P., Pal,Z., Xu,Z. & Zan,H. DNA repair in antibody somatic hypermutation. *Trends in Immunology* **27**, 313-321 (2006).
68. Cassese,G. *et al.* Plasma cell survival is mediated by synergistic effects of cytokines and adhesion-dependent signals. *The Journal of Immunology* **171**, 1684 (2003).
69. Castillo-Méndez,S.I. *et al.* Characterization of the spleen B-cell compartment at the early and late blood-stage *Plasmodium chabaudi* malaria. *Scandinavian Journal of Immunology* **66**, 309 (2007).
70. Caton,A.J., Brownlee,G.G., Yewdell,J.W. & Gerhard,W. The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). *Cell* **31**, 417-427 (1982).
71. Cavanagh,D.R. *et al.* A longitudinal study of type-specific antibody responses to *Plasmodium falciparum* merozoite surface protein-1 in an area of unstable malaria in Sudan. *The Journal of Immunology* **161**, 347 (1998).
72. Cazac,B.B. & Roes,J. TGF- β receptor controls B cell responsiveness and induction of IgA in vivo. *Immunity* **13**, 443-451 (2000).
73. Chapdelaine,Y., Smith,D.K., Pedras-Vasconcelos,J.A., Krishnan,L. & Sad,S. Increased CD8⁺ T cell memory to concurrent infection at the expense of increased erosion of pre-existing memory: the paradoxical role of IL-15. *The Journal of Immunology* **171**, 5454 (2003).
74. Chevrier,S. *et al.* CD93 is required for maintenance of antibody secretion and persistence of plasma cells in the bone marrow niche. *Proceedings of the National Academy of Sciences* **106**, 3895 (2009).
75. Choi,Y.S. *et al.* ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6. *Immunity* **34**, 932-946 (2011).
76. Chu,V.T., Beller,A., Nguyen,T.T.N., Steinhauser,G. & Berek,C. The Long-Term Survival of Plasma Cells. *Scandinavian Journal of Immunology* **73**, 508-511 (2011).
77. Clatworthy,M.R. Targeting B Cells and Antibody in Transplantation. *American Journal of Transplantation* **11**, 1359-67 (2011).
78. Clatworthy,M.R. *et al.* Systemic lupus erythematosus-associated defects in the inhibitory receptor Fc γ RIIb reduce susceptibility to malaria. *Proceedings of the National Academy of Sciences* **104**, 7169 (2007).
79. Coban,C. *et al.* Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin. *The Journal of Experimental Medicine* **201**, 19 (2005).

80. Coban,C., Ishii,K.J., Horii,T. & Akira,S. Manipulation of host innate immune responses by the malaria parasite. *Trends in Microbiology* **15**, 271-278 (2007).
81. Coffman,R.L. *et al.* The role of helper T cell products in mouse B cell differentiation and isotype regulation. *Immunological Reviews* **102**, 5-28 (1988).
82. Cohen,S., McGregor,I.A. & Carrington,S. Gamma-globulin and acquired immunity to human malaria. *Nature* **192**, 733 (1961).
83. Coiffier,B. *et al.* CHOP chemotherapy plus rituximab compared with CHOP alone in elderly patients with diffuse large-B-cell lymphoma. *New England Journal of Medicine* **346**, 235-242 (2002).
84. Cooke,G.S. *et al.* Association of Fc γ receptor IIa (CD32) polymorphism with severe malaria in West Africa. *The American Journal of Tropical Medicine and Hygiene* **69**, 565-568 (2003).
85. Cooney,E.L. *et al.* Safety of and immunological response to a recombinant vaccinia virus vaccine expressing HIV envelope glycoprotein. *The Lancet* **337**, 567-572 (1991).
86. Cooper,N. & Arnold,D.M. The effect of rituximab on humoral and cell mediated immunity and infection in the treatment of autoimmune diseases. *British Journal of Haematology* **149**, 3-13 (2010).
87. Couper,K.N., Phillips,R.S., Brombacher,F. & Alexander,J. Parasite-specific IgM plays a significant role in the protective immune response to asexual erythrocytic stage *Plasmodium chabaudi* AS infection. *Parasite Immunology* **27**, 171-180 (2005).
88. Couper,K.N. *et al.* Macrophage-mediated but gamma interferon-independent innate immune responses control the primary wave of *Plasmodium yoelii* parasitemia. *Infection and Immunity* **75**, 5806 (2007).
89. Couper,K.N. *et al.* IL-10 from CD4⁺ CD25⁻ Foxp3⁻ CD127⁻ adaptive regulatory T cells modulates parasite clearance and pathology during malaria infection. *Plos Pathogens* **4**, e1000004 (2008).
90. Craig,A.G. *et al.* The Role of Animal Models for Research on Severe Malaria. *Plos Pathogens* **8**, e1002401 (2012).
91. Crompton,P.D. *et al.* The TLR9 ligand CpG promotes the acquisition of *Plasmodium falciparum*-specific memory B cells in malaria-naive individuals. *The Journal of Immunology* **182**, 3318 (2009).
92. Crompton,P.D. *et al.* A prospective analysis of the Ab response to *Plasmodium falciparum* before and after a malaria season by protein microarray. *Proceedings of the National Academy of Sciences* **107**, 6958 (2010).

93. Crosnier,C. *et al.* Basigin is a receptor essential for erythrocyte invasion by *Plasmodium falciparum*. *Nature* **480**, 534-537 (2011).
94. Crotty,S. *et al.* Cutting edge: long-term B cell memory in humans after smallpox vaccination. *The Journal of Immunology* **171**, 4969 (2003).
95. Crotty,S., Aubert,R.D., Glidewell,J. & Ahmed,R. Tracking human antigen-specific memory B cells: a sensitive and generalized ELISPOT system. *Journal of Immunological Methods* **286**, 111-122 (2004).
96. Crotty,S. & Ahmed,R. Immunological memory in humans. 16(3), 197-203. 2004. Elsevier.
Ref Type: Conference Proceeding
97. Crotty,S. Follicular Helper CD4 T Cells (Tfh). *Annual Review of Immunology* **29**, 621-663 (2011).
98. Cunnington,A.J., de Souza,J.B., Walther,M. & Riley,E.M. Malaria impairs resistance to Salmonella through heme-and heme oxygenase-dependent dysfunctional granulocyte mobilization. *Nature Medicine* **18**, 120-127 (2011).
99. Dörner,T. & Radbruch,A. Antibodies and B cell memory in viral immunity. *Immunity* **27**, 384-392 (2007).
100. Dal Porto,J.M., Haberman,A.M., Shlomchik,M.J. & Kelsoe,G. Antigen drives very low affinity B cells to become plasmacytes and enter germinal centers. *The Journal of Immunology* **161**, 5373 (1998).
101. Dal Porto,J.M., Haberman,A.M., Kelsoe,G. & Shlomchik,M.J. Very low affinity B cells form germinal centers, become memory B cells, and participate in secondary immune responses when higher affinity competition is reduced. *The Journal of Experimental Medicine* **195**, 1215 (2002).
102. Dall'Era,M. *et al.* Reduced B lymphocyte and immunoglobulin levels after atacicept treatment in patients with systemic lupus erythematosus: Results of a multicenter, phase ib, double-blind, placebo-controlled, dose-escalating trial. *Arthritis & Rheumatism* **56**, 4142-4150 (2007).
103. Daly,T.M. & Long,C.A. Humoral response to a carboxyl-terminal region of the merozoite surface protein-1 plays a predominant role in controlling blood-stage infection in rodent malaria. *The Journal of Immunology* **155**, 236 (1995).
104. Davies,D.H. *et al.* Vaccinia virus H3L envelope protein is a major target of neutralizing antibodies in humans and elicits protection against lethal challenge in mice. *Journal of Virology* **79**, 11724 (2005).
105. de Souza,J.B. *et al.* Neutralization of Malaria Glycosylphosphatidylinositol In Vitro by Serum IgG from Malaria-Exposed Individuals. *Infection and Immunity* **78**, 3920 (2010).

106. Defrance,T., Taillardet,M. & Genestier,L. T cell-independent B cell memory. *Current Opinion in Immunology* **23**, 330-336 (2011).
107. del Portillo,H.A. *et al.* The role of the spleen in malaria. *Cellular Microbiology* **14**, 343-355 (2011).
108. Dengler,H.S. *et al.* Distinct functions for the transcription factor Foxo1 at various stages of B cell differentiation. *Nature Immunology* **9**, 1388-1398 (2008).
109. Der Weid,T., Honarvar,N. & Langhorne,J. Gene-targeted mice lacking B cells are unable to eliminate a blood stage malaria infection. *The Journal of Immunology* **156**, 2510 (1996).
110. der Weld,T. & Langhorne,J. Altered response of CD4+ T cell subsets to *Plasmodium chabaudi chabaudi* in B cell-deficient mice. *International Immunology* **5**, 1343 (1993).
111. Di Noia,J.M. & Neuberger,M.S. Molecular mechanisms of antibody somatic hypermutation. *Annu. Rev. Biochem.* **76**, 1-22 (2007).
112. DiLillo,D.J. *et al.* Maintenance of long-lived plasma cells and serological memory despite mature and memory B cell depletion during CD20 immunotherapy in mice. *Journal of Immunology* **180**, 361-371 (2008).
113. Dogan,I. *et al.* Multiple layers of B cell memory with different effector functions. *Nature Immunology* **10**, 1292-1299 (2009).
114. Doherty,P.C. Anatomical environment as a determinant in viral immunity. *The Journal of Immunology* **155**, 1023 (1995).
115. Doolan,D.L., Dobano,C. & Baird,J.K. Acquired immunity to malaria. *Clinical Microbiology Reviews* **22**, 13 (2009).
116. Dorfman,J.R. *et al.* B cell memory to 3 *Plasmodium falciparum* blood-stage antigens in a malaria-endemic area. *Journal of Infectious Diseases* **191**, 1623 (2005).
117. Douglas,A.D. *et al.* The blood-stage malaria antigen PfRH5 is susceptible to vaccine-inducible cross-strain neutralizing antibody. *Nature Communications* **2**, 601 (2011).
118. Drakeley,C.J. *et al.* Estimating medium-and long-term trends in malaria transmission by using serological markers of malaria exposure. *Proceedings of the National Academy of Sciences of the United States of America* **102** , 5108 (2005).
119. Edwards,J.C.W. *et al.* Efficacy of B-cell–targeted therapy with rituximab in patients with rheumatoid arthritis. *New England Journal of Medicine* **350**, 2572-2581 (2004).

120. Egan,A., Waterfall,M., Pinder,M., Holder,A. & Riley,E. Characterization of human T-and B-cell epitopes in the C terminus of *Plasmodium falciparum* merozoite surface protein 1: evidence for poor T-cell recognition of polypeptides with numerous disulfide bonds. *Infection and Immunity* **65**, 3024 (1997).
121. Egan,A.F., Burghaus,P., Druilhe,P., Holder,A.A. & Riley,E.M. Human antibodies to the 19 kDa-terminal fragment of *Plasmodium falciparum* merozoite surface protein 1 inhibit parasite growth in vitro. *Parasite Immunology* **21**, 133-139 (1999).
122. Eichelberger,M., Allan,W., Zijlstra,M., Jaenisch,R. & Doherty,P.C. Clearance of influenza virus respiratory infection in mice lacking class I major histocompatibility complex-restricted CD8+ T cells. *The Journal of Experimental Medicine* **174**, 875 (1991).
123. Eichelberger,M.C., Wang,M.L., Allan,W., Webster,R.G. & Doherty,P.C. Influenza virus RNA in the lung and lymphoid tissue of immunologically intact and CD4-depleted mice. *J. Gen. Virol* **72**, 1695-1698 (1991).
124. Elliott,S.R. *et al.* Inhibition of dendritic cell maturation by malaria is dose dependent and does not require *Plasmodium falciparum* erythrocyte membrane protein 1. *Infection and Immunity* **75**, 3621-3632 (2007).
125. Ellyard,J.I., Avery,D.T., Mackay,C.R. & Tangye,S.G. Contribution of stromal cells to the migration, function and retention of plasma cells in human spleen: potential roles of CXCL12, IL-6 and CD54. *European Journal of Immunology* **35**, 699-708 (2005).
126. Erickson,L.D. *et al.* Short-circuiting long-lived humoral immunity by the heightened engagement of CD40. *Journal of Clinical Investigation* **109**, 613-620 (2002).
127. Etchart,N. *et al.* Intranasal immunisation with inactivated RSV and bacterial adjuvants induces mucosal protection and abrogates eosinophilia upon challenge. *European Journal of Immunology* **36**, 1136-1144 (2006).
128. Everly,M.J., Terasaki,P.I., Hopfield,J., Trivedi,H.L. & Kaneku,H. Protective immunity remains intact after antibody removal by means of proteasome inhibition. *Transplantation* **90**, 1493 (2010).
129. Fahey,J.L. & Sell,S. The immunoglobulins of mice. *The Journal of Experimental Medicine* **122**, 41 (1965).
130. Fairfax,K.A., Kallies,A., Nutt,S.L. & Tarlinton,D.M. Plasma cell development: from B-cell subsets to long-term survival niches. 20(1), 49-58. 2008. Elsevier.
Ref Type: Conference Proceeding
131. Fang,Y., Xu,C., Fu,Y.X., Holers,V.M. & Molina,H. Expression of complement receptors 1 and 2 on follicular dendritic cells is necessary for the generation of a strong antigen-specific IgG response. *The Journal of Immunology* **160**, 5273 (1998).

132. Favaloro,E.J. Differential expression of surface antigens on activated endothelium. *Immunology and Cell Biology* **71**, 571 (1993).
133. Fazekas,G., Rosenwirth,B., Dukor,P., Gergely,J. & Rajnavolgyi,E. Igg Isotype Distribution of Local and Systemic Immune-Responses Induced by Influenza-Virus Infection. *European Journal of Immunology* **24**, 3063-3067 (1994).
134. Fazilleau,N., Mark,L., McHeyzer-Williams,L.J. & McHeyzer-Williams,M.G. Follicular helper T cells: lineage and location. *Immunity* **30**, 324-335 (2009).
135. Feng,J.Q. *et al.* Influenza A virus infection engenders a poor antibody response against the ectodomain of matrix protein 2. *Virology Journal* **3**, 102 (2006).
136. Ferreira,M.U., Kimura,E.A.S., De Souza,J.M. & Katzin,A.N.O.A. The isotype composition and avidity of naturally acquired anti-*Plasmodium falciparum* antibodies: differential patterns in clinically immune Africans and Amazonian patients. *Am. J. Trop. Med. Hyg* **55**, 315-323 (1996).
137. Finney,O.C., Riley,E.M. & Walther,M. Regulatory T cells in malaria-friend or foe? *Trends in Immunology* **31**, 63-70 (2010).
138. Flynn,K.J. *et al.* Virus-Specific CD8+ T Cells in Primary and Secondary Influenza Pneumonia. *Immunity* **8**, 683-691 (1998).
139. Fonager,J. *et al.* Reduced CD36-dependent tissue sequestration of *Plasmodium*-infected erythrocytes is detrimental to malaria parasite growth in vivo. *The Journal of Experimental Medicine* **209**, 93-107 (2012).
140. Fonjungo,P.N. *et al.* A Longitudinal Study of Human Antibody Responses to *Plasmodium falciparum* Rhoptry-Associated Protein 1 in a Region of Seasonal and Unstable Malaria Transmission. *Infection and Immunity* **67**, 2975-2985 (1999).
141. Foote,J.B. Polysaccharide specific B cells: A study of their development and function. (2010).
142. Forrest,B.D. *et al.* Correlation of cellular immune responses with protection against culture-confirmed influenza virus in young children. *Clinical and Vaccine Immunology* **15**, 1042-1053 (2008).
143. Fröhlich,A. *et al.* Eosinophils are required for the maintenance of plasma cells in the bone marrow. *Nature Immunology* **12**, 151-159 (2011).
144. Frazer,I.H. Measuring serum antibody to human papillomavirus following infection or vaccination. *Gynecologic Oncology* **118**, S8-S11 (2010).
145. Freeman,R.R. & Parish,C.R. Polyclonal B-cell activation during rodent malarial infections. *Clinical and Experimental Immunology* **32**, 41 (1978).
146. Freeman,R.R., Trejdosiewicz,A.J. & Cross,G.A.M. Protective monoclonal antibodies recognising stage-specific merozoite antigens of a rodent malaria parasite. *Nature* **284**, 366-368 (1980).

147. Freitas do Rosário,A.P. *et al.* Gradual decline in malaria-specific memory T cell responses leads to failure to maintain long-term protective immunity to *Plasmodium chabaudi* AS despite persistence of B cell memory and circulating antibody. *The Journal of Immunology* **181**, 8344 (2008).
148. Frevort,U. *et al.* Malaria circumsporozoite protein binds to heparan sulfate proteoglycans associated with the surface membrane of hepatocytes. *The Journal of Experimental Medicine* **177**, 1287 (1993).
149. Friesen,J. *et al.* Natural immunization against malaria: causal prophylaxis with antibiotics. *Sci. Transl. Med* **2**, 40-49 (2010).
150. Fu,Y.X., Huang,G., Matsumoto,M., Molina,H. & Chaplin,D.D. Independent signals regulate development of primary and secondary follicle structure in spleen and mesenteric lymph node. *Proceedings of the National Academy of Sciences* **94**, 5739 (1997).
151. Gavin,A.L., Barnes,N., Dijstelbloem,H.M. & Hogarth,P.M. Cutting edge: identification of the mouse IgG3 receptor: implications for antibody effector function at the interface between innate and adaptive immunity. *The Journal of Immunology* **160**, 20 (1998).
152. Gerhard,W., Mozdzanowska,K., Furchner,M., Washko,G. & Maiese,K. Role of the B–cell response in recovery of mice from primary influenza virus infection. *Immunological Reviews* **159**, 95-103 (1997).
153. GeurtsvanKessel,C.H. *et al.* Both conventional and interferon killer dendritic cells have antigen-presenting capacity during influenza virus infection. *Plos One* **4**, e7187 (2009).
154. GeurtsvanKessel,C.H. *et al.* Dendritic cells are crucial for maintenance of tertiary lymphoid structures in the lung of influenza virus–infected mice. *The Journal of Experimental Medicine* **206**, 2339 (2009).
155. Giha,H.A. *et al.* Nine-year longitudinal study of antibodies to variant antigens on the surface of *Plasmodium falciparum*-infected erythrocytes. *Infection and Immunity* **67**, 4092 (1999).
156. Gilks,C.F., Walliker,D. & Newbold,C.I. Relationships between sequestration, antigenic variation and chronic parasitism in *Plasmodium chabaudi chabaudi*–a rodent malaria model. *Parasite Immunology* **12**, 45-64 (1990).
157. Gong,Q. *et al.* Importance of cellular microenvironment and circulatory dynamics in B cell immunotherapy. *The Journal of Immunology* **174**, 817 (2005).
158. Good,K.L., Avery,D.T. & Tangye,S.G. Resting human memory B cells are intrinsically programmed for enhanced survival and responsiveness to diverse stimuli compared to naive B cells. *The Journal of Immunology* **182**, 890 (2009).

159. Good,M.F., Xu,H., Wykes,M. & Engwerda,C.R. Development and regulation of cell-mediated immune responses to the blood stages of malaria: implications for vaccine research. *Annu. Rev. Immunol.* **23**, 69-99 (2005).
160. Goronzy,J.J. *et al.* Value of immunological markers in predicting responsiveness to influenza vaccination in elderly individuals. *Journal of Virology* **75**, 12182-12187 (2001).
161. Goumard,P., Vu,D.N., Maurois,P. & Camus,D. Influence of malaria on a pre-existing antibody response to heterologous antigens. 133(3), 313. 1982.
Ref Type: Conference Proceeding
162. Grüner,A.C. *et al.* Sterile protection against malaria is independent of immune responses to the circumsporozoite protein. *Plos One* **2**, e1371 (2007).
163. Gray,D. Immunological memory. *Annual Review of Immunology* **11**, 49-77 (1993).
164. Gray,D. A role for antigen in the maintenance of immunological memory. *Nature Reviews Immunology* **2**, 60-65 (2002).
165. Greenwood,B.M., Palit,A., Bradley-Moore,A.M. & Bryceson,A.D.M. Immunosuppression in children with malaria. *The Lancet* **299**, 169-172 (1972).
166. Greenwood,B.M. Possible role of a B-cell mitogen in hypergammaglobulinaemia in malaria and trypanosomiasis. *The Lancet* **303**, 435-436 (1974).
167. Groux,H. & Gysin,J. Opsonization as an effector mechanism in human protection against asexual blood stages of *Plasmodium falciparum*: functional role of IgG subclasses. *Research in Immunology* **141**, 529-542 (1990).
168. Grun,J.L. & Weidanz,W.P. Immunity to *Plasmodium chabaudi adami* in the B-cell-deficient mouse. *Nature* **290**, 143-145 (1981).
169. Guevara Patiño,J.A., Holder,A.A., McBride,J.S. & Blackman,M.J. Antibodies that inhibit malaria merozoite surface protein-1 processing and erythrocyte invasion are blocked by naturally acquired human antibodies. *The Journal of Experimental Medicine* **186**, 1689 (1997).
170. Gupta,S., Snow,R.W., Donnelly,C.A., Marsh,K. & Newbold,C. Immunity to non-cerebral severe malaria is acquired after one or two infections. *Nature Medicine* **5**, 340-343 (1999).
171. Hafalla,J.C., Silvie,O. & Matuschewski,K. Cell biology and immunology of malaria. *Immunological Reviews* **240**, 297-316 (2011).
172. Hafalla,J.C.R. *et al.* The CTLA-4 and PD-1/PD-L1 Inhibitory Pathways Independently Regulate Host Resistance to *Plasmodium*-induced Acute Immune Pathology. *Plos Pathogens* **8**, e1002504 (2012).

173. Hammarlund,E. *et al.* Duration of antiviral immunity after smallpox vaccination. *Nature Medicine* **9**, 1131-1137 (2003).
174. Han,S. *et al.* Enhanced differentiation of splenic plasma cells but diminished long-lived high-affinity bone marrow plasma cells in aged mice. *The Journal of Immunology* **170**, 1267 (2003).
175. Hannum,L.G., Haberman,A.M., Anderson,S.M. & Shlomchik,M.J. Germinal center initiation, variable gene region hypermutation, and mutant B cell selection without detectable immune complexes on follicular dendritic cells. *The Journal of Experimental Medicine* **192**, 931-942 (2000).
176. Hargreaves,D.C. *et al.* A coordinated change in chemokine responsiveness guides plasma cell movements. *The Journal of Experimental Medicine* **194**, 45 (2001).
177. Hauser,A.E. *et al.* Chemotactic responsiveness toward ligands for CXCR3 and CXCR4 is regulated on plasma blasts during the time course of a memory immune response. *The Journal of Immunology* **169**, 1277 (2002).
178. He,X.S. *et al.* T cell-dependent production of IFN-gamma by NK cells in response to influenza A virus. *J Clin Invest* **114**, 1812-1819 (2004).
179. Helmbj,H., Jönsson,G. & Troye-Blomberg,M. Cellular changes and apoptosis in the spleens and peripheral blood of mice infected with blood-stage *Plasmodium chabaudi chabaudi* AS. *Infection and Immunity* **68**, 1485-1490 (2000).
180. Hendrikx,L.H. *et al.* Identifying long-term memory B-cells in vaccinated children despite waning antibody levels specific for *Bordetella pertussis* proteins. *Vaccine* **29**, 1431-1437 (2010).
181. Herold,S. *et al.* Alveolar epithelial cells direct monocyte transepithelial migration upon influenza virus infection: impact of chemokines and adhesion molecules. *The Journal of Immunology* **177**, 1817 (2006).
182. Ho,M. & Webster,H.K. T cell responses in acute *falciparum* malaria. *Immunology Letters* **25**, 135-138 (1990).
183. Hofer,T. *et al.* Adaptation of humoral memory. *Immunological Reviews* **211**, 295-302 (2006).
184. Hoffman,S.L. *et al.* Protection of humans against malaria by immunization with radiation-attenuated *Plasmodium falciparum* sporozoites. *Journal of Infectious Diseases* **185**, 1155 (2002).
185. Holder,A.A. & Freeman,R.R. Immunization against blood-stage rodent malaria using purified parasite antigens. *Nature* **294**, 361-364 (1981).
186. Holder,A.A. *et al.* Primary structure of the precursor to the three major surface antigens of *Plasmodium falciparum* merozoites. *Nature* **317**, 270-273 (1985).

187. Holland,W.G. *et al.* The effect of *Trypanosoma evansi* infection on pig performance and vaccination against classical swine fever. *Veterinary Parasitology* **111**, 115-123 (2003).
188. Horikawa,K. *et al.* Enhancement and suppression of signaling by the conserved tail of IgG memory-type B cell antigen receptors. *The Journal of Experimental Medicine* **204**, 759 (2007).
189. Horimoto,T. & Kawaoka,Y. Influenza: lessons from past pandemics, warnings from current incidents. *Nature Reviews Microbiology* **3**, 591-600 (2005).
190. Hou,S. & Doherty,P.C. Clearance of Sendai virus by CD8+ T cells requires direct targeting to virus-infected epithelium. *European Journal of Immunology* **25**, 111-116 (1995).
191. Hu,C. *et al.* Treatment with CD20-specific antibody prevents and reverses autoimmune diabetes in mice. *The Journal of Clinical Investigation* **117**, 3857 (2007).
192. Hu,C., Wong,F.S. & Wen,L. Translational Mini-Review Series on B Cell-Directed Therapies: B cell-directed therapy for autoimmune diseases. *Clinical & Experimental Immunology* **157**, 181-190 (2009).
193. Hu,C.C.A., Dougan,S.K., McGehee,A.M., Love,J.C. & Ploegh,H.L. XBP-1 regulates signal transduction, transcription factors and bone marrow colonization in B cells. *The EMBO journal* **28**, 1624-1636 (2009).
194. Huang,H., Benoist,C. & Mathis,D. Rituximab specifically depletes short-lived autoreactive plasma cells in a mouse model of inflammatory arthritis. *Proceedings of the National Academy of Sciences* **107**, 4658 (2010).
195. Huang,K.H.G. *et al.* B-cell depletion reveals a role for antibodies in the control of chronic HIV-1 infection. *Nature Communications* **1**, 102 (2010).
196. Huard,B. *et al.* APRIL secreted by neutrophils binds to heparan sulfate proteoglycans to create plasma cell niches in human mucosa. *The Journal of Clinical Investigation* **118**, 2887 (2008).
197. Hyland,L., Sangster,M., Sealy,R. & Coleclough,C. Respiratory virus infection of mice provokes a permanent humoral immune response. *Journal of Virology* **68**, 6083 (1994).
198. Ingold,K. *et al.* Identification of proteoglycans as the APRIL-specific binding partners. *The Journal of Experimental Medicine* **201**, 1375 (2005).
199. Iwakoshi,N.N. *et al.* Plasma cell differentiation and the unfolded protein response intersect at the transcription factor XBP-1. *Nature Immunology* **4**, 321-329 (2003).

200. Jafarshad,A. *et al.* A novel antibody-dependent cellular cytotoxicity mechanism involved in defense against malaria requires costimulation of monocytes Fc γ RII and Fc γ RIII. *The Journal of Immunology* **178**, 3099 (2007).
201. Jakobsen,P.H. *et al.* Anti-phospholipid antibodies in patients with *Plasmodium falciparum* malaria. *Immunology* **79**, 653 (1993).
202. Jarra,W. Studies on the induction and expression of protective immunity in rodent malaria *Plasmodium b. berghei* and *Pc chabaudi* infections of inbred mice. (1982).
203. Jayawardena,A.N., Targett,G.A., Leuchars,E. & Davies,A.J. The immunological response of CBA mice to *P. yoelii*. II. The passive transfer of immunity with serum and cells. *Immunology* **34**, 157 (1978).
204. Jegerlehner,A., Schmitz,N., Storni,T. & Bachmann,M.F. Influenza A vaccine based on the extracellular domain of M2: weak protection mediated via antibody-dependent NK cell activity. *The Journal of Immunology* **172**, 5598 (2004).
205. Jennings,R.M. *et al.* Imported *Plasmodium falciparum* malaria: are patients originating from disease-endemic areas less likely to develop severe disease? A prospective, observational study. *The American Journal of Tropical Medicine and Hygiene* **75**, 1195 (2006).
206. Jones,P.D. & Ada,G.L. Influenza virus-specific antibody-secreting cells in the murine lung during primary influenza virus infection. *Journal of Virology* **60**, 614 (1986).
207. Jones,P.D. & Ada,G.L. Persistence of influenza virus-specific antibody-secreting cells and B-cell memory after primary murine influenza virus infection. *Cellular Immunology* **109**, 53-64 (1987).
208. Joo,H.M., He,Y. & Sangster,M.Y. Broad dispersion and lung localization of virus-specific memory B cells induced by influenza pneumonia. *Proceedings of the National Academy of Sciences* **105**, 3485 (2008).
209. June,C.H., Contreras,C.E., Perrin,L.H., Lambert,P.H. & Miescher,P.A. Circulating and tissue-bound immune complex formation in murine malaria. *The Journal of Immunology* **122**, 2154 (1979).
210. Kafuko,G.W. & Burkitt,D.P. Burkitt's lymphoma and malaria. *International Journal of Cancer* **6**, 1-9 (1970).
211. Kallies,A. *et al.* Plasma cell ontogeny defined by quantitative changes in blimp-1 expression. *The Journal of Experimental Medicine* **200**, 967 (2004).
212. Kallies,A. *et al.* Initiation of plasma-cell differentiation is independent of the transcription factor Blimp-1. *Immunity* **26**, 555-566 (2007).

213. Kassiotis,G., Gray,D., Kiafard,Z., Zwirner,J. & Stockinger,B. Functional specialization of memory Th cells revealed by expression of integrin CD49b. *The Journal of Immunology* **177**, 968 (2006).
214. Kasturi,S.P. *et al.* Programming the magnitude and persistence of antibody responses with innate immunity. *Nature* **470**, 543-U136 (2011).
215. Kesmir,C. & De Boer,R.J. A mathematical model on germinal center kinetics and termination. *The Journal of Immunology* **163**, 2463 (1999).
217. Keystone,E. *et al.* Safety and efficacy of additional courses of rituximab in patients with active rheumatoid arthritis: An open-label extension analysis. *Arthritis & Rheumatism* **56**, 3896-3908 (2007).
218. Kim,S.K. & Welsh,R.M. Comprehensive early and lasting loss of memory CD8 T cells and functional memory during acute and persistent viral infections. *The Journal of Immunology* **172**, 3139-3150 (2004).
219. Kinyanjui,S.M., Conway,D.J., Lanar,D.E. & Marsh,K. IgG antibody responses to *Plasmodium falciparum* merozoite antigens in Kenyan children have a short half-life. *Malar J* **6**, 82 (2007).
220. Kitano,M. *et al.* Bcl6 protein expression shapes pre-germinal center B cell dynamics and follicular helper T cell heterogeneity. *Immunity* **34**, 961-972 (2011).
221. Klein,U., Rajewsky,K. & Küppers,R. Human immunoglobulin (Ig) M+ IgD+ peripheral blood B cells expressing the CD27 cell surface antigen carry somatically mutated variable region genes: CD27 as a general marker for somatically mutated (memory) B cells. *The Journal of Experimental Medicine* **188**, 1679-1689 (1998).
222. Klein,U. *et al.* Transcription factor IRF4 controls plasma cell differentiation and class-switch recombination. *Nature Immunology* **7**, 773-782 (2006).
223. Klotz,C. & Frevert,U. *Plasmodium yoelii* sporozoites modulate cytokine profile and induce apoptosis in murine Kupffer cells. *International Journal for Parasitology* **38**, 1639-1650 (2008).
224. Koni,P.A. *et al.* Distinct roles in lymphoid organogenesis for lymphotoxins [alpha] and [beta] revealed in lymphotoxin [beta]-deficient mice. *Immunity* **6**, 491-500 (1997).
225. Kumagai,Y. *et al.* Alveolar Macrophages Are the Primary Interferon-[alpha] Producer in Pulmonary Infection with RNA Viruses. *Immunity* **27**, 240-252 (2007).
226. Kumar,K.A., Baxter,P., Tarun,A.S., Kappe,S.H.I. & Nussenzweig,V. Conserved protective mechanisms in radiation and genetically attenuated uis3 (-) and uis4 (-) *Plasmodium* sporozoites. *Plos One* **4**, e4480 (2009).
227. Kumararatne,D.S., Phillips,R.S., Sinclair,D., Parrott,M.V. & Forrester,J.B. Lymphocyte migration in murine malaria during the primary patent parasitaemia of

- Plasmodium chabaudi* infections. *Clinical and Experimental Immunology* **68**, 65 (1987).
228. Kurosaki,T., Aiba,Y., Kometani,K., Moriyama,S. & Takahashi,Y. Unique properties of memory B cells of different isotypes. *Immunological Reviews* **237**, 104-116 (2010).
229. Kurtzhals,J.A.L. *et al.* Reversible suppression of bone marrow response to erythropoietin in *Plasmodium falciparum* malaria. *British Journal of Haematology* **97**, 169-174 (1997).
230. Kurtzhals,J.A.L. *et al.* Low plasma concentrations of interleukin 10 in severe malarial anaemia compared with cerebral and uncomplicated malaria. *The Lancet* **351**, 1768-1772 (1998).
231. Lalvani,A. *et al.* Rapid effector function in CD8+ memory T cells. *The Journal of Experimental Medicine* **186**, 859 (1997).
232. Lamb,T.J., Brown,D.E., Potocnik,A.J. & Langhorne,J. Insights into the immunopathogenesis of malaria using mouse models. *Expert Reviews in Molecular Medicine* **8**, 1-22 (2006).
233. Langhorne,J., Evans,C.B., Asofsky,R. & Taylor,D.W. Immunoglobulin isotype distribution of malaria-specific antibodies produced during infection with *Plasmodium chabaudi adami* and *Plasmodium yoelii*. *Cellular Immunology* **87**, 452-461 (1984).
234. Langhorne,J., Kim,K.J. & Asofsky,R. Distribution of immunoglobulin isotypes in the nonspecific B-cell response induced by infection with *Plasmodium chabaudi adami* and *Plasmodium yoelii*. *Cellular Immunology* **90**, 251-257 (1985).
235. Langhorne,J., Gillard,S., Simon,B., Slade,S. & Eichmann,K. Frequencies of CD4+ T cells reactive with *Plasmodium chabaudi chabaudi*: distinct response kinetics for cells with Th1 and Th2 characteristics during infection. *International Immunology* **1**, 416 (1989).
236. Langhorne,J., Cross,C., Seixas,E., Li,C. & Der Weid,T. A role for B cells in the development of T cell helper function in a malaria infection in mice. *Proceedings of the National Academy of Sciences* **95**, 1730 (1998).
237. Langhorne,J., Quin,S.J. & Sanni,L.A. Mouse models of blood-stage malaria infections: immune responses and cytokines involved in protection and pathology. *Chem. Immunol* **80**, 204-228 (2002).
238. Langhorne,J. *et al.* Dendritic cells, pro-inflammatory responses, and antigen presentation in a rodent malaria infection. *Immunological Reviews* **201**, 35-47 (2004).
239. Langhorne,J., Ndungu,F.M., Sponaas,A.M. & Marsh,K. Immunity to malaria: more questions than answers. *Nature Immunology* **9**, 725-732 (2008).

240. Lanzavecchia,A. *et al.* Understanding and making use of human memory B cells. *Immunological Reviews* **211**, 303-309 (2006).
241. Lawrence,C.W. & Braciale,T.J. Activation, differentiation, and migration of naive virus-specific CD8+ T cells during pulmonary influenza virus infection. *The Journal of Immunology* **173**, 1209 (2004).
242. Le Goffic,R. *et al.* Cutting Edge: Influenza A virus activates TLR3-dependent inflammatory and RIG-I-dependent antiviral responses in human lung epithelial cells. *The Journal of Immunology* **178**, 3368 (2007).
243. Lee,K.S. *et al.* *Plasmodium knowlesi*: reservoir hosts and tracking the emergence in humans and macaques. *Plos Pathogens* **7**, e1002015 (2011).
244. Leyendeckers,H. *et al.* Correlation analysis between frequencies of circulating antigen specific IgG bearing memory B cells and serum titers of antigen specific IgG. *European Journal of Immunology* **29**, 1406-1417 (1999).
245. Liang,B., Hyland,L. & Hou,S. Nasal-associated lymphoid tissue is a site of long-term virus-specific antibody production following respiratory virus infection of mice. *Journal of Virology* **75**, 5416-5420 (2001).
246. Lim,K.J. *et al.* Humoral responses against the C-terminal region of merozoite surface protein 1 can be remembered for more than 30 years in persons exposed to *Plasmodium vivax*. *Parasitology Research* **92**, 384-389 (2004).
247. Lim,S.H. *et al.* B-cell depletion for 2 years after autologous stem cell transplant for NHL induces prolonged hypogammaglobulinemia beyond the rituximab maintenance period. *Leukemia & Lymphoma* **49**, 152-153 (2008).
248. Ling,I.T., Ogun,S.A. & Holder,A.A. Immunization against malaria with a recombinant protein. *Parasite Immunology* **16**, 63-67 (1994).
249. Linterman,M.A. *et al.* IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses. *The Journal of Experimental Medicine* **207**, 353-363 (2010).
250. Looareesuwan,S. *et al.* Dynamic alteration in splenic function during acute falciparum malaria. *New England Journal of Medicine* **317**, 675-679 (1987).
251. Lyke,K.E. *et al.* Serum Levels of the Proinflammatory Cytokines Interleukin-1 Beta (IL-1 {beta}), IL-6, IL-8, IL-10, Tumor Necrosis Factor Alpha, and IL-12 (p70) in Malian Children with Severe *Plasmodium falciparum* Malaria and Matched Uncomplicated Malaria or Healthy Controls. *Infection and Immunity* **72**, 5630 (2004).
252. Macallan,D.C. *et al.* B-cell kinetics in humans: rapid turnover of peripheral blood memory cells. *Blood* **105**, 3633-3640 (2005).

253. MacLennan,I.C.M. Germinal centers. *Annual Review of Immunology* **12**, 117-139 (1994).
254. MacPherson,G.G., Warrell,M.J., White,N.J., Looareesuwan,S. & Warrell,D.A. Human cerebral malaria. A quantitative ultrastructural analysis of parasitized erythrocyte sequestration. *The American Journal of Pathology* **119**, 385 (1985).
255. Maggio-Price,L., Brookoff,D. & Weiss,L. Changes in hematopoietic stem cells in bone marrow of mice with *Plasmodium berghei* malaria. *Blood* **66**, 1080-1085 (1985).
256. Maloney,D.G. *et al.* IDEC-C2B8 (Rituximab) anti-CD20 monoclonal antibody therapy in patients with relapsed low-grade non-Hodgkin's lymphoma. *Blood* **90**, 2188-2195 (1997).
257. Mamani-Matsuda,M. *et al.* The human spleen is a major reservoir for long-lived vaccinia virus–specific memory B cells. *Blood* **111**, 4653 (2008).
258. Mandelboim,O. *et al.* Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. *Nature* **409**, 1055-1060 (2001).
259. Manicassamy,B. *et al.* Analysis of in vivo dynamics of influenza virus infection in mice using a GFP reporter virus. *Proceedings of the National Academy of Sciences* **107**, 11531 (2010).
260. Manz,R.A., Thiel,A. & Radbruch,A. Lifetime of plasma cells in the bone marrow. *Nature* **388**, 133-134 (1997).
261. Manz,R.A., Lohning,M., Cassese,G., Thiel,A. & Radbruch,A. Survival of long-lived plasma cells is independent of antigen. *International Immunology* **10**, 1703-1711 (1998).
262. Manz,R.A., Hauser,A.E., Hiepe,F. & Radbruch,A. Maintenance of serum antibody levels. *Annu. Rev. Immunol.* **23**, 367-386 (2005).
263. Markwell,M.A. & Paulson,J.C. Sendai virus utilizes specific sialyloligosaccharides as host cell receptor determinants. *Proceedings of the National Academy of Sciences* **77**, 5693 (1980).
264. Marsh,K. & Kinyanjui,S. Immune effector mechanisms in malaria. *Parasite Immunology* **28**, 51-60 (2006).
265. Maruyama,M., Lam,K.P. & Rajewsky,K. Memory B-cell persistence is independent of persisting immunizing antigen. *Nature* **407**, 636-642 (2000).
266. Matsumoto,M. *et al.* Role of lymphotoxin and the type I TNF receptor in the formation of germinal centers. *Science* **271**, 1289 (1996).
267. Mauad,T. *et al.* Lung pathology in fatal novel human influenza A (H1N1) infection. *American Journal of Respiratory and Critical Care Medicine* **181**, 72 (2010).

268. McHeyzer-Williams,L.J. & McHeyzer-Williams,M.G. Antigen-specific memory B cell development. *Annu. Rev. Immunol.* **23**, 487-513 (2005).
269. McHeyzer-Williams,M., Okitsu,S., Wang,N. & McHeyzer-Williams,L. Molecular programming of B cell memory. *Nature Reviews Immunology* **12**, 24-34 (2011).
270. McIntosh,R.S. *et al.* The importance of human Fc γ RI in mediating protection to malaria. *Plos Pathogens* **3**, e72 (2007).
271. McLaughlin,P. *et al.* Rituximab chimeric anti-CD20 monoclonal antibody therapy for relapsed indolent lymphoma: half of patients respond to a four-dose treatment program. *Journal of Clinical Oncology* **16**, 2825-2833 (1998).
272. McLean,S.A., Pearson,C.D. & Phillips,R.S. *Plasmodium chabaudi*: antigenic variation during recrudescence parasitaemias in mice. *Experimental Parasitology* **54**, 296-302 (1982).
273. Meding,S.J., Cheng,S.C., Simonhaarhaus,B. & Langhorne,J. Role of Gamma Interferon During Infection with *Plasmodium-Chabaudi-Chabaudi*. *Infection and Immunity* **58**, 3671-3678 (1990).
274. Meyer-Bahlburg,A., Khim,S. & Rawlings,D.J. B cell-intrinsic TLR signals amplify but are not required for humoral immunity. *The Journal of Experimental Medicine* **204**, 3095 (2007).
275. Migot,F. *et al.* Anti-malaria antibody-producing B cell frequencies in adults after a *Plasmodium falciparum* outbreak in Madagascar. *Clinical & Experimental Immunology* **102**, 529-534 (1995).
276. Miller,L.H., Baruch,D.I., Marsh,K. & Doumbo,O.K. The pathogenic basis of malaria. *Nature* **415**, 673-679 (2002).
277. Millington,O., Caterina Di Lorenzo,R.P., Garside,P. & Brewer,J. Suppression of adaptive immunity to heterologous antigens during *Plasmodium* infection through hemozoin-induced failure of dendritic cell function. *Journal of Biology* **5**, 5 (2006).
278. Milpied,N. *et al.* Humanized anti-CD20 monoclonal antibody (Rituximab) in post transplant B-lymphoproliferative disorder: a retrospective analysis on 32 patients. *Annals of Oncology* **11**, S113 (2000).
279. Minoprio,P.M., Coutinho,A., Joskowicz,M., D'Imperio Lima,M.R. & Eisen,H. Polyclonal lymphocyte responses to murine *Trypanosoma cruzi* infection. *Scandinavian Journal of Immunology* **24**, 669-679 (1986).
280. Moens,L., Wuyts,M., Meyts,I., De Boeck,K. & Bossuyt,X. Human memory B lymphocyte subsets fulfill distinct roles in the anti-polysaccharide and anti-protein immune response. *The Journal of Immunology* **181**, 5306 (2008).
281. Mohr,E. *et al.* IFN- γ produced by CD8 T cells induces T-bet-dependent and -independent class switching in B cells in responses to alum-precipitated protein

- vaccine. *Proceedings of the National Academy of Sciences* **107**, 17292-17297 (2010).
282. Moisini,I. & Davidson,A. BAFF: a local and systemic target in autoimmune diseases. *Clinical & Experimental Immunology* **158**, 155-163 (2009).
283. Mori,H., Natarajan,K., Betschart,B., Weiss,N. & Franklin,R.M. Polyclonal B-cell activation and autoantibody formation during the course of mosquito-transmitted *Plasmodium berghei* infection in mice. *Tropical Medicine and Parasitology: official organ of Deutsche Tropenmedizinische Gesellschaft and of Deutsche Gesellschaft fur Technische Zusammenarbeit (GTZ)* **38**, 157 (1987).
284. Moser,K., Tokoyoda,K., Radbruch,A., MacLennan,I. & Manz,R.A. Stromal niches, plasma cell differentiation and survival. *Current Opinion in Immunology* **18**, 265-270 (2006).
285. Mota,M.M., Jarra,W., Hirst,E., Patnaik,P.K. & Holder,A.A. *Plasmodium chabaudi*-infected erythrocytes adhere to CD36 and bind to microvascular endothelial cells in an organ-specific way. *Infection and Immunity* **68**, 4135 (2000).
286. Mota,M.M. *et al.* Migration of *Plasmodium* sporozoites through cells before infection. *Science* **291**, 141 (2001).
287. Moyron-Quiroz,J.E. *et al.* Role of inducible bronchus associated lymphoid tissue (iBALT) in respiratory immunity. *Nature Medicine* **10**, 927-934 (2004).
288. Muehlinghaus,G. *et al.* Regulation of CXCR3 and CXCR4 expression during terminal differentiation of memory B cells into plasma cells. *Blood* **105**, 3965-3971 (2005).
289. Mueller,A.K., Labaied,M., Kappe,S.H.I. & Matuschewski,K. Genetically modified *Plasmodium* parasites as a protective experimental malaria vaccine. *Nature* **433**, 164-167 (2004).
290. Mueller,A.K. *et al.* Genetically attenuated *Plasmodium berghei* liver stages persist and elicit sterile protection primarily via CD8 T cells. *The American Journal of Pathology* **171**, 107 (2007).
291. Murali-Krishna,K. *et al.* Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. *Immunity* **8**, 177-187 (1998).
292. Murali-Krishna,K. *et al.* Persistence of memory CD8 T cells in MHC class I-deficient mice. *Science* **286**, 1377 (1999).
293. Muramatsu,M. *et al.* Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell* **102**, 553-563 (2000).
294. Mwangi,D.M., Munyua,W.K. & Nyaga,P.N. Immunosuppression in caprine trypanosomiasis: Effects of acute *Trypanosoma congolense* infection on antibody

- response to anthrax spore vaccine. *Tropical Animal Health and Production* **22**, 95-100 (1990).
295. Nanan,R., Heinrich,D., Frosch,M. & Kreth,H.W. Acute and long-term effects of booster immunisation on frequencies of antigen-specific memory B-lymphocytes. *Vaccine* **20**, 498-504 (2001).
296. Nduati,E. *et al.* The Plasma Concentration of the B Cell Activating Factor Is Increased in Children With Acute Malaria. *Journal of Infectious Diseases* **204**, 962-970 (2011).
297. Nduati,E.W. *et al.* Distinct Kinetics of Memory B-Cell and Plasma-Cell Responses in Peripheral Blood Following a Blood-Stage *Plasmodium chabaudi* Infection in Mice. *Plos One* **5**, (2010).
298. Ndungu,F.M. *et al.* Functional Memory B Cells and Long-Lived Plasma Cells Are Generated after a Single *Plasmodium chabaudi* Infection in Mice. *Plos Pathogens* **5**, (2009).
299. Nimmerjahn,F., Bruhns,P., Horiuchi,K. & Ravetch,J.V. Fc [gamma] RIV: a novel FcR with distinct IgG subclass specificity. *Immunity* **23**, 41-51 (2005).
300. Nimmerjahn,F. & Ravetch,J.V. Fc γ receptors as regulators of immune responses. *Nature Reviews Immunology* **8**, 34-47 (2008).
301. Nishio,M. *et al.* Hypogammaglobulinemia with a selective delayed recovery in memory B cells and an impaired isotype expression after rituximab administration as an adjuvant to autologous stem cell transplantation for non-Hodgkin lymphoma. *European Journal of Haematology* **77**, 226-232 (2006).
302. Nossal,G.J.V., Abbot,A. & Mitchell,J. Antigens in immunity. *The Journal of Experimental Medicine* **127**, 263 (1968).
303. Novak,E.J., Liu,A.W., Nepom,G.T. & Kwok,W.W. MHC class II tetramers identify peptide-specific human CD4⁺ T cells proliferating in response to influenza A antigen. *Journal of Clinical Investigation* **104**, R63 (1999).
304. Nurieva,R.I. *et al.* Bcl6 mediates the development of T follicular helper cells. *Science* **325**, 1001 (2009).
305. Nussenzweig,R.S., Vanderberg,J.P., Most,H. & Orton,C. Specificity of protective immunity produced by X-irradiated *Plasmodium berghei* sporozoites. *Nature* **222**, 488-489 (1969).
306. O'Brien,J., Wilson,I., Orton,T. & Pognan,F. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *European Journal of Biochemistry* **267**, 5421-5426 (2000).

307. O'Brien,K.L., Hochman,M. & Goldblatt,D. Combined schedules of pneumococcal conjugate and polysaccharide vaccines: is hyporesponsiveness an issue? *The Lancet Infectious Diseases* **7**, 597-606 (2007).
308. O'Connor,B.P., Cascalho,M. & Noelle,R.J. Short-lived and long-lived bone marrow plasma cells are derived from a novel precursor population. *The Journal of Experimental Medicine* **195**, 737 (2002).
309. O'Connor,B.P. *et al.* BCMA is essential for the survival of long-lived bone marrow plasma cells. *The Journal of Experimental Medicine* **199** , 91 (2004).
310. Obukhanych,T.V. & Nussenzweig,M.C. T-independent type II immune responses generate memory B cells. *The Journal of Experimental Medicine* **203**, 305 (2006).
311. Odendahl,M. *et al.* Generation of migratory antigen-specific plasma blasts and mobilization of resident plasma cells in a secondary immune response. *Blood* **105**, 1614 (2005).
312. Odumade,O.A. *et al.* Primary Epstein-Barr virus infection does not erode preexisting CD8+ T cell memory in humans. *The Journal of Experimental Medicine* **209**, 471-478 (2012).
313. Omer,F.M., de Souza,J.B. & Riley,E.M. Differential induction of TGF- α regulates proinflammatory cytokine production and determines the outcome of lethal and nonlethal *Plasmodium yoelii* infections. *The Journal of Immunology* **171**, 5430 (2003).
314. Omi,K. *et al.* Fc γ receptor IIA and IIIB polymorphisms are associated with susceptibility to cerebral malaria. *Parasitology International* **51**, 361-366 (2002).
315. Onodera,T. *et al.* Memory B cells in the lung participate in protective humoral immune responses to pulmonary influenza virus reinfection. *Proceedings of the National Academy of Sciences* **109**, 2485-2490 (2012).
316. Oprea,M. & Perelson,A.S. Somatic mutation leads to efficient affinity maturation when centrocytes recycle back to centroblasts. *The Journal of Immunology* **158**, 5155 (1997).
317. Othoro,C. *et al.* A low interleukin-10 tumor necrosis factor- α ratio is associated with malaria anemia in children residing in a holoendemic malaria region in western Kenya. *Journal of Infectious Diseases* **179**, 279 (1999).
318. Owusu-Agyei,S. *et al.* Incidence of symptomatic and asymptomatic *Plasmodium falciparum* infection following curative therapy in adult residents of northern Ghana. *The American Journal of Tropical Medicine and Hygiene* **65**, 197-203 (2001).
319. Ozaki,K. *et al.* A critical role for IL-21 in regulating immunoglobulin production. *Science* **298**, 1630 (2002).

320. Ozaki,K. *et al.* Regulation of B cell differentiation and plasma cell generation by IL-21, a novel inducer of Blimp-1 and Bcl-6. *The Journal of Immunology* **173**, 5361 (2004).
321. Palframan,R.T., Collins,P.D., Williams,T.J. & Rankin,S.M. Eotaxin induces a rapid release of eosinophils and their progenitors from the bone marrow. *Blood* **91**, 2240-2248 (1998).
322. Palladino,G., Mozdzanowska,K., Washko,G. & Gerhard,W. Virus-neutralizing antibodies of immunoglobulin G (IgG) but not of IgM or IgA isotypes can cure influenza virus pneumonia in SCID mice. *Journal of Virology* **69**, 2075 (1995).
323. Pang,I.K. & Iwasaki,A. Inflammasomes as mediators of immunity against influenza virus. *Trends in Immunology* **32**, 34-41 (2010).
324. Panum,P.L., Petersen,J.J. & Dimot,J. Observations made during the epidemic of measles on the Faroe Islands in the year 1846. Delta Omega Society, (1940).
325. Pape,K.A. *et al.* Visualization of the genesis and fate of isotype-switched B cells during a primary immune response. *The Journal of Experimental Medicine* **197**, 1677 (2003).
326. Pape,K.A., Catron,D.M., Itano,A.A. & Jenkins,M.K. The humoral immune response is initiated in lymph nodes by B cells that acquire soluble antigen directly in the follicles. *Immunity* **26**, 491-502 (2007).
327. Pape,K.A., Taylor,J.J., Maul,R.W., Gearhart,P.J. & Jenkins,M.K. Different B cell populations mediate early and late memory during an endogenous immune response. *Science* **331**, 1203 (2011).
328. Park,S.R. *et al.* HoxC4 binds to the promoter of the cytidine deaminase AID gene to induce AID expression, class-switch DNA recombination and somatic hypermutation. *Nature Immunology* **10**, 540-550 (2009).
329. Paul,J.R., Riordan,J.T. & Melnick,J.L. Antibodies to three different antigenic types of poliomyelitis virus in sera from North Alaskan Eskimos. *American Journal of Epidemiology* **54**, 275 (1951).
330. Paus,D. *et al.* Antigen recognition strength regulates the choice between extrafollicular plasma cell and germinal center B cell differentiation. *The Journal of Experimental Medicine* **203**, 1081 (2006).
331. Paust,S. & von Andrian,U.H. Natural killer cell memory. *Nature Immunology* **12**, 500-508 (2011).
332. Peng,S.L., Szabo,S.J. & Glimcher,L.H. T-bet regulates IgG class switching and pathogenic autoantibody production. *Proceedings of the National Academy of Sciences* **99**, 5545 (2002).

333. Pepper,M. & Jenkins,M.K. Origins of CD4+ effector and central memory T cells. *Nature Immunology* **131**, 467-471 (2011).
334. Pereira,J.P., Kelly,L.M., Xu,Y. & Cyster,J.G. EB12 mediates B cell segregation between the outer and centre follicle. *Nature* **460**, 1122-1126 (2009).
335. Phan,T.G. *et al.* High affinity germinal center B cells are actively selected into the plasma cell compartment. *The Journal of Experimental Medicine* **203**, 2419 (2006).
336. Phan,T.G., Green,J.A., Gray,E.E., Xu,Y. & Cyster,J.G. Immune complex relay by subcapsular sinus macrophages and noncognate B cells drives antibody affinity maturation. *Nature Immunology* **10**, 786-793 (2009).
337. Phillips,R.S., Brannan,L.R., Balmer,P. & Neuville,P. Antigenic variation during malaria infection—the contribution from the murine parasite *Plasmodium chabaudi*. *Parasite Immunology* **19**, 427-434 (1997).
338. Pichlmair,A. *et al.* RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. *Science* **314**, 997 (2006).
339. Piqueras,B., Connolly,J., Freitas,H., Palucka,A.K. & Banchereau,J. Upon viral exposure, myeloid and plasmacytoid dendritic cells produce 3 waves of distinct chemokines to recruit immune effectors. *Blood* **107**, 2613 (2006).
340. Pleass,R.J. *et al.* Novel antimalarial antibodies highlight the importance of the antibody Fc region in mediating protection. *Blood* **102**, 4424-4430 (2003).
341. Plebanski,M. *et al.* Direct processing and presentation of antigen from malaria sporozoites by professional antigen-presenting cells in the induction of CD8+ T-cell responses. *Immunology and Cell Biology* **83**, 307-312 (2005).
342. Prakash,D. *et al.* Clusters of cytokines determine malaria severity in *Plasmodium falciparum*-infected patients from endemic areas of Central India. *Journal of Infectious Diseases* **194**, 198 (2006).
343. Pulendran,B. Modulating vaccine responses with dendritic cells and Toll-like receptors. *Immunological Reviews* **199**, 227-250 (2004).
344. Pulendran,B. & Ahmed,R. Translating innate immunity into immunological memory: implications for vaccine development. *Cell* **124**, 849-863 (2006).
345. Pulendran,B. Learning immunology from the yellow fever vaccine: innate immunity to systems vaccinology. *Nature Reviews Immunology* **9**, 741-747 (2009).
346. Pulendran,B. & Ahmed,R. Immunological mechanisms of vaccination. *Nature Immunology* **12**, 509-517 (2011).
347. Purtha,W.E., Tedder,T.F., Johnson,S., Bhattacharya,D. & Diamond,M.S. Memory B cells, but not long-lived plasma cells, possess antigen specificities for viral escape mutants. *The Journal of Experimental Medicine* **208**, 2599-2606 (2011).

348. Putrianti,E.D., Silvie,O., Kordes,M., Borrmann,S. & Matuschewski,K. Vaccine-like immunity against malaria by repeated causal-prophylactic treatment of liver-stage Plasmodium parasites. *Journal of Infectious Diseases* **199**, 899 (2009).
349. Qi,H., Egen,J.G., Huang,A.Y.C. & Germain,R.N. Extrafollicular activation of lymph node B cells by antigen-bearing dendritic cells. *Science* **312**, 1672 (2006).
350. Qi,H., Cannons,J.L., Klauschen,F., Schwartzberg,P.L. & Germain,R.N. SAP-controlled T–B cell interactions underlie germinal centre formation. *Nature* **455**, 764-769 (2008).
351. Quin,S.J. & Langhorne,J. Different regions of the malaria merozoite surface protein 1 of *Plasmodium chabaudi* elicit distinct T-cell and antibody isotype responses. *Infection and Immunity* **69**, 2245 (2001).
352. Radbruch,A. *et al.* Competence and competition: the challenge of becoming a long-lived plasma cell. *Nature Reviews Immunology* **6**, 741-750 (2006).
353. Radwanska,M. *et al.* Trypanosomiasis-induced B cell apoptosis results in loss of protective anti-parasite antibody responses and abolishment of vaccine-induced memory responses. *Plos Pathogens* **4**, e1000078 (2008).
354. Randall,K.L. *et al.* Dock8 mutations cripple B cell immunological synapses, germinal centers and long-lived antibody production. *Nature Immunology* **10**, 1283-1291 (2009).
355. Rangel-Moreno,J. *et al.* Role of CXC chemokine ligand 13, CC chemokine ligand (CCL) 19, and CCL21 in the organization and function of nasal-associated lymphoid tissue. *The Journal of Immunology* **175**, 4904 (2005).
356. Rankin,A.L. *et al.* IL-21 receptor is critical for the development of memory B cell responses. *The Journal of Immunology* **186**, 667 (2011).
357. Ranuncolo,S.M. *et al.* Bcl-6 mediates the germinal center B cell phenotype and lymphomagenesis through transcriptional repression of the DNA-damage sensor ATR. *Nature Immunology* **8**, 705-714 (2007).
358. Rastetter,W., Molina,A. & White,C.A. Rituximab: expanding role in therapy for lymphomas and autoimmune diseases. *Annu. Rev. Med.* **55**, 477-503 (2004).
359. Ravetch,J.V. & Kinet,J.P. Fc receptors. *Annual Review of Immunology* **9**, 457-492 (1991).
360. Ravetch,J.V. & Bolland,S. Igg fc receptors. *Annual Review of Immunology* **19**, 275-290 (2001).
361. Reff,M.E. *et al.* Depletion of B cells in vivo by a chimeric mouse human monoclonal antibody to CD20. *Blood* **83**, 435 (1994).

362. Reimold,A.M. *et al.* Plasma cell differentiation requires the transcription factor XBP-1. *Nature* **412**, 300-307 (2001).
363. Reinhardt,R.L., Liang,H.E. & Locksley,R.M. Cytokine-secreting follicular T cells shape the antibody repertoire. *Nature Immunology* **10**, 385-393 (2009).
364. Reis e Sousa, Sher,A. & Kaye,P. The role of dendritic cells in the induction and regulation of immunity to microbial infection. *Current Opinion in Immunology* **11**, 392-399 (1999).
365. Renegar,K.B. & Small Jr,P.A. Immunoglobulin A mediation of murine nasal anti-influenza virus immunity. *Journal of Virology* **65**, 2146 (1991).
366. Ridderstad,A. & Tarlinton,D.M. Kinetics of establishing the memory B cell population as revealed by CD38 expression. *The Journal of Immunology* **160**, 4688 (1998).
367. Robinson,L.J. *et al.* Cellular tumor necrosis factor, gamma interferon, and interleukin-6 responses as correlates of immunity and risk of clinical *Plasmodium falciparum* malaria in children from Papua New Guinea. *Infection and Immunity* **77**, 3033 (2009).
368. Rodriguez,A. *et al.* Requirement of bic/microRNA-155 for normal immune function. *Science* **316**, 608 (2007).
369. Roestenberg,M. *et al.* Protection against a malaria challenge by sporozoite inoculation. *New England Journal of Medicine* **361**, 468-477 (2009).
370. Rogers,N.J., Hall,B.S., Obiero,J., Targett,G.A.T. & Sutherland,C.J. A model for sequestration of the transmission stages of *Plasmodium falciparum*: adhesion of gametocyte-infected erythrocytes to human bone marrow cells. *Infection and Immunity* **68**, 3455 (2000).
371. Roland,J. *et al.* NK cell responses to *Plasmodium* infection and control of intrahepatic parasite development. *The Journal of Immunology* **177**, 1229 (2006).
372. Rom+ín,E. *et al.* CD4 Effector T Cell Subsets in the Response to Influenza. *The Journal of Experimental Medicine* **196**, 957-968 (2002).
373. Roozendaal,R. *et al.* Conduits mediate transport of low-molecular-weight antigen to lymph node follicles. *Immunity* **30**, 264-276 (2009).
374. Rosado,M.M. *et al.* Switched memory B cells maintain specific memory independently of serum antibodies: the Hepatitis B example. *European Journal of Immunology* **41**, 1800-1808 (2011).
375. Rosenberg,E.B., Strickland,G.T., Yang,S.L. & Whalen,G.E. IgM antibodies to red cells and autoimmune anemia in patients with malaria. *The American Journal of Tropical Medicine and Hygiene* **22**, 146-152 (1973).

376. Rosenberg, Y.J. Autoimmune and polyclonal B cell responses during murine malaria. *Nature* **274**, 170-172 (1978).
377. Rotman, H.L., Daly, T.M., Clynes, R. & Long, C.A. Fc receptors are not required for antibody-mediated protection against lethal malaria challenge in a mouse model. *The Journal of Immunology* **161**, 1908 (1998).
378. Rowe, J.A., Claessens, A., Corrigan, R.A. & Arman, M. Adhesion of *Plasmodium falciparum*-infected erythrocytes to human cells: molecular mechanisms and therapeutic implications. *Expert Reviews in Molecular Medicine* **11**, e16 (2009).
379. Rozanski, C.H. *et al.* Sustained antibody responses depend on CD28 function in bone marrow-resident plasma cells. *The Journal of Experimental Medicine* **208**, 1435 (2011).
380. Rurangirwa, F.R., Musoke, A.J., Nantulya, V.M. & Tabel, H. Immune depression in bovine trypanosomiasis: effects of acute and chronic *Trypanosoma congolense* and chronic *Trypanosoma vivax* infections on antibody response to *Brucella abortus* vaccine. *Parasite Immunology* **5**, 267-276 (1983).
381. Sabbagh, L., Snell, L.M. & Watts, T.H. TNF family ligands define niches for T cell memory. *Trends in Immunology* **28**, 333-339 (2007).
382. Sallusto, F., Geginat, J. & Lanzavecchia, A. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu. Rev. Immunol.* **22**, 745-763 (2004).
383. Sanders, C.J., Doherty, P.C. & Thomas, P.G. Respiratory epithelial cells in innate immunity to influenza virus infection. *Cell and Tissue Research* **343**, 13-21 (2011).
384. Sanni, L.A., Fonseca, L.F. & Langhorne, J. Mouse models for erythrocytic-stage malaria. *Methods in Molecular Medicine* **72**, 57-76 (2002).
385. Sanz, I., Wei, C., Lee, F. & Anolik, J. Phenotypic and functional heterogeneity of human memory B cells. 20(1), 67-82. 2008. Elsevier.
Ref Type: Conference Proceeding
386. Sawyer, W.A. Persistence of yellow fever immunity. *J Prev Med* **5**, 413-428 (1931).
387. SberroΓCÉSoussan, R. *et al.* Bortezomib as the Sole Post-Renal Transplantation Desensitization Agent Does Not Decrease Donor-Specific Anti-HLA Antibodies. *American Journal of Transplantation* **10**, 681-686 (2010).
388. Schofield, L. *et al.* γ Interferon, CD8+ T cells and antibodies required for immunity to malaria sporozoites. *Nature* **330**, 664-666 (1987).
389. Schwickert, T.A. *et al.* In vivo imaging of germinal centres reveals a dynamic open structure. *Nature* **446**, 83-87 (2007).

390. Schwickert,T.A. *et al.* A dynamic T cell–limited checkpoint regulates affinity-dependent B cell entry into the germinal center. *The Journal of Experimental Medicine* **208**, 1243 (2011).
391. Seixas,E., Cross,C., Quin,S. & Langhorne,J. Direct activation of dendritic cells by the malaria parasite, *Plasmodium chabaudi chabaudi*. *European Journal of Immunology* **31**, 2970-2978 (2001).
392. Seixas,E. & Ostler,D. *Plasmodium chabaudi chabaudi* (AS): Differential cellular responses to infection in resistant and susceptible mice. *Experimental Parasitology* **110**, 394-405 (2005).
393. Selin,L.K. & Welsh,R.M. Plasticity of T cell memory responses to viruses. *Immunity* **20**, 5-16 (2004).
394. Shaffer,A.L. *et al.* Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. *Immunity* **17**, 51-62 (2002).
395. Shapiro-Shelef,M. & Calame,K. Regulation of plasma-cell development. *Nature Reviews Immunology* **5**, 230-242 (2005).
396. Sharpe,R.T., Langley,A.M., Mowat,G.N., Macaskill,J.A. & Holmes,P.H. Immunosuppression in bovine trypanosomiasis: response of cattle infected with *Trypanosoma congolense* to foot-and-mouth disease vaccination and subsequent live virus challenge. *Research in Veterinary Science* **32**, 289 (1982).
397. Shearer,J.D., Siemann,L., Gerkovich,M. & House,R.V. Biological activity of an intravenous preparation of human vaccinia immune globulin in mouse models of vaccinia virus infection. *Antimicrobial Agents and Chemotherapy* **49**, 2634-2641 (2005).
398. Shi,Y.P. *et al.* Fcγ receptor IIa (CD32) polymorphism is associated with protection of infants against high-density *Plasmodium falciparum* infection. VII. Asembo Bay Cohort Project. *Journal of Infectious Diseases* **184**, 107 (2001).
399. Sidjanski,S. & Vanderberg,J.P. Delayed migration of *Plasmodium* sporozoites from the mosquito bite site to the blood. *The American journal of tropical medicine and hygiene* **57**, 426 (1997).
400. Silverman,G.J. & Weisman,S. Rituximab therapy and autoimmune disorders: prospects for anti–B cell therapy. *Arthritis & Rheumatism* **48**, 1484-1492 (2003).
401. Silverman,P.H., Schooley,J.C. & Mahlmann,L.J. Murine malaria decreases hemopoietic stem cells. *Blood* **69**, 408 (1987).
402. Silvie,O. *et al.* Effects of irradiation on *Plasmodium falciparum* sporozoite hepatic development: implications for the design of pre–erythrocytic malaria vaccines. *Parasite Immunology* **24**, 221-223 (2002).

403. Silvie,O., Mota,M.M., Matuschewski,K. & Prudêncio,M. Interactions of the malaria parasite and its mammalian host. *Current Opinion in Microbiology* **11**, 352-359 (2008).
404. Slifka,M.K. & Ahmed,R. Long-term humoral immunity against viruses: Revisiting the issue of plasma cell longevity. *Trends in Microbiology* **4**, 394-400 (1996).
405. Slifka,M.K., Antia,R., Whitmire,J.K. & Ahmed,R. Humoral immunity due to long-lived plasma cells. *Immunity* **8**, 363-372 (1998).
406. Smalley,M.E., Abdalla,S. & Brown,J. The distribution of *Plasmodium falciparum* in the peripheral blood and bone marrow of Gambian children. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **75**, 103 (1981).
407. Smith,K.G.C., Light,A., Nossal,G.J.V. & Tarlinton,D.M. The extent of affinity maturation differs between the memory and antibody-forming cell compartments in the primary immune response. *The EMBO journal* **16**, 2996-3006 (1997).
408. Snapper,C.M. & Paul,W.E. Interferon-gamma and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. *Science* **236**, 944 (1987).
409. Soulard,V. *et al.* An early burst of IFN- γ induced by the pre-erythrocytic stage favours *Plasmodium yoelii* parasitaemia in B6 mice. *Malaria Journal* **8**, 128 (2009).
410. Sponaas,A.M. *et al.* Malaria infection changes the ability of splenic dendritic cell populations to stimulate antigen-specific T cells. *The Journal of Experimental Medicine* **203**, 1427 (2006).
411. Stasi,R., Pagano,A., Stipa,E. & Amadori,S. Rituximab chimeric anti-CD20 monoclonal antibody treatment for adults with chronic idiopathic thrombocytopenic purpura. *Blood* **98**, 952 (2001).
412. Stavnezer,J., Guikema,J.E.J. & Schrader,C.E. Mechanism and regulation of class switch recombination. *Annual Review of Immunology* **26**, 261 (2008).
413. Steinhauer,D.A. Role of hemagglutinin cleavage for the pathogenicity of influenza virus. *Virology* **258**, 1-20 (1999).
414. Stephens,R., Ndungu,F.M. & Langhorne,J. Germinal centre and marginal zone B cells expand quickly in a second *Plasmodium chabaudi* malaria infection producing mature plasma cells. *Parasite Immunology* **31**, 20-31 (2009).
415. Stephens,R., Culleton,R.L. & Lamb,T.J. The contribution of *Plasmodium chabaudi* to our understanding of malaria. *Trends in Parasitology* (2011).
416. Stevenson,M.M., Tam,M.F., Wolf,S.F. & Sher,A. IL-12-induced protection against blood-stage *Plasmodium chabaudi* AS requires IFN-gamma and TNF-alpha and occurs via a nitric oxide-dependent mechanism. *The Journal of Immunology* **155**, 2545 (1995).

417. Stevenson,M.M. & Riley,E.M. Innate immunity to malaria. *Nature Reviews Immunology* **4**, 169-180 (2004).
418. Strambachová-McBride,J. & Micklem,H.S. Immunosuppression in murine malaria. IV. The secondary response to bovine serum albumin. *Parasite Immunology* **1**, 141-157 (1979).
419. Sturm,A. *et al.* Manipulation of host hepatocytes by the malaria parasite for delivery into liver sinusoids. *Science* **313**, 1287 (2006).
420. Sturm,A. & Heussler,V. Live and let die: manipulation of host hepatocytes by exoerythrocytic *Plasmodium* parasites. *Medical Microbiology and Immunology* **196**, 127-133 (2007).
421. Su,Z. & Stevenson,M.M. Central role of endogenous gamma interferon in protective immunity against blood-stage *Plasmodium chabaudi* AS infection. *Infection and Immunity* **68**, 4399-4406 (2000).
422. Subramaniam,K. *et al.* IgM+ Memory B Cell Expression Predicts HIV-Associated Cryptococcosis Status. *Journal of Infectious Diseases* **200**, 244 (2009).
423. Svensson,M. & Kaye,P.M. Stromal-cell regulation of dendritic-cell differentiation and function. *Trends in immunology* **27**, 580-587 (2006).
424. Sze,D.M.Y., Toellner,K.M., de Vinuesa,C.G., Taylor,D.R. & MacLennan,I. Intrinsic constraint on plasmablast growth and extrinsic limits of plasma cell survival. *The Journal of Experimental Medicine* **192**, 813 (2000).
425. Taillardet,M. *et al.* The thymus-independent immunity conferred by a pneumococcal polysaccharide is mediated by long-lived plasma cells. *Blood* **114**, 4432 (2009).
426. Takahashi,Y., Dutta,P.R., Cerasoli,D.M. & Kelsoe,G. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl) acetyl. V. Affinity maturation develops in two stages of clonal selection. *The Journal of Experimental Medicine* **187**, 885 (1998).
427. Takahashi,Y. *et al.* Relaxed negative selection in germinal centers and impaired affinity maturation in bcl-xL transgenic mice. *The Journal of Experimental Medicine* **190**, 399 (1999).
428. Takai,T., Li,M., Sylvestre,D., Clynes,R. & Ravetch,J.V. FcR [gamma] chain deletion results in pleiotropic effector cell defects. *Cell* **76**, 519-529 (1994).
429. Takai,T., Ono,M., Hikida,M., Ohmori,H. & Ravetch,J.V. Augmented humoral and anaphylactic responses in FcγRII-deficient mice. *Nature* **379**, 346-349 (1996).
430. Tamura,S. *et al.* Antibody-forming cells in the nasal-associated lymphoid tissue during primary influenza virus infection. *Journal of General Virology* **79**, 291 (1998).

431. Tangye,S.G., Ferguson,A., Avery,D.T., Ma,C.S. & Hodgkin,P.D. Isotype switching by human B cells is division-associated and regulated by cytokines. *The Journal of Immunology* **169**, 4298 (2002).
432. Tangye,S.G. & Good,K.L. Human IgM+ CD27+ B cells: memory B cells or "memory" B cells? *The Journal of Immunology* **179**, 13 (2007).
433. Tarlinton,D. B-cell memory: are subsets necessary? *Nature Reviews Immunology* **6**, 785-790 (2006).
434. Tarlinton,D., Radbruch,A., Hiepe,F. & Dorner,T. Plasma cell differentiation and survival. *Current Opinion in Immunology* **20**, 162-169 (2008).
435. Tarlinton,D.M. & Smith,K.G.C. Dissecting affinity maturation: a model explaining selection of antibody-forming cells and memory B cells in the germinal centre. *Immunology Today* **21**, 436-441 (2000).
436. Tarun,A.S. *et al.* Protracted sterile protection with *Plasmodium yoelii* pre-erythrocytic genetically attenuated parasite malaria vaccines is independent of significant liver-stage persistence and is mediated by CD8+ T cells. *Journal of Infectious Diseases* **196**, 608 (2007).
437. Taylor,R.R., Allen,S.J., Greenwood,B.M. & Riley,E.M. IgG3 antibodies to *Plasmodium falciparum* merozoite surface protein 2 (MSP2): increasing prevalence with age and association with clinical immunity to malaria. *The American Journal of Tropical Medicine and Hygiene* **58**, 406-413 (1998).
438. Terstappen,L.W., Johnsen,S., Segers-Nolten,I.M. & Loken,M.R. Identification and characterization of plasma cells in normal human bone marrow by high-resolution flow cytometry. *Blood* **76**, 1739 (1990).
439. Thai,T.H. *et al.* Regulation of the germinal center response by microRNA-155. *Science* **316**, 604 (2007).
440. Tian,J.H. *et al.* Genetic regulation of protective immune response in congenic strains of mice vaccinated with a subunit malaria vaccine. *The Journal of Immunology* **157**, 1176 (1996).
441. Todd,D.J. *et al.* XBP1 governs late events in plasma cell differentiation and is not required for antigen-specific memory B cell development. *The Journal of Experimental Medicine* **206**, 2151-2159 (2009).
442. Tokoyoda,K., Egawa,T., Sugiyama,T., Choi,B.I. & Nagasawa,T. Cellular niches controlling B lymphocyte behavior within bone marrow during development. *Immunity* **20**, 707-718 (2004).
443. Tokoyoda,K. *et al.* Professional memory CD4+ T lymphocytes preferentially reside and rest in the bone marrow. *Immunity* **30**, 721-730 (2009).

444. Tomayko, M.M. *et al.* Systematic comparison of gene expression between murine memory and naive B cells demonstrates that memory B cells have unique signaling capabilities. *The Journal of Immunology* **181**, 27 (2008).
445. Tonegawa, S. Somatic generation of antibody diversity. *Nature* **302**, 575-581 (1983).
446. Topham, D.J., Tripp, R.A. & Doherty, P.C. CD8+ T cells clear influenza virus by perforin or Fas-dependent processes. *The Journal of Immunology* **159**, 5197 (1997).
447. Topham, D.J. & Doherty, P.C. Clearance of an influenza A virus by CD4+ T cells is inefficient in the absence of B cells. *Journal of Virology* **72**, 882 (1998).
448. Toyama, H. *et al.* Memory B cells without somatic hypermutation are generated from Bcl6-deficient B cells. *Immunity* **17**, 329-339 (2002).
449. Traggiai, E., Puzone, R. & Lanzavecchia, A. Antigen dependent and independent mechanisms that sustain serum antibody levels. *Vaccine* **21**, S35-S37 (2003).
450. Tran, T.H. *et al.* B cell-specific and stimulation-responsive enhancers derepress Aicda by overcoming the effects of silencers. *Nature Immunology* **11**, 148-154 (2009).
451. Traore, B. *et al.* The TLR9 agonist CpG fails to enhance the acquisition of *Plasmodium falciparum*-specific memory B cells in semi-immune adults in Mali. *Vaccine* **27**, 7299-7303 (2009).
452. Tsubaki, T. *et al.* Accumulation of plasma cells expressing CXCR3 in the synovial sublining regions of early rheumatoid arthritis in association with production of Mig/CXCL9 by synovial fibroblasts. *Clinical & Experimental Immunology* **141**, 363-371 (2005).
453. Tsuji, M. *et al.* Gamma delta T cells contribute to immunity against the liver stages of malaria in alpha beta T-cell-deficient mice. *Proceedings of the National Academy of Sciences* **91**, 345 (1994).
454. Tumpey, T.M. *et al.* Pathogenicity of influenza viruses with genes from the 1918 pandemic virus: functional roles of alveolar macrophages and neutrophils in limiting virus replication and mortality in mice. *Journal of Virology* **79**, 14933-14944 (2005).
455. Urban, B. & Todryk, S. Malaria pigment paralyzes dendritic cells. *Journal of Biology* **5**, 4 (2006).
456. Urban, B.C. *et al.* *Plasmodium falciparum*-infected erythrocytes modulate the maturation of dendritic cells. *Nature* **400**, 73-77 (1999).
457. van der Kolk, L.E., Baars, J.W., Prins, M.H. & van Oers, M.H.J. Rituximab treatment results in impaired secondary humoral immune responsiveness. *Blood* **100**, 2257 (2002).

458. Van Lent,P. *et al.* The inhibitory receptor Fc γ RII reduces joint inflammation and destruction in experimental immune complex-mediated arthritides not only by inhibition of Fc γ RI/III but also by efficient clearance and endocytosis of immune complexes. *The American Journal of Pathology* **163**, 1839 (2003).
459. Van Trung,C. *et al.* Eosinophils are required for the maintenance of plasma cells in the bone marrow. *Nature immunology* **12**, 151 (2011).
460. Vezys,V. *et al.* Memory CD8 T-cell compartment grows in size with immunological experience. *Nature* **457**, 196-199 (2008).
461. Victora,G.D. *et al.* Germinal center dynamics revealed by multiphoton microscopy with a photoactivatable fluorescent reporter. *Cell* **143**, 592-605 (2010).
462. Vieira,P. & Rajewsky,K. The half-lives of serum immunoglobulins in adult mice. *European Journal of Immunology* **18**, 313-316 (1988).
463. Vigorito,E. *et al.* microRNA-155 regulates the generation of immunoglobulin class-switched plasma cells. *Immunity* **27**, 847-859 (2007).
464. Viki,B. & Nathalie,G. Acute Disruption of Bone Marrow B Lymphopoiesis and Apoptosis of Transitional and Marginal Zone B Cells in the Spleen following a Blood-Stage *Plasmodium chabaudi* Infection in Mice. *Journal of Parasitology Research* **2011**, 534697 (2011).
465. Vo,A.A. *et al.* Rituximab and intravenous immune globulin for desensitization during renal transplantation. *New England Journal of Medicine* **359**, 242-251 (2008).
466. Vukovic,P., Hogarth,P.M., Barnes,N., Kaslow,D.C. & Good,M.F. Immunoglobulin G3 antibodies specific for the 19-kilodalton carboxyl-terminal fragment of *Plasmodium yoelii* merozoite surface protein 1 transfer protection to mice deficient in Fc- γ RI receptors. *Infection and Immunity* **68**, 3019 (2000).
467. Waffarn,E.E. & Baumgarth,N. Protective B Cell Responses to Flu Γ ÇöNo Fluke! *The Journal of Immunology* **186**, 3823 (2011).
468. Wahrmann,M. *et al.* Effect of the proteasome inhibitor bortezomib on humoral immunity in two presensitized renal transplant candidates. *Transplantation* **89**, 1385 (2010).
469. Waki,S., Uehara,S., Kanbe,K., Nariuch,H. & Suzuki,M. Interferon- γ and the induction of protective IgG2a antibodies in non-lethal *Plasmodium berghei* infections of mice. *Parasite Immunology* **17**, 503-508 (1995).
470. Wang,K. *et al.* Expression and purification of an influenza hemagglutinin--one step closer to a recombinant protein-based influenza vaccine. *Vaccine* **24**, 2176-2185 (2006).

471. Wang,Y. & Carter,R.H. CD19 regulates B cell maturation, proliferation, and positive selection in the FDC zone of murine splenic germinal centers. *Immunity* **22**, 749-761 (2005).
472. Ward,C.L. *et al.* Design and performance testing of quantitative real time PCR assays for influenza A and B viral load measurement. *Journal of Clinical Virology* **29**, 179-188 (2004).
473. Ward,E.S., Zhou,J., Ghetie,V. & Ober,R.J. Evidence to support the cellular mechanism involved in serum IgG homeostasis in humans. *International Immunology* **15**, 187 (2003).
474. Ward,P.A. & Kibukamusoke,J.W. Evidence for soluble immune complexes in the pathogenesis of the glomerulonephritis of quartan malaria. *The Lancet* **293**, 283-285 (1969).
475. Weiss,G.E. *et al.* Atypical memory B cells are greatly expanded in individuals living in a malaria-endemic area. *The Journal of Immunology* **183**, 2176 (2009).
476. Weiss,G.E. *et al.* The *Plasmodium falciparum*-specific human memory B cell compartment expands gradually with repeated malaria infections. *Plos Pathogens* **6**, e1000912 (2010).
477. Weiss,G.E. *et al.* A positive correlation between atypical memory B cells and *Plasmodium falciparum* transmission intensity in cross-sectional studies in Peru and Mali. *Plos One* **6**, e15983 (2011).
478. Welsh,R.M., Selin,L.K. & Szomolanyi-Tsuda,E. Immunological memory to viral infections 1. *Annu. Rev. Immunol.* **22**, 711-743 (2004).
479. White,W.I., Evans,C.B. & Taylor,D.W. Antimalarial antibodies of the immunoglobulin G2a isotype modulate parasitemias in mice infected with *Plasmodium yoelii*. *Infection and Immunity* **59**, 3547 (1991).
480. Whitelaw,D.D. *et al.* Immunosuppression in bovine trypanosomiasis: studies with louping-ill vaccine. *Research in Veterinary Science* **26**, 102 (1979).
481. Whittle,H.C. *et al.* T-cell control of Epstein–Barr virus-infected B cells is lost during *P. falciparum* malaria. *Nature* **312**, 449-450 (1984).
482. Wickramasinghe,S.N., Phillips,R.E., Looareesuwan,S., Warrell,D.A. & Hughes,M. The Bone-Marrow in Human Cerebral Malaria - Parasite Sequestration Within Sinusoids. *British Journal of Haematology* **66**, 295-306 (1987).
483. Wickramasinghe,S.N. & Abdalla,S.H. Blood and bone marrow changes in malaria. *Best Practice & Research Clinical Haematology* **13**, 277-299 (2000).
484. Wijburg,O.L.C., DiNatale,S., Vadolas,J., vanRooijen,N. & Strugnell,R.A. Alveolar macrophages regulate the induction of primary cytotoxic T-lymphocyte responses during influenza virus infection. *Journal of Virology* **71**, 9450-9457 (1997).

485. Wiley,D.C., Wilson,I.A. & Skehel,J.J. Structural identification of the antibody-binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation. *Nature* **289**, 373 (1981).
486. Williamson,W.A. & Greenwood,B.M. Impairment of the immune response to vaccination after acute malaria. *The Lancet* **311**, 1328-1329 (1978).
487. Wilson,N.S. *et al.* Systemic activation of dendritic cells by Toll-like receptor ligands or malaria infection impairs cross-presentation and antiviral immunity. *Nature Immunology* **7**, 165-172 (2006).
488. Winkelmann,R. *et al.* B cell homeostasis and plasma cell homing controlled by Krnppel-like factor 2. *Proceedings of the National Academy of Sciences* **108**, 710 (2011).
489. Wipasa,J. *et al.* Long-lived antibody and B Cell memory responses to the human malaria parasites, *Plasmodium falciparum* and *Plasmodium vivax*. *Plos Pathogens* **6**, e1000770 (2010).
490. Wirths,S. & Lanzavecchia,A. ABCB1 transporter discriminates human resting naive B cells from cycling transitional and memory B cells. *European Journal of Immunology* **35**, 3433-3441 (2005).
491. Wols,H.A.M., Underhill,G.H., Kansas,G.S. & Witte,P.L. The role of bone marrow-derived stromal cells in the maintenance of plasma cell longevity. *The Journal of Immunology* **169**, 4213 (2002).
492. Wykes,M.N., Zhou,Y.H., Liu,X.Q. & Good,M.F. *Plasmodium yoelii* can ablate vaccine-induced long-term protection in mice. *The Journal of Immunology* **175**, 2510 (2005).
493. Xiang,Z. *et al.* Fc γ RIIb controls bone marrow plasma cell persistence and apoptosis. *Nature immunology* **8**, 419-429 (2007).
494. Xu,H. *et al.* The mechanism and significance of deletion of parasite-specific CD4+ T cells in malaria infection. *The Journal of Experimental Medicine* **195**, 881 (2002).
495. Xu,L. *et al.* Cutting edge: pulmonary immunopathology mediated by antigen-specific expression of TNF- α by antiviral CD8+ T cells. *The Journal of Immunology* **173**, 721 (2004).
496. Yamauchi,L.M., Coppi,A., Snounou,G. & Sinnis,P. *Plasmodium* sporozoites trickle out of the injection site. *Cellular Microbiology* **9**, 1215-1222 (2007).
497. Yoneto,T. *et al.* A critical role of Fc receptor-mediated antibody-dependent phagocytosis in the host resistance to blood-stage *Plasmodium berghei* XAT infection. *The Journal of Immunology* **166**, 6236 (2001).

498. Yoshida,T. *et al.* Memory B and memory plasma cells. *Immunological Reviews* **237**, 117-139 (2010).
499. Yu,S., Dunn,R., Kehry,M.R. & Braley-Mullen,H. B cell depletion inhibits spontaneous autoimmune thyroiditis in NOD. H-2h4 mice. *The Journal of Immunology* **180**, 7706 (2008).
500. Yu,X. *et al.* Neutralizing antibodies derived from the B cells of 1918 influenza pandemic survivors. *Nature* **455**, 532 (2008).
501. Yurdakul,P. *et al.* Compartment-Specific Remodeling of Splenic Micro-Architecture during Experimental Visceral Leishmaniasis. *The American Journal of Pathology* (2011).
502. Zingman,B.S. & Viner,B.L. Splenic complications in malaria: case report and review. *Clinical Infectious Diseases* **16**, 223 (1993).
503. Zinkernagel,R.M. *et al.* On immunological memory. *Annual Review of Immunology* **14**, 333-367 (1996).
504. Zinkernagel,R.M. On differences between immunity and immunological memory - Opinion. *Current Opinion in Immunology* **14**, 523-536 (2002).
505. Zotos,D. *et al.* IL-21 regulates germinal center B cell differentiation and proliferation through a B cell–intrinsic mechanism. *The Journal of Experimental Medicine* **207**, 365-378 (2010).
506. Zuñiga,E., Motran,C.C., Montes,C.L., Yagita,H. & Gruppi,A. *Trypanosoma cruzi* infection selectively renders parasite-specific IgG⁺ B lymphocytes susceptible to Fas/Fas ligand-mediated fratricide. *The Journal of Immunology* **168**, 3965 (2002).
507. Zuercher,A.W., Coffin,S.E., Thurnheer,M.C., Fundova,P. & Cebra,J.J. Nasal-associated lymphoid tissue is a mucosal inductive site for virus-specific humoral and cellular immune responses. *The Journal of Immunology* **168**, 1796 (2002).