



IMMUNE RESPONSES TO VACCINES AGAINST MALARIA

Thesis submitted for the degree of Doctor of Philosophy

Hilary Term 2017

Carly May Bliss

St Edmund Hall

Word count: ~50,000 (excluding tables, figures, legends, references and appendices)

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Carly May Bliss, St Edmund Hall, University of Oxford

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Abstract

The development of a malaria vaccine is necessary for disease eradication. Successful vaccine candidates to date have targeted the asymptomatic, pre-erythrocytic stage of the disease, however even the most efficacious vaccines are only partially protective. Research undertaken in our laboratory has demonstrated that one such regimen, using an 8 week prime-boost viral vector approach of ChAd63 ME-TRAP and MVA ME-TRAP, induces sterile efficacy in 21% of vaccinees, with a key role identified for TRAP-specific CD8⁺ T cells. The work described in this thesis explores the most immunogenic regimen by which to administer these two pre-erythrocytic malaria vaccines. A shortening of the prime-boost interval from 8 to 4 weeks, and the addition of an extra ChAd63 ME-TRAP priming vaccination, both demonstrated improved T cell immunogenicity over the standard 8 week regimen. Further to this, novel assays were developed to aid the evaluation of vaccine-induced immune responses. Adaptations of the existing methodology for ELISpot analysis and to whole blood flow cytometry techniques, enabled more detailed analyses of paediatric vaccine-induced T cell responses in The Gambia. This work also permitted the comparison of vaccine immunogenicity in this paediatric population, with malaria-naïve and malaria-exposed adult vaccinees. The results suggest that vaccine-induced T cell responses in infants of 8 weeks and older are comparable to that of adults. A second approach involved the development of a novel functional assay. This assay quantitatively measured the *in vitro* inhibition of intrahepatic *Plasmodium* parasite development using T cells from ChAd63.MVA ME-TRAP vaccinated volunteers. The assay demonstrated the ability of CD8⁺ T cells to inhibit parasite development in a TRAP-specific manner, and provides a platform with which to further explore pre-erythrocytic immune responses.

Acknowledgements

Firstly, I would like to thank my wonderful DPhil supervisors Dr. Alexandra Spencer, Dr. Katie Ewer and Prof. Adrian Hill. Your guidance and encouragement have been invaluable and I am hugely grateful for the advice and opportunities you have given me during my time at the Jenner Institute. You have challenged me, inspired me and helped develop my confidence as a scientist, which I will take forward into my academic career.

Secondly I wish to thank the teams across the multiple clinical trials' sites for their contributions to the many vaccine trials referenced in this thesis, including the clinical teams and volunteers at the Centre for Clinical Vaccinology and Tropical Medicine in Oxford, the Wellcome Trust Clinical Research Facility in Southampton, NIHR/Wellcome Trust Imperial CRF at Hammersmith Hospital in London, the Medical Research Council in The Gambia, and the CNRFP/URC-B clinical units in Burkina Faso.

Many thanks also for the technical support provided by several members of the Jenner Institute, particularly Julie Furze for keeping the lab running smoothly and to Andrew Worth for the extensive cell sorting undertaken on my behalf. Also to Marta Ulaszewska for your patience in the FGF and to the MVT liver stage team Georgina Bowyer, Danny Wright, Jonathan Powlson, Richard Morter and Sophie Jamieson for your work on numerous clinical trials. Thank you also to Rhea Longley, Ahmed Salman and Ana Rodriguez-Galan for your help with assay development, to Alex Fyfe and Sumi Biswas of the Jenner Insectary, and staff at Wellcome Trust FGF for your assistance throughout my project.

Thank you to collaborators at Leiden University Medical Centre, Dr. Shahid Khan and Dr. Chris Janse, for the transgenic parasites that contributed to my work. I would also like to thank Dr. Valérie Souldard and Prof. Dominique Mazier for welcoming me to CIMI-PARIS and for the warm welcome by staff at the MRC in the Gambia during my research visit. These were wonderful opportunities to do research overseas, for which I am very grateful.

Very special thanks to my colleagues and fellow students at the Jenner Institute: Lynda Coughlan, Elena Stylianou, Federica Cappuccini, Sean Elias, Rachel Tanner, Adam Walters, Nick

Edwards, Florian Brod, Joe Illingworth, Janet Pashricha, Christos Krastev, Benedict Halbroth, Steph Tombs, Iona Taylor, Sarah Silk and Simone de Cassan for making my time at the Jenner Institute hugely enjoyable, predominantly through coffee, cakes and chats. Thanks also to Matthew O'Shea for scientific discussions, and to Pawan Dulal for support throughout the thesis writing process. I would also not be where I am today without the support of Prof Martin Maiden, my first mentor in Oxford, and to whom I owe so very much.

And finally, the most heartfelt thank you to my wonderful family, who think I'm far more intelligent than I will ever be, and to my amazing friends who have supported and encouraged me thorough out my studies. To my team mates, who inspire and motivate me daily, and to Tess Braunerova, Lydia Houldsworth and Naomi Bull who are there through thick and thin. And finally to my wonderful parents, Maureen and Barry Bliss, to whom the biggest thanks must be given, for your continual support and unwavering belief in the things I can achieve.

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List of abbreviations

AAV	Adeno-associated virus
ACT	Artemisinin-based combination therapy
AdHu	Human adenovirus
AMA1	Apical membrane antigen 1
ANOVA	Analysis of variance
APC	Antigen presenting cell
APC	Allophycocyanin
ATCC	American Type Culture Collection
BCIP/NBT	5-Bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium
BCR	B cell receptor
BSA	Bovine serum albumin
C-1	One day prior to CHMI
CD	Cluster of differentiation
ChAd63	Chimpanzee adenovirus serotype 63
CHMI	Controlled human malaria infection
CMV	Cytomegalovirus
CSP	Circumsporozoite protein
CTL	Cytotoxic lymphocytes
CTL pool	Pool of cytotoxic CD8 ⁺ T cell epitopes
CXCL	CXC chemokine ligand
DAMP	Damage-associated molecular patterns
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DDT	Dichlorodiphenyltrichloroethane
DiD	Vybrant DiD cell-labelling solution
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTP	Diphtheria, tetanus and pertussis
E:T	Effector to target ratio
EBV	Epstein-Barr virus
ECACC	European Collection of Cell Cultures
EEF	Exo-erythrocytic form
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-Linked ImmunoSpot
EPI	Expanded Programme on Immunisation
ER	Endoplasmic reticulum
EXP 1	Exported protein 1
F	FLIFFDLFL(V) peptide
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FCS	Forward scatter
FGF	Functional genetics facility
FITC	Fluorescein isothiocyanate

FP	Fowlpox virus
G	GILGFVFTL peptide
G	Gravitational acceleration
GAP	Genetically attenuated parasites
GC	Germinal centre
GCLP	Good clinical laboratory practice
GCP	Good clinical practice
GFP	Green fluorescent protein
GIA	Growth inhibition assay
GM-CSF	Granulocyte macrophage colony-stimulating factor
gMFI	Geometric mean fluorescence intensity
GMP	Good manufacturing practice
GPI	Glycosylphosphatidylinositol
GrA	Granzyme A
GrB	Granzyme B
GSK	GlaxoSmithKline
HBsAg	Hepatitis B surface antigen
HepB	Hepatitis B
Hib	<i>Haemophilus influenzae</i> type B
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPI	Hours post infection
HSPGS	Heparan sulphate proteoglycans
i.d.	Intradermal
i.m.	Intramuscular
i.p.	Intraperitoneal
i.v.	Intravenous
ICAM	Intracellular adhesion molecule
ICS	Intracellular cytokine staining
IFN- γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
ILSDA	Inhibition of liver stage development assay
iNOS	Inducible nitric oxide synthase
IPT	Intermittent preventative therapy
IQR	Interquartile range
iRBC	Infected red blood cell
IRS	Indoor-residual spraying
ISI	Inhibition of sporozoite invasion
ITN	Insecticide treated bednets
LLD	Lower limit of detection
LSA	Liver stage antigen
LSAP	Liver stage associated protein
LSEC	Liver sinusoidal endothelial cell
M1	Matrix protein 1
MACS	Magnetic activated cell sorting

ME	Multi-epitope string
MHC	Major histocompatibility complex
MIATA	Minimal information about T cell assays
MIF	Migration inhibitory factor
MSP	Merozoite surface protein
MVA	Modified Vaccinia Ankara
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHP	Non-human primate
NK	Natural killer cell
NO	Nitric oxide
NOD	Nucleotide oligomerization domain-like receptor
PAMP	Pathogen associated molecular pattern
<i>Pb</i>	<i>Plasmodium berghei</i>
PBMC	Peripheral blood mononuclear cell
<i>PbPf</i> [antigen]	Transgenic <i>P. berghei</i> expressing the <i>P. falciparum</i> antigen
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
<i>Pf</i>	<i>P. falciparum</i>
pfu	Plaque forming units
PHA-L	Phytohemagglutinin-L
PHH	Primary human hepatocytes
PRR	Pattern recognition receptor
PV	Parasitophorous vacuole
PVM	Parasitophorous vacuole membrane
<i>Py</i>	<i>P. yoelii</i>
qPCR	Quantitative polymerase chain reaction
RAS	Radiation attenuated sporozoites
RBC	Red blood cell
RESA	Ring infected erythrocyte antigen
RH5	Reticulocyte binding protein homolog 5
s.c.	Subcutaneous
SAP1	Sporozoite Asparagine-rich Protein 1
SD	Standard deviation
SEB	Staphylococcus enterotoxin B
SFC	Spot forming cells
SP	Sulphadoxine plus pyrimethamine
SPECT	Sporozoite microneme protein essential for cell traversal
Spz	Sporozoites
SSC	Side scatter
SSP2	Sporozoite surface protein 2
TAP	Transporter associated with antigen processing
TBV	Transmission blocking vaccine
TCR	T cell receptor
Tfh	T follicular helper cell
Tg	Transgenic

Th	T helper
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TNF- α	Tumour necrosis factor alpha
TRAP	Thrombospondin related adhesion protein
Treg	Regulatory T cell
TT	Thrombospondin related adhesion protein T9/96 strain
UIS	Upregulated in sporozoites
VLP	Virus like particle
vp	Viral particles
VSA	Variant surface antigen
WHO	World Health Organisation
β 2M	Beta-2 microglobulin

Papers published from these studies

The following paper includes work presented in this thesis:

Carly M. Bliss, Abdoulie Drammeh, Georgina Bowyer, Guillaume S. Sanou, Ya Jankey Jagne, Oumarou Ouedraogo, Nick J. Edwards, Casimir Tarama, Nicolas Ouedraogo, Mireille Ouedraogo, Jainaba Njie-Jobe, Amidou Diarra, Muhammed O. Afolabi, Alfred B. Tiono, Jean Baptiste Yaro, Uche J. Adetifa, Susanne H. Hodgson, Nicholas A. Anagnostou, Rachel Roberts, Christopher J.A. Duncan¹⁰, Riccardo Cortese, Nicola K. Viebig, Odile Leroy, Alison M. Lawrie, Katie L. Flanagan¹¹, Beate Kampmann, Egeruan B. Imoukhuede, Sodiomon B. Sirima, Kalifa Bojang, Adrian V.S. Hill, Issa Nébié, Katie J. Ewer. ***Viral Vector Malaria Vaccines Induce High-Level T Cell and Antibody Responses in West African Children and Infants.*** Published online: Molecular Therapy December 14, 2016

Authorship statement

This thesis has been composed by Carly Bliss, and all work presented is that of Carly Bliss, unless stated otherwise.

Chapter 1

Introduction

1 Introduction

1.1 Malaria

1.1.1 Global health threat

With almost half the world's population at risk, malaria is a huge global burden. In 2015, there were an estimated 212 million cases and 429,000 deaths from malaria. While this is a 21% decrease in incidence and 29% reduction in mortality since 2010 [8], malaria still carries a huge burden both socially [9, 10] and economically [10-12]. Children under 5 are particularly susceptible to infection, severe disease and death, and shockingly account for approximately 70% of the mortality from malaria, predominantly in Africa [8]. Furthermore, there is a striking correlation between malaria incidence and poverty, meaning treatment costs outweigh the financial provisions of endemic countries [13-15].

Five species of malaria parasite are known to infect humans: *Plasmodium falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*, with *P. falciparum* and *P. vivax* posing the greatest global threat. At approximately 90%, a disproportionately high share of the world malaria burden is centred in sub-Saharan Africa, where *P. falciparum* is the most prevalent *Plasmodium* parasite. *P. vivax* still poses a significant risk, and is the dominant malaria parasite outside of sub-Saharan Africa, with a wide geographical distribution affecting both South-East Asia and Latin America [16, 17]. *P. vivax* malaria is also present in Africa, however is largely restricted by the high proportion of individuals negative for the Duffy blood group antigen, which renders red blood cells resistant to infection by both the *P. vivax* and *P. knowlesi* parasites [18-20]. The disease is transmitted via the bite of an infected female mosquito from the *Anopheles* genus, of which there are over 450 different species [21], 70 with the capacity to transmit human malaria and 41 that are considered dominant vector species of malaria transmission at a level of major public health concern [22].

1.1.2 Life cycle

This thesis focusses on cellular immune responses induced by candidate malaria vaccines against *P. falciparum*, and uses rodent *P. berghei* as a model to study responses to *P. falciparum* antigens. Other malaria parasites are capable of infecting rodents (*P. yoelii*, *P. chabaudi* and *P. vinckei*), however the availability of transgenic *P. berghei* parasites expressing *P. falciparum* antigens determined the use of this model. The stages of the malaria lifecycle are outlined below, with particular reference to *P. falciparum* and *P. berghei*, and summarised in Figure 1.1.

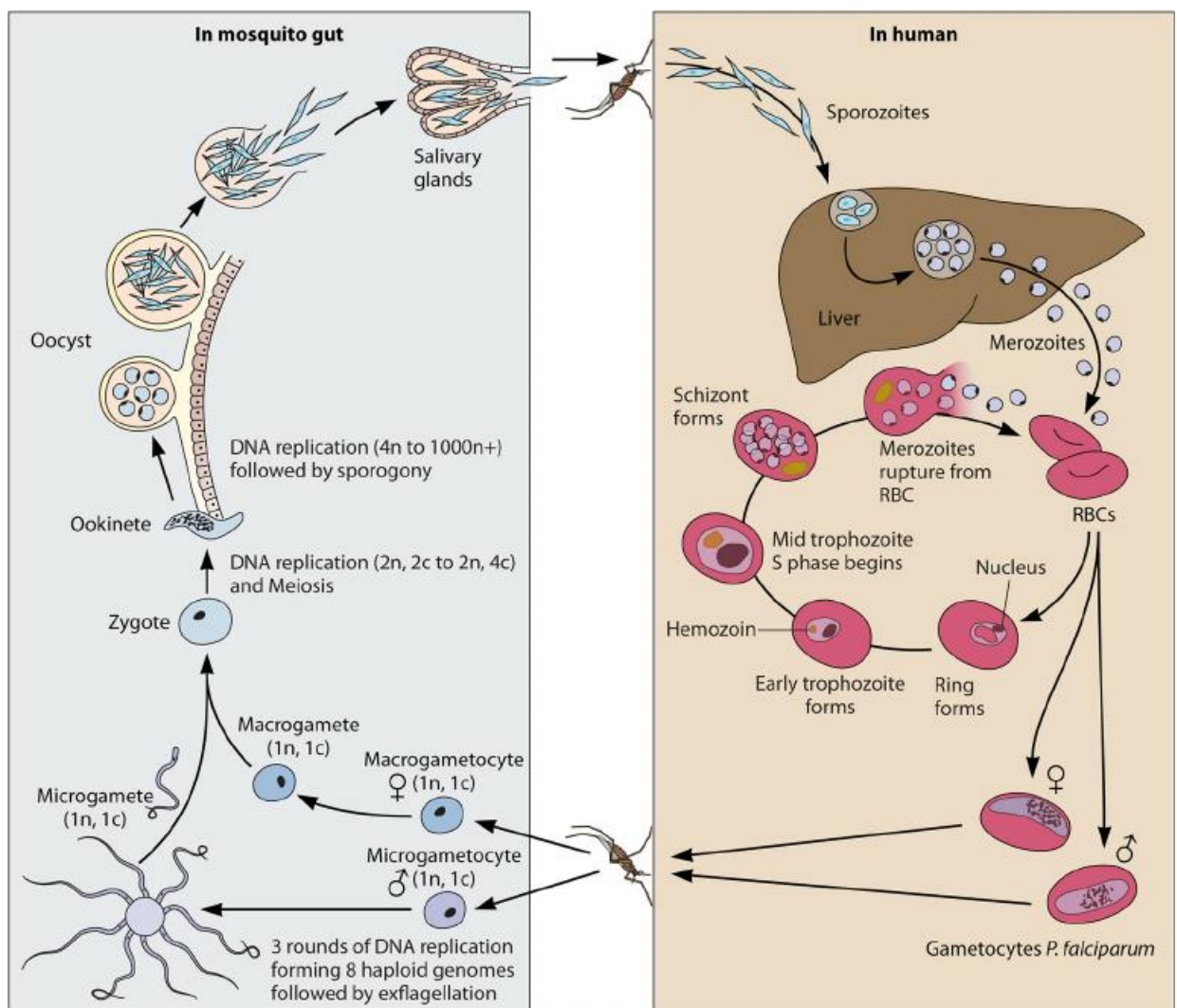


Figure 1.1 The *Plasmodium* life cycle [1, 2].

Summary of sporozoite infection of the human host: the pre-erythrocytic liver stage; the asexual blood stage with generation of gametocytes; and the completion to sexual stages via parasite gametes and oocyst formation in the mosquito vector.

1.1.2.1 Pre-erythrocytic stage

Mosquito saliva contains anticoagulants and local anaesthetic, which is injected into the skin of the host prior to the mosquito taking a blood meal [23, 24]. Malaria infection begins when *Plasmodium* sporozoites from an infected female *Anopheles* mosquito are injected into a host in this manner. The sporozoite inoculation load can vary depending on salivary gland load, but is predicted to be in the region of tens to low hundreds per bite from natural exposure in the field [25, 26]. However, in a laboratory setting, this can be much higher. Recent studies have shown that sporozoites can spend up to several hours at the inoculation site [27], before crossing the endothelial barrier into either the lymphatic system (~20%) where they drain to lymph nodes and can interact with the immune system, or into the blood stream (~70%) [28-30]. Some sporozoites remain in the skin.

Once in the bloodstream, sporozoites accumulate in the liver due to the highly sulphated heparan-sulphate proteoglycans (HSPGs) expressed on cells in the liver, and their interaction with the major surface protein of sporozoites, circumsporozoite protein (CSP) [31, 32]. There is strong evidence to support the gateway hypothesis, whereby sporozoites traverse Kupffer cells in order to cross the sinusoidal wall of the liver, which is predominantly made up of liver sinusoidal endothelial cells (LSECs) [33-35]. Once in contact with the hepatocytes inside the liver sinusoid, sporozoites can breach the cell membrane of hepatocytes, glide through the cytosol and re-exit the cell, a process known as cell traversal [36]. It is questioned whether cell traversal is an essential process prior hepatocyte infection, as genes involved in cell traversal (e.g. Sporozoite Microneme Protein Essential for Cell Traversal (SPECT1 and SPECT2)) can be disrupted without eliminating the ability of sporozoites to infect hepatocytes, as long as the sporozoite can first cross the liver sinusoidal wall [37]. Cell traversal is distinct from the hepatocyte infection. During infection, the sporozoite forms a parasitophorous vacuole (PV) in the hepatocyte, leaving no wound in the cell membrane [38].

Once inside a hepatocyte, the sporozoite undergoes transformation inside the PV and develops as a liver stage parasite, also referred to as an exo-erythrocytic form. The parasite resides adjacent to the nucleus and is highly metabolically active, growing to the size exceeding that of an

uninfected hepatocyte [39]. This liver stage growth phase is one of the main differences between the life cycle of *P. falciparum* and *P. berghei*, with the liver stage lasting approximately 5.5 -8 days for *P. falciparum*, and just 48-52 hours for *P. berghei* (Table 1). During this time, the parasite develops into the mature schizont, which can contain several thousand merozoites. The hepatocyte ruptures, releasing the merozoites into the bloodstream giving rise to the clinical manifestations of malaria.

Thrombospondin-Related Adhesion Protein (TRAP), also referred to as Sporozoite Surface Protein 2 (SSP2), is involved in the gliding motility of sporozoites and the infection of hepatocytes. It provides the link between the parasites cytoskeleton and the host cell, through linkage to parasite actin through its cytoplasmic tail while binding sulphated glycoconjugates on the hepatocyte via its extracellular portion [40]. Studies using *P. berghei* indicate that TRAP is necessary for both gliding motility and infection of hepatocytes by sporozoites [41, 42], as demonstrated through TRAP knockout experiments *in vivo* and antibody blocking of TRAP *in vitro* [40, 43].

1.1.2.2 Erythrocytic stage

Merozoites released from ruptured hepatocytes infect red blood cells (RBCs). Inside the RBC, merozoites undergo asexual reproduction forming ring stage parasites, which evolve into trophozoites. Trophozoites then mature into erythrocytic schizonts, which rupture the RBC releasing 16 to 32 daughter merozoites back into the bloodstream [44, 45]. These merozoites then complete cycles of re-infection of other uninfected red blood cells. Analogous to the shorter liver stage phase of *P. berghei* compared to *P. falciparum*, the erythrocytic cycle of *P. falciparum* lasts twice as long as that of *P. berghei* (Table 1).

1.1.2.3 Sexual stage

During the erythrocytic stage, some merozoites develop into sexual stage male and female gametocytes, a process known as gametocytogenesis. It is unclear what process triggers gametocytogenesis, however gametocytes appear in the blood of the infected human host between

7-15 days after the initiation of the blood stage of infection [46]. To complete the lifecycle, these gametocytes are ingested by an uninfected female *Anopheles* mosquito during a blood meal, which is followed shortly by the transformation of these stages to male and female gametes in the midgut of the mosquito. Fertilization of the spherical, female gamete by the flagellated male gamete occurs in the mosquito midgut, forming a diploid zygote. This zygote develops into a mobile ookinete that penetrates the midgut wall, where it forms an oocyst. Sporozoites are formed within this oocyst, a process called sporogony. Upon oocyst rupture, the sporozoites migrate to the mosquito salivary glands, where they are able to infect the next human host [47].

Table 1 Summary of lifecycle stage duration across *Plasmodium* species

Data combined from [4, 6, 7].

	Human <i>Plasmodium</i> species				Rodent <i>Plasmodium</i> species
	<i>P. vivax</i>	<i>P. ovale</i>	<i>P. malariae</i>	<i>P. falciparum</i>	<i>P. berghei</i>
Pre-erythrocytic phase (days)	8	9	14-15	5.5-8	48-52 hours
Erythrocytic cycle (hours)	48	50	72	48	22-24
Sporogony (days)	8- 9	12- 14	14-15	9-10	13-14

1.1.3 Pathogenesis

P. falciparum infection in the human host has a range of pathologies, from totally asymptomatic at the pre-erythrocytic stage of infection, to potentially fatal during the erythrocytic stage. Severity of the disease is dependent on several factors, including host genetics, parasite species, and degree of pre-existing immunity (as discussed in section 1.2). Common symptoms of mild malaria at the erythrocytic stage include fever, headache, nausea, vomiting, fatigue and diarrhoea, which coincide with the cycles of RBC rupture. *P. ovale*, *P. malariae* and *P. knowlesi* often only cause such mild to moderate symptoms. *P. falciparum* and *P. vivax* are the major cause of

severe malaria, although *P. vivax* has significantly lower mortality in the key age group of 0-5 years [48], however several relapses of *P. vivax* malaria from dormant liver stage hypnozoites add to its degree of pathology upon an individual. This summary will focus on the pathogenesis of *P. falciparum* malaria.

Severe malaria can have life threatening symptoms: including severe anaemia, coma, acute respiratory distress and renal impairment. These symptoms often occur as a result of both the host inflammatory response to the blood stage infection, referred to as immunopathology, as well as the parasite interactions with host cells.

1.1.3.1 Immunopathology

Immunopathology results from two major functions: increased splenic removal of parasitised or altered RBCs from the bloodstream, which contributes to malarial anaemia [49, 50]; and the production of pro-inflammatory cytokines, which in itself can be triggered by macrophage activation and cytokine production in the spleen, as well as directly triggered by parasites in the blood. This pro-inflammatory response is also thought to contribute to many of the other symptoms of severe malaria [51, 52]. The pro-inflammatory response includes the production of a range of cytokines, such as tumour necrosis factor alpha (TNF- α), macrophage migration inhibitory factor (MIF) and interferon-gamma (IFN- γ), in addition to the release of mediators such as oxygen free radicals and nitric oxide (NO). These factors have been implicated in cerebral malaria, placental and hepatic dysfunction, the underlying cause of fever, and linked to the respiratory distress and renal impairment as a symptom of underlying metabolic acidosis [53, 54]. MIF also exacerbates malarial anaemia, through inhibition of erythropoiesis [55]. It is believed that the timing and balance of the pro-inflammatory and anti-inflammatory cytokine responses, as well as the presence of a regulatory T cell response, in addition to the parasite burden and associated parasite toxins such as haemozoin and glycosylphosphatidylinositol (GPI) that are released during the blood stage of infection, all determine disease progression and parasite clearance [56].

1.1.3.2 Host-parasite interactions

The main host-parasite interaction thought to contribute to the broad range of symptoms of *P. falciparum* malaria disease is the ability of parasite infected RBCs (iRBCs) to adhere to host molecules expressed on the surface of the endothelial cells lining the small blood vessels or organs, including the heart, liver, lung, kidney and in the case of cerebral malaria, the brain. The appearance of knobs on *P. falciparum*-infected RBCs around 12-16 hours following erythrocyte infection, consist of adhesive proteins such as PfEMP1, that bind host receptors in these tissues, leading to RBC sequestration. Sequestration can be tissue-specific, with Intracellular Adhesion Molecule 1 (ICAM-1) particularly responsible for parasite sequestration in the brain and CD36, P-selectin, E-selectin PECAM/CD31 and CR1 expressed on other host tissues [56-59]. Host receptors such as chondroitin sulphate A, which are expressed on cells of the placenta, also bind PfEMP1 variant VAR2CSA, thus increasing the severity of the disease in pregnant women [60]. Such sequestration in organs can lead to oxygen starvation and organ dysfunction. Infected erythrocytes can also adhere to each other (platelet-mediated agglutination) [61] or uninfected RBCs (rosetting) [62], further blocking blood flow in the vessels and restricting blood flow to organs.

It is widely accepted that sequestration of red blood cells leads to endothelial dysfunction, tissues inflammation, and in the case of the brain, the symptoms of cerebral malaria including seizures, loss of consciousness, brain damage, coma and even death [63]. There is however, some debate whether there is a simple direct link between reduced microvasculature flow caused by sequestration of infected RBCs and the pathologies seen in severe malaria, as sequestration of parasitised red blood cells are prominent in some tissues but absent in others, with equal functional loss [51] (reviewed in [64]). Regardless, sequestration clearly enables the parasites in the infected RBCs to evade immune clearance by the spleen, and in parallel to the immunopathology of the disease, leads to a vast array of clinical symptoms.

1.1.4 Current control measures

There is a renewed focus on both the elimination and eradication of malaria [65]. The former relates to the cessation of local transmission of malaria within a defined geographical area, whilst eradication would be the global disappearance of one or more malaria species [65]. Current control measures for prevention of malaria include ownership and use of insecticide treated bed nets (ITNs), indoor residual spraying (IRS) of insecticide, larval control at water sources and intermittent preventative therapy (IPT) for high risk groups, such as pregnant women and young infants during high transmission periods of the year [66]. The burden of malaria in the countries of sub-Saharan Africa has declined with scaling up of these prevention methods, alongside improved treatment and diagnosis of malaria. Although in vast areas of central Africa, poor infrastructure to support routine case reporting hinders progress tracking [67].

Certain areas have shown more promise of malaria elimination than others, for instance several countries in Southern Africa have shown substantial, lasting declines linked to the scale-up of such interventions [67]. In some regions, incidence decline was not considered a direct effect of ITNs or IRS: in The Gambia and Kilifi district of Kenya, the fall in malaria incidence began at a similar time to a switch in first line drug treatment, due to increasing chloroquine resistance [68, 69]. The switch to sulphadoxine plus pyrimethamine (SP) or SP plus chloroquine likely improved malaria cure rates, in addition to providing a period of chemoprevention against new malaria infections that could last for up to 6 weeks [70]. This chemo-preventative effect may be especially important in areas such as The Gambia, where malaria transmission is restricted to the June-September rainy season and immediate period following [71]: a transmission period much shorter than year round transmission in countries such as Ghana [72]. Similarly, the fall in malaria incidence in the early 2000s in Zanzibar was linked to the introduction of Artemisinin-based combination therapies (ACTs), as opposed to the increased use of ITNs or IRS several years later [73].

In several countries, malaria reduction is more strongly associated with the scale-up of preventative interventions. The Zambian National Malaria Control Programme has achieved substantial success in scaling up the use of ITNs and IRS, in addition to SP intermittent preventive

treatment in pregnant women [74]. Paediatric malaria parasite prevalence declined by 53% during this period between 2006 and 2008, and moderate to severe anaemia fell by 69% [75].

Interestingly, the decline in malaria incidence in some countries began several years before scale-up of malaria control, for example in Rwanda, where the decline in confirmed malaria cases occurred shortly before or at the same time as mass distribution of long-lasting ITNs and ACT use in 2006–2007 [76].

From the 1600s, infusions of Cinchona bark were the only effective antimalarial treatment until its active component, quinine, was isolated in 1820 [77]. *P. falciparum* malaria treatment subsequently consisted of chloroquine and primaquine, which is one of only two reported drugs to also eliminate *P. vivax* hypnozoites [78], alongside Tafenoquine [79]. However, the development and use of therapeutic drugs for malaria is a continual race against the development of drug-resistant parasites. Resistance to chloroquine emerged in the late 1950s in Southeast Asia [80] and spread to Africa by the late 1970s [81]. This sparked a new line of combination therapies such as SP and mefloquine, however SP resistance soon originated in the same region of South East Asia as that of chloroquine resistance [82], spreading even more rapidly to Sub-Saharan Africa. Mefloquine resistance also emerged in Thailand, Cambodia and Vietnam in the 1990s [83]. ACTs are now recommended by the World Health Organisation (WHO) as the first-line treatment of uncomplicated *P. falciparum* malaria in all malaria endemic regions [84], however many reports confirm resistance to ACT in several areas of Southeast Asia, again originating in Western Cambodia [85-87].

Further to drug resistance, insecticides used for ITNs and IRS have seen similar rounds of resistance, starting with *Anopheles gambiae* resistance to dichlorodiphenyltrichloroethane (DDT) in 1955 [88]. Insecticide resistance is now widespread among many malaria-transmitting mosquito species [89], and has developed to all 4 classes of insecticides recommended for public use, including carbamates, organochlorines, organophosphates and pyrethroids [8, 90-92].

Malaria therefore remains a global, medical, political and economic challenge and despite the implementation of the measures outlined above, several areas of sub-Saharan Africa still see rising incidence and transmission rates of malaria, suggesting that efforts using these interventions alone will not be sufficient to eliminate or eradicate malaria.

1.1.5 The requirement for an effective vaccine

Due to the increased prevalence of antimalarial drug-resistant parasites and insecticide-resistant mosquitoes, the current control methods for malaria are now only partially effective. While new classes and generations of antimalarial drugs, in particular, are being discovered [93-95], development of a prophylactic vaccine is considered necessary for malaria eradication [96], with vaccination proving successful in elimination programmes against smallpox [97] and polio [98]. A highly effective malaria vaccine against the most lethal malaria species, *P. falciparum*, could help to save half a million lives each year [99]. The primary target population for a vaccine is young infants in sub-Saharan Africa because from 6 months of age, infants and children in this region bear the greatest burden of malaria mortality [99].

1.2 Immunity

Despite our incomplete understanding of host immune responses to malaria, immunity to malaria has been measured in several forms. A key aspect of developing more efficacious vaccines is through understanding the mechanisms behind innate, adaptive and naturally acquired immunity to malaria, and applying these concepts to improve vaccination strategies.

1.2.1 Innate

The innate line of the immune system is considered the first line of defence after the skin barrier has been breached by a pathogen, and provides an immune response within a few hours of pathogen exposure, despite no prior sensitisation to the invading pathogen. The innate response is rapid but broad, and acts to clear the infection. The innate immune system is stimulated through the recognition of pathogen-associated molecular patterns (PAMPs) on invading microorganisms. Examples of PAMPs include lipopolysaccharide from gram-negative bacteria, bacterial DNA, viral RNA and glucans from fungal cells walls. These PAMPs are recognised by pathogen-recognition receptors (PRRs), including Toll-like receptors (TLRs) on innate cells, such as neutrophils, dendritic cells (DCs) and macrophages, basophils and mast cells. PAMPs can also be recognised by soluble PRRs in the blood, which initiate the complement pathway, which acts to opsonize infected cells, trigger an inflammatory response, and induce and complement the action of antibodies, in addition to phagocytic cells. The complement system together with the innate cellular components, work in tandem to produce soluble pro-inflammatory mediators, recruit immune cells to the site of infection, activate the complement cascade and activate the adaptive arm of the immune system.

Innate responses can initiate in the dermis and draining lymph node, where sporozoites can be detected shortly after infection. Innate response toward the whole sporozoite is a largely unexplored area [100], however using the *P. berghei* rodent model, it was recently discovered that *Plasmodium* liver-stage parasite RNA is sensed by the host, activating a type I IFN response, with hepatocytes identified as the primary source of expression [101] (see Figure 1.2). Using a *P. yoelii*

model, it was identified that NK and NKT cells were recruited into the liver as a result of this innate response from hepatocytes, where they subsequently set up a IFN- γ dependent inhibitory response against liver stage parasites [102]. Finally, upon traversal of hepatocytes, the release of host cytosolic material can result in innate stimulation through TLR4, activating the transcription factor NF- κ B. This in turn results in the induction of the inducible nitric oxide synthase (iNOS): a molecule detrimental to development for exo-erythrocytic forms (EEFs) [103-105].

The parasite has inhibitory mechanisms to evade such host responses. Activation of NF- κ B in the aforementioned study was only measured in surrounding cells, not traversed cells, which is consistent with findings that CSP can translocate to the hepatocyte nucleus and inhibit the NF- κ B pathway [106]. Furthermore, shedding of TRAP and CSP induces a suppressive signalling cascade that inhibits Kupffer cell activation and respiratory burst [100, 107]. Thus while shedding of parasite proteins during cell traversal may be deemed to be an opportunity for immune sensing, in many respects the process is an immune suppressor.

Innate responses towards the blood stage of infection have been studied somewhat more extensively, although is still quite a limited area of investigation. Extracellular blood stage parasites can lead to the induction of high amounts of pro-inflammatory cytokines and nitric oxide (NO) production. While these contribute to host defence and control of parasite growth, they are also proposed to contribute to malaria immunopathology and the rapid onset of clinical symptoms [108, 109]. Many studies have shown the complement cascade is triggered by merozoites and gametocytes, following egress from infected RBCs [110-112]. Furthermore, parasite metabolites haemozoin and GPI have both been reported to initiate innate responses via TLR receptors 2, 4, 9, and inflammasome activation, again contributing to the production of various pro-inflammatory cytokines and NO [113-118].

1.2.1.1 Genetic effects

Certain genetic variants have been selected for in malaria-endemic populations, due to the evolutionary protective advantage they provide against malaria. One such genetic trait is the sickle-cell allele, whereby heterozygotes for the sickle cell gene are relatively protected against death from malaria, due to the increased destruction of 'sickling' infected RBCs by macrophages [119]. Other examples of protective genetic traits include thalassemia, glucose-6-phosphate dehydrogenase 22 deficiency and the Duffy-negative phenotype [120, 121]. While these are not innate immune response, they are driven by evolutionary selection pressures due to their protective or partially protective effects.

1.2.2 Adaptive immune system

As previously mentioned, the innate immune system can initiate the activation of the adaptive immune system. This arm of the immune system is highly specialised, and relies on pre-exposure to pathogen antigens in order to form long lasting immunological memory. The adaptive response is made up of a humoral response, driven by antibody production by B lymphocytes following stimulation of their B cell receptor (BCR), and by cellular cytotoxic responses from T cells, stimulated via their respective T cell receptor (TCR). Once the specific antigen is recognised by either of these cell types, the naïve lymphocytes are activated and undergo rapid clonal expansion and differentiate into effector cells. A subset of these antigen-specific cells can persist long after antigen has been eliminated via pathogen clearance. These cells are thought to be precursors of antigen-specific long-lived memory cells, which are able to respond rapidly upon re-infection [122].

1.2.3 Cell-mediated immunity

The cellular response to malaria is comprised of T lymphocytes that recognise peptides displayed by major histocompatibility complex (MHC) molecules in the presence of co-stimulatory molecules. T cells are composed of two classes based on the presence of the surface markers CD4 and CD8, whereby expression is related to function.

CD4⁺ T cells, also termed T helper cells for their supportive role in immune responses, mainly recognise exogenous antigen presented by MHC class II molecules. The primary source of MHC class II presentation is professional antigen presenting cells (APCs) such as DCs, mononuclear phagocytes, some endothelial cells, thymic epithelial cells and B cells. Non-APCs can also express MHC class II, including atypical APCs such as mast cells, basophils, eosinophils and innate lymphoid cells, where MHC class II can be upregulated under certain conditions [123]. For MHC class II presentation, exogenous peptides are generated within APC endosomes, from exogenous proteins internalised by endocytic pathways. The MHC class II molecules are synthesised in the endoplasmic reticulum (ER) and delivered to the endo-lysosomal compartment, where they are loaded with peptide with specificity to their MHC class II peptide binding groove. This MHC-peptide complex is then transported to the cell surface for presentation to CD4⁺ T cells via their TCR [122].

Primed CD4⁺ T cells can be divided into subclasses based on their phenotype and functional characteristics. These classes include Th1, Th2, Th17, regulatory T cells (Treg) and T follicular helper cells (Tfh). This differentiation into different T helper classes and the effector functions is influenced by the cytokines and transcription factors present during clonal expansion and is indicative of their later functions as a T helper cell [124]. The two major subsets of CD4⁺ T cells are Th1, which produce interleukin-2 (IL-2), IFN- γ and TNF- α , and Th2 cells, which produce cytokines such as IL-4, IL-5, IL-6, IL-10 [125].

In contrast, CD8⁺ T cells recognise mainly endogenous antigen processed and presented by MHC class I molecules, which are expressed in all nucleated cells of the body, including APCs. Following direct infection of a cell by a pathogen, foreign proteins are identified and tagged for destruction by ubiquitination. These proteins are then degraded by the cell proteasome into peptide

fragments, and transported to the ER via the Transporter associated with Antigen-Processing (TAP) proteins, TAP1 and TAP2. MHC class I molecules are loaded with peptides with specificity to their MHC class I peptide binding groove. The MHC-peptide complex is formed in the ER, and upon loss of their chaperone proteins, transported via the Golgi apparatus to the cell surface [126, 127] for presentation to CD8⁺ T cells via their TCR. CD8⁺ T cells can also recognise exogenous peptide antigens through cross-presentation onto MHC class I molecules, usually on DCs [101][128].

The liver is an autonomous priming site for CD8⁺ T cells, in that it does not require infiltration of APCs in order to present antigen. Hepatic DCs, stellate cells, LSECs and liver-resident macrophages (termed Kupffer cells) all present MHC class I and II [129-131]. Like most nucleated cells, hepatocytes express MHC class I, but can also express MHC class II under inflammatory conditions [131, 132]. This is consistent with reports of *in vitro* CD4⁺ T cell recognition of parasite-derived peptides on the surface of infected hepatocytes in an MHC class II restricted manner [133, 134].

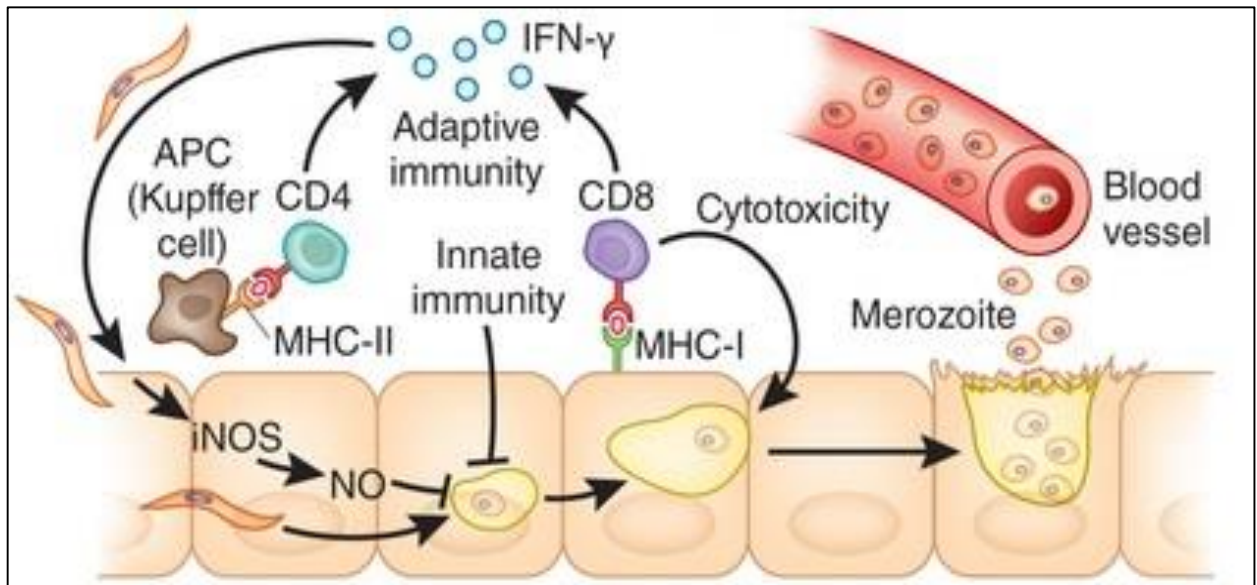


Figure 1.2 Innate and adaptive immune interactions with *Plasmodium* parasites in the liver

Image adapted from [5]. Sporozoites enter the liver and cross the endothelial barrier to traverse/infect hepatocytes. This infection can trigger innate signalling from within hepatocytes, including a type I IFN response. One proposed mechanism of the adaptive immune system is an antigen-specific IFN-γ response, triggering further effector functions. This IFN-γ response could be mediated by CD8⁺ T cells, stimulated via MHC class I presentation of endogenous parasite antigens from infected hepatocytes. MHC class II signalling to CD4⁺ T cells from Kupffer cells could also be the source of an IFN-γ response. IFN-γ can trigger NO production within the hepatocytes and inhibiting parasite development. Equally, CD8⁺ T cells could exert their own cytotoxic effects upon infected cells. Any mature liver stage schizonts that are not destroyed via the innate or adaptive arms of the immune system go on to release merozoites into the bloodstream.

1.2.4 Humoral immunity

The activation of B cells and their differentiation into antibody-secreting plasma cells is triggered by antigen. The process often requires T cell help, although T-independent B cell responses do exist [135]. Both linear and conformational epitopes can be recognised through the BCR. BCR binding to cognate antigen initiates a cascade of intracellular signalling, which leads to the internalisation of antigen for processing and presentation to CD4⁺ T cells via the exogenous MHC class II presentation pathway [136]. Recognition from a CD4⁺ T helper cell activates the B cell, which then undergoes clonal expansion and antibody secretion. This process takes place in lymph node germinal centres (GCs).

Inside the GCs, proliferating B cells may differentiate into short-lived plasma cells, which migrate to the periphery to secrete their antigen-specific antibodies. Alternatively, B cells may remain in the GC and undergo affinity maturation and somatic hypermutation to generate high affinity antibodies. Such affinity matured B cells can differentiate into long-lived plasma cells, which also migrate to the periphery, however they secrete high-affinity antibodies over many years. Affinity matured B cells can also differentiate into memory B cells, which provide immunological memory. These cells can respond rapidly upon re-exposure to the same antigen, resulting in a faster immune response. Newly generated antibodies following secondary antigen exposure are of switched isotypes and have higher affinity than the antibodies generated in the primary humoral response. Class switching of antibody immunoglobulin (Ig) from IgM to IgG, IgE or IgA is dependent on the cytokine environment and influence of CD4⁺ T cells.

1.2.5 Naturally-acquired immunity against malaria

In humans, three types of naturally-acquired immunity against *Plasmodium* have been defined: anti-disease immunity, which provides protection against clinical disease and the associated morbidity; anti-parasite immunity, which confers protection against high parasitaemia; and premunition, which provides protection against new *Plasmodium* infections through the maintenance of low-level, sub-clinical parasitaemia [137-139]. Sterilising natural immunity against infection is never fully achieved and has not been reported [3]. These aspects of naturally-acquired immunity to malaria involve both humoral and cell-mediated immunity, and while the exact mechanisms underlying this have not been fully elucidated, individuals living in endemic-regions develop such immunity largely in an age-dependent manner (Figure 1.3).

It has frequently been reported that malaria-sensitized individuals have no or relatively low cellular responses to pre-erythrocytic and blood stage parasite antigens, although responses to relatively few antigens have been assessed [140-142] (discussed later). Longitudinal studies in exposed populations suggest that immune responses to the pre-erythrocytic stages likely have limited involvement in the acquisition of natural immunity, due the re-emergence of sub-clinical

parasitaemia following drug clearance [143]. Thus humoral immune responses are considered to be the cornerstone of naturally-acquired immunity, with the potential for T helper responses to assist the antibody response. Furthermore, it is reported that naturally acquired antibody responses to sporozoites do not protect against malaria [144], so antibody responses to the blood stage of infection underpin the naturally acquired immunity seen in semi-immune adults.

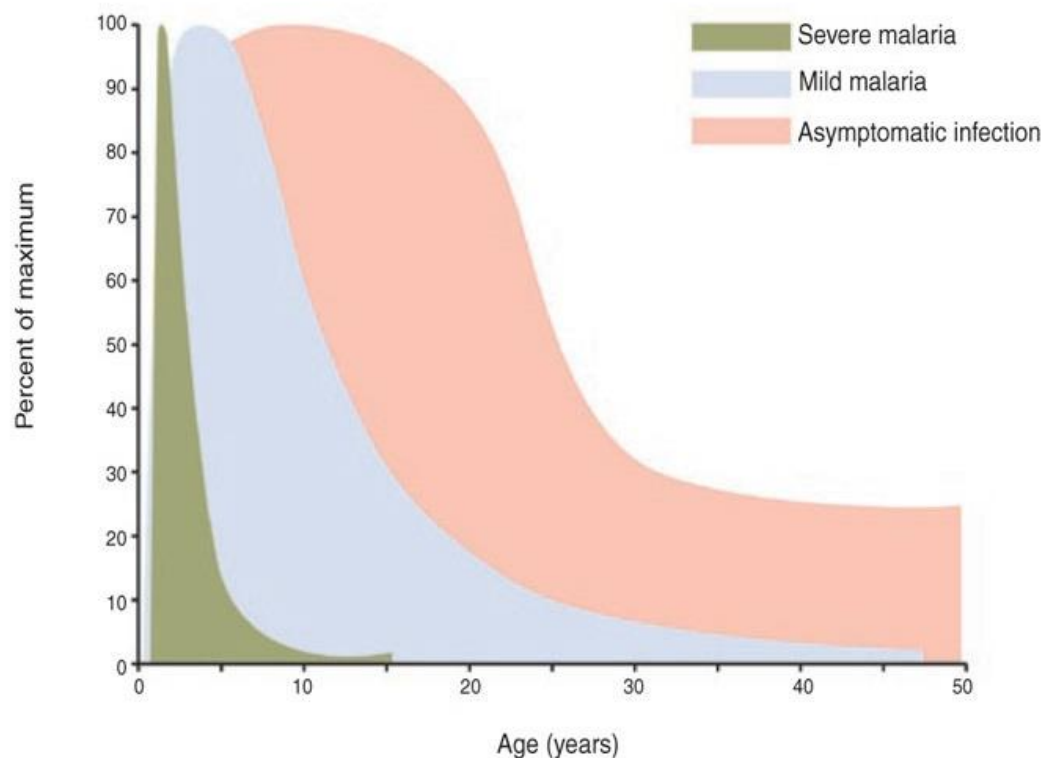


Figure 1.3 Age-dependent naturally acquired immunity to *P. falciparum* [3, 4]

Change over time of various malaria indices in a population living in an endemic area of *P. falciparum* transmission. Asymptomatic infection (pink); mild disease/febrile malaria illness (blue); severe/life-threatening disease (green). Data normalized and presented as the percent of maximum cases for each population index.

In 1961, a key study found that passive transfer of serum from immune adults to children suffering from severe clinical malaria provided obvious therapeutic effects [145]. This finding was independently verified in adults, with clearance of parasites and symptoms as fast as, or faster, than with drug treatment [146]. Together these results provide long-standing evidence that naturally acquired immunity is largely mediated by IgG against the blood stage of the disease. However, such blood stage immunity can take as long as 10-15 years to develop [147] and wanes on cessation of

exposure. Antibodies to merozoite antigens are believed to act by directly inhibiting merozoite invasion of erythrocytes or opsonising merozoites for phagocytosis [148, 149]. Specifically, Osier and colleagues found that a broad antibody response was strongly associated with protection from clinical malaria and the strongest associations with protection were found with combined responses to Merozoite Surface Proteins 2 and 3 (MSP2 and MSP3) [150]. Antibodies can also bind parasite-induced molecules on the surface in the infected RBC, such as PfEMP1, leading to greater infected cell clearance, with several studies having concluded that antibody responses against parasite encoded variant surface antigens (VSA) that are present on the surface of infected RBCs, are a key defence in the prevention of clinical disease [151-153].

There have been few links identified between natural exposure to malaria and the generation of protective cellular responses, which is most likely due to the small liver burden of each infection, immune evasion by the parasite and the immunosuppressive [154] or immunostimulatory [155] effects of concurrent blood stage infections. Reports of naturally-acquired cellular responses include detection of cytotoxic cells and cytokine responses to Liver Stage Antigen-1 (LSA-1) and their association with parasite clearance and protection from clinical malaria [156, 157] [158]. Studies of the TRAP antigen reveal that memory T cell responses against TRAP are associated with a significantly reduced incidence of malaria [159], however *ex vivo* IFN- γ response do not correlate with protection, indicating the importance of the memory phenotype generated [159, 160]. Finally, a report of naturally-acquired immunity to a CSP epitope was shown to be predictive of protection against infection and disease. This consisted of a memory CD4⁺ T cell response, but again did not correlate with *ex vivo* IFN- γ ELISpot response [161].

Low level cellular responses to erythrocytic antigens have also been reported [162, 163] and have been associated with reduced malaria morbidity and protection from disease. It is generally believed that the existence of malaria-specific CD4⁺ T cells in humans is linked to the induction of *P. falciparum*-specific antibodies, via Th2-type helper cells [164, 165]. In a similar manner to liver stage antigens, these responses are generally low, perhaps because they are often directed against the more polymorphic antigenic regions, suggesting parasite mutation and selection has occurred in response to immune pressure from T cells [166-168], however such polymorphism may impose an

obstacle for vaccine development. Cellular immune responses have also been detected against blood stage antigens, such as Merozoite Surface Protein 1 (MSP1), which correlated with number of previous malaria episodes in adults and protection against *P. falciparum* in children [157, 169].

Together these data suggest that in order to provide significant protection against infection or disease, a protective vaccine will require antibody and/or T cell induction of a much greater magnitude, breadth, phenotype or affinity than induced by natural parasite exposure. However the antigens associated with naturally acquired immune responses may provide indications as to where these responses should be directed.

1.3 Vaccination

Vaccination is one of the most cost-effective health care interventions available [170]. Most licensed vaccines are administered during infancy and protect primarily through the induction of antibodies [97, 171-173]. The exception to this is BCG, where protection appears to involve mainly CD4⁺ T cells [174]. However there is a range of diseases affecting infants for which vaccines are yet to be developed, where an ability to induce potent CD8⁺ T cell responses could be important. These include respiratory syncytial virus (RSV), human immunodeficiency virus (HIV), tuberculosis (TB) and vaccines targeting malaria parasites at the liver stage of infection [175]. A successful vaccine against malaria is likely to require induction of both cellular and humoral immunity [176-178], with the cellular arm providing cytotoxic cell-mediated protection against pre-erythrocytic liver stage of the disease, in addition to CD4⁺ T cell help and B cell antibody induction against the sporozoite and blood stages of infection [179].

Vaccines against eukaryotic parasites are inherently difficult to produce, due to the genetically and biologically complex nature of these organisms. *P. falciparum* parasites are no exception, with over 5000 genes and an elaborate sexual and asexual lifecycle across two hosts. There are currently no licensed human vaccines against any parasite, and only a handful of veterinary vaccines against parasites are commercially available, including protozoan vaccines for bovine babesiosis, poultry coccidiosis and ovine toxoplasmosis, of which are generally whole parasite vaccines with a degree of attenuation [180].

The 2013 Malaria Roadmap by WHO aims to license vaccines targeting *P. falciparum* and *P. vivax* by 2030. These vaccines aim to have protective efficacy of at least 75 percent against clinical malaria, include a transmission blocking element and must be suitable for administration to the at-risks groups in endemic regions [99]. Current vaccination strategies range from live whole sporozoite vaccines, to protein-in-adjuvant vaccines and subunit vaccines. These approaches are discussed below, along with key antigenic targets at each stage of the disease, and the clinical development of particularly successful strategies to date.

1.3.1 Pre-erythrocytic vaccine targets

Pre-erythrocytic vaccines target both the sporozoite and liver stage of infection. The brief period of the sporozoite in the bloodstream between breaching the skin barrier and infecting the liver is a target for a humoral response [181]. The aim is to eliminate sporozoites before infecting the liver. The time frame from the sporozoites entering the bloodstream (where exposure to such antibodies begins), to the point of liver stage infection can be as brief as 30 minutes [182]. Therefore, for sterilising pre-erythrocytic antibody-mediated immunity against malaria, it is believed the titre of antibodies against sporozoite surface antigens, such as CSP, needs to be very high [183]. Antibody-mediated protection at the liver stage is also reported, whereby antibodies that remain bound to sporozoites following liver stage infection can still suppress liver stage parasite development [184]. Prime targets for pre-erythrocytic antibodies are the dominant sporozoite surface antigens, CSP and TRAP. However the sporozoite proteome consists of almost 2000 proteins, so there is potential for other vaccine-induced antibody targets to be identified and developed [185].

The intracellular liver stage of infection is a second pre-erythrocytic parasite target for vaccination. It is a prime target for vaccination as the stage which lasts between 5.5 and 8 days in humans [126, 186, 187], thus prolonging the opportunity for antigen-specific cell-mediated killing. Furthermore, very few parasites are present at the liver stage of infection, relative to the blood stage, thus providing a bottleneck in the infection where the immune system has fewer targets to eliminate. Adaptive immune responses at the liver stage of infection rely on antigen presentation of parasite peptides via MHC molecules (see Figure 1.2). Infected hepatocytes express MHC class I, and are therefore ideal targets of cytotoxic CD8⁺ T cells and their associated protective immunity [188]. Furthermore, liver-resident APCs can present exogenous parasite antigens via MHC class II and cross present via MHC class I.

Key liver stage proteins identified thus far are either involved in hepatocyte infection, such as CSP, TRAP and Apical Membrane Antigen 1 (AMA1) [40, 41, 189, 190], or are expressed inside the PV or on the PV membrane, such as LSA1, LSA3 and Exported Protein 1 (EXP1) [191-193].

1.3.2 Pre-erythrocytic vaccine development

1.3.2.1 Whole sporozoite vaccines

Immunization with attenuated malaria sporozoites (dried and irradiated sporozoites) first achieved success in the early 1940s, in the *P. gallinaceum* whole sporozoite vaccination strategy of chickens [194]. It was later discovered that irradiated *P. falciparum* mosquitoes delivering sporozoites by mosquito bite provided sterile protection to animals [195, 196] and humans [197-200], providing the first evidence that a pre-erythrocytic vaccine is indeed possible. While not currently a deployable vaccine platform, due to over 1000 mosquito bites needed to confer protection in humans, it does provide a basis to study protective liver stage immune responses to malaria and remains the gold standard for induction of sterile protection.

Radiation attenuated sporozoites (RAS) are capable of invading hepatocytes, but development is arrested prior to merozoite release. This provides a repertoire of naturally expressed pre-erythrocytic antigens, against which an immune response can be directed [201]. Despite some earlier studies implicating neutralising antibodies as the protective mechanism [202, 203], more recent studies in mice and primates have shown dependence on CD8⁺ T cells [104, 204, 205]. Such immunity has been demonstrated towards infected hepatocytes, through murine CD8⁺ T cell depletion following vaccination [178]. Observations in murine studies indicate that CD8⁺ cells against defined *P. berghei* and *P. yoelii* CSP epitopes strongly inhibit liver stage parasite development [206]. Furthermore, adoptively transferred CD8⁺ T cells generated by vaccination with radiation attenuated sporozoites confer protection [207] and radiation attenuated sporozoites do not protect mice deficient in CD8⁺ T cells either as a result of *in vivo* depletion of these cells [178, 208] or depletion of β 2M [209] or MHC class I (K^bD^b) disruption [210].

One of these studies, a large murine study with 7 inbred strains and 1 outbred strain, initially explored the effects of CD8⁺ T cell, CD4⁺ T cell, NK cell, IFN- γ , IL-12 and NO depletion in vaccinated mice [211]. Interestingly, in all 8 mouse strains, protection was absolutely dependent on CD8⁺ T cells. In 4/7 inbred strains and the outbred strain, CD4⁺ T cells were not sufficient or required for the underlying effector mechanism. However in the other 3 inbred strains, an absolute requirement for

CD4⁺ T cells in protection was measured. Furthermore, an absolute requirement of IFN- γ in 6/7 inbred strains was identified, of which 5/6 also had an absolute requirement for NO. This is consistent with previous hypotheses that IFN- γ activates NO synthase, subsequently inducing the L-arginine-dependent NO pathway and production of high levels of NO. A study 6 years earlier identified that induction of NO activity was necessary but not sufficient for protection against sporozoites in the RAS model [104], further expanding the hypothesis for an alternative mediator of effector function triggered by IFN- γ (see Figure 1.2).

Finally, a definitive requirement for NK cells or IL-12, or effectors perforin, Granzyme B or Fas ligand was not identified. However, a triple knockout mouse for perforin, Granzyme B and Fas ligand would be required to rule out redundant contributions of each of these mechanisms. The study also highlighted that while some findings are consistent across all mouse strains following whole sporozoite vaccination, protection measured in different mouse strains with different vaccination platforms (comparing whole sporozoite to DNA vaccination) can add an additional layer of complexity to drawing firm conclusions, which may indeed be the case when exploring correlates of protection in diverse human populations.

The RAS approach progressed with the advancements made by biotech company, Sanaria, whereby non-replicating, irradiated sporozoites are injected intravenously by needle and syringe to elicit the same response. The strategy is to dissect and cryopreserve sporozoites for injection, providing a clinically deliverable product over that of mosquito bites [197, 212-214]. Theoretically this approach should achieve similar immunity to that of irradiated sporozoites. However, the logistics of delivering a vaccine that requires a liquid nitrogen cold chain, in addition to the cost and vast resources needed to raise and manually dissect mosquitoes under sterile conditions are clear limitations of this approach. Sporozoite administration methods are not yet optimal to mimic the efficiency of sporozoites delivered by mosquito bite, with the likelihood of intravenous injection required, over that of subcutaneous or intradermal injection, for optimal vaccine-induced immunity [214, 215]. This vaccination route potentially poses problems for administration to young infants. Additionally, the efficacy of such an approach has yet to be substantially validated, with only small sample sizes tested by controlled human malaria infection (CHMI) [216]. Finally, up to 5

vaccinations, each with a high dose of sporozoites (up to 1.35×10^5 'PfSPZ') are required for sterilising protection. However in one CHMI study, not all of the naïve controls became infected, potentially indicating a weak sporozoite challenge [214].

A second whole sporozoite vaccine immunises using non-attenuated sporozoites delivered by mosquito bite, whilst under chloroquine chemoprophylaxis (CPS). Chloroquine is an antimalarial drug that functions at only the blood stage of infection, and while the CPS approach is not strictly a vaccine, the strategy again delivers a broad spectrum of antigens presented through the full pre-erythrocytic infection and into the blood stage. In the first human study in 2009, Chloroquine was administered for a total of 13 weeks, while 12-15 *P. falciparum*-infected mosquitoes were allowed to bite on 3 separate occasions, at monthly intervals. Protection was measured in 100% (15/15) of volunteers, following CHMI 8 weeks post final immunisation [217], with all naïve controls becoming parasitaemic. Protective efficacy was measured again after 2 years: 67% (4/6) of volunteers exhibited sterile protection, with the remainder demonstrating a delay to parasitaemia [218]. A further study demonstrated this immunity was due to pre-erythrocytic, as opposed to blood stage immunity [219]. An earlier mouse study supported these findings, with liver stage drug Primaquine abrogating protection due to the lack of liver stage parasite development during vaccination. Furthermore, the corresponding murine study under Chloroquine administration used passive transfer of sera from immunised mice, in addition to CD4⁺ T cell- and CD8⁺ T cell- depletions, and concluded a role for both CD4⁺ and CD8⁺ T cells, but not antibodies [220]. Finally, in line with many studies using RAS vaccination, reversal of protection was not observed when neutralizing antibodies against IL-6, IL-12, and TNF- α were used, whereas injection of anti-IFN- γ antibody partially reversed protection. Equally, protection was also dependent on NO, with protection completely abrogated when a potent inhibitor of iNOS was administered 24 hours post challenge [220].

The CPS vaccination approach again delivers a high degree of long lasting sterile protection and requires vastly fewer mosquito bites in order to achieve similar level of protection. The difficulty would arise with mass administration, and the guarantee that anti-malarial drugs were being administered alongside the 3 rounds of mosquito bites, in order to prevent breakthrough infections.

Concurrent infections in endemic areas could also interfere with the 8 week vaccination strategy, especially if other anti-malaria drugs were administered concurrently.

Following the success of RAS vaccination, genetic attenuation of parasites was developed as an alternative form of whole sporozoite vaccination. Instead of irradiated sporozoites, this approach genetically deletes or mutates key antigens within the parasite, resulting in the parasite arresting during the liver stage of infection. This approach gave protection in rodent challenge models [221-224], with genetic attenuation of genes including Upregulated in Infective Sporozoites genes 3 and 4 (UIS3, UIS4), and members of the 6-Cys domain protein family: p36 and p52. Protection was again dependent on CD8⁺ T cells in an MHC dependent manner, with mice deficient in $\beta_2 m$ not protected [225]. It has been suggested that the GAP approach has an advantage over the RAS approach, because proteins expressed at the late liver-stage can be targeted for attenuation, therefore increasing the time parasites are present in the liver and thus allowing the development of a broader immune response [226]. The first human study to assess the safety and immunogenicity of this approach used deletion of pre-erythrocytic stage-expressed genes p52 and p36. One low bite dose (5 mosquito bites) and 1 high bite dose (~200 mosquito bites) of mosquitoes infected with *P. falciparum* *p52*⁻/*p36*⁻ showed good safety following low exposure dose, but saw breakthrough parasitaemia in 1/6 volunteers following the high exposure dose, indicating that full liver stage attenuation had not been achieved with the double gene knockout [227]. New GAP parasites are being developed, including triple gene attenuated parasites, and tested *in vitro* and in liver humanised mice *in vivo* [228, 229]. Most recently, success with triple gene knockout of p52, p36 and Sporozoite Asparagine-rich Protein 1 (SAP1) has achieved no breakthrough infection in humans, even with 200 mosquito bites [230].

The risk of breakthrough infections associated with the GAP vaccination strategy has already been observed once in human trials, with incomplete attenuation previously identified as a risk [231]. Going forward, a GAP parasite needs to demonstrate complete attenuation in order to be a viable clinical product. In addition to GAP vaccination, chemically attenuated parasites have been tested in mouse models only [232, 233]. These have again demonstrated long lasting sterile protection, transcending across strains. The risks associated with this form of vaccination are similar

to that of GAP vaccination, however data so far supports testing of this vaccine approach in humans [234].

1.3.2.2 Subunit vaccines

In 1986 the Hepatitis B surface antigen vaccine was the first subunit vaccine to be licensed [235]. Since then, subunit vaccines have been licensed for many diseases ranging from diphtheria and tetanus, to *Haemophilus influenzae* type b [236]. Subunit vaccines include synthetic peptide vaccines, DNA vaccines, recombinant protein-in-adjuvant vaccines and vaccines that are virally vectored. Many of these subunit approaches have been used as vaccine platforms against malaria as next generation vaccines, following on from whole sporozoite vaccines. Malaria subunit vaccine efforts traditionally focused on CSP, as this was one of the first parasite antigens identified, is the dominant surface antigen of sporozoites and was initially thought to mediate protection of irradiated sporozoite vaccines [237], 238, 239]. A number of other antigens have also been assessed as vaccine candidates in clinical trials of subunit vaccines, at both the pre-erythrocytic stage (e.g. TRAP, LSA1, LSA3, EXP1) and the blood stage (e.g. MSP1, MSP3, AMA1, RH5) [240]. Outlined below are the main pre-erythrocytic developments using subunit vaccines.

1.3.2.2.1 Protein-in-adjuvant

Adjuvants are usually delivered alongside protein vaccines, in order to increase the immunogenicity of the protein, often through stimulation of the innate arm of the immune response. Adjuvants licensed for human use include aluminium salts, emulsions such as MF59, liposomes, virosomes and Lipid A analogues, with most reported to enhance the humoral response. This is observed with the low CD8⁺ T cell responses of RTS,S, the leading subunit CSP-vaccine that use TLR4 agonists AS01 or AS02 [241-243], where strong CD4⁺ T cell and antibody responses are measured (discussed in 1.3.2.2.3). A similar response phenotype was measured when recombinant LSA1 protein was administered with AS01 or AS02 [244]. While adjuvants in clinical testing also enhance

CD8⁺ T cell responses [245], few have been used in malaria vaccines and are generally not the approach used to induce strong cell-mediated responses required by liver stage vaccines, however their use in enhancing antibody induction in sporozoite stage vaccines is extensive, as discussed in 1.3.2.2.3.

1.3.2.2.2 DNA and viral vectors

DNA and viral vector vaccines both work by delivering a gene sequence to host cells, which is subsequently transcribed and translated, resulting in the expression by that cell of the encoded protein. These vaccines are therefore very effective at inducing T cell responses as the endogenous protein can be processed and presented on MHC molecules, particularly MHC class I. Furthermore, with viral vectors, the vector itself can also act as an adjuvant [30, 246].

The first malaria DNA vaccine tested was *P.yoelii* CSP vaccination in mice, which resulted in a strong CD8⁺ mediated response and efficacy against homologous *P.yoelii* challenge [247]. Such strong immunogenicity did not translate into humans [248, 249], however DNA vaccination provided a good priming basis for boosting with a viral vectors or recombinant protein-in-adjuvant in heterologous prime-boost approaches [250], with many human studies performed with TRAP and/or CSP antigens [251-253].

The use of adenoviruses and poxviruses as vaccine vectors has been explored, as the viruses are highly immunogenic, stable and easy to manipulate [254]. Adenoviruses are non-enveloped DNA viruses and poxviruses are enveloped DNA viruses. Both human and chimpanzee adenoviruses have been used as vaccine vectors in many human clinical vaccine trials, as well as poxviruses such as the fowlpox FP9 virus and Modified Vaccinia Ankara (MVA). These vaccine trials have been against a range of diseases, including hepatitis C [255, 256], HIV [257-259], Ebola virus disease [260, 261], TB [262, 263], influenza [264, 265] and malaria [252, 266-270].

Several human and simian adenoviruses have been assessed for their suitability for use in human vaccination [271] and have been found to induce high levels of both T cells and antibodies, particularly CD8⁺ effector and effector memory responses [266, 272]. It is also reported that

following MVA vaccination, infected APCs rapidly migrate to draining lymph nodes presenting antigen to naïve CD8⁺ T cells as soon as 6 hours after inoculation [273]. MVA is therefore also able to effectively stimulate the cytotoxic arm of the adaptive immune response and induce accelerated development of CD8⁺ T cell memory [274], in addition to robust antibody responses [275]. Memory phenotyping of T cell responses to malaria antigen, TRAP, following adenovirus and MVA vaccination in mice demonstrated that adenoviruses induces a strong CD8⁺ T cell effector memory response and little CD8⁺ T cell central memory, with the opposite induced after MVA vaccination [276], further highlighting the combined use of these vectors in a prime-boost approach. The ability of these vaccines to stimulate both the humoral and cell-mediated arms of the adaptive immune system have led to their further use in Phase I and II clinical vaccine studies.

Two major concerns surrounding the use of viral vectored vaccines were safety and the impact of pre-existing immunity in vaccine immunogenicity. MVA is a replication incompetent, and was derived from Chorioallantois Vaccinia virus Ankara following over 500 passages through chicken embryo fibroblast cells [277], and adenovirus vectors for use in malaria vaccine studies undergo deletion of their E1 and E3, rendering viruses replication incompetent as well as reducing viral immune evasion through interference with signalling and MHC peptide presentation [278]. Furthermore, safety concerns have largely been addressed with the excellent safety profile of adenoviruses, particularly chimpanzee adenovirus 63 (ChAd63) and human adenovirus 35 (AdHu35), and poxviruses FP9 and MVA in human clinical vaccine trials against malaria in both malaria naïve adults, and vaccinated adults and children from malaria endemic countries [252, 266-269, 279-288]. The effects of pre-existing immunity against viral vectors, particularly common adenovirus serotypes such as AdHu5, with particularly wide seroprevalence, and higher neutralising antibody levels found in sub-Saharan Africa and parts of South East Asia [289, 290], have been circumvented through the use of chimpanzee adenovirus vectors. These vectors including ChAd63 and ChAd3, have much rarer seroprevalence in human populations, and low cross-reactivity with other human and chimpanzee adenoviruses [271]. For example, in cohort of Kenyan children aged 1-6 years, 23% had high-titre neutralizing antibodies to AdHu5 but only 4% had high-titre neutralizing antibodies to ChAd63 [291].

In a CSP plus AMA1 vaccination approach delivered using a DNA prime and AdHu5 boost, a statistically non-significant effect of 27% sterile protection was measured and associated with cell-mediated immunity against AMA1. AMA1 is expressed at the sporozoite, pre-erythrocytic and erythrocytic stages of infection, but it was demonstrated that vaccinees exhibiting sterile protection did so at the liver stage of infection, with no blood stage parasitaemia measured, contrasting with the unvaccinated controls and non-protected vaccinees [292].

The most immunogenic prime-boost vaccination strategy to date uses ChAd63 and MVA. This prime boost regimen has been tested with many pre-erythrocytic and blood stage antigens, including single and multiple antigen approaches using CSP, TRAP, AMA1 and MSP1 [266, 267, 286], resulting in strong T cell and antibody responses. A Phase I clinical study assessing ChAd63.MVA CSP reports good safety to both vaccines, and a median post boost peak *ex vivo* IFN- γ ELISpot response of 1524 SFC / 10^6 PBMC [286]. However in a Phase II CHMI study, only 8% (1/15) of vaccinees exhibited sterile protection following CHMI, with a delay to time of treatment in 20% (3/15) [285].

The most successful candidate antigen using the ChAd63 prime-MVA boost regimen is ME-TRAP: TRAP is a membrane protein essential for sporozoite motility and liver cell invasion [41]. The antigen is fused to a multi-epitope string (ME) of predominantly CD8⁺ T cell epitopes from several malaria antigens, including pre-erythrocytic antigens LSA1, CS, STARP, LSA3, TRAP and EXP1, in addition to some CD4⁺ T cell and B cell epitopes [293]. ME-TRAP is encoded in ChAd63 and MVA viral vectors, and administered in an 8-week prime-boost schedule: ChAd63.MVA ME-TRAP. In Phase I and II trials, ChAd63.MVA ME-TRAP has proven safe and immunogenic, both in malaria naive [266] and malaria exposed adults [269, 294], with a median post-boost peak *ex vivo* IFN- γ ELISpot response of >2400 SFC / 10^6 PBMC [287].

Heterologous strain CHMI studies in healthy UK adults measured sterile protection in 21% (3/14) of vaccinees, with delay to treatment in a further 36% [287]. These findings were replicated to a similar level in a second heterologous CHMI study, exhibiting 13% (2/15 vaccinees) sterile protection and a delay to time of treatment in a further 33% (5/15) of vaccinees [285]. Monofunctional IFN- γ producing CD8⁺ T cells positively correlate with protection [287]. This biomarker suggests infected hepatocytes present TRAP peptide via MHC class I to vaccine-induced

CD8⁺ T cells. Finally, in a recent field trial in Kenyan adult vaccinees, ChAd63.MVA ME-TRAP measured 67% efficacy against malaria infection. Again, a T cell correlate of efficacy was observed [295]. Significant anti-TRAP IgG titres after heterologous prime-boost with ChAd63.MVA ME-TRAP have also been reported, and could additionally contribute to vaccine efficacy [285].

1.3.2.2.3 Particle vaccines

Particle vaccines, or virus-like particles (VLPs), are multiprotein structures that mimic the organisation and conformation of authentic viruses, but lack the viral genome. They make recombinant proteins more immunogenic than they would be administered as protein or peptide vaccines [296]. RTS,S is the only VLP in clinical malaria vaccine testing and is the most advanced malaria vaccine to date. Developed by GlaxoSmithKline and the Walter Reid Army Institute of Research (WRAIR), RTS,S is a recombinant yeast-expressed subunit vaccine that uses the hepatitis B surface antigen (HBsAg) as a matrix carrier for fused epitopes derived from CSP. This CS fusion protein consists of the C-terminal region of CS, which contains 19 NANP repeats ("R") and is the dominant B cell epitope in CSP [297], and T cell epitopes in the C-terminal flanking region of CSP ("T") [298, 299]. Fusion to HBsAg ("S") and expression in an excess of HBsAg, results in the particle vaccine RTS,S, which is administered with adjuvant AS01 or AS02 [300].

RTS,S has shown good efficacy against CHMI in adults in the USA [301, 302], however efficacy against clinical malaria observed among 6-12 week old infants in a large Phase 3 clinical trial was around 30% over 12 months of follow up [303, 304], and declined thereafter, well below the target of 75% efficacy against clinical malaria as laid down in the Malaria Vaccine Technology Roadmap [99, 305]. RTS,S/AS01 efficacy is mediated by IgG antibodies and CD4⁺ T cells against CSP [225, 242, 306]. The vaccine fails to induce significant CD8⁺ T cell responses [243], possibly because VLPs may be poor primers of CD8⁺ T cells.

1.3.3 Blood stage vaccine targets

The aim for a blood stage vaccine is to improve upon the protective immunity against severe disease measured with naturally-acquired humoral immunity, by eliminating merozoites before they can cause clinical symptoms. The progress of blood stage vaccine development has been slower than recent pre-erythrocytic approaches, and with less success in inducing protective immunity [240, 307]. One difficulty with antibody-inducing vaccines is the requirement to express antigens with correct conformational folding. A further challenge is the necessity to induce the exceptionally high titres of antibody required to clear the blood stage infection, which is currently not being achieved with the current vaccine platforms and adjuvants available. However, with antibody levels induced by vaccination far surpassing levels measured by natural exposure, but not providing significant protection, the question is posed regarding the breadth of antibody responses needed against a variety of blood stage antigens. Finally, due to the naturally acquired immune selection pressures on the blood stage of infection, blood stage antigens are often highly polymorphic [308]. Vaccine studies of polymorphic antigens, such as AMA1 and MSP1, suggest variant-specific effects can be achieved [240], but the requirement to include more than one allele in a vaccine may hamper the approach further.

1.3.4 Blood stage vaccine targets and vaccine development

Most blood stage vaccines are protein-in-adjuvant vaccines, using antigens such as MSP1 and AMA1. Three clinical approaches in humans have shown some non-significant levels of efficacy. In a protein-in-adjuvant trial of AMA1 in AS01B/AS02A resulted in a possible reduction in parasite densities following sporozoite CHMI [309]. A Phase II field trial of the AS02A formulation saw no efficacy against the primary end point of clinical malaria episodes, however did show some strain-specific protection [310]. A Phase 1/IIb field trial in Papua New Guinea of 3 proteins (MSP1, MSP1 and Ring-Infected Erythrocyte Surface Antigen (RESA)) formulated in oil-based adjuvant Montanide ISA720 measured a slight but non-significant reduction in parasite burden following vaccination,

which was again driven by a strain-specific response [311]. Finally, a Phase I/IIa CHMI trial of an influenza virosome-based vaccine formulation including CS and AMA1 malaria antigens: PEV3A, significantly reduced mean rate of blood stage parasite growth in vaccinees, suggesting a substantial blood stage effect [312].

In 2011, basigin and Reticulocyte Binding Protein Homolog 5 (RH5), were identified as essential and non-redundant in RBC invasion by blood stage merozoites [313]. Encouragingly in 2015, a *P. falciparum* CHMI study in *Aotus* monkeys showed significant strain-transcending blood stage efficacy, which strongly correlated with anti-PfRH5 antibody concentration, when RH5 was administered alongside Freund's adjuvant or encoded in an 8 week prime boost regimen using viral vectors ChAd63 and MVA [314]. As this is the only blood stage antigen to show heterologous parasite strain efficacy in non-human primates, ChAd63 RH5 alone and the ChAd63.MVA RH5 prime-boost regimen has been taken forward to Phase I human clinical vaccine trials (NCT02181088, unpublished data) and remains the most promising blood stage candidate to date.

Interestingly, the whole sporozoite vaccination approach has been tested for efficacy against blood stage malaria, whereby cellular immune responses against blood stage infection have showed protection. There are several reports of low dose iRBC vaccination with dead parasites or with drug treatment in murine and non-human primates (reviewed in [315]), however in 2002, the approach was tested in 5 volunteers [162]. Low dose iRBCs were administered followed by drug treatment while the infection was still subclinical. After 3 rounds of this vaccine treatment, volunteers were given a blood stage challenge, and protection was measured in 75% (3/4) volunteers. Parasite-specific antibodies were not induced, but proliferative CD4⁺ and CD8⁺ T cell responses were measured, alongside IFN- γ responses and NO induction in PBMC, indicating protection was due to cell-mediated responses. As a blood stage vaccine, this has showed encouraging protection, however it is extremely unlikely this will become a clinical product, due to the risks associated with administering a donated blood product, in addition to the risk of clinical malaria infection and the requirement to grow large numbers of blood stage parasites.

1.3.5 Transmission blocking vaccine targets and vaccine development

Transmission-blocking vaccines (TBVs) aim to induce antibodies to blood stage or mosquito stage parasite antigens in order to block transmission of the parasites other individuals by stopping parasite growth in the mosquito. As such, transmission blocking vaccines do not protect the vaccinee, rather their application has an altruistic impact on communities. This has obvious disadvantages as a vaccine strategy, as high vaccine coverage in a community is required, and with no direct benefit to the individual vaccinee, it is unknown how high vaccine uptake would be. Unlike pre-erythrocytic and blood stage vaccines, if mosquito stage antigens are used in the TBV, no natural re-boosting of antibody levels would occur. However, this novel approach to vaccination selects mosquito antigens that have not previously been under any immune selection pressure from the host, and are therefore less polymorphic [5, 316], although regions of polymorphism have been identified in some key antigens [317, 318].

Phase I human clinical vaccine trials of *P. falciparum* and *P. vivax* surface proteins, Pfs25 and Pvs25, have induced antigen-specific antibodies in vaccinees through protein-in-adjuvant vaccination. These antibodies correlated with transmission blocking activity in the standardised readout, which is a membrane feeding assay. Efforts have been made to increase the immunogenicity of Pfs25 in an adjuvanted viral vector approach [319], in addition to the identification of other vaccine candidate antigens, including Pfs48/45, for which a Phase I study is on the horizon [320].

More work is required in the development of transmission blocking vaccines. However with elimination of malaria from communities a renewed and achievable target, the inclusion of a transmission blocking element in a multi-stage vaccine could provide the extra control upon malaria transmission needed to achieve this target [65].

1.3.6 Multi stage vaccine development

It is hypothesised that partially protective vaccines could be combined to achieve an additive or even synergistic protective effect. A key question in the development of multi-stage vaccines is regarding the way in which they will work together. Partially protective vaccines can reduce the number of infected hepatocytes, thus decreasing the number of merozoites released into the bloodstream from the liver [285]. However, does this in turn permit more time for blood-stage immunity to develop before the clinical symptoms appear, or indeed for a blood stage response to mount from a multi-stage vaccine? If pre-erythrocytic and blood stage vaccines could work with such an additive effect, combining *P. falciparum* antigens that target these stages may further decrease the probability of reaching the disease threshold.

The counter argument to this is the potential for naturally acquired blood stage immunity to be lost, or acquired at a much slower rate, due to decreased exposure to blood stage antigens through natural infection. This is because blood stage immunity relies of frequent exposure in order to maintain levels. Thus the true consequences of partially protective vaccines is not fully understood, however they will undoubtedly have potential to provide some protection.

To this end, the ChAd63.MVA ME-TRAP viral vector regimen has been combined with the 3 shot RTS,S regimen, in order to assess the combination of 2 partially protective vaccines. The staggered administration of the 5 vaccinations was safe, and vaccine efficacy was evaluated by CHMI 2-4 weeks following final vaccination, comparing efficacy against a control group receiving only the 3 vaccination RTS,S regimen. Both groups demonstrated statistically significant vaccine efficacy against CHMI, with sterile protection in the control group measured in 75% (12/16) of RTS,S vaccinees, compared to 82.4% (14/17) of vaccinees in the ChAd63.MVA ME-TRAP + RTS,S group. This significant effect was also seen 6 months later, when a proportion of vaccinees from each group were re-challenged by CHMI. However, there was no significant enhancement of vaccine efficacy in the combined vaccine group, compared to RTS,S alone [284], possibly due to the high levels of efficacy from by RTS,S alone in this study. Encouragingly, anti-CSP antibody induction between group 1 and 2 were comparable, and T cell responses against TRAP in group 1 were comparable to previous studies

of ChAd63.MVA ME-TRAP administration alone [287], indicating no interference between vaccine-induced immunogenicity when the vaccines were administered in a staggered regimen. While these vaccines did not obviously interfere with one another, a substantial additive effect of each of the strategies was not measured to level of statistical significance. This study does, however, warrant further investigation of such multi-stage vaccines.

1.3.7 Models for vaccine evaluation

1.3.7.1 *Controlled human malaria infection*

Compared with other infectious diseases of major global importance, such as HIV and TB, one of the key factors contributing to the progress of malaria vaccines has been the availability of a clinical challenge model of controlled human malaria infection with *P. falciparum*, enabling definitive Phase II vaccine evaluation through direct human assessment. CHMI for evaluation of pre-erythrocytic candidate vaccines is traditionally conducted by exposure of a volunteer to laboratory reared *P. falciparum*-infected *Anopheles* mosquitoes [321], however more recently the concept of CHMI through administration of commercially available cryopreserved sporozoites by needle and syringe has been explored [322, 323]. Patient parasitaemia can be monitored by quantitative real-time polymerase chain reaction (qPCR) [267, 324, 325] with endpoints based upon detection of blood stage parasites by microscopy combined with presentation of clinical symptoms and/or positive qPCR result. Volunteers are then drug treated to clear parasites [321].

While CHMI is an extremely powerful tool that allows immunogenic vaccines in Phase I studies to be tested for small-scale efficacy before larger scale, expensive field efficacy studies are embarked upon, it provides little or no indication of mechanism responsible for the protection measured. Furthermore, human vaccine studies are generally limited to sampling of the leukocytes from peripheral circulation, which do not account for tissue-resident T cells that likely play a critical role in the liver immunity [215, 326, 327]. However, CHMI efficacy data can be combined with peripheral immunogenicity data to explore correlates of protection. Concurrent experimentation in

animal models or functional *in vitro* models is then required to elucidate the exact mechanisms behind the protection measured.

1.3.7.2 *In vivo and in vitro models*

In vivo and *in vitro* models can be useful tools in both down-selecting vaccine candidates for human testing, in addition to further exploring the optimal administration of vaccination regimens and the underlying mechanisms of protection, following the measurement of vaccine efficacy by CHMI. *In vivo* models using mice and NHPs will never truly replicate human immune responses to *P. falciparum* following vaccination, thus there is renewed interest in using novel transgenic models and functional *in vitro* assays to study such areas.

Murine models of *P. berghei*, *P. yoelii*, *Plasmodium vinckei* and *P. chabaudi* infection permit a basis for such *in vivo* experimentation and evaluation of malaria vaccines. The *P. berghei* infection model has been used for over 60 years [328], and has been used extensively to study vaccine-induced immune responses to malaria [104, 221-225, 329-333]. *P. yoelii*, and to a lesser extent *P. vinckei* and *P. chabaudi*, provide models with different disease outcomes and levels of susceptibility in inbred mouse strains.

At the hepatic stage, BALB/c mice show low susceptibility of infection to *P. berghei* sporozoites, with only ~0.1% of inoculated sporozoites infecting a hepatocyte [334]. The same mouse strain is 2000 fold more susceptible to *P. yoelii* parasites, however they show great variability between BALB/cJ and BALB/cByJ mice [335]. Generally speaking the innate immune response in rodents is characterized by an influx of mononuclear cells, neutrophils and eosinophils around late stage schizonts and emerging merozoites. This is particularly seen with *P. berghei*, whereby the innate inflammatory response initiates as soon as 24 hours post inoculation [334, 336]. This inevitably undermines the accuracy with which adaptive immune-responses to vaccination can be evaluated.

It is argued that inbred mouse strains are poor predictors of the diversity measured in human populations [337], and therefore may only be representative of a very small area of the immune

responses measured in humans. Among the rodent models, *P. berghei* and *P. yoelii* have been the most commonly used in liver stage vaccine research, however it is clear that careful consideration is needed for the selection of an appropriate mouse model for each experimental purpose. Mouse models did accurately identify the role of CD8⁺ T cells in liver stage malaria, and while mice have often accurately identified vaccination regimens that will not be protective in humans, positive results in mice do not always translate into humans. A key example is the high murine immunogenicity of DNA vaccines that was not replicated in human vaccinees [249, 338].

An alternative model to mice, is that of non-human primate (NHPs). *Aotus* monkeys can be infected with *P. falciparum* and *P. vivax* [314, 339-342], while rhesus macaques (*Macaca mulatta*) can be infected with *P. coatneyi*, *P. knowlesi* and *P. cynomolgi*, with the latter capable of generating persisting EEFs that produce relapses, [343] mimicking that of human *P. vivax* malaria with dormant liver forms [344]. In addition, experimental infections of chimpanzees with *P. falciparum* have been achieved multiple times under laboratory conditions [345, 346]. At present, *Aotus* monkeys are the recommended NHP model for *P. falciparum* [347], however protective efficacy measured in this model is not necessarily indicative of protection in humans [348].

With particular reference to studying cell-mediated immunity, there are key differences in the MHC of both mice and NHPs to that of humans, which inevitably affect host immune responses. Comparative analysis of humans and mice reveal similarity in MHC function, but not in genetic sequence, which is unsurprising considering the evolutionary split of rodent and primate ancestors approximately 60 million years ago [349]. Furthermore, unlike human MHC class I, mouse class I molecules also act as ligands for NK cells. There are also biological drawbacks to NHP models, such as the more diverse MHC in NHPs, the absence of HLA-A2 in chimpanzees and lack of HLA-C loci in rhesus macaques [350-352]. In addition, the cost of NHP studies are extremely high and can be hindered by lack of immunological tools and reagents to probe the immune response to the extent achieved in rodent models [353]. Thus, while rodent and NHP models permit scientists to use most appropriate model for their particular research question [354], there are many limitations to using other species to model human immune responses. Since human immunity to *Plasmodium* species or

the exact mechanics of parasite development within human hepatocytes is not fully understood, it is difficult to determine which model, if any, best reflect human immune responses.

To this end, the study of protective human immune responses has expanded with the use of humanised mouse models incorporating human hepatocytes [355, 356], erythrocytes [357] or immune cells [358]. In addition, the use of chimeric or transgenic parasites [359-361] aid the advancement of *in vivo* and *in vitro* studies going forward.

In vitro models can provide functional data on human immune responses. In the setting of malaria immunity, only two such models exist: the inhibition of sporozoite invasion (ISI) assay; and the inhibition of liver stage development assay (ILSDA) [184, 362]. Both of these assays measure the functional capacity of human antibodies to inhibit liver stage invasion and development. No such assay currently exists for the evaluation of human T cells in liver stage infection, however a murine model has been recently developed at The Jenner Institute [363]. The advancement of these assays, and the assay developed in this thesis, is underpinned by the development of transgenic parasites. Initially, the transgene introduced resulted in fluorescent parasites [364, 365]. Since these early studies, transgenic rodent malaria parasites that express specific *P. falciparum* antigens were developed [366-368], and advanced to transgenic parasites that can both fluoresce and express *P. falciparum* antigens. These parasites are generated by either allelic replacement of the *P. berghei* gene with the *P. falciparum* homolog or integration of the *P. falciparum* gene at a disposable point in the *P. berghei* genome, by double homologous recombination [360].

1.4 Thesis aims and outline

There has been much progress in the development of a protective subunit malaria vaccine, with pre-erythrocytic candidate ChAd63.MVA ME-TRAP currently the most advanced viral vector approach. Much of the clinical testing so far has included Phase I and Phase II studies, evaluating safety and immunogenicity in malaria naïve and malaria-exposed adults, and assessing vaccine efficacy through small scale CHMI studies, which led to the identification of a single immune correlate of protection measured through immunogenicity assays performed on peripheral blood. The underlying effector mechanism, including whether this CD8⁺ T cell correlate is associated with direct inhibition of parasites at the liver stage of infection, have yet to be explored, as there is currently no direct functional assay to measure T cell-mediated liver stage parasite inhibition.

Re-boosting of the 8 week ChAd63.MVA ME-TRAP regimen has previously been tested, however optimal regimens using these viral vectors have never been explored in human volunteers, relating to prime-boost duration and administration of multiple priming or boosting vaccinations. Finally, until now this vaccination regimen has not been tested in infants or children in malaria endemic countries, which are the firm target population for a licensed malaria vaccine.

This thesis aims to address these under explored areas of malaria vaccine immunology, through examination of immune responses to vaccination, using both *in vivo* and *in vitro* systems.

1.4.1 Aims

- Optimise a flow cytometric assay with intracellular cytokine staining suitable for use at field laboratories with limited resources, and utilise the assay alongside the standard *ex vivo* IFN- γ ELISpot readout to assess the immunogenicity of ChAd63.MVA ME-TRAP across age de-escalating populations living in a malaria-endemic country.
- Evaluate the immunogenicity of seven novel schedules of ChAd63 ME-TRAP and MVA ME-TRAP administration, through assessment of enhanced cell-mediated immunogenicity and the associated effects of anti-vector immunity.
- Develop a functional *in vitro* assay suitable for measuring human T cell inhibition of liver stage *Plasmodium* parasites.
- Use this novel assay to quantify parasite inhibition by ChAd63.MVA ME-TRAP vaccinee T cells, and explore the associated effector mechanisms.

1.4.2 Outline

Chapter 2 describes the materials and methods employed throughout this study.

Chapter 3 investigates the immunogenicity of viral vectored vaccines ChAd63.MVA ME-TRAP through age de-escalation clinical vaccine trials.

Chapter 4 describes the different immune responses generated through novel scheduling of the candidate vaccines ChAd63 ME-TRAP and MVA ME-TRAP.

Chapter 5 optimises an *in vitro* parasite inhibition assay, using an infection model of transgenic *P. berghei* parasites expressing an additional copy of *P. falciparum* TRAP (*PbPfTRAP*) in human hepatocellular carcinoma cell lines. This model is used to study the effects of T cells from human clinical trials vaccinees.

Chapter 6 measures parasite inhibition from vaccinee T cells in the *in vitro* parasite inhibition model optimised in chapter 5. TRAP-specific inhibition is quantified, and correlates and mechanisms of protection are explored.

Chapter 7 summarises the findings of this work and discusses future directions.

Chapter 2

Materials and Methods

2 Materials and methods

2.1 Materials

2.1.1 Reagents

Table 2 Commercially available reagents used throughout this thesis

Product	Supplier	Catalogue number
2.0mL Screw-Cap Tubes	Sarstedt	72.694.006
4',6-Diamidino-2-Phenylindole Dihydrochloride (DAPI)	Sigma-Aldrich Co Ltd	32670-5MG-F
40um Cell Strainer For 50mL Falcon Tube	Scientific Laboratory Supplies Limited	352340
96 Flat Well/Half Area Tissue Culture Cluster	Appleton Woods Ltd	CC220
96 Well Microplate, Black, µclear, Case of 40	Greiner Bio-One Ltd	655096
A*02:01 - FILIFFDVFL Pentamer PE 150 Tests	ProlImmune Ltd	Custom pentamer
A*02:01 GILGFVFTL Pentamer PE 150 Tests	ProlImmune Ltd	007
Albumin Solution From Bovine Serum	Sigma-Aldrich Co Ltd	A8412
Alexa Fluor® 700 Anti-Human HLA-A2 (clone BB7.2)	BioLegend	343317
Anti-CD28 Unlabelled 500ug	eBioscience UK Ltd	16-0289-85
Anti-CD49d Unlabelled 500ug	eBioscience UK Ltd	16-0499-85
Anti-Human CD14 eFluor® 450 (clone 61D3)	eBioscience UK Ltd	48-0149-42
Anti-Human CD19 eFluor® 450 (clone SJ25-C1)	eBioscience UK Ltd	48-0199-42
Anti-Human CD3 Alexa Fluor® 700 (clone UCHT1)	eBioscience UK Ltd	56-0038-82
Anti-Human CD4 APC (clone Leu-3)	eBioscience UK Ltd	17-0049-42
Anti-human CD4 Qdot® 605 (clone S3.5)	Invitrogen	Q10008
Anti-Human CD45 APC (clone HI30)	BioLegend Inc	304012
Anti-Human CD56 eFluor® 450 (clone TLUY56)	eBioscience UK Ltd	48-0566-42
Anti-Human CD56 PE-Cy7 (clone B159)	BD Biosciences	557747
Anti-Human CD8a APC-eFluor® 780 (clone RPAT8)	eBioscience UK Ltd	47-0088-42
Anti-Human HLA-A2 APC (clone BB7.2)	BioLegend Inc	343307
Anti-Human HLA-DR APC (clone L243)	BioLegend Inc	307609
Anti-Human IFN gamma FITC (clone 4S.B3)	eBioscience UK Ltd	11-7319-82
Anti-Human IL-2 PE (clone MQ1-17H12)	eBioscience UK Ltd	12-7029-82
Anti-Human TNFa PE-Cy7 (clone MAb11)	eBioscience UK Ltd	25-7349-82
Anti-human TNFa APC (clone MAb11)	BD Biosciences	554514

APC Mouse IgG2a, kappa Isotype Ctrl (clone MOPC-173)	BioLegend Inc	400220
APC Mouse IgG2b, kappa Isotype Ctrl (clone MPC-11)	BioLegend Inc	400320
Aqua Live/Dead Reg Fixable Aqua Dead Cell Stain Kit, For 405 nm Excitation	Life Technologies Ltd	L34957
ArC Amine Reactive Compensation Bead Kit	Life Technologies Ltd	A10346
ATCC-CRL-8024 PLC/PRF/5` Hepatoma` Human (Homo sapiens)	LGC Standards	ATCC-CRL-8024 PLC/PRF/5
Atovaquone	Sigma-Aldrich Co Ltd	A7986
BCIP/NBT (Plus) Solution	Europa Bioproducts Ltd	M0711A-1000
BD Microfine Insulin Syringes Complete With Needles 0.5mL	Nu-care Products Ltd	M4892
BD Microfine Insulin Syringes Complete With Needles 1mL	Nu-care Products Ltd	M4891
Benzonase® Nuclease, Purity > 99%	Merck Chemicals Ltd	70664-3
Bovine Serum Albumin	Sigma Aldrich Company Ltd	A7906
Bovine Serum Albumin Solution 7.5% In Dpbs, Sterile-Filtered, Bioxtra, Suitable For Cell Culture	Sigma-Aldrich Co Ltd	A8412-100ML
Brefeldin A Solution (1000X)	eBioscience UK Ltd	00-4506-51
Carbonate-Bicarbonate Buffer Capsule	Sigma-Aldrich Co Ltd	C3041-
Casein In PBS Blocker 1L	Fisher Scientific	10496134
Casy Cups	Sedna Scientific	43003
Casyclean	Sedna Scientific	43008
Casyton Fluid	Sedna Scientific	43001
CD4 MicroBeads sample kit	Miltenyi Biotec Ltd	130-045-100
CD56 MicroBeads sample kit, human	Miltenyi Biotec Ltd	130-050-400
CD8 T Cell Isolation Kit, Human	Miltenyi Biotec Ltd	130-096-495
Cluster Tubes Individual Tube Format Nonsterile (For 96 Well)	Scientific Laboratory Supplies Limited	4401
Cryobaby Labels-Laser Cryo-Tags, 33 X 13 mm	Web Scientific	LCRY-1700
Custom Luminex Configuration	Bio-Techne (R&D Systems)	xM3YTyIT
Cytofix/Cytoperm	Becton, Dickinson U.K. Limited	554722
Cytometer Set-Up And Tracking Beads	BD Biosciences	642412
D-Fructose	Sigma Aldrich Company Ltd	F0127
DMSO Hybri-Max™, Sterile-Filtered, BioReagent, Suitable For Hybridoma, ≥99.7%	Sigma-Aldrich Co Ltd	D2650
Dulbeccos MEM High Glucose (DMEM)	Sigma Aldrich Company Ltd	D6546
Dulbecco'S Phosphate Buffered Saline, Mo	Sigma Aldrich Company Ltd	D8537
EDTA	Fisher Scientific	AM9260G
Ethanol Absolute	Sigma Aldrich Company Ltd	24103-2.5L-R
FasL (Human) (Multimeric) (Rec.)	Caltag Medsystems Ltd	AG-40B-0130-C010
Fast Read Cell Counting Chambers	VWR	BVS100

Fisherbrand™ Borosilicate Glass Homogenizers	Fisher Scientific UK Ltd	11512443
Foetal Bovine Serum / Foetal Calf Serum	Labtech International	FCS-SA/500
Giemsa Stain	Sigma Aldrich Company Ltd	GS1L-1L
Goat Anti-Mouse IgG Alexa Fluor 488	Invitrogen	A-11001
Grace'S Insect Medium, Unsupplemented, Powder	Life Technologies Ltd	11300-027
Homogeniser Mortar And Pestle Mini Borosilicate Glass Chamber Length 31Mm / Pestle Diameter 3.65mm 0.1mL Fisherbrand	Fisher Scientific UK Ltd	11512443
Human IFN-γ ELISpot kit (ALP)	Mabtech AB	3420-2A
Immersion Oil	Sigma Aldrich Company Ltd	I0890
Ketaset (100mg/ml Ketamine)	Oxford University Veterinary Services	N/A
LB Broth Tablets	Sigma Aldrich Company Ltd	L7275
Leibovitz'S L-15 Medium (1X), Liquid [With L-Glutamine] - 500ML	Life Technologies Ltd	11415049
Leucosep Tubes	Greiner-Bio-One Ltd	227290
L-Glutamine 200Mm	Sigma Aldrich Company Ltd	G-7513
Lymphoprep	Stemcell Technologies	7851
Lymphoprep	Alere Ltd	NYC-1114547
Methanol	Fisher Scientific UK Ltd	10606652
Ms Columns	Miltenyi Biotec Ltd	130-042-201
Multiscreen-IP 96 Separation System, Clear Plate Color, Acrylic Plate Material, 0.45Mm Immobilon-P PVDF Membrane, Sterile	Millipore UK Ltd	MAIPS4510
Nitrophenyl Phosphate In Diethanolamine Buffer	Pierce	37621
Nunc-Immuno 96 Well Plates	Fisher Scientific UK Ltd	10547781
OneComp eBeads	eBioscience UK Ltd	01-1111-42
P-Amino Benzoic Acid (PABA)	Sigma	A9878-25G
Paraformaldehyde 16% W/V Methanol Free.	Alfa Aesar	43368
Penicillin-Streptomycin Sterile-Filtered	Sigma-Aldrich Co Ltd	P0781
Peptides	NEO Peptide	Customised
Percoll	Sigma Aldrich Company Ltd	P1644
Perm Wash Buffer 10X	Becton, Dickinson U.K. Limited	554723
Phosphate Buffered Saline Tablets	Sigma Aldrich Company Ltd	P4417
Phosphate Buffered Saline Tablets (Dulbecco 'A' Tablets) Oxoid	Fisher Scientific UK Ltd	10209252
Phosphate Buffered Saline+Tween 20	Sigma-Aldrich Co Ltd	P3563
Phytohemagglutinin-L	Sigma Aldrich Company Ltd	L4144
Polypropylene FACS Tubes	Scientific Laboratory Supplies Limited	352063

Polystyrene FACS Tubes With Cell Strainer Cap	Scientific Laboratory Supplies Limited	352235
Propan-2-ol 99.5+% (GLC) (iso-Propanol)	Fisher Scientific	10070320
Propidium Iodide Solution	Cambridge Bioscience Ltd	421301
Purified Mouse IgG2b, κ Isotype Ctrl Antibody	BioLegend	400301
QIAmp DNA mini kit	QIAGEN Ltd	51304
Rat Tail Collagen, Type 1	BD Biosciences	354236
RBC Lysis Solution (1000 ml)	QIAGEN Ltd	158904
Recombinant Human IFN- γ (carrier-free)	BioLegend Inc	570204
Recombinant Human IL-6 (carrier-free)	BioLegend Inc	570802
Recombinant Human Interleukin-2	Bio-Rad AbD Serotec Limited	PHP042A
Recombinant Human TNF- α (carrier-free)	BioLegend Inc	570102
Rompun (2% Xylazine)	Oxford University Veterinary Services	
RPMI	Sigma Aldrich Company Ltd	R0883-6X500ml
Secondary Antibody (Goat Anti-Human IgG Conjugated To Horseradish Peroxidase)	ADI	H-HuG.211
Secondary Antibody (Goat Anti-Human Whole IgG Conjugated To Alkaline Phosphatase)	Sigma Aldrich Company Ltd	A1543
Sodium Azide	Sigma Aldrich Company Ltd	S2002
Staphylococcal Enterotoxin B From Staphylococcus Aureus	Sigma-Aldrich Co Ltd	S4881-1MG
Stop Solution	ADI	80101
Titer Tops Microplate Film	Scientific Laboratory Supplies Limited	MIC5000
TMB Substrate	ADI	80091
Trypan Blue Solution 0.4%, Liquid, Sterile-Filtered, Cell Culture Tested	Sigma Aldrich Company Ltd	T8154-100ML
TrypLE Express	Life Technologies	12605010
Tween 20	Sigma Aldrich Company Ltd	P7949-500ml
Vybrant Reg DiD Cell-Labeling Solution	Life Technologies Ltd	V22887
Water, Double-Processed Cell Culture*Tes	Sigma Aldrich Company Ltd	W3500
Williams E Medium	Fisher Scientific	32551-020

2.1.2 Solutions and buffers

Coating buffer: 15mM sodium carbonate and 35mM sodium bicarbonate capsules dissolved in dH₂O and autoclaved. Stored at room temperature.

Exflagellation medium: RPMI-1640 supplemented with 25mM HEPES, 20% FCS, 10mM sodium bicarbonate and 50µM xanthurenic acid. pH adjusted to 8.3. Stored at room temperature.

FACS buffer: 0.1 % BSA and 0.01% sodium azide added to autoclaved PBS solution. Stored at 4°C.

FACS Lysing Solution: 10x solution diluted 1:10 in dH₂O. Stored at room temperature in the dark.

Giemsa: 10% Giemsa in dH₂O. Made fresh each day.

Hepatoma cell thawing medium: 500mL DMEM supplemented with 5mL L-glutamine (2mM), 5mL Penicillin/Streptomycin (100U penicillin, 100µg streptomycin) and 50mL of heat inactivated FCS (10%). Stored at 4°C.

Hepatoma cell culture medium: 500mL RPMI supplemented with 5mL L-glutamine (2mM), 5mL Penicillin/Streptomycin (100U penicillin, 100µg streptomycin) and 50mL of heat inactivated FCS (10%). Stored at 4°C.

Hepatoma cell recovery buffer: 500mL PBS supplemented with 50mL FCS (10%). Stored at 4°C.

MACS Buffer: 2.5g BSA (0.5%) and 2ml 0.5M EDTA (2mM) added to 500ml sterile PBS, and sterile filtered prior to use. Stored at 4°C.

Lysogeny broth: 1 tablet dissolved in 50mL dH₂O and autoclaved. Stored at room temperature.

Grace's insect medium: 1 pot Grace's powder resuspended in 950mL dH₂O and pH buffered to 6.8-7.0 using 5M NaOH and 2M HCl. Volume topped up to 1L with dH₂O, and supplemented with 10g BSA (1%). Vacuum filtered through 0.22µM filter. Stored at 4°C.

Matrigel solution: Matrigel diluted 1:2 in DMEM.

Mosquito fructose: 80g fructose and 0.5g of PABA resuspended in 1L dH₂O, and autoclaved. Stored at 4°C.

Mouse anaesthetic for i.m. administration: 100 µL of 2% Rompun solution (Bayer – active ingredient is xylazine 20mg/mL), 200 µL of Ketaset (100 mg/mL ketamine) and 300 µL of PBS yield a working

stock of 3.3mg/mL Xylazine and 33mg/mL Ketaset. Administer i.m. at 2.5mL/kg. Stored at room temperature for 1 week.

MVA ELISA blocking buffer: 1% Casein in PBS supplemented with 10% *E. coli* lysate. Stored at 4°C.

Paraformaldehyde 1%: 10mL of 16% paraformaldehyde diluted 1:16 in 150mL PBS. Stored at 4°C.

PBS (0.1M): 0.138M NaCl, 0.0027M KCl, pH 7.4; tablets dissolved in dH₂O and autoclaved. Stored at room temperature.

PBS/Tween: NaCl 0.138M, KCl 0.0027M, pH 7.4 Tween-20 0.05% sachets dissolved in dH₂O. Stored at room temperature.

Perm wash: 10x Perm wash buffer diluted 1:10 in dH₂O. Made fresh each day. Stored at 4°C.

Iso-osmotic Percoll: 2.7mL Percoll and 300μL 10X PBS were diluted in 7mL DMEM. Stored at 4°C.

PHH culture medium: 500mL Williams E medium (1% Glutamax) supplemented with 50mL FCS (10%), 5mL Penicillin/Streptomycin (100U penicillin, 100μg streptomycin) and 5μg/mL insulin.

Rat Tail Collagen 1: Collagen diluted 5μg/mL in acetic acid. Stored at 4°C.

R0 Medium: 500mL RPMI-1640 supplemented with 5mL L-glutamine (2mM) and 5mL Penicillin/Streptomycin (100U penicillin, 100μg streptomycin). Stored at 4°C.

R2 Medium: 500mL RPMI-1640 was supplemented with 5mL L-glutamine (2mM) and 5mL Penicillin/Streptomycin (100U penicillin, 100μg streptomycin), and 10mL (2%) heat inactivated FCS. Stored at 4°C.

R10 Medium: 500mL RPMI-1640 was supplemented with 5mL L-glutamine (2mM), 5mL Penicillin/Streptomycin (100U penicillin, 100μg streptomycin) and 50mL (10%) of heat inactivated FCS. Stored at 4°C.

2.1.3 Vaccines

Manufacture of ChAd63 ME-TRAP by the Clinical Biomanufacturing Facility, University of Oxford and MVA ME-TRAP by IDT Biologika, Rosslau, Germany were performed under Good Manufacturing Practice conditions. A Local Safety Monitor provided safety oversight and GCP compliance was externally monitored.

2.2 Clinical trials

This thesis utilises PBMCs and serum from the Jenner Institute's clinical vaccine trials, identified by their National Clinical Trial number:

Mal 34 (NCT00890760) – samples processed and *ex vivo* IFN- γ ELISpot assays performed by Dr. Katie Ewer and research assistants at the Jenner Institute, Oxford, UK.

Vac 41 (NCT01739036) – samples processed and *ex vivo* IFN- γ ELISpot assays performed by C. Bliss and research assistants at Medical Research Council (MRC) Laboratories, Fajara, The Gambia.

Vac 42 (NCT01450293) – samples processed and *ex vivo* IFN- γ ELISpot and ICS assays performed by C. Bliss and research assistants at MRC Laboratories, Fajara, The Gambia.

Vac 43 (NCT01364883)- samples processed and *ex vivo* IFN- γ ELISpot and ICS assays performed by C. Bliss, Dr. Katie Ewer and research assistants at the Jenner Institute, Oxford, UK.

Vac 45 (NCT01623557)– samples processed and *ex vivo* IFN- γ ELISpot and ICS assays performed by C. Bliss, Dr. Katie Ewer and research assistants at the Jenner Institute, Oxford, UK.

Vac 48 (NCT01669512) - samples processed and *ex vivo* IFN- γ ELISpot and ICS assays performed by C. Bliss, Dr. Katie Ewer and research assistants at the Jenner Institute, Oxford.

Vac 50 (NCT01635647)- samples processed and *ex vivo* IFN- γ ELISpot assays performed Dr. Issa Nèbié Ouédraogo and research assistants at Unité de Recherche Clinique de Banfora (URC-B), Banfora, Burkina Faso.

Vac 52 (NCT01739036) – samples processed and *ex vivo* IFN- γ ELISpot assays performed by C. Bliss, Dr. Katie Ewer and research assistants at the Jenner Institute, Oxford, UK.

Vac 55 (NCT01883609) - samples processed and *ex vivo* IFN- γ ELISpot and ICS assays performed by C. Bliss, Dr. Katie Ewer and research assistants at the Jenner Institute, Oxford, UK.

Vac 58 (NCT02083887) - samples processed and *ex vivo* IFN- γ and ICS assays performed by Dr. Sophie Roetynck and research assistants at MRC Laboratories, Fajara, The Gambia.

For all trials, clinical staff at the respective clinical trial clinic(s) were responsible for volunteer vaccinations, phlebotomy and CHMI procedures.

2.2.1 Clinical trial overview

All clinical vaccine trials consisted of the intramuscular administration of 5×10^{10} vp (viral particles) ChAd63-METRAP followed by intramuscular 2×10^8 pfu (plaque forming units) MVA-ME-TRAP 8 weeks later (ChAd63.MV ME-TRAP), unless stated otherwise. Trials sites consisted CCVTM (Centre for Clinical Vaccinology and Tropical Medicine), Old Road, Headington, Oxford, OX3 7LJ, UK; MRC Laboratories, Fajara, The Gambia; WTCRF (Wellcome Trust Clinical Research Facility), Southampton University Hospitals NHS Trust, Southampton, SO16 6YD; Infection & Immunity Section, Sir Alexander Fleming Building, Imperial College of Science, Technology and Medicine, Imperial College Road, London, SW7 2AZ; NIHR/Wellcome Trust Imperial CRF, Hammersmith Hospital, Imperial College NHS Trust, London, W12 0HS; Centre National de Recherche et de Formation sur le Paludisme (CNRFP)/ Unité de Recherche Clinique de Banfora (URC-B), 01 BP 2208 Ouagadougou 01, Burkina Faso.

2.2.1.1 Mal 34

A Phase I/IIa sporozoite challenge trial to assess protection against malaria in healthy adults vaccinated with AdCh63 ME-TRAP alone, and as a heterologous boost with MVA ME-TRAP, in healthy adults aged 18 – 50. CCVTM, Oxford. Only groups 1 and 5 included in this thesis.

Group 1: 9 adult volunteers, aged 18-50, ChAd63 ME-TRAP 5×10^{10} vp intramuscularly and 1 dose MVA ME-TRAP 2×10^8 pfu intradermally 8 weeks later, followed by sporozoite challenge 2 weeks later.

Group 5: 6 adult volunteers, aged 18-50, receiving priming vaccination with ChAd63 ME-TRAP 5×10^{10} vp intramuscularly, boosted with MVA ME-TRAP 2×10^8 pfu intradermally at an interval of 8 weeks (range 6-12 weeks), followed by sporozoite challenge 3 weeks later (range 2-5.5 weeks).

2.2.1.2 Vac 41

A Phase I study of safety and immunogenicity of a heterologous prime-boost vaccine strategy with AdCh63 ME-TRAP and MVA ME-TRAP in healthy adults and children in a malaria endemic area. MRC Laboratories, Fajara, The Gambia. Only dosing groups 1B and 3A included in this thesis.

Group 1B: 10 adult male volunteers, aged 18-50, receiving standard ChAd63.MVA ME-TRAP regime.

Group 3A: 6 paediatric volunteers, aged 2-6, receiving ChAd63 ME-TRAP 5×10^{10} vp and boosted with MVA ME-TRAP 1×10^8 pfu 8 weeks later.

2.2.1.3 Vac 42

A Phase I study of safety and immunogenicity of heterologous prime-boost vaccination with the candidate malaria vaccines ChAd63 ME-TRAP and MVA ME-TRAP in healthy infants in a malaria-endemic area. MRC Laboratories, Fajara, The Gambia. Only dosing groups 1B and 1E included in this thesis.

Group 1B: 12 paediatric volunteers, aged 5-12 months, receiving ChAd63 ME-TRAP 5×10^{10} vp and boosted with MVA ME-TRAP 1×10^8 pfu 8 weeks later.

Group 1E: 12 paediatric volunteers, aged 10 weeks, receiving ChAd63 ME-TRAP 5×10^{10} vp and boosted with MVA ME-TRAP 1×10^8 pfu 8 weeks later.

2.2.1.4 Vac 43

A Phase I study of safety and immunogenicity of novel schedules for vaccination with the candidate malaria vaccines ChAd63 ME-TRAP and MVA ME-TRAP, in healthy adults aged 18 – 50. CCVTM, Oxford; and WTCRF, Southampton. Vaccination schedules summarised in Table 3.

Table 3 Summary of Vac 43 ChAd63 ME-TRAP and MVA ME-TRAP vaccine regimens

“A” denotes intramuscular administration of 5×10^{10} virus particles of ChAd63 MVA ME-TRAP, “M” denotes intramuscular administration of 2×10^8 plaque forming units of MVA ME-TRAP, “_” denotes an 8 week interval, no underscore denotes a 4 week interval.

Group (n=6)	Time point of vaccine administration						Schedule abbreviation
	Day 0	4 weeks	8 weeks	12 weeks	16 weeks	24 weeks	
1	ChAd63 ME-TRAP	ChAd63 ME-TRAP	ChAd63 ME-TRAP		MVA ME- TRAP		AAA_M
2	ChAd63 ME-TRAP		ChAd63 ME-TRAP		MVA ME- TRAP	MVA ME- TRAP	A_A_M_M
3	ChAd63 ME-TRAP	ChAd63 ME-TRAP	MVA ME- TRAP	MVA ME- TRAP			AAMM
4	ChAd63 ME-TRAP		MVA ME- TRAP		MVA ME- TRAP	MVA ME- TRAP	A_M_M_M
5	ChAd63 ME-TRAP	MVA ME- TRAP	ChAd63 ME-TRAP		MVA ME- TRAP		AMA_M
6	ChAd63 ME-TRAP	MVA ME- TRAP	ChAd63 ME-TRAP	MVA ME- TRAP			AMAM
7	ChAd63 ME-TRAP		MVA ME- TRAP		ChAd63 ME-TRAP	MVA ME- TRAP	A_M_A_M
Standard Regime (G4+7)	ChAd63 ME-TRAP		MVA ME- TRAP				A_M
Shortened Regime (G5+6)	ChAd63 ME-TRAP	MVA ME- TRAP					AM

2.2.1.5 Vac 45

A Phase I/IIa sporozoite challenge study to assess the protective efficacy of 2 prime-boost malaria vaccine candidates: ChAd63 and MVA encoding ME-TRAP and the same viral vectors encoding CS, in healthy adults aged 18 – 45. CCVTM, Oxford; WTCRF, Southampton; Imperial College of Science, London. Only vaccine group 2 (ME-TRAP) included in this thesis.

Group 2: 15 volunteers receiving standard ChAd63.MVA ME-TRAP regimen, followed by controlled human malaria infection 14-28 days later.

2.2.1.6 Vac 48

A Phase I safety and immunogenicity study of ChAd63 ME-TRAP / MVA ME-TRAP heterologous prime boost malaria vaccination, adjuvanted with Matrix M™, in healthy adults aged 18 – 50. Centre for Clinical Vaccinology and Tropical Medicine, Old Road, Headington, Oxford, OX3 7LJ, UK. Only group 3 included in this thesis.

Group 3: 8 adult volunteers, receiving ChAd63 ME-TRAP 5×10^{10} vp mixed with Matrix M-1 50µg and boosted MVA ME-TRAP 2×10^8 pfu mixed with Matrix M-1 50µg 8 weeks later.

2.2.1.7 Vac 50

A Phase 1/IIb double blind randomised controlled trial of the efficacy, safety and immunogenicity of heterologous prime-boost immunisation with the candidate malaria vaccines ChAd63 ME-TRAP and MVA ME-TRAP in 5-17 month old Burkinabe infants and children. CNRFP/URC-B, Burkina Faso. Only safety lead-in arm of trial included in this thesis.

Phase I safety lead in group: 30 paediatric volunteers, aged 5-17 months, receiving ChAd63 ME-TRAP 5×10^{10} vp and boosted with MVA ME-TRAP 1×10^8 pfu 8 weeks later.

2.2.1.8 Vac 52

A Phase I/IIa sporozoite challenge study to assess the efficacy of candidate combination malaria vaccine approaches using the ChAd63 and MVA vectors encoding the antigens ME-TRAP, CS and AMA1, aged 18-45. CCVTM, Oxford; WTCRF, Southampton; and Imperial College of Science, London. Only group 1 (CS and ME-TRAP) included in this thesis.

Group 1: 15 adult volunteers, receiving a mixture of ChAd63 CS 5×10^{10} vp and ChAd63 ME-TRAP 5×10^{10} vp and boosted with a mixture of MVA CS 2×10^8 pfu and MVA ME-TRAP 2×10^8 pfu 8 weeks later.

2.2.1.9 Vac 55

A Phase I/IIa sporozoite challenge study to assess the safety and protective efficacy of the combination malaria vaccine candidate regimen of RTS,S/AS01_B + ChAd63 and MVA encoding ME-TRAP and also RTS,S/AS01_B alone, in adults aged 18-45. CCVTM, Oxford; WTCRF, Southampton; NIHR/Wellcome Trust Imperial CRF, London; Imperial College of Science, London. Only group 1 (ME-TRAP + RTS,S) included in this thesis.

Group 1: 20 adult volunteers receiving RTS,S/AS01_B (50mcg RTS,S + AS01: 50mcg MPL + 50mcg QS21), ChAd63 ME-TRAP at week 2, RTS,S/AS01_B at week 4 and 8, MVA ME-TRAP at week 10, followed by controlled human malaria infection 14 days later.

2.2.1.10 Vac 58

A Phase Ib study to assess the safety and immunogenicity of ChAd63 ME-TRAP and MVA ME-TRAP heterologous prime-boost vaccination co-administered with EPI vaccines in Gambian infants. MRC Laboratories, Fajara, The Gambia. See Table 4.

Group 1: 15 paediatric volunteers, aged 16 weeks, receiving ChAd63 ME-TRAP 5×10^{10} vp and boosted with MVA ME-TRAP 1×10^8 pfu 8 weeks later.

Group 2: 15 paediatric volunteers, aged 8 weeks, receiving ChAd63 ME-TRAP 5×10^{10} vp and boosted with MVA ME-TRAP 1×10^8 pfu 8 weeks later.

Group 3: 15 paediatric volunteers, aged 1 week, receiving ChAd63 ME-TRAP 5×10^{10} vp and boosted with MVA ME-TRAP 1×10^8 pfu 8 weeks later.

Group 4 (Control): 20 infants receiving EPI vaccinations only.

Table 4 Vaccination list of the World Health Organization's Expanded Program on Immunization

Vectored vaccines co-administered alongside EPI vaccines, but not at same injection site.

*1 week following birth

Vaccine	Age								
	Birth	2 mths	3 mths	4 mths	18 wks	6 mths	9 mths	12 mths	18 mths
BCG	X								
Hepatitis B	X								
Oral Polio Vaccine	X	X	X	X			X		X
Pneumococcal conjugate vaccine		X	X	X					
DTP Hib HepB		X	X	X					
Vitamin A supplementation						X		X	X
Measles vaccine							X		
Yellow fever							X		
Rotavirus vaccine (Rotateq)		X	X	X					
Group 1				ChAd63 ME-TRAP		MVA ME-TRAP			
Group 2		ChAd63 ME-TRAP		MVA ME-TRAP					
Group 3	*ChAd63 ME-TRAP	MVA ME-TRAP							

2.3 Clinical trial immunological assays

All clinical protocols and record keeping performed were subject to GCP.

2.3.1 Blood separation

Blood samples for PBMC separation were collected in lithium heparin Vacutainer® blood collection systems and separated on a centrifugation gradient using Lymphoprep® within 6 hours of venepuncture. Blood samples for serum separation were collected in plain Vacutainer® blood collection tubes, and serum collected following centrifugation at 706G for 5 minutes. Serum was stored at -80°C.

2.3.2 PBMC freezing

Remaining PBMCs following the setup of fresh primary immunological assays were frozen at $5-12 \times 10^6$ cells per vial using pre-chilled Mr. Frosty Cryocontainers plus isopropanol, or using CoolCell containers. PBMCs were centrifuged at 706G for 5 minutes at room temperature, the supernatant discarded and pellet resuspended in 0.5mL of FCS per vial to be frozen. Resuspensions were chilled at 4°C for 30 minutes, before the addition of an equal volume of 20% DMSO in FCS. PBMCs were immediately aliquoted into cryovials and placed in freezing container. Containers were then stored at -80°C overnight, with vials transferred to liquid nitrogen within 72 further hours.

2.3.3 PBMC thawing

Frozen PBMCs were thawed by immersion in a 37°C water bath. When only small frozen pellet remained, each vial of cells was added drop-wise into 10ml pre-warmed R10 in a Falcon tube. Cells were centrifuged at 706G for 5 minutes and resuspended in 2mL R10 per 5×10^6 PBMCs. 1µL of

25U/mL benzonase was added per million cells. Cells were incubated at 37°C 5% CO₂ in a humidified incubator for at least 2 hours prior to use.

2.3.4 Peptides

Peptides were manufactured to >70% purity. Peptides covering the full length of TRAP were 20 amino acids in length and overlapped by 10 amino acids (Table S1). Peptides to the multi-epitope string were equivalent in length to their corresponding epitope (Table S2). Hexon peptides were pooled according to peptides specific to ChAd63 (termed “specific”) and common to both ChAd63 and AdHu4 adenovirus (termed “common”), (Table S3). CTL megapool consists of known CD8⁺ T cell epitopes from influenza, cytomegalovirus and Epstein-Barr virus (Table S4). Peptides were reconstituted in DMSO at 50-200mg/mL and pooled in R10 for ELISpot and flow cytometry assays.

2.3.5 *Ex vivo* IFN- γ ELISpot assay

Ex vivo IFN- γ ELISpot to ME+TRAP were performed at several immunologically relevant post-vaccination time points. For all trials of the 8 week regimen ChAd63.MVA ME-TRAP, this included a pre-vaccination time point (day 0), a post Chad63 ME-TRAP time point of 21-28 days post vaccination (day 21/day 28), a pre-MVA ME-TRAP time point on day of boost vaccination (day 56) and a post MVA ME-TRAP time point (day 63). Challenge trials also included a time point 1 day prior to challenge (C-1), and several post challenge time points: challenge + 7, 21 (if protected), 35, 90 and 150 (Mal 34 only) days post challenge. Vac 43 had a different bleed schedule for each group, matched on time point post vaccination: pre-vaccination, 4 weeks and 8 weeks (if no 4 week vaccination) post Chad63 ME-TRAP; 1 week and 4 weeks post MVA ME-TRAP, 8 weeks post MVA ME-TRAP (if 8 week MVA to MVA); and 24 weeks post final MVA ME-TRAP. *Ex vivo* IFN- γ ELISpot against ChAd63 total hexon on VAC 43 groups 1 and 2 only.

Assays were performed using Multiscreen IP ELISpot plates coated using 10 μ g/mL human IFN- γ and developed using SA-ALP antibody kits and BCIP NBT-plus chromogenic substrate. Cells were

cultured in R10 for 18-20 hours. Antigens were tested at 10 µg/mL in duplicate, with 2.5×10^5 PBMC added to each well (2.0×10^5 /well for paediatric trials) of the *ex vivo* ELISpot plate in a final volume of 100 µL. Peptides covered 6 pools of 7–10 peptides for TRAP, 1 pool for ME. 1 pool for ChAd63 specific peptides, 1 pool for ChAd63 common peptides and 1 pool for CTL peptides. Responses to TRAP megapool were also tested. Responses were averaged across duplicates, and responses in unstimulated (negative control) wells were subtracted and responses in individual pools summed for the TRAP antigen, plus ME. Staphylococcal enterotoxin B (0.02 µg/ mL) and phytohaemmagglutinin-L (10 µg/mL) were pooled and used as a positive control. Plates were counted using an AID automated ELISpot counter (AID Diagnostika GmbH, algorithm C). Identical settings for all plates were used per site, with calibration testing performed between sites. Counts were adjusted only to remove artefacts.

2.3.5.1 *ELISpot QC*

ELISpot QC passed assays with >800 SFC per million PBMC in at least one of the positive control wells and <80 SFC per million PBMC or <50% of the antigen-specific response in the negative control wells. Responses of greater than (median + 2 standard deviations of unstimulated wells) were considered positive. The lower limit of detection for the assay was 28 SFC SFC/ 10^6 PBMC for ME-TRAP ELISpots and 16 SFC/ 10^6 PBMC for hexon ELISpots. Hexon ELISpots were performed on total pool hexon peptides (summed ChAd63-specific and common peptides).

2.3.5.2 *Area-under-curve interpretation of ex vivo IFN-γ ELISpot data*

Area under curve (AUC) analysis was performed for each individual vaccinee response time course, using Prism 6 software. The median AUC response was then calculated using the AUC values for each group. When time courses covered different periods of time, the AUC values were scaled by the number of days in the time course, in order to make direct comparisons between groups (AUC/days).

2.3.5.3 Full blood count

Full blood count with differentials was measured at each immunogenicity time point as part of the standard safety evaluation and follow up. Monocyte and lymphocyte counts were either measured as absolute values, or calculated by using the total white blood count multiplied by the respective white blood cell differential. These data were then used to calculate ELISpot response/mL whole blood.

2.3.6 PBMC flow cytometry with intracellular cytokine staining

1-3×10⁶ freshly isolated PBMC in 1 ml of medium containing 1µg/mL anti-CD28 and anti-CD49d and 1:200 CD107a-PeCy5 were stimulated in 5 mL polypropylene FACS tubes for 18-20 hours at 37°C in 5% CO₂. For TRAP-specific responses, PBMC were stimulated with 2µg/mL 56 peptides spanning the T9/96 strain of the TRAP antigen (Supplementary table S1). For anti-ChAd63 hexon responses, peptides at 2µg/mL spanning only the ChAd63 hexon and with no homology to AdHu4 were tested, as highlighted in table S3. Unstimulated PBMC and 1µg/mL Staphylococcal enterotoxin B (SEB) stimulated PBMC were run in parallel as negative and positive controls, respectively. 10µg/mL Brefeldin A and 1:1000 monensin were added for the last 16-18 hours of stimulation.

After stimulation, cells were washed FACS buffer and PBMC separated from an unstimulated tube to act as an unstained control. PBMC were then incubated with 1:500 Aqua amine reactive dye at 4°C for 10 minutes and then stained using a 2-step staining protocol of surface staining, followed by permeabilisation (300µL Fix/Perm for 20 minutes at room temperature) and then ICS (Table 5, Vac 43 only), or a 1-step protocol of permeabilisation then ICS (Table 6, PBMC and whole blood protocol for all other trials). Samples were then washed in 1x Perm Wash and resuspended in 1% paraformaldehyde. Samples were filtered through 5mL polystyrene FACS tubes with cell strainer lids, then immediately acquired on an LSRII cytometer (BD Biosciences) using FACSDiva v6.2 (BD Biosciences) software, with the relevant single fluorochrome compensation controls and Photo Multiplier Tube voltages set by daily acquisition of Cytometer Set Up and Tracking beads.

Table 5 Two-step PBMC staining: Surface staining panel and intracellular cytokine staining panel and titrations for PBMC flow cytometry

Used on Vac 43 PBMC only.

Antibody	Final titration (Surface cocktail)
CD14 eFluor 450	1:100
CD19 eFluor 450	1:100
CD4 Qdot 605	1:100

Antibody	Final titration (Intracellular cocktail)
CD3 Alexa Fluor 700	1:100
CD8 APC-eFluor 780	1:13
IFN- γ FITC	1:500
TNF α APC	1:100
CD56 PE-Cy7	1:100

Table 6 One-step staining: Intracellular cytokine staining panel and titrations for PBMC and whole blood flow cytometry

Antibody	Final titration (Intracellular cocktail)
CD14 eFluor 450	1:100
CD19 eFluor 450	1:100
CD3 Alexa Fluor 700	1:100
CD4 APC	1:50
CD8 APC-eFluor 780	1:20
IFN- γ FITC	1:200
IL-2 PE	1:100
TNF α PE-Cy7	1:1000

For short stimulation effector cell quantification, a similar 1-step protocol was used, however cells were stimulated for 1 hour prior to the addition of Brefeldin A and monensin, followed by 6 further hours of incubation, followed by immediate Aqua staining and ICS using the panel outlines in Table 7.

Table 7 Intracellular cytokine staining panel and titrations for short stimulation PBMC ICS for effector quantification

Antibody	Final titration (Intracellular cocktail)
CD14 eFluor 450	1:100
CD19 eFluor 450	1:100
CD3 Alexa Fluor 700	1:100
CD4 PE	1:25
CD8 APC-eFluor 780	1:13
IFN- γ FITC	1:500
TNF α APC	1:100
CD56 PE-Cy7	1:100

2.3.7 Whole blood flow cytometry with intracellular cytokine staining

The whole blood clinical protocol was optimised on adult blood samples. Due to the higher cell numbers in paediatric samples and the limited blood volumes available for immunological testing, blood volumes and reagents were titrated. Adult titrations are listed, with paediatric titrations in square brackets.

Blood samples were collected using the heparinised Vacutainer® system and stored at room temperature prior to processing within 6 hours of venepuncture. 500 μ L [350 μ L] of whole blood were distributed in 2ml screw-cap tubes (Sarstedt, Germany) and stimulated in the presence of 0.5 μ g/mL of anti-CD49d and anti-CD28 monoclonal antibodies and 2 μ g/mL of 56 TRAP 20mers, overlapping by 10 amino acids (Supplementary Table S1). PBMC stimulated by 2 μ g/mL SEB or an equivalent volume of R10 were used as positive and negative controls, respectively.

Tubes were incubated at 37°C in 5% CO₂ for 2 to 4 hours, prior to the addition of 1 μ g/mL Brefeldin A and further incubation at 37°C for 16 hours. At the end of the culture, samples were then incubated with 2mM EDTA for 15 minutes to remove adherent cells, washed in PBS (800G, 5 minutes) then treated for 2 x10 minutes with 4mL Red Cell Lysis Buffer, with PBS washing in-between each step. Cells were again washed in PBS, then stained at room temperature for 20 minutes in the dark with 1:125 (0.4 μ L) Aqua amine reactive dye for live cell identification. Cells were again washed in PBS, then fixed with 1mL 1% paraformaldehyde for 5 minutes. Cells were washed in PBS, then

frozen in 0.5mL FCS containing 10% DMSO and stored at -80°C until batched thawing and staining analysis.

Cells were thawed and stained in batches within 4 months of the date they were frozen. For Vac 58, corresponding day 21 and day 63 samples for each vaccinee were analysed on the same day for consistency. After thawing, cells were transferred in 5mL polypropylene FACS tubes and washed with FACS buffer. After 20 minutes of permeabilisation in Cytofix/Cytoperm buffer at room temperature in the dark, cells were washed once in 1x Perm Wash Buffer. Cells were then stained at room temperature for 30 minutes with a combined surface and intracellular cocktail (see Table 6). Samples were washed in 1x Perm/Wash Buffer and resuspended in 1% paraformaldehyde in PBS for acquisition on a BD LSR Fortessa Cell Analyzer (Becton Dickinson). Compensation was calculated for each fluorochrome on each acquisition run using OneComp beads for surface and intracellular antibodies and ARC beads for Aqua reactive dye.

2.3.8 Staining of single colour compensation controls

Single colour compensation controls for each fluorochrome were prepared using an identical protocol for all flow cytometry assays. OneComp beads were vortexed and 1 drop of beads added per cluster tube. A matching titration of each antibody as used in cell staining protocol was added per tube. Tubes were vortexed and incubated in the dark for 15 minutes. 300µL FACS buffer was added per tube, and tubes were microfuged at 344G for 5 minutes, before flicking off the supernatant and resuspending in 50µL 1% PFA for ICS experiments or in R2 buffer for cell sorting experiments. ARC beads were unavailable for use in Vac 43.

Single colour controls for dead cell identification were as follows: For Aqua compensation, ARC positive beads were vortexed and 1 drop added to cluster tube. Tube was stained and washed as above, with one drop of vortexed ARC negative beads added immediately prior to acquisition. Propidium iodide (PI) compensation was performed on cells resuspended in R2 with 1:500 (1µg/mL) PI, and acquired without washing.

2.3.9 Flow cytometry QC

Data were analysed using FlowJo v9.6.2 (Treestar) with a hierarchical gating strategy. A sample gating strategy is shown in supplementary figure S1. Responses to peptide were determined after subtraction of the response in the unstimulated control for each sample. Samples where a response to the positive control of >1% cytokine positive CD4⁺ or CD8⁺ T cells after subtraction of the unstimulated control could not be detected were excluded from the analysis. All samples had a lower level of detection identified, based on the minimum number of CD4⁺ and CD8⁺ T cells acquired for the antigen-stimulated and unstimulated samples in the dataset. A response was classified as positive if the subtracted response was greater than the medium control for the corresponding sample (QC = TRAP-Unstimulated>Unstimulated). A minimum of 30 counts was acquired through each cytokine gate, where counts data was available.

2.3.10 Anti-TRAP ELISA

As performed by C. Snudden and G. Bowyer. Serum was taken in Vac 43 for antibody analysis at D0, D28, D56, D84, D112 and 24 weeks post last vaccination for all volunteers. Serum was additionally taken at 1 and/or 4 weeks after MVA vaccinations according to the vaccination schedule in each group. Antibody responses to TRAP were measured using total IgG Enzyme Linked Immunosorbant Assay (ELISA) as previously published [285].

Briefly, Nunc-Immuno 96 well plates were coated with 0.5µg/mL of TRAP antigen in carbonate-bicarbonate coating buffer at 4°C overnight. Plates were washed 6 times with PBS-Tween and blocked with 1% BSA in PBS-Tween for 1 hour at room temperature. Serum dilutions were made using 0.2% BSA in PBS/Tween, added to the plate in triplicate and incubated for 2 hours at room temperature. Secondary antibody (goat anti-human whole IgG conjugated to alkaline phosphatase) was added at a dilution of 1:1000 in PBS-Tween with 0.2% BSA for 1 hour at room temperature. Plates were washed a final time and developed using p-NitrophenylPhosphate in diethanolamine buffer. Optical density (OD) was read at 405nm on an ELx800 microplate reader (Biotek) with Gen5 software (version 1.10). A positive reference pool of TRAP-positive serum was used to form a

standard curve on each plate. Reference serum was added in duplicate at an initial dilution of 1:100 (in PBS-Tween with 0.2% BSA) and diluted 2-fold 10 times. The initial dilution was assigned an arbitrary 20 antibody units and OD values were fitted to a 4 parameter logistic curve. A 1:800 dilution (in PBS-Tween with 0.2% BSA) of this reference pool was also included in triplicate on each plate as an internal control. ELISA units were calculated from their OD (optical density) values using the parameters estimated from the standard curve.

2.3.11 Anti-MVA ELISA

As performed by A. Grobbelaar and G. Bowyer. Anti-MVA ELISAs were conducted using serum taken at baseline (D0) and 4 weeks after each MVA vaccination for all volunteers in Vac 43. Pre-coated and pre-blocked 96-well ELISA plates were kindly donated by Dr. Philip Felgner, University of California, Irvine. Plates were coated with MVA protein WR113/D8L, blocked with 1% Casein in PBS, dried and stored at 4°C until use. Samples were diluted 1:200 in MVA ELISA blocking buffer. After incubating in blocking buffer at room temperature for 30 minutes to block anti-*E. coli* reactivity, samples were added to the plate in duplicate, 50µL per well. Plates were incubated for 45 minutes at room temperature before washing 6 times with PBS. Secondary antibody (goat anti-human IgG conjugated to Horseradish Peroxidase, ADI, H-HuG.211) was added 100µL/well at a dilution of 1:100 in PBS and plates were incubated at room temperature for 45 minutes. Plates were washed 6 times in PBS before adding 100µL of TMB substrate per well and covering plates to protect from light. After 10 minutes, the reaction was stopped by adding 100µL/well of stop solution. OD was read at 450nm on an ELx800 microplate reader (Biotek) with Gen5 software (version 2.07).

2.4 *In vitro* assay components

2.4.1 Hepatoma cell lines

2.4.1.1 *DNA extraction*

DNA extraction was performed on whole hepatoma cells, using QIAmp DNA mini kit. Briefly cell suspension at 7×10^5 /mL was washed in PBS (500G, 5 minutes) and resuspended in 500 μ L PBS in an O-ring cryovial. According to QIAgen SOP, 40 μ L protease was added and the suspension vortexed, followed by the addition of 400 μ L lysis buffer AZ. The solution was pulse vortexed for 15 seconds, and then incubated in a water bath at 56°C for 15 minutes. 400 μ L of 100% ethanol was added and pulse vortexed for 15 seconds. Half the prep (650 μ L) was loaded onto the spin column, and microfuged at 6797G for 1 minute, then repeated for second half of prep. 650 μ L AW1 buffer was then added to the column and centrifuged for 2 x 6797G for 1 minute. 650 μ L AW2 buffer was then added to the spin column, and left for 2 minutes to remove salts, before microfuging at 6796G for 1 minute. Microfuge for a further 3 minutes on 20817G to dry the spin column membrane, the 50 μ L nuclease-free H₂O, incubated for 2 minutes, then microfuge into a fresh collection tube, at 900rpm for 90 seconds. DNA was quantified by NanoDrop and diluted to 200-500ng/mL in dH₂O, before being sent for HLA typing.

2.4.1.2 *Plating of hepatoma cells*

Media was removed from a single culture flask of hepatoma cells. Cells were washed twice in PBS, then incubated in 4mL TrypLE Express for 8-12 minutes (or until cells were detaching from one another). The incubation time varied between cell lines. Cells were recovered in 20mL R10 for counting by Trypan blue exclusion on a haemocytometer. Cell suspensions were washed once in R10 (491G for 5 minutes), then resuspended at the desired concentration in 100 μ L plating volume. The optimised plating density was 20,000 HC-04/well, for 96 well half-surface area plates. Plates were gently agitated to disperse cells, then incubated at 37°C 5% CO₂ before being used in further experimentation.

2.4.1.3 Counting viable hepatoma cell number post-plating

Cells were detached from 96 well plates using a single 120µL/well PBS wash, followed by the addition of 30µL/well Tryple Express. Plates were incubated at 37°C for ~10 minutes, then cells collected in 10% FCS in PBS and counted immediately using Trypan blue exclusion dye on a haemocytometer slide. Mean live cell number from 6 wells counted.

2.4.1.4 Staining hepatoma cell lines using DiD and CFSE

Hepatoma cell lines were labelled with DiD or CFSE following Trypan blue counting. Cells were resuspended at 5×10^6 /mL in R10 and stained with 10µL DiD or resuspended at 1×10^7 /mL in pre-warmed 0.1% BSA in PBS and stained with a final concentration of 10µM CFSE. Immediately following addition of stain, cells were mixed and incubated in a 37°C water bath for 10 minutes, inverting the tube every 2-3 minutes. Tubes were topped up with 4 times the volume of respective media (chilled 0.1% BSA in PBS for CFSE), and washed twice by centrifugation at 491G for 5 mins. Cells were then ready for plating, as described in 2.4.1.2.

2.4.1.5 Infection of hepatoma cells

Plated hepatoma cells were left to settle for 24 hours before infection with *PbPf*TRAP. Mosquitoes were kept on ice prior to dissection. Salivary glands were dissected into supplemented Grace's dissection medium, and kept in a glass homogeniser on ice, before homogenisation using glass homogenising stick to release sporozoites. Sporozoites were counted using a haemocytometer and microscope (x40) and then diluted to desired concentration in R10 medium (~150,000/mL) and added to hepatoma cells at 100µL/well. Plates were centrifuged at 368G for 3 mins with low brake, then incubated at room temperature for 30 minutes, followed by 37°C with 5% CO₂ for 2.5 hours. Sporozoites were then removed, wells washed with 120µL R10, and then left to incubate in 100µL R10 at 37°C with 5% CO₂ before subsequent experimentation.

2.4.2 T cell protocols

2.4.2.1 T cell culture

On day 0, PBMC were thawed as per the standard protocol described in 2.3.3. Cells were counted by Trypan blue exclusion and resuspended at $\sim 5 \times 10^6/\text{mL}$ in R10. 1mL of cell suspension was added per well of a 12 well tissue culture plate. Relevant peptides for culture were diluted to $4\mu\text{g}/\text{mL}$ in R10, and 1mL added each well, giving a final peptide concentration of $2\mu\text{g}/\text{mL}$. For the final optimised protocol, recombinant human IL-2 (rhIL-2) was added at day 3 and day 7 at a final concentration of 10U/mL. To achieve this, 1mL of cell culture supernatant was carefully removed per well, and replaced with 1mL of 20U/mL rh IL-2. On day 8, cells were removed from each well using a Pasteur pipette and up to 6 washes per well using R10. Each culture was washed 3 times in R10 (706G for 5 minutes) to remove excess IL-2, followed by an additional wash the following morning before use in subsequent assays.

2.4.2.2 CD8⁺ T cell enrichment by MACS

PBMC were labelled with a cocktail of biotin-conjugated monoclonal anti-human antibodies against CD4, CD15, CD16, CD19, CD34, CD36, CD56, CD123, TCR γ/δ and CD235a (Glycophorin A), and microbeads conjugates to monoclonal anti-CD14 antibody (isotype: mouse IgG2a), anti-CD61 antibody (isotype: mouse IgG1), and anti-biotin antibody (isotype: mouse IgG1) (Miltenyi Biotec), as per the manufacturer's instructions. Unlabelled CD8⁺ T cells were separated from labelled PBMC by MACS, washed in R10 and ready for use in subsequent assays.

2.4.2.3 Staining cultured T cells for pentamer cell sort – Oxford

Cultured PBMC were counted by CasyCounter, split into $1-4 \times 10^6$ cells/mL in polypropylene FACS tubes and washed in PBS. A small fraction of cells from the sample with the highest cell count was removed for use as a single colour compensation control for PI dead cell stain at 1:500.

Pentamers were centrifuges in a chilled microfuge at 14000G for 10 minutes, then 15µL (15µL for MoFlo sort, 10µL for FACS Aria II sort) supernatant added to each corresponding T cell culture, and incubated in the dark for 10 minutes at room temperature. Cells were washed in 2-3mL of 0.1% BSA in PBS wash buffer and centrifuged at 500G for 5 minutes at 10°C. 50µL of surface cocktail (Table 8) was added per tube and incubated for 30 mins in the fridge, before washing in 0.1% BSA in PBS at 500G for 5 minutes. Cells were resuspended in R2 medium (20x10⁶ cells/mL for sorting by MoFlo in chapter 4, 35x10⁶ cells/mL for FACS Aria II sort in chapter 6) and 1:500 PI added. Cells were stored in the dark and on ice until sorting within 6 hours of staining. Single colour compensation controls were used. Cells were sorted into FCS lined FACS tubes containing R10 media, and kept at room temperature or 37°C for 6 hours following the cell sort, before use in subsequent assays.

Table 8 Surface staining panel and titrations for Oxford-based cell sorting by MoFlo and FACS Aria II

Antibody	Final titration	
	Mo Flo (Chapter 5 experiments)	FACS Aria II (Chapter 6 <i>PbPf</i> TRAP experiments)
CD8 APC-eFlour780	1:10	1:13
CD56 PE Cy7	1:67	1:100
CD3 Alexa Ffuor 700	N/A	1:100
CD4 APC	N/A	1:50

2.4.2.4 Staining cultured T cells for pentamer cell sort – Paris

A staining protocol identical to 2.4.2.3, with the exception of dead cell staining using 1:500 Aqua prior to surface staining panel (Table 9), was used for primary human hepatocytes experiments in Paris (chapter 6).

Table 9 Surface staining panel and titrations for Paris-based cell sorting by FACS Aria II

Used on *P. falciparum* experiment, chapter 6

Antibody	Final titration
CD8 APC-eFluor 780	1:10
CD56 PE Cy7	1:67

2.4.2.5 Staining fresh PBMC for NK cell sort - Oxford

PBMC were thawed according to 2.3.3 and rested overnight. The following day, cells were split into $1-4 \times 10^6$ cells/mL in polypropylene FACS tubes and washed in PBS (500G, 5 minutes), then washed in 0.1% BSA in PBS wash buffer (500G, 5 minutes at 10°C) alongside T cells being stained in pentamer staining protocol. 50µL of surface cocktail (see Table 8) was added per tube and incubated for 30 mins in the fridge, before washing in wash buffer at 500G for 5 minutes. Cells were resuspended in R2 (20×10^6 for sorting by MoFlo in chapter 4, 35×10^6 cells/mL for FACS Aria II sort in chapter 6) and 1:500 PI added. Cells were stored in the dark and on ice until sorting within 6 hours. Single colour compensation controls were used. Cells were sorted into FCS lined FACS tubes containing R10 media, and kept at room temperature or 37°C for 6 hours before use in assays.

2.4.2.6 CD45 staining of hepatoma and T cell monolayers

Using a multichannel pipette, 90µl of 100µl supernatant removed from each well and stored at -20°C for Luminex analysis. Wells were washed with 120µL PBS + 1% BSA wash buffer, then 30µL of 1:60 CD45-APC (diluted in wash buffer) was added and incubated at 37°C for 10 minutes. Antibody was removed, and wells were washed with 100µL PBS, followed by the addition of 30µL TrypLE Express solution. Plates were incubated at 30°C for approx. 12 minutes (or until cells began to detach from one another). Recover cells into racked cluster tubes, using 2 vigorous pipetting washes, each of 30µL 10% FCS in PBS. Store in the dark and on ice until acquired by FACS.

2.4.3 Assessment of infectivity by FACS

Hepatoma cells in cluster tubes were stored on ice. 5µL DAPI (1µg/ml) was added per tube immediately prior to acquisition on LSRII cytometer, to identify dead cells with disrupted cell membranes.

2.4.4 Quantification of parasite inhibition by T cells

Hepatoma cells were gated as per gating strategy (see Figure 5.11). Background GFP of uninfected cells was subtracted from test samples if the median count per experiment >1, or frequency >0.05%. These values never exceeded 3 or 0.1%, respectively. Median number of infected cells were used to calculate the frequency of infected cells, which was then multiplied by 30,000 (approximate number of HC-04 cells at time of infection), indicating the total number of infected cells when T cells were added. These constitute the 'target' cells, used in the calculation of E:T. Number of effector cells was determined by the cell sort, and was equivalent to the total number of CD8⁺, CD4⁺ or NK cells added per well. Inhibition was measured using the gating strategy in Figure 5.11, without the CD45 gate, as this was not required for counting number of infected cells per sample. The difference in gating strategy permitted absolute number of infected cells in wells with- and without- T cells to be counted without bias.

Number of test wells acquired was always >8000 HC-04 (mean = 14,242 HC-04). Percentage inhibition of CD8⁺ pentamer⁺, CD8⁺ pentamer⁻ and CD4⁺ cells was calculated using the following equation, where:

Mn = mean number of infected HC-04 in infected-only control wells

Nf = number of infected HC-04 in FLIFFDLFL test well

Ng = number of infected HC-04 in GILGFVFTL test well

$$\% \text{ FLIFFDLFL inhibition} = \left[\frac{(Mn - Nf)}{Mn} \right] \times 100$$

$$\% \text{ GILGFVFTL inhibition} = \left[\frac{(Mn - Ng)}{Mn} \right] \times 100$$

Percentage inhibition using CD56⁺ NK cells used percentage infection as the readout, as there was no cellular control well. Number of test wells acquired was always >6000 HC-04 (mean = 11,649

HC-04). Percentage infectivity in NK test wells was therefore compared directly against the infected-only control well, and percentage inhibition calculated, using the following equation, where:

Mp= mean percentage number of infected HC-04 in infected-only control wells

NKp = percentage number of infected HC-04 in NK test wells

$$\% \text{ NK cell inhibition} = \left[\frac{(Mp - NKp)}{Mp} \right] \times 100$$

2.4.5 Staining of HLA-A2

Using a multichannel pipette, 90µL of 100µL supernatant removed from each well. Wells were washed with 120µL PBS + 1% BSA wash buffer, then either: 30µL of 1:80 (2.5µg/mL) HLA-A2 IgG2b or 30uL of 1:80 (2.5µg/mL) isotype control antibody IgG2b, or 30µL of 1:20 (12.5µg/mL) HLA-DR IgG2a or 30µL of 1:160 (12.5µg/mL) isotype control antibody IgG2a (diluted in wash buffer) was added and incubated at 37°C for 10 minutes. Antibody was removed, and wells were washed with 100µL PBS, followed by the addition of 30µL TrypLE Express solution. Plates were incubated at 37°C for 12 minutes (or until cells began to detach from one another). Cells were recovered into racked cluster tubes, using 2 vigorous pipetting washes, each of 30µL 10% FCS in PBS, stored in the dark and on ice until acquired by FACS. Identical getting strategy to inhibition assay, with percentage positivity for MHC or isotype calculated on infected or uninfected populations within the same well.

2.4.6 Luminex assay

See Jenner Protocol J414. Supernatants were collected and stored at -20°C following wells overnight incubation of parasite inhibition assay. Supernatant plates were centrifuged at 706G for 5 minutes to pellet any cellular material. Supernatant wells were then combined from all replicate in the *in vitro* parasite inhibition assay, and split across 2 duplicates wells in the Luminex assay, where

possible. Assay conducted on black, flat bottom plates. A set of standards was prepared as per the manufacturer's instructions, and included on each plate for generation of a standard curve, and for QC purposes. 50µL micro particle cocktail was added per well and incubated at room temperature in the dark on a horizontal plate shaker set at 800+/- 50rpm for 2 hours. Plates were washed manually in wash buffer using a magnetic plate washer. The magnet was attached for 1 minute, before flicking off the wash buffer. Diluted biotin antibody cocktail (1:17) was then added per well and incubated at room temperature in the dark on a horizontal plate shaker set at 800+/- 50rpm for 1 hour. The plates were washed 3 times using the magnetic plate washer, before the addition of diluted streptavidin-PE (1:17) and incubated at room temperature in the dark on a horizontal plate shaker set at 800+/- 50rpm for 30 minutes. Plates were washed 3 times, and resuspended in 100µL wash buffer before acquisition on Luminex 100 running eXponent 3.1 software.

2.4.6.1 Luminex QC

Standard curves passed 1:3 dilution QC for every analyte in the panel. Test values fell within the curve for all analytes, except for IL-8, which was subsequently excluded from the analysis. Mean of duplicate wells calculated, and autologous infected-only well subtracted from each test well for each analyte (or peptide pulsed-only wells for peptide pulsing experimental analysis) and negative values corrected to zero to give the test well response. Responses were considered positive if greater than 10pg/mL was detected, which corresponds to 1pg per test well in the *in vitro* parasite inhibition assay. Responses <10pg/mL were still plotted for reference.

2.4.7 Peptide pulse assay

HC-04 cells were pulsed overnight with 100µg/mL FLIFFDLFLV peptide and labelled with DiD membrane dye. Unpulsed HC-04 cells were labelled with CFSE, as per standard CFSE protocol. The pulsed DiD⁺ and unpulsed CFSE⁺ cells were plated in a 50:50 ratio and left to settle for 6 – 24 hours, before plating of T cells.

2.4.8 Experimentation using primary human hepatocytes

All experiments in this thesis using *P. falciparum* were undertaken at CIMI-Paris, alongside Dr Valérie Soulard and her research team.

2.4.8.1 Thawing and maintenance of primary human hepatocytes

Black flat bottom 96 well plates were coated with 100µL of 5µg/mL rat tail collagen 1 in acetic acid and incubated overnight at room temperature. Plates were then washed twice with 200µL PBS and once with primary human hepatocyte (PHH) culture medium. A vial of PHH was removed from liquid nitrogen and cleaned with 70% ethanol, then thawed in a 37°C water bath until an ice crystal was loose but visible. Vial contents were then poured into pre-warmed culture medium in a 15mL Falcon tube, and the vial rinsed twice to remove residual cells. PHH were centrifuged at 180G for 1 minute at room temperature, supernatant aspirated, then resuspended in 3mL culture medium. This solution was then slowly layered onto 4ml of 30% iso-osmotic Percoll on a 15mL Falcon tube and centrifuged at 180G for 1 minute at room temperature before aspirating supernatant and resuspending in 3mL seeding medium. PHH were then counted by Trypan blue exclusion. PHH were diluted in 800,000/mL and 100µL plated per well (80,000 PHH/well) and incubated at 37°C. After 24 hours, wells were washed with 200µL culture medium and 100µL culture medium added and incubated at 37°C. A media change with no washing step was performed every 24 hours.

2.4.8.2 Infection of primary human hepatocytes

Four days following thawing, *P. falciparum*-infected mosquitoes were drenched in ethanol, then washed through 3 Petri dishes containing Leibovitz's L15 medium, before being hand dissected to remove salivary glands. Mechanical disruption was used to release sporozoites. Sporozoites were counted and resuspended at 400,000spz/mL, and 50µL (20,000 spz) plated per well with 50µL culture medium. Plates were incubated at room temperature for 3 hours, and at 37°C for a further 3 hours,

before removal of sporozoites. Plates were washed with 200µL culture medium and 40µL of Matrigel was added per well and incubated at 37°C for 45 minutes, before the addition of 100µL/well of culture medium on top of Matrigel. Plates were incubated at 37°C, with a media change performed every 24 hours. Cell sort fractions were added the next day.

2.4.8.3 Readout of infected primary human hepatocytes

Cells and infected PHH were left to incubate for 48 hours. The PHH were then fixed with cold 100% methanol, and stained with 50µL 1:1500 anti-HSP70 diluted in PBS 1% BSA for 1 hour at 37°C, followed by 3 PBS washes. 50µL/well goat anti-mouse Alexa Fluor 488 (1:1000) and DAPI (1:500, 1µg/mL) diluted in PBS 1% BSA was added and incubated for 1 hour at 37°C. Plates were again washed 3 times with PBS, then resuspended in 200µL PBS 0.1% sodium azide. Plates were analysed using a CellInsight NXT (Fisher Scientific), assessing monolayer intactness via DAPI staining and number of infected wells via HSP70 staining. Data was analysed using the same methodology as transgenic *PbPf*TRAP assays.

2.5 Animals and parasitology

2.5.1 Mice

BALB/c mice (Harlan) or equivalent were used for all experiments. All animal work was conducted in accordance with the UK Animals (Scientific Procedures) Act 1986 and approved by the University of Oxford Animal Care and Ethical Review Committee for use under Project License PPL 30/2889. Animals were group housed in individually ventilated cages under specific pathogen free conditions, with constant temperature and humidity, and with a 12:12 light to dark cycle (8am to 8pm). All animals were humanely sacrificed at the end of each experiment by an approved Schedule 1 method of cervical dislocation. All efforts were made to minimize animal suffering.

2.5.2 Parasites

Transgenic *P. berghei* parasite strain 2281 clone 1: PfTRAP_{Pbuis4} GFP::Luc_{Pbeef1a_230p} (*PbPfTRAP*) from Leiden University Medical Centre was used in all experiments in this thesis.

2.5.3 Sporozoite generation

See Jenner Protocol: J138, J145. Mice were injected intraperitoneally with 80-130µL of Infected red blood cell stock. Blood stage parasitaemia was measured from day 4 post infection, by tail tip bleed, and preparation of a thin blood film. Slides were air dried and then fixed in methanol for 30 seconds. Slide was then stained in 10% Giemsa for at least 15 minutes, followed by washing in water, air drying and counting of infected red cells by light microscope at 100x under oil immersion. Once the mouse's parasitaemia reached 5%, mosquito pots were fed on water overnight or starved for 4 hours prior to the mouse feed. Mice were anaesthetised with 50µL of intramuscular anaesthetic, and then placed on mosquito pot for 10 minutes. Maximum 2 pots per mouse. Fed mosquito pots were placed on their side until dry, then maintained at 19-21°C in a humidified incubator with a 12 hour day-night cycle, and every second day a cotton wool soaked in

Fructose/PABA solution was replaced. At day 10-12 post feed, 3 mosquitoes were removed from the pot to check for parasite oocyst development via midgut dissection. Oocysts could be visualised by fluorescence microscopy, with 488nm laser activation. At day 18-28 post feed, salivary glands were dissected from infected mosquitoes into Grace's insect medium and collected in a glass homogeniser and kept on ice.

2.6 SYFPEITHI epitope prediction

The SYFPEITHI epitope prediction software uses published motifs and takes into consideration the amino acids in the anchor and auxiliary anchor positions of MHC binding, as well as other frequent amino acids. The SYFPEITHI epitope prediction score is calculated according to the following rules: Amino acids of a certain peptide are given a specific value depending on whether they are anchor, auxiliary anchor or a preferred residue. Ideal anchors are given 10 points, unusual anchors 6-8 points, auxiliary anchors 4-6 and preferred residues 1-4 points. Amino acids that are regarded as having a negative effect on the binding ability are given values between -1 and -3 [369].

2.7 Statistical analyses

All statistical testing was performed in Prism 6. Statistical tests performed depended on the type of data, and whether they were of a Normal distribution and could therefore be tested using a parametric statistical test. Grouped data with more than one parameter for assessment were analysed by a 2-way ANOVA, assessing the contributions of each variable in addition to the interaction between the 2. Column data were assessed by D'Agostino-Pearson Normality test. If data were of a Normal distribution, a t-test was performed (if just 2 groups), or a 1 way ANOVA (if 3 or more groups). Means were plotted. If data were not of a Normal distribution, or there were too few data points to accurately assess the distribution, then a Mann Whitney test was performed (for 2 groups), or a Kruskal-Wallis test (if 3 or more groups). Non-parametric data for paired analysis were analysed by a Friedman test. Medians were plotted, or geometric mean plotted for FACS data. For all tests with 3 or more groups, a post test was applied to correct for multiple tests between groups or against a single control group. Specific details are outlined in each individual analysis, with p-value stated according to: * <0.05 , ** <0.01 , *** <0.001 , **** <0.0001 . Correlations were performed using Spearman r , reporting r and p for statistically significant correlations.

2.8 Summary of finalised protocol for *in vitro* parasite inhibition assay:

2.8.1 Preparatory work

- 16 days:** Inject mice i.p. with 100 μ L *PbPf*(TRAP) blood stock.
- 12 days :** Begin daily thin blood smear of mice, until parasitaemia reaches 4-5%, then perform mosquito feed on infected mouse, and a second feed on a naive mouse 1 week later, to aid sporozoite production. Sporozoites will be ready for dissection 21 days later (ideal range 19-26 days)
- ~ -6 days:** Thaw HC-04 hepatoma cell line and stabilise line in culture, aiming to use in the *in vitro* assay before passage number 40.

2.8.2 Assay set up

- Day 0:** Thaw post-vaccination PBMC for testing and set up FLIFFDLFLV and GILGFVFTL T cell cultures, at $\sim 5 \times 10^6$ cells in 2 μ g/mL peptide
- Day 3:** Add rh IL-2 to cultures at final concentration 10U/mL
- Day 7 (am):** Add rh IL-2 to cultures at final concentration 10U/mL
- Day 8 (pm):** Remove T cells from culture by 3 x wash. Count and plate 20,000 HC-04 /well in half surface area 96 well plates. Aim to plate 24 hours prior to infection.
- Day 9 (am):** Stain T cell cultures using pentamer equivalent to culture peptide, plus surface marker panel and propidium iodide. Cell sort CD8⁺ pentamer⁺, CD8⁺ pentamer⁻ and CD4⁺ populations by FACS.
- Day 9 (pm):** Dissect *PbPf*(TRAP) sporozoites into Grace's buffer and infect 80/96 wells per plate at $\sim 15,000$ spz/well, avoiding edge wells. 1 plate per volunteer being tested. Determine plate layout based on cell counts from sort. At >6 hours post cell sort and ~ 3 -5 hours post infection, add cells to infected plate.
- Day 10 (pm):** ~ 24 hours post infection, perform CD45 staining of monolayers, then acquire samples by FACS (25 - 32 hours post infection).

Chapter 3

Immunogenicity of ChAd63.MVA ME-TRAP in paediatric populations

3 Immunogenicity of ChAd63.MVA ME-TRAP in paediatric populations

3.1 Introduction

Adult, paediatric and newborn immunity differ in many ways, and a vaccination regimen developed through clinical testing in adults, such as ChAd63.MVA ME-TRAP, may be suboptimal in paediatric vaccinees. It is therefore unknown how T cell vaccine immunogenicity and efficacy will translate from adults into paediatric vaccinees. The WHO's current EPI (Expanded Program on Immunization) vaccines administered to infants and children rely predominantly on antibody-mediated efficacy, with the role of T cells generally not considered essential. However a role of T cells has been identified in several instances (reviewed in [370]) and provides a scientific rationale basis for the development of protective T cell vaccines [371].

Innate immunity in newborns is limited at birth and takes a period of months to fully mature [372]. There is evidence that neonates become biased *in utero* against producing a strong pro-inflammatory response, and as a residual effect of having recently been in the intra-uterine environment, newborn Th1 responses can be impaired, particularly with respect to production of IFN- γ and IL-12 (reviewed in [373-375]). Neonatal innate responses to viruses are often lower than those in older children and adults, due to limited interferon α/β secretion by plasmacytoid DCs and the hypo-responsiveness of NK cells left over from *in utero* conditions [376, 377]. However, more recent studies have shown that newborn myeloid DCs are capable of secreting concentrations of pro-inflammatory cytokines comparable to, or even in excess of, that of adults [378, 379].

Adaptive immune responses in newborns and young infants are phenotypically and functionally different to those of adults in several ways. At birth, nearly all T cells carry the CD45RA glycoprotein, typical of naive T cells, which have never encountered foreign antigen and many of these CD4⁺ CD45RA⁺ T cells are of a regulatory phenotype, again due to the self-tolerance required *in utero* [380]. Neonatal T cell responses are also often Th2 skewed [381] and unchallenged by defective IL-12 production by DCs [382]. High numbers of $\gamma\delta$ T cells are also often circulating and

capable of producing significant amounts of IFN- γ , likely compensating in part for the under-development of the classical Th1-type response required against intracellular pathogens [383]. However following vaccination, these cells cannot contribute to cytotoxic CD8⁺ T cell priming or generation of a cellular effector memory response required for protection.

For the first 6-12 months of life, infants are protected from many pathogens by maternal antibodies that cross the placenta in the third trimester of pregnancy. Additionally, maternal antibodies can be transferred thorough breastfeeding. Antibody-mediated vaccine-induced immunity is generally strong in infants, although induction of adaptive antibody responses can be hindered at a subclinical level by these maternal antibodies. Evidence of maternal antibody inhibition of T cell responses is rare, with most studies reporting minimal effects on T cell induction [384]. In the setting of ChAd63.MVA ME-TRAP vaccination, maternal antibody responses against the ChAd63 and MVA vectors could impact upon vaccine-induced responses.

In contrast, maternal T cells do not usually cross the placenta, so T cell immunity needs to be developed by the child postnatally. T cell priming in newborns and infants is a hugely understudied area. Extensive literature in murine immunology documents the variability of CD8⁺ T cell induction in newborn mice depending on the mode of antigen delivery, suggesting that generation of such T cell responses in human infants may not be straightforward [372, 385-389]. Furthermore, the associated memory responses may be inferior to those generated in adulthood [390]. Limited studies in infants demonstrate reduced or defective secretion of Th1 cytokines in response to oral polio, hepatitis B and protein-in-adjuvant vaccines, such as diphtheria, tetanus and pertussis [372, 391-393], however data are generally limited on CD8⁺ T cell induction in early infancy. Comparable Th1 responses following BCG vaccination have been reported in newborns and adults [392, 394-396], as have strong CD4⁺ and CD8⁺ T cell responses to *Bordetella pertussis* infection in 1-4 month and 2-month old whole cell pertussis vaccinees [397]. Additionally, CD8⁺ T cell responses that develop in newborns following congenital human cytomegalovirus infection are functional and have similar characteristics to those responses measured in adults [398]. There is evidence that the manner in which a vaccine is delivered can overcome the Th2 bias of young infants [399]. Thus it is argued that despite differences in the infant and adult immune system, robust and life-long responses to many childhood

pathogens and vaccinations can be, and indeed are, generated and the antigen-delivery platform, timing of delivery and use of boosting vaccinations and adjuvants are all likely to have a substantial impact upon this. These parameters of vaccine delivery are likely linked to the quality of the signal delivered by APCs to T cells, with level of DC activation previously demonstrated to be a critical factor associated with this [400, 401].

Efficacy of leading malaria vaccine-candidate RTS,S is mediated by anti-CS antibodies and CS-specific CD4⁺ T cells. Immunogenicity from large RTS,S Phase III efficacy trials demonstrate that levels of anti-CS antibodies induced in 6-12 week old infants were 3-fold lower than in 5-17 month olds, suggesting that RTS,S/AS01 is less immunogenic in young infants. This was mirrored in the reported efficacy of RTS,S to protect infants from clinical malaria, whereby 55.8% efficacy was measure in the 5-17 month olds over the 12 month period following vaccination. The equivalent efficacy in 6-12 week olds was 31.3%. CD4⁺ T cell immunogenicity following vaccination was not reported [304, 402, 403].

Clinical vaccine studies of ChAd63.MVA ME-TRAP have been performed in malaria-exposed African adults, children and infants in West Africa, in addition to malaria naïve adults in the UK. Given each of these clinical trials were small Phase I or II studies, combining the datasets together into a single analysis facilitates the observation of trends across age-groups and between cohorts with differing malaria exposure. Two clinical studies were Phase II CHMI studies in UK adults, whereby protective efficacy was measure in 57% of ChAd63.MVA ME-TRAP vaccinees [287]. Three clinical trials were carried out in adults, children and infants in Sukuta, in the western region of The Gambia [269, 283, 294], where modest malaria transmission is still detectable following seasonal rains, despite a substantial decline in incidence since 2003 [68]. A sixth study was undertaken in infants in Burkina Faso, where the incidence rate of malaria is higher than in The Gambia, averaging more than one confirmed clinical episode per child per year [404] [405]. These clinical studies are summarised in Table 10, whereby only the vaccine groups incorporated in this thesis are listed.

Using these clinical vaccine studies, the research aims were to accurately report vaccine-induced T cell immunogenicity in paediatric populations. This necessitated the development of novel assays and methodologies to effectively measure and interpret paediatric immunogenicity in the

field, and reliably compare these paediatric immune responses to those measured previously in adult vaccinees.

Table 10 Summary of clinical vaccine trials presented in Chapter 3.

Clinical trial number	Group	Age	Trial site	N	ChAd63 ME-TRAP dose, vp	MVA ME-TRAP dose, pfu	Other trial info
Mal 34	1	18-50 yr	UK	15	5×10^{10}	2×10^8	Phase I/IIa CHMI study
Vac 55	2	18-50 yr	UK	20	5×10^{10}	2×10^8	Phase I/IIa CHMI study. Staggered administration alongside RTS,S/AS01B: 50mcg RTS,S + Standard dose AS01 (0.5ml containing 50mcg MPL + 50mcg QS21).
Vac 41	3	18-50 yr (male)	The Gambia	10	5×10^{10}	2×10^8	Phase Ib
Vac 41	4	2-6 yr	The Gambia	6	5×10^{10}	1×10^8	Phase Ib
Vac 42	5	5-12 mo	The Gambia	12	5×10^{10}	1×10^8	Phase Ib
Vac 42	6	10 wk	The Gambia	12	5×10^{10}	1×10^8	Phase Ib
Vac 58	7	16 wk	The Gambia	15	5×10^{10}	1×10^8	Phase Ib. Chad63.MVA ME-TRAP administered alongside EPI vaccines
Vac 58	8	8 wk	The Gambia	15	5×10^{10}	1×10^8	Phase Ib. Chad63.MVA ME-TRAP administered alongside EPI vaccines
Vac 58	9	1 wk	The Gambia	15	5×10^{10}	1×10^8	Phase Ib. Chad63.MVA ME-TRAP administered alongside EPI vaccines
Vac 50	10	5-17 mo.	Burkina Faso	30	5×10^{10}	1×10^8	Phase Ib (part of Phase II study)

3.2 Results

3.2.1 Vaccine-induced immunogenicity by *ex vivo* IFN- γ ELISpot is comparable between adult and paediatric populations

Peak ELISpot responses at 7 days post MVA ME-TRAP boost were compared between groups in these age de-escalation studies with those from previously published adult Phase I trials in the UK [287] and The Gambia [294]. A trend towards lower T cell immunogenicity in paediatric vaccinees compared with adult vaccinees was measured, with responses in Burkinabe children significantly reduced. Notably, responses in 10 week old Gambian infants were comparable to those in adults and higher than in Burkinabe children aged 5-12 months and 13-17 months (Figure 3.1 A, $p < 0.0001$ Kruskal-Wallis test). No differences were measured between responses in UK and Gambian adults (data not shown).

Due to the observation that children under 17 months have significantly higher lymphocyte counts per millilitre of blood than older children and adults (Figure 3.1 B, $p < 0.0001$ Kruskal-Wallis test), expressing ELISpot responses using the number of peripheral blood mononuclear cells (PBMC) as the denominator may not accurately reflect immunogenicity across age groups. ELISpot responses were expressed as spot-forming cells (SFC) per millilitre of blood by integrating lymphocyte counts collected during routine haematology tests at each time point, using the following formula:

$$\begin{aligned} & \text{(No. lymphocytes/mL whole blood)} \times (\text{ELISpot response SFC}/10^6 \text{ PBMC}) \\ & \qquad \qquad \qquad = \text{ELISpot response SFC/mL whole blood} \end{aligned}$$

Using this arguably more physiologically relevant denominator, T cell responses were comparable between adults, children and older infants. Furthermore, responses in 10 week old infants were almost 3-fold higher than UK and Gambian adults (Figure 3.1 C, $p = 0.0017$, Kruskal-Wallis test). No differences were measured between responses in UK and Gambian adults (data not shown). Responses remained higher in Gambian 10 week old infants compared with both Burkinabe age groups ($p = 0.0007$, Kruskal-Wallis test).

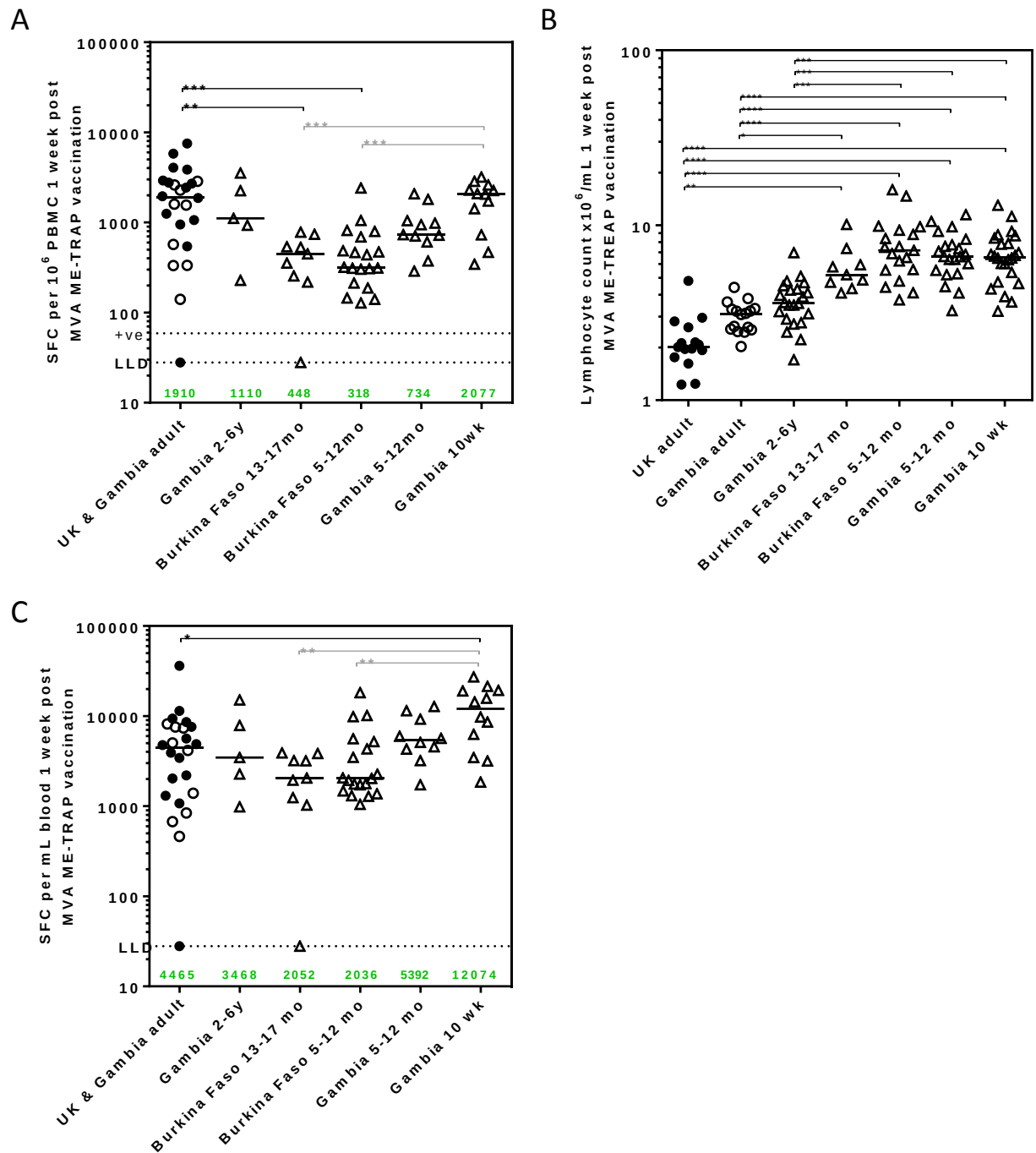


Figure 3.1 Immunogenicity summary of ChAd63.MVA ME-TRAP vaccine trials with age de-escalation.

A) Comparison of *ex vivo* IFN- γ ELISpot responses 7 days post MVA ME-TRAP, expressed as SFC per million PBMC. Adult groups combined, due to comparable immunogenicity. Kruskal-Wallis test with Dunn's correction for multiple comparisons to adult control group denoted by black bars, Kruskal-Wallis test with Dunn's correction for multiple comparisons between all paediatric groups denoted by grey bars. B) Lymphocyte counts per millilitre of blood for all vaccinated volunteers 7 days post MVA ME-TRAP. Kruskal-Wallis test with Dunn's correction for multiple comparisons between all groups. Adult groups not combined due to difference by 2-tailed Mann-Whitney test, $p=0.0009$. C) *Ex vivo* IFN- γ ELISpot responses per millilitre of blood. Kruskal-Wallis test with Dunn's correction for multiple comparisons to adult control group denoted by black bars, Kruskal-Wallis test with Dunn's correction for multiple comparisons between all paediatric groups denoted by grey bars. Dotted lines show LLD (lower limit of assay detection) and positive threshold for ELISpot response. Medians displayed in green, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Baseline monocyte count, and respective monocyte to lymphocyte ratio (M:L) were recorded for most vaccinee groups. Pre-vaccination M:L was significantly higher in UK adults compared with all of the paediatric populations with the exception of 13-17 month old Burkinabe infants (Figure 3.2 A, $p=0.0247$ Kruskal-Wallis test). These trends continued post-vaccination (data not shown). Using monocyte and lymphocyte data available at the peak immunogenicity time point, a correction factor can be calculated for the ELISpot response per millilitre of blood, which normalises for the proportion of monocytes in the ELISpot assay across all vaccine groups and thus prevents a skew due to different number of monocytes in the ELISpot assay. This was calculated through the enumeration of lymphocytes per million PBMC that contribute to the ELISpot response, using monocyte and lymphocyte differential data. The correction factor was then calculated using the following formula, and then used to multiple up the ELISpot response per million PBMC to per mL blood:

$$\text{M:L correction factor} = \frac{\text{Lymphocytes per mL blood}}{\text{Number of lymphocytes in 1 million PBMC}}$$

The same trends in the 'ELISpot per mL' dataset are seen both with and without the correction factor applied (Figure 3.2 B, NSD by Kruskal-Wallis test), thus supporting the assumption that there is no requirement to correct for monocyte count in the ELISpot per mL conversion calculation.

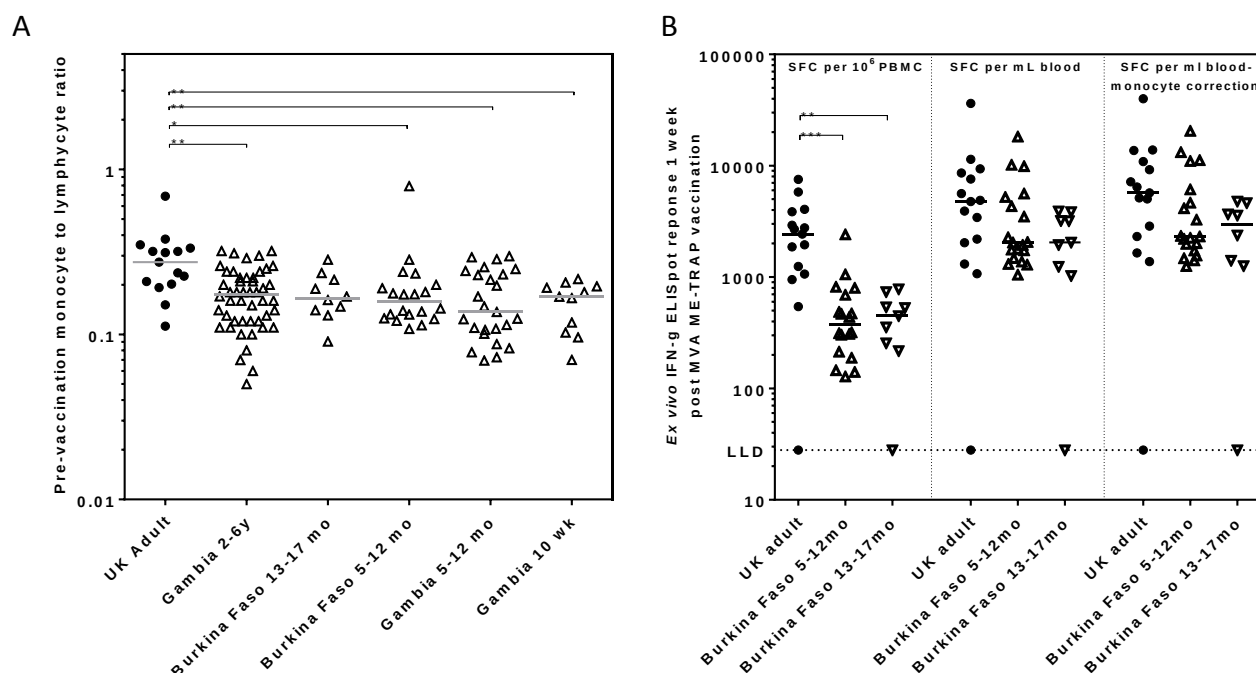


Figure 3.2 Incorporation of monocyte data into ELISpot readout.

A) Baseline monocyte to lymphocyte ratio for all enrolled volunteers. B) Comparative analysis of ELISpot readout in SFC/ 10^6 , SFC/mL and SFC/mL with monocyte correction factor applied. Kruskal-Wallis tests with Dunn's correction for comparisons against UK adult control group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.2.2 Challenges associated with intracellular cytokine staining of PBMC

Analysis of paediatric immunogenicity using flow cytometry with ICS on fresh PBMC samples has proven difficult in recent clinical vaccine studies in The Gambia, with incomplete sample preparation and staining, in addition to QC failure in over half of the data produced. These issues were linked to lack of resources, including personnel, and non-adherence to protocols. As a result, frozen PBMC samples were shipped to Oxford from The Gambia and Burkina Faso. Flow cytometry with ICS was performed on all samples available from 5-12 month old Gambian infants (Vac 42) and on 13 of the 30 5-17 month old Burkinabe infants (Vac 50), selected on the ELISpot response to TRAP T9/96 megapool >248 SFC/ 10^6 PBMC at 1 week post MVA ME-TRAP (median 348 SFC/ 10^6 PBMC). Mean cell viability upon thawing was 70% and 57%, and live cell staining by FACS 79% and 77%, in Vac 42 and Vac 50 vaccinees, respectively. Assessment of cytokine expression 7 days post boost with

MVA ME-TRAP showed detectable IFN- γ , IL-2 and TNF- α secretion from CD4⁺ and CD8⁺ T cells (Figure 3.3). Positive cytokine responses were measured in 89% of volunteers tested, however polyfunctional responses were not calculated due to low cell numbers in the respective Boolean gates. Expression of the degranulation marker CD107a on CD8⁺ T cells was also measured in 15% of volunteers tested.

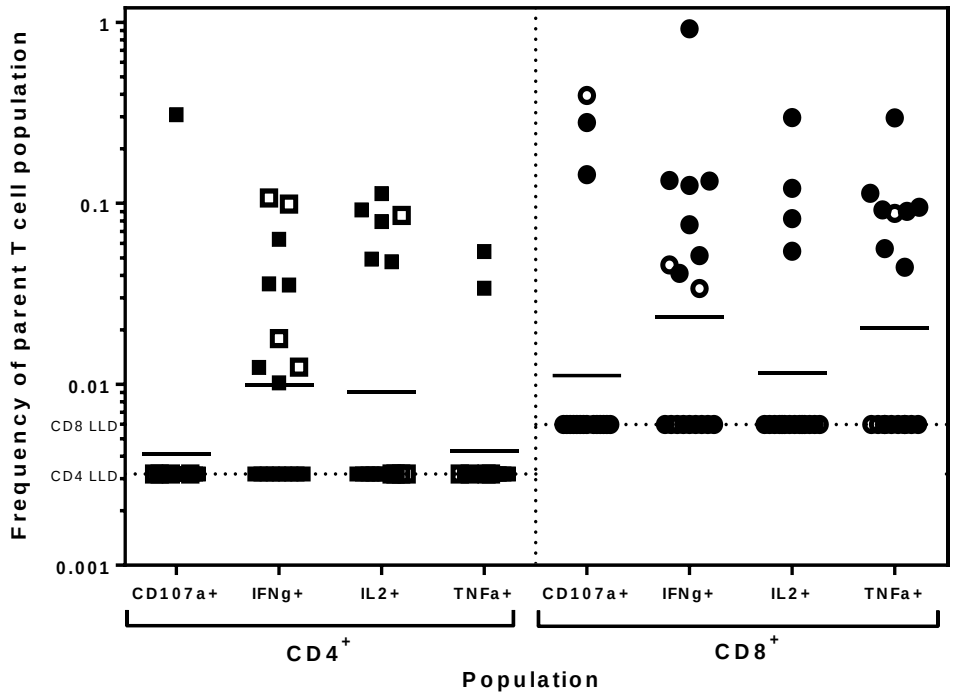


Figure 3.3 Flow cytometry with intracellular cytokine staining

Frequencies of cytokine-producing CD4⁺ and CD8⁺ T cells performed on thawed PBMC from paediatric volunteers in Vac 42 (open) and Vac 50 (closed). Geometric mean displayed. CD4 and CD8 LLD aligned for separate trials and displayed as a dotted line. Minimum of 29,000 live CD3⁺ cells acquired per sample.

These data prompted the development of a whole blood assay, whereby fresh whole blood could be stimulated and subsequently frozen at field sites, with limited sample handling and processing. Such a process would enable batched in-house analysis, or shipment to a central laboratory for staining and acquisition, with the overall aim of capturing the higher antigen-specific responses associated with fresh sample stimulation, without compromising the robustness and quality of the data acquired.

3.2.3 Development of a novel flow cytometry assay for use at field sites

A whole blood ICS assay could overcome many of the issues encountered at field site laboratories, however current whole blood ICS protocols do not permit staining of dead cells [406-409], due to the simultaneous cell fixation and RBC lysis within the protocol using FACS Lysing solution® (Becton Dickinson). The importance of dead cell exclusion and the identification of dead cells within a whole blood ICS assay protocol are explored below.

3.2.3.1 Inclusion of dead cells increases background ICS signal

To assess the effect of dead cells upon background responses, 16 blood samples were incubated overnight with costimulatory antibodies anti-CD28 and anti-CD49d, and Brefeldin A. Red cells were lysed twice with 4mls of Qiagen Red Cell Lysis Buffer, then stained with Aqua amine-reactive dye for dead cell identification before being frozen and subsequently thawed and stained with the standard clinical trials' ICS panel. Background responses were analysed with and without dead cell exclusion (Figure 3.4, $p < 0.0001$ 2-way ANOVA). Significantly higher background responses of IFN- γ from CD4⁺ and CD8⁺ T cells were measured when dead cells were included in the analysis.

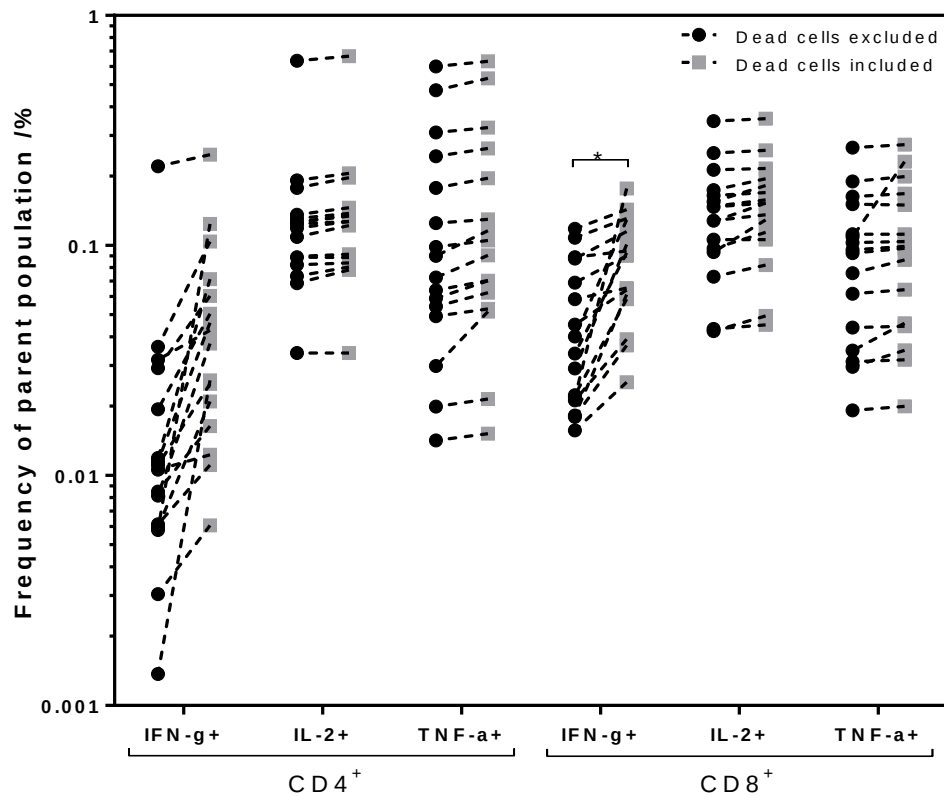


Figure 3.4 Effect of dead cells on background cytokine measurements

A single dataset of fresh, unstimulated whole blood samples, gated with and without a dead cell exclusion gate. Use of dead cell exclusion significantly affects background IFN- γ detection, 2-way ANOVA * $p < 0.05$.

3.2.3.2 Evaluation of red cell lysis buffers

Red cell lysis following whole blood stimulation is a key step before staining of T cells in published whole blood ICS protocols, due to elevated degree of non-specific binding of antibodies to red cells in whole blood. Lytic capacity of 3 non-fixative containing buffers was evaluated by 3 rounds of lysis and washing. Relative lysis was calculated by size analysis by automated cell counting of the remaining fractions present in the sample following lysis. A cell debris fraction was counted, in addition to a total count that included cell debris in addition to red cells and lymphocytes. Lysis between samples was compared by calculating relative lysis:

$$\text{Relative lysis} = \frac{\text{Cell debris count (0.75-6.25uM)}}{\text{Total count (0.475-15uM)}}$$

As the buffers target red cells, the relative size of the cell debris fraction increases relative to the total fraction, thus a higher relative lysis value represents more efficient red cell lysis (Figure 3.5 A+B). Generally 4mls of buffer gave a higher relative lysis value, and 3 rounds of lysis resulted in significantly higher red cell lysis compared with a single lysis step, regardless of buffer or volume (Figure 3.5 C, $p=0.0002$ Kruskal-Wallis test). In order to keep the protocol of reasonable length and use reagents already in mainstream use in field site laboratories, a double lysis with 4mls Qiagen buffer was taken forwards for further testing in the protocol.

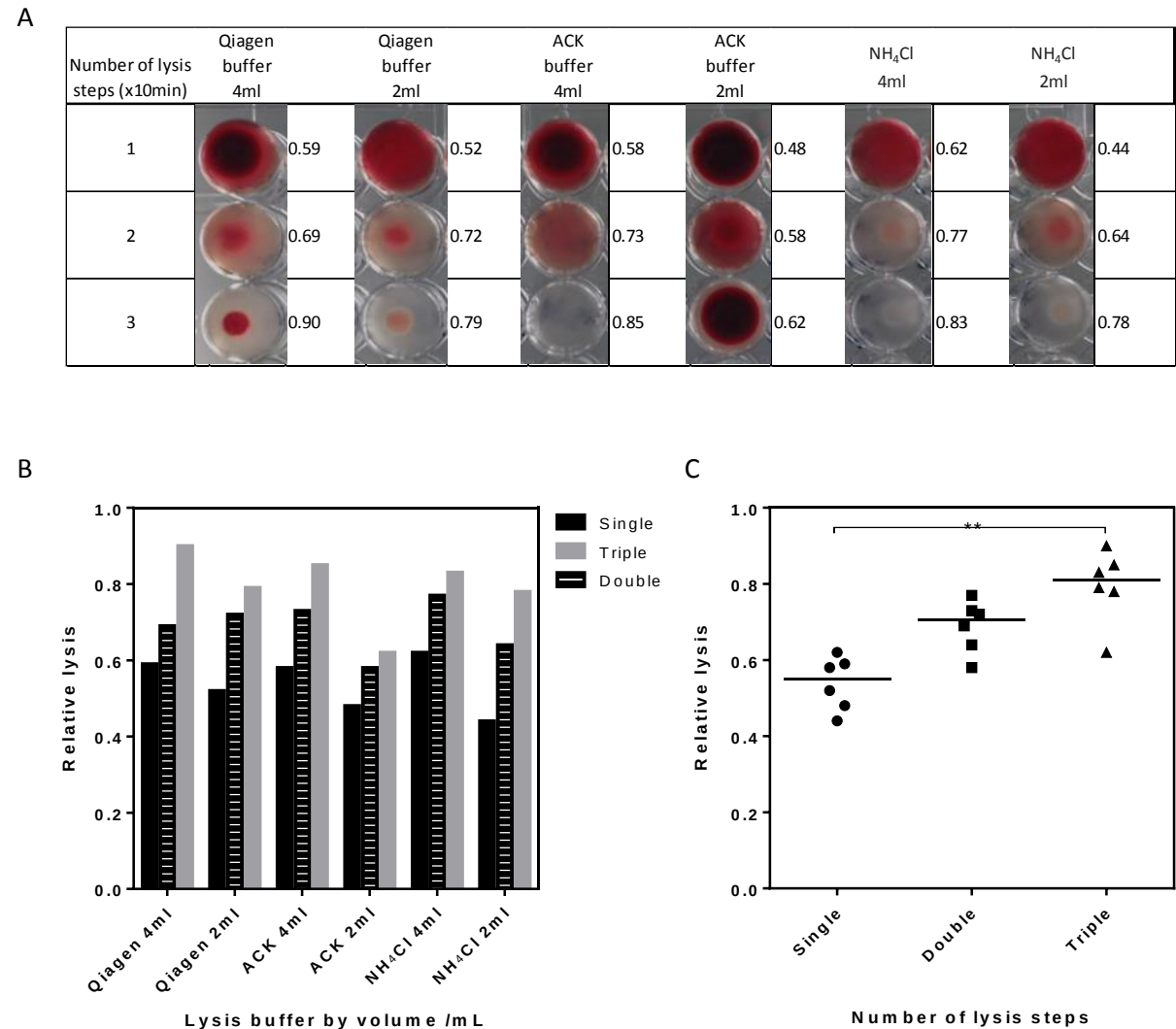


Figure 3.5 Relative red cell lysis of whole blood.

A) Representative images and B) relative lysis of 500uL of whole blood by Qiagen RBC lysis buffer, ACK buffer and ammonium chloride, measured after a single, double or triple lysis treatment with either 2mls or 4mls buffer. C) Buffer types and volumes combined for analysis of number of lysis steps, Kruskal-Wallis tests with Dunn's correction for comparisons between all groups, ** $p<0.01$.

3.2.3.3 Aqua dead cell staining using selected lysis buffer

Fixative-containing red cell lysis buffers, such as FACS Lysing Buffer®, exhibit good red cell lysis, but eliminate the ability to subsequently stain for cell viability (Figure 3.6 C). Fixative-free red cell lysis buffers permit live cell identification following red cell lysis (Figure 3.6 D). Following this red cell lysis and subsequent staining with Aqua amine reactive dye, the dye is retained during a single freeze-thaw cycle under all stimulation conditions (unstimulated, antigen-stimulated, SEB-stimulated. Figure 3.7).

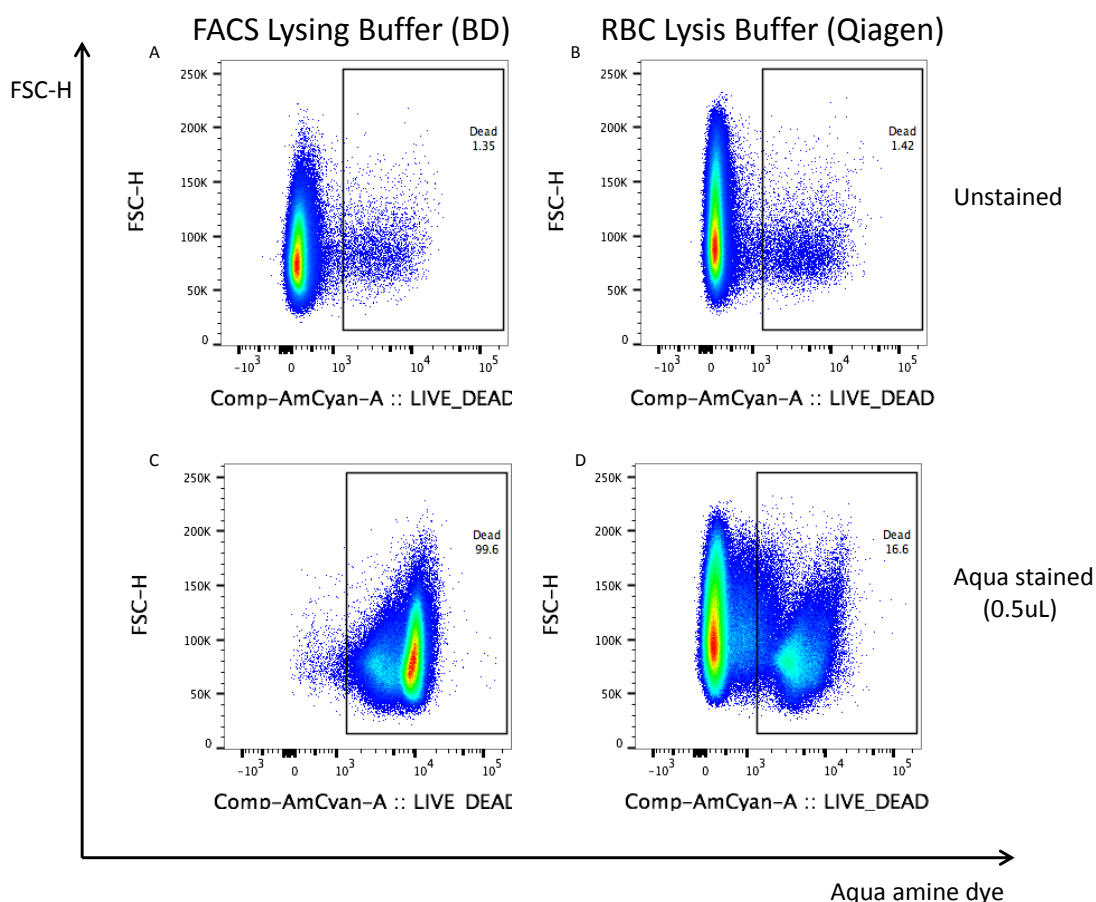


Figure 3.6 Aqua staining of fixed and non-fixed cells

Unstained and Aqua staining of leukocytes following whole blood red cell lysis with A+C) BD FACS Lysing Solution® with fixative, B+D) Qiagen Red Cell Lysis Solution® without fixative.

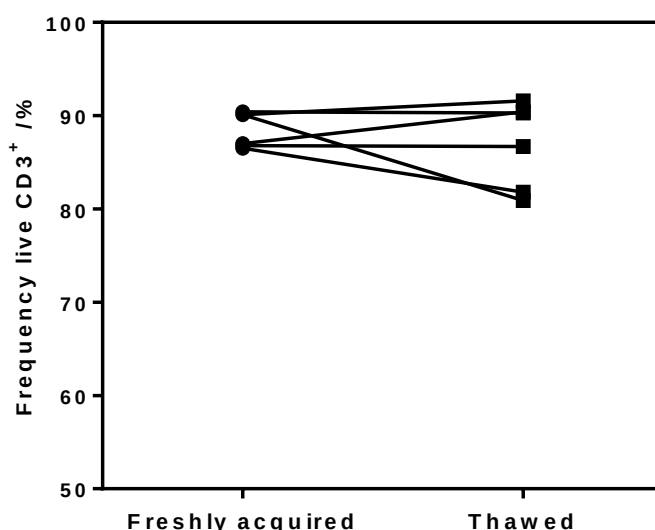


Figure 3.7 Retention of Aqua dye during freeze-thaw process.

Aqua stained leukocytes, subsequently fixed and either freshly acquired on the same day, or acquired following a single freeze-thaw cycle. NSD by Wilcoxon matched-pairs signed rank test.

3.2.4 Agreement between fresh PBMC ICS and frozen whole blood ICS

3.2.4.1 Comparable cell phenotypes (validation 1)

Frequencies and absolute numbers of live CD3⁺ populations, in addition to CD4⁺ and CD8⁺ T cell populations were measured using the fresh PBMC assay and the frozen whole blood assay, and compared in order to begin validating the new whole blood ICS staining procedure. Frequencies of live CD3⁺ cells were slightly higher using the whole blood assay, with frequencies of all 3 populations within 3% in each assay. Absolute numbers of live CD3⁺ cells were higher with the PBMC assay. This was to be expected, as 2.0x10⁶ PBMC were stimulated, compared with 500μL of whole blood, which in this adult population contains approx. 0.5-1.0x10⁶ PBMC. The ratio between the acquired counts is therefore consistent with cell numbers that were stimulated. Interestingly, the standard deviations in cell numbers from the whole blood assay were lower, indicating this assay reliably produces more consistent cell numbers for analysis, compared with the PBMC assay equivalent, despite the inclusion of a freezing step (Figure 3.8). PBMC ICS assays performed by Malaria Vaccine Trials team.

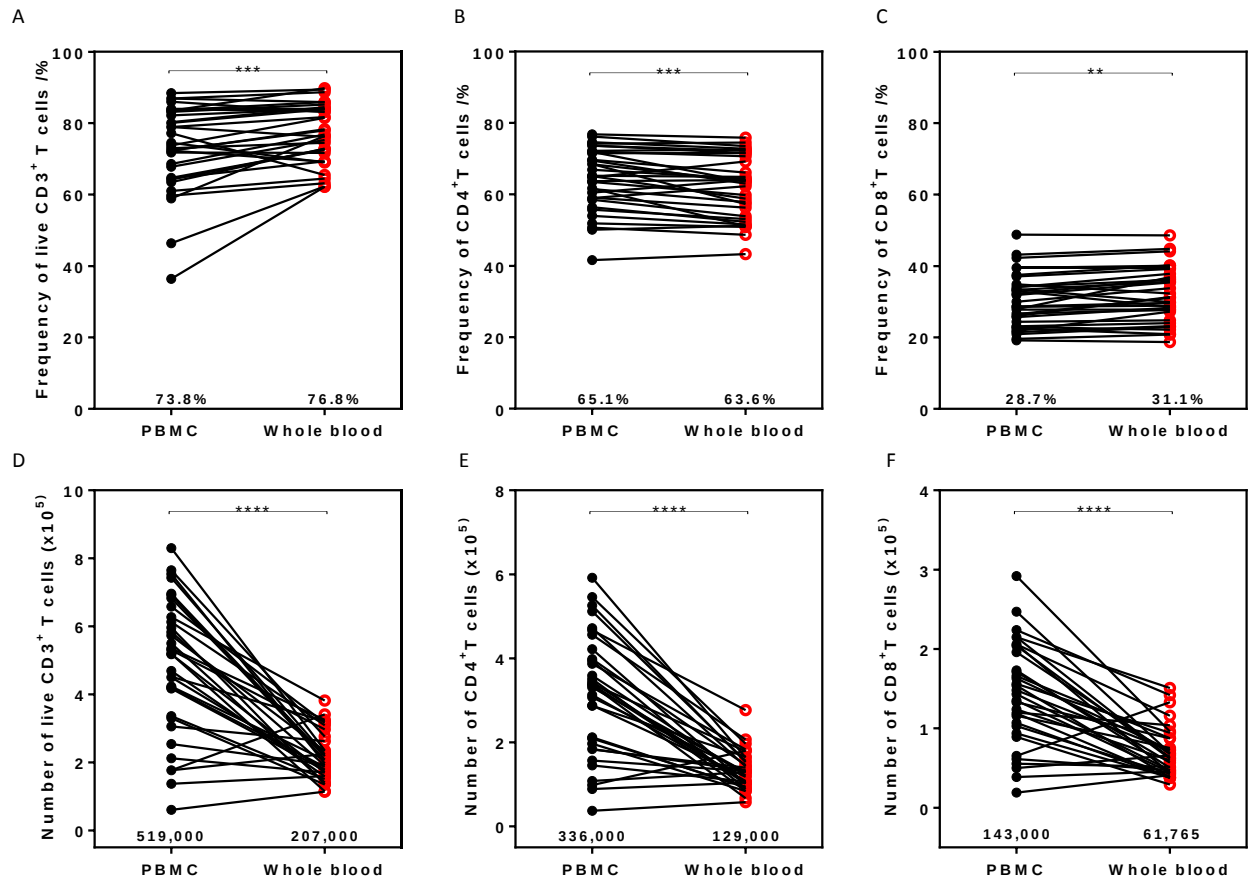


Figure 3.8 Basic phenotypes identified using fresh PBMC and frozen whole blood assay platforms.

A-C) Cell frequencies and D-F) cell counts of live CD3⁺ T cells, CD4⁺ T cells and CD8⁺ T cells. Wilcoxon matched-pairs signed rank test performed on paired samples, ** p<0.01, *** p<0.001, **** p<0.0001. Mean values displayed. Standard deviation of PBMC and whole blood counts respectively: Live CD3⁺ 200,177 and 70,093, CD4⁺ T cell 143,669 and 45,247, CD8⁺ T cell 64,490 and 31,774.

3.2.4.2 Comparable cytokine responses by intracellular cytokine staining of fresh PBMC and frozen whole blood (validation 2)

PBMC and whole blood samples from a Phase I/IIa clinical vaccine trial evaluating vaccine efficacy of the combination RTS,S/AS01_B + ChAd63. MVA ME-TRAP and of RTS,S/AS01_B alone (Vac 55) were used to compare cytokine responses measured by the 2 different ICS protocols at the C-1 time point (one day prior to challenge). PBMC stimulation, staining and acquisition were performed on fresh PBMC samples in 2 batches. Whole blood samples were stimulated in 2 batches and subsequently frozen down for staining and acquisition across 3 batches. Responses to peptides covering TRAP T9/96 and 3D7 strains and to CSP were measured in group 1, and to CSP only in group 2 (Figure 3.9).

Through alignment of assay LLDs (lower limits of detection), cytokine responses from each assay were compared head-to-head (Figure 3.10). Most variability between the assays was measured when responses were <0.05%, highlighted by the disagreement between background responses from unstimulated (background) samples and volunteers with lower responses, predominantly CS-specific responses. This frequency is not only low, but approaches the lower limit of detection for both assays, and variability at the lower end of the assay is to be expected. Background responses were generally comparable across the 2 assays for all parameters, with the exception of TNF- α production from CD4⁺ T cells and IL-2 production from CD8⁺ T cells, with assay type accounting for 3.6% of the variability measured in background responses (Figure 3.10 D, $p < 0.0001$ 2-way ANOVA). Antigen-specific responses were generally comparable, or higher in the whole blood assay for certain antigen-specific CD8⁺ T cell responses (Figure 3.10 A-C). A small proportion of the variation in antigen-specific responses was due to assay type (<2.2%), with volunteer and cytokine responsible for 48-87% of the variation in ICS response (Figure 3.10 A-C, 2-way ANOVA)

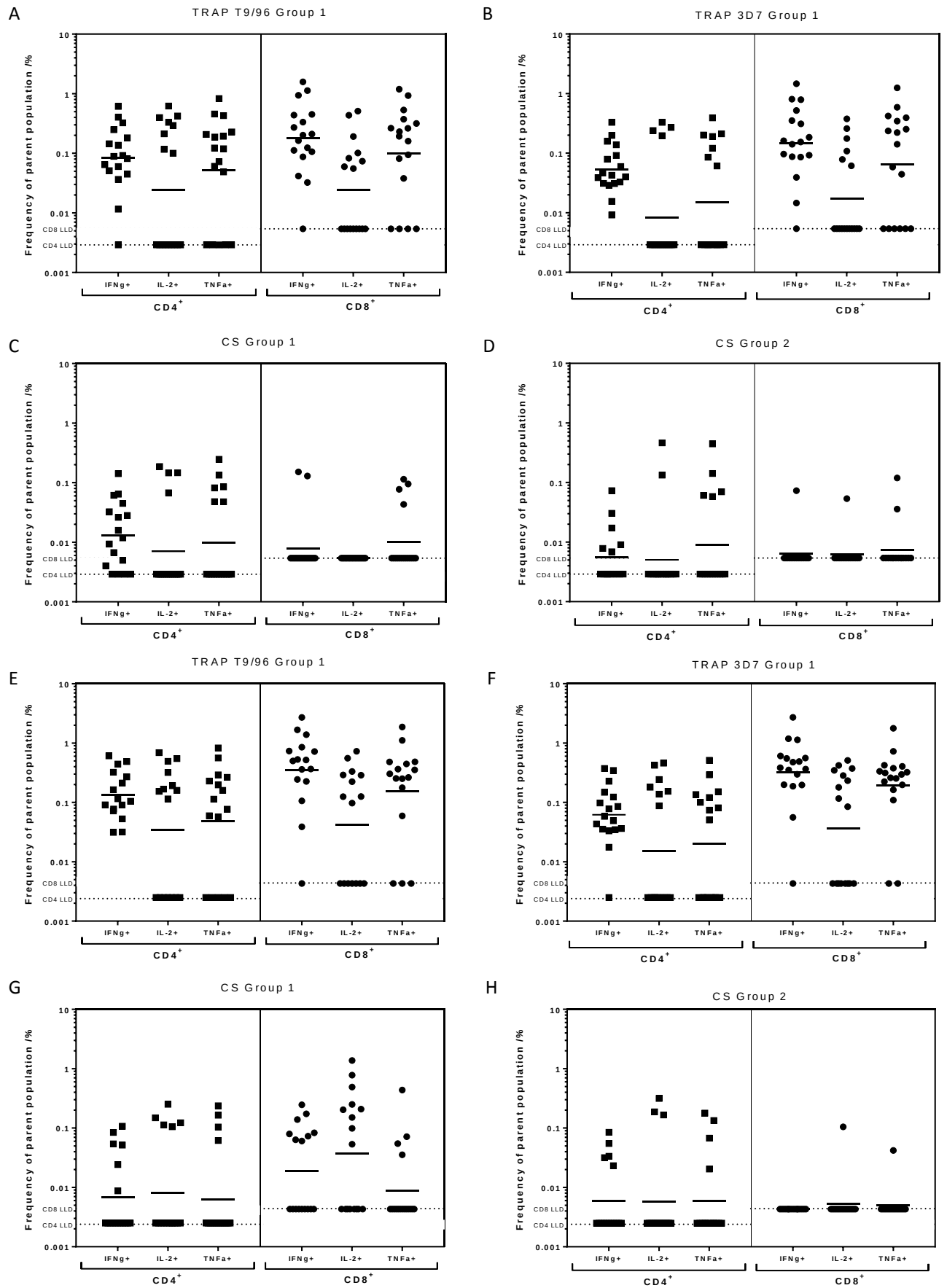


Figure 3.9 Flow cytometry with ICS performed on Vac 55 UK adults at challenge-1 time point

Flow cytometry with ICS on stimulated A-D) fresh PBMC and E-H) thawed whole blood. Geometric means displayed. Dead cells excluded from all analyses. Live CD3⁺ T cell count acquired from PBMC >56800, from whole blood >82,500. CD4 and CD8 LLD aligned and displayed as dotted line.

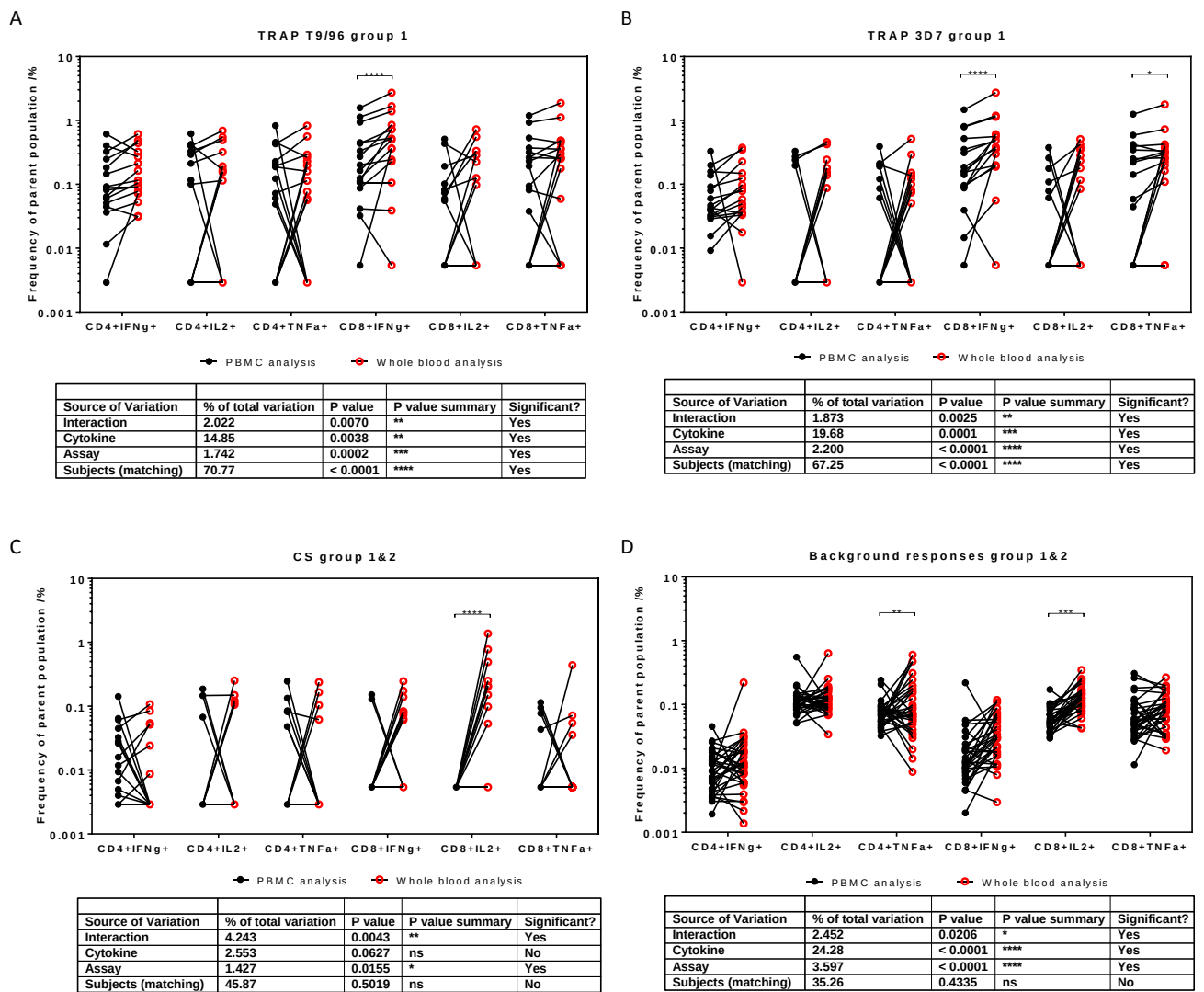


Figure 3.10 Head-to-head comparison of fresh PBMC ICS and frozen whole blood ICS from Figure 3.9 Responses to A)TRAP T9/96 group 1, B) TRAP 3D7 group 1, C) CSP group 1 and D) CSP group 2. CD4 and CD8 LLD aligned for analysis. 2-way ANOVA on paired samples, to assess effects of assay type, cytokine and subject on the variation measured in ICS response.

3.2.4.3 Correlation between fresh PBMC ELISpot and fresh PBMC/frozen whole blood ICS (validation 3)

IFN- γ responses measured by flow cytometry with ICS at C-1 were correlated with *ex vivo* IFN- γ ELISpot data performed using fresh PBMC stimulated with the same pool of TRAP T9/96 peptides. As expected, the ELISpot response correlated with the PBMC ICS for both CD4⁺ IFN- γ ⁺, CD8⁺ IFN- γ ⁺ and the combined CD4⁺ and CD8⁺ response (Figure 3.11 A-C, Spearman r). Whole blood ICS responses correlated in the CD8⁺ T cell compartment (Figure 3.11 A, Spearman r) and the combined CD4⁺ IFN- γ ⁺ + CD8⁺ IFN- γ ⁺ response (Figure 3.11 C, Spearman r), with similar r values to that of PBMC ICS.

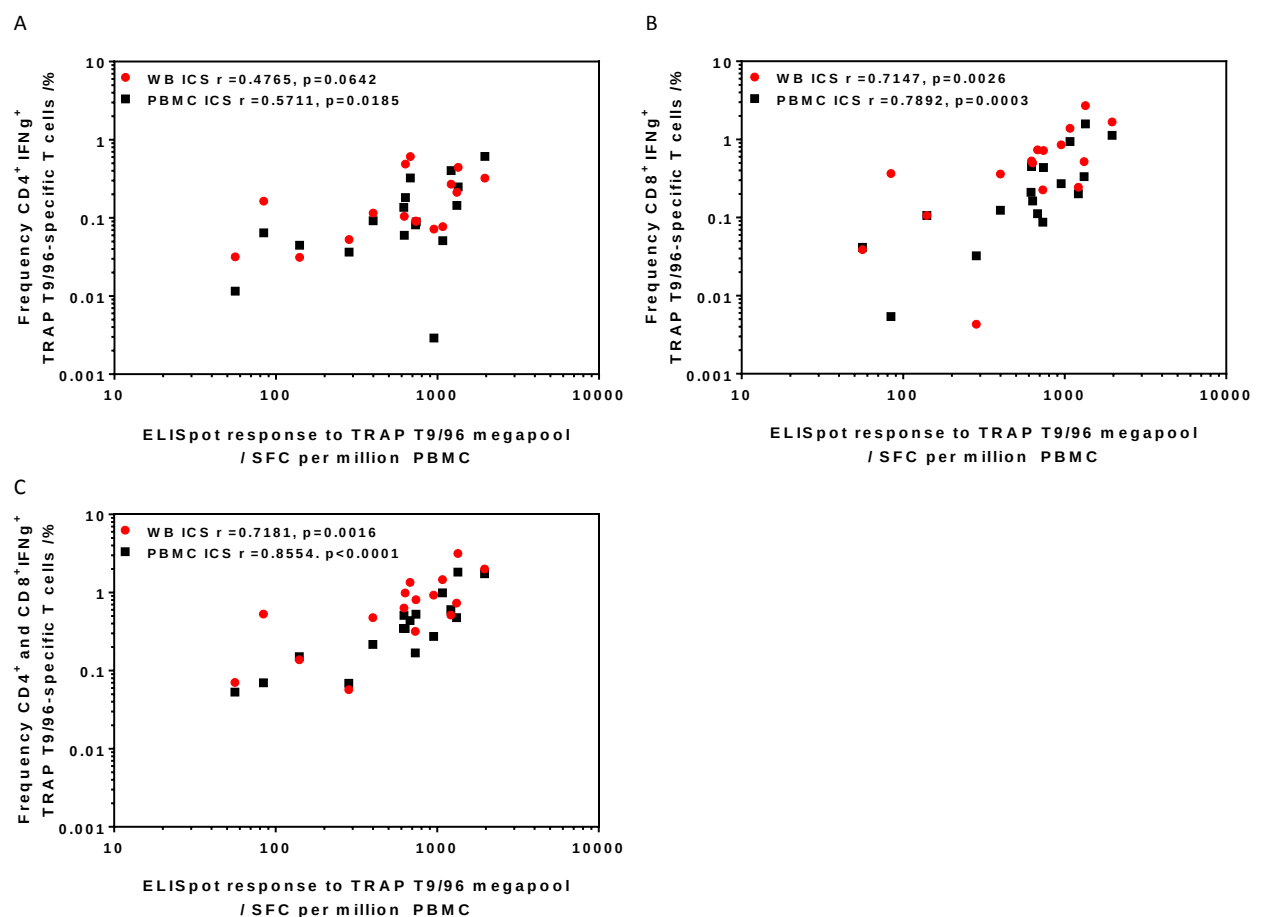


Figure 3.11 Correlations between PBMC/whole blood ICS and ELISpot response

Correlation between IFN- γ response to TRAP T9/96 megapool by *ex vivo* IFN- γ ELISpot and A) CD4⁺ IFN- γ ⁺ response B) CD8⁺ IFN- γ ⁺ response and C) summed CD4⁺ IFN- γ ⁺ and CD8⁺ IFN- γ ⁺ response to the equivalent TRAP T9/96 peptide pool by ICS. Correlations measured by Spearman rank test.

3.2.5 A validated whole blood ICS assay assessed on samples from paediatric

ChAd63.MVA ME-TRAP vaccinees

The whole blood ICS assay, validated as described above, was deemed appropriate for the evaluation of vaccine-specific responses in a Phase I study assessing the safety and immunogenicity of the ChAd63.MVA ME-TRAP vaccination regimen administered alongside standard EPI vaccines in Gambian infants. Three age de-escalating cohorts were vaccinated with ChAd63 ME-TRAP at age 16 weeks, 8 weeks and 1 week, followed by MVA ME-TRAP 8 weeks later (Table 11). Previous assessment of TRAP-specific responses in malaria exposed individuals revealed negligible response by ICS pre-vaccination, thus immunogenicity by ICS was measured 3 weeks post-prime (day 21) and 1 week post-boost (day 63). ICS assays performed by S. Roetynck. *Ex vivo* IFN- γ ELISpots were performed pre-vaccination and at day 21, 63, 112 and 168, and were carried out by V. Mensah.

Table 11 Vaccination list of the World Health Organization's Expanded Program on Immunization Vectored vaccines co-administered alongside EPI vaccines, but not at same injection site. (DTP=Diphtheria, tetanus and pertussis, HiB – *Haemophilus influenza* type B, HepB = hepatitis B).

*1 week following birth

Vaccine	Age								
	Birth	2 mths	3 mths	4 mths	18 wks	6 mths	9 mths	12 mths	18 mths
BCG	X								
Hepatitis B	X								
Oral Polio Vaccine	X	X	X	X			X		X
Pneumococcal conjugate vaccine		X	X	X					
DTP Hib HepB		X	X	X					
Vitamin A supplementation						X		X	X
Measles vaccine							X		
Yellow fever							X		
Rotavirus vaccine (Rotateq)		X	X	X					
Group 1				ChAd63 ME-TRAP		MVA ME-TRAP			
Group 2		ChAd63 ME-TRAP		MVA ME-TRAP					
Group 3	*ChAd63 ME-TRAP	MVA ME-TRAP							

Post-prime CD4⁺ and CD8⁺ T cell responses were measured in all age groups, with a response rate of 93%, 100% and 100% for CD4⁺ T cells and 67%, 100% and 87% for CD8⁺ T cells producing any-of-3 cytokines (IFN- γ , IL-2 or TNF- α) in the age de-escalating groups, respectively. Significant boosting of the TRAP-specific response was measured in CD4⁺ T cells in 16 week olds, and in CD8⁺ T cells for 16 and 8 week olds, specifically in their production of IFN- γ (Figure 3.12 A-F, $p < 0.05$ and $P < 0.01$ Kruskal-Wallis test). Any-of-3 analyses revealed significant boosting in the CD8⁺ antigen-specific T cell response of 8 week old infants (Figure 3.12 H), and a significantly higher “any-of-3” (IFN- γ , IL-2, TNF- α) CD4⁺ T cells response at day 63 in the 16 week old infants compared with 1 week olds (Figure 3.12 G). The response rate by day 63 was 100% for the older infants of 16 and 8 weeks, and above 90% in the 1 week old vaccinees.

When stratified by individual cytokine, a trend towards a higher CD4⁺ TNF- α ⁺ T cell response was measured 1 week post-boost in the oldest cohort, compared with the youngest, but overall no differences in frequency of antigen-specific cells were detected between different age groups at day 21 or day 63 for individual cytokines (Figure 3.13 and Figure 3.14).

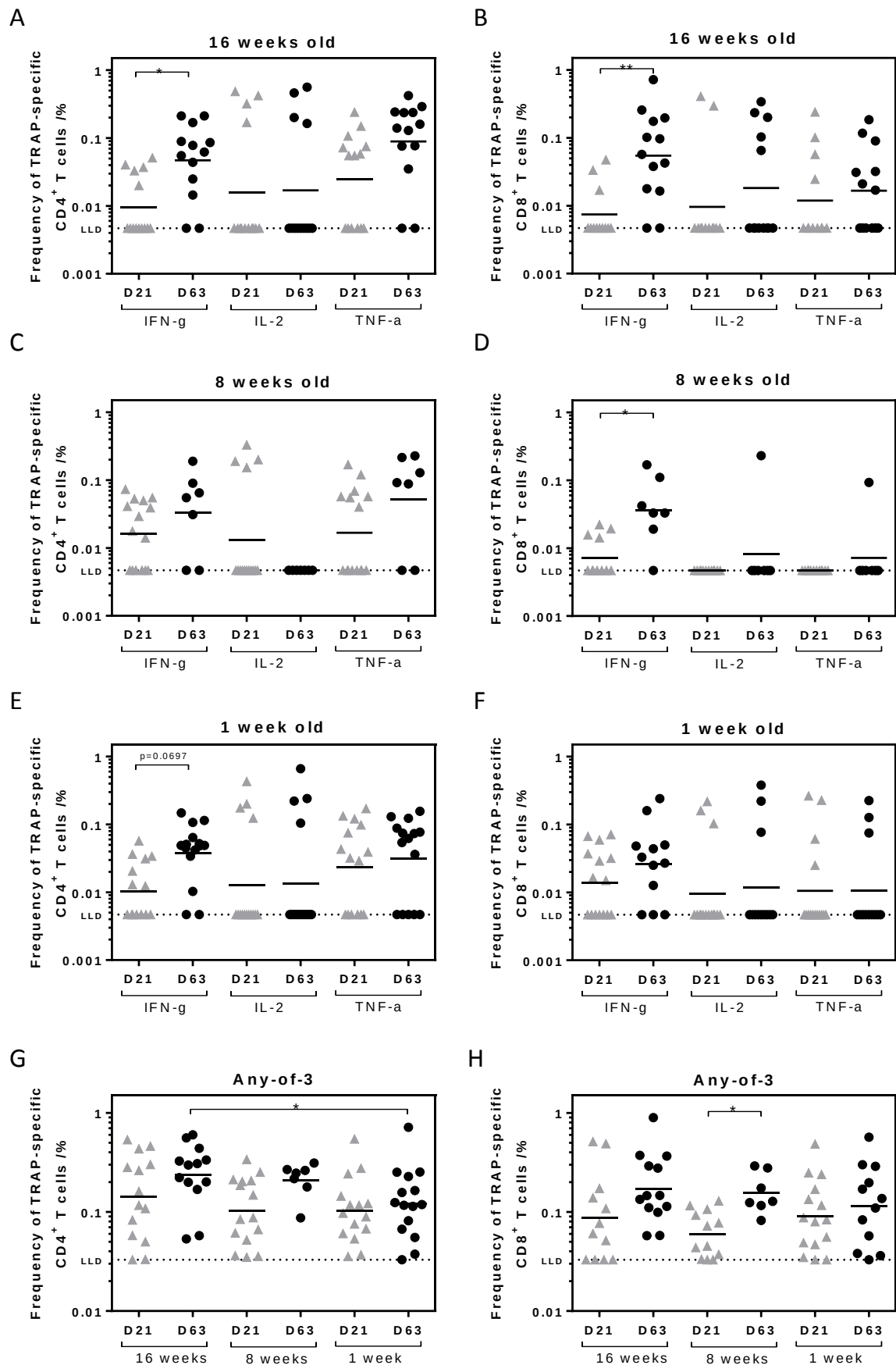


Figure 3.12 Infant immunogenicity by whole blood ICS

A-F) Single cytokine and G-H) any-of-3 cytokine analyses from the whole blood ICS assay at 21 days post ChAd63 ME-TRAP vaccination (day 21) and 7 days post MVA ME-TRAP vaccination (day 63). Kruskal-Wallis test with Dunn's correction for multiple comparisons between day 21 and day 63 responses, and between comparable time points of different groups (G+H only), * $p < 0.05$, ** $p < 0.01$.

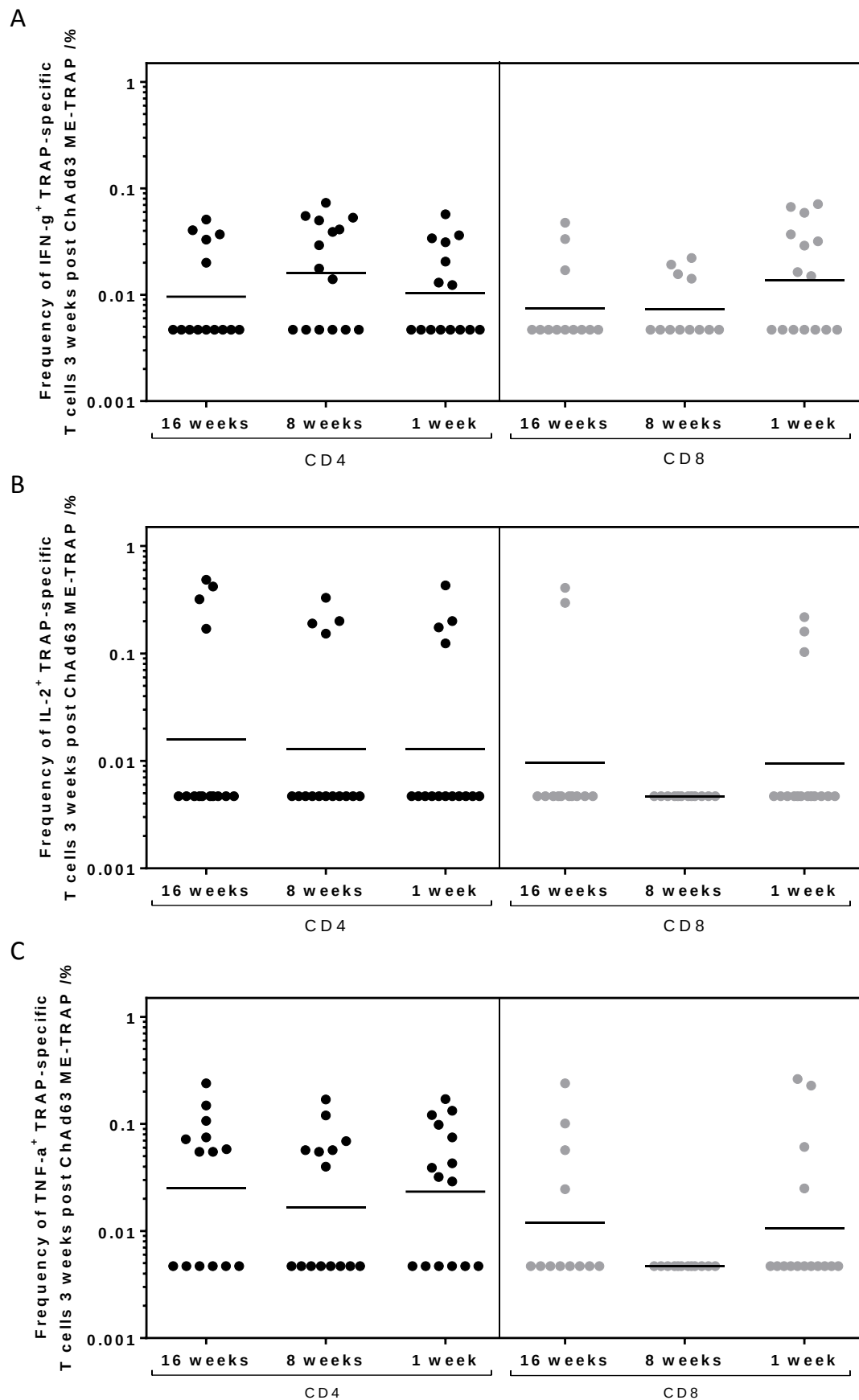


Figure 3.13 Analysis of post-ChAd63 ME-TRAP response between age groups

Whole blood ICS responses compared between 16 week, 8 week and 1 week old vaccinees for A) IFN- γ ⁺, B) IL-2⁺ and C) TNF- α ⁺ TRAP-specific T cells. No significant difference between groups by Kruskal-Wallis test with Dunn's correction for multiple comparisons between groups.

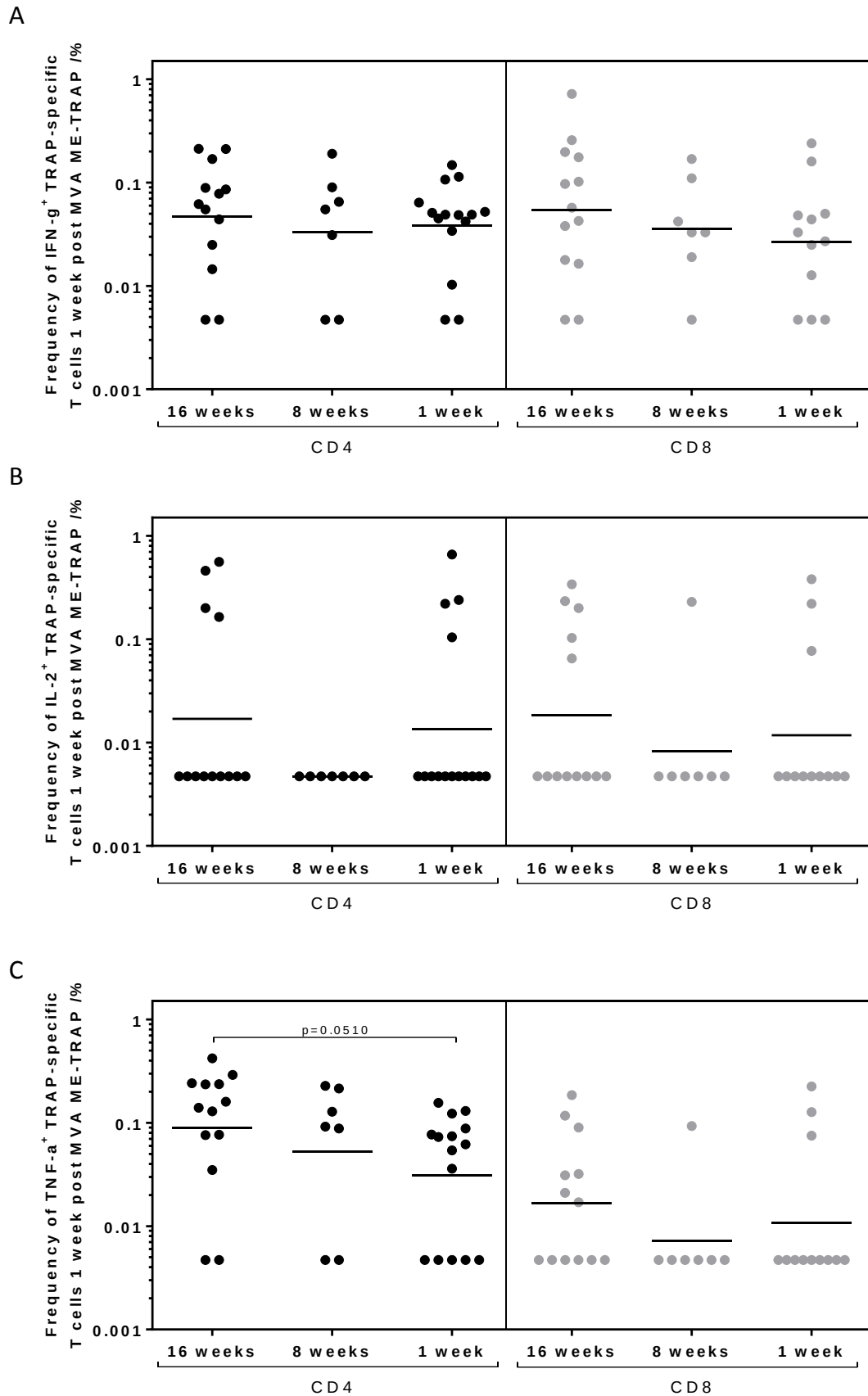


Figure 3.14 Analysis of post-MVA ME-TRAP response between age groups

Whole blood ICS responses compared between 16 week, 8 week and 1 week old vaccinees for A) IFN- γ ⁺, B) IL-2⁺ and C) TNF- α ⁺ TRAP-specific T cells. Kruskal-Wallis test with Dunn's correction for multiple comparisons between groups.

3.2.5.1 Correlation between fresh PBMC ELISpot and frozen whole blood ICS in target population (validation 4)

A final validation of the whole blood ICS assay was performed on immunogenicity data from the Vac 58 infant vaccinees. IFN- γ responses measured by whole blood flow cytometry with ICS were correlated with *ex vivo* IFN- γ ELISpot data performed using fresh PBMC stimulated with the same pool of TRAP T9/96 peptides. In this setting, the *ex vivo* IFN- γ ELISpot response correlated significantly with both CD4⁺ IFN- γ ⁺ and CD8⁺ IFN- γ ⁺ T cell responses, as well as the combined CD4⁺ and CD8⁺ response (Figure 3.15). This provides assay validation in the target population the whole blood ICS assay was optimised for, in addition to the paediatric malaria-exposed population toward which malaria vaccines are targeted. The highest *ex vivo* IFN- γ ELISpot response to the TRAP T9/96 megapool was 314 SFC/10⁶ PBMC, indicated by a dotted line at 0.0314%. The response measured by whole blood ICS is clearly higher than that of the *ex vivo* IFN- γ ELISpot to a single peptide pool.

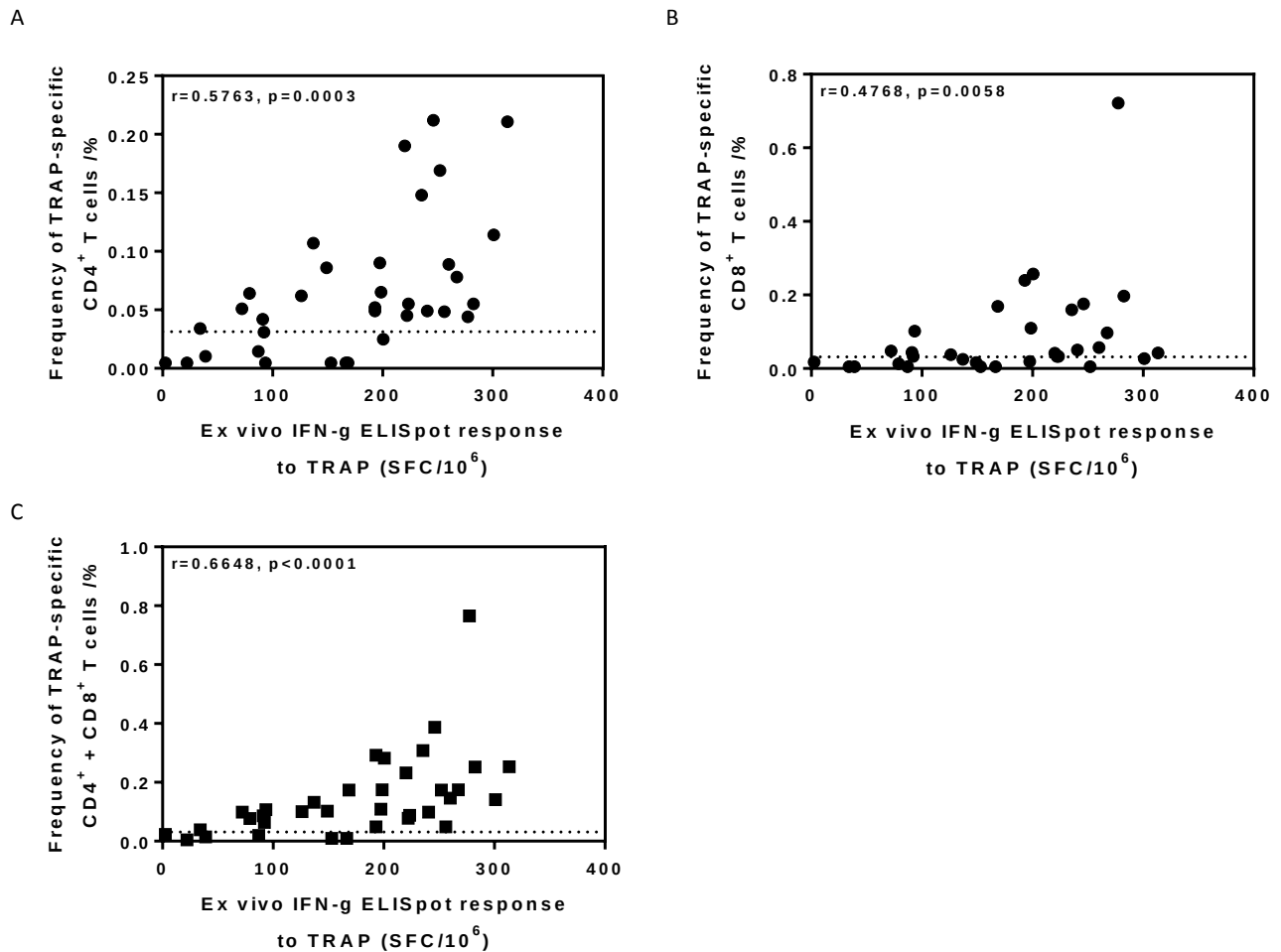


Figure 3.15 Correlations between PBMC/whole blood ICS and ELISpot response

Correlation between IFN- γ response to TRAP T9/96 megapool by *ex vivo* IFN- γ ELISpot and A) CD4⁺ IFN- γ ⁺ response B) CD8⁺ IFN- γ ⁺ response and C) summed CD4⁺ IFN- γ ⁺ and CD8⁺ IFN- γ ⁺ response to the equivalent TRAP peptide pool by whole blood ICS. Dotted line represents antigen-specific response of 0.314%, equivalent to the highest response by *ex vivo* IFN- γ ELISpot. Correlations measured by Spearman rank test.

3.2.6 Vaccine-induced CD4⁺ T cell immunogenicity is comparable between adult and paediatric populations by whole blood ICS

The validation experiments performed on UK adults for the whole blood ICS assay (Figure 3.9 E-H) provide the first comparison of multiple cytokine immunogenicity in malaria-exposed infants and malaria-naïve adults. Following ChAd63 ME-TRAP vaccination, IL-2 and TNF- α production by both CD4⁺ and CD8⁺ T cells were comparable between adults and infants. IFN- γ responses were significantly lower in infants vaccinees (Figure 3.16 A, 2-way ANOVA). Despite significant boosting in

16 and 8 week olds, IFN- γ ⁺ CD8⁺ T cells remained lower than that of UK adult vaccinees post-MVA ME-TRAP vaccination. A similar difference was measured with CD8⁺ TNF- α ⁺ T cells (Figure 3.16 B, 2-way ANOVA).

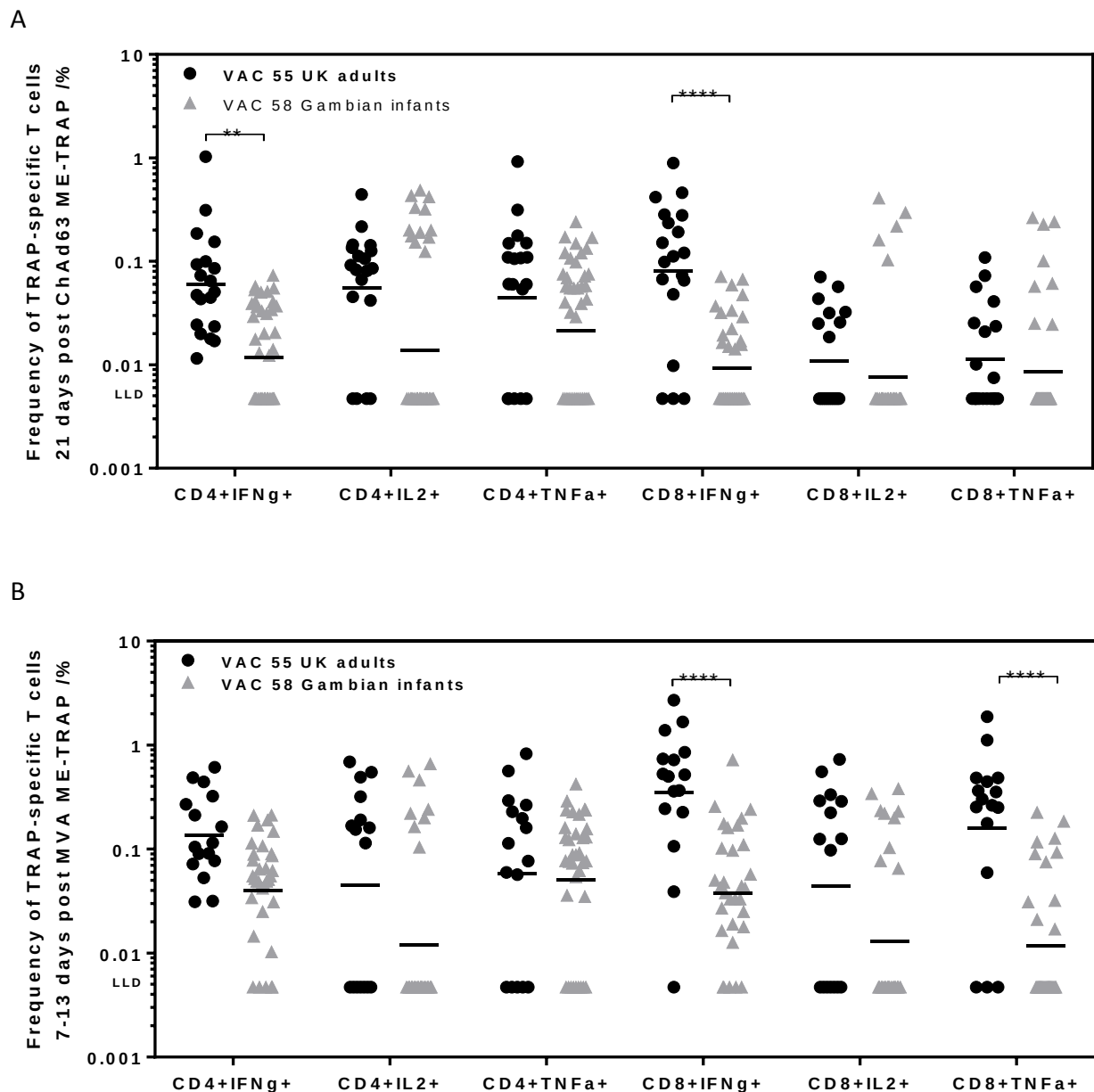


Figure 3.16 Comparison of whole blood ICS responses in UK adults and Gambian infants

Whole blood ICS at A) 3 weeks post ChAd63 ME-TRAP vaccination, B) 7 days and 13 days following MVA ME-TRAP vaccination for Gambian infants UK adults, respectively. ** $p < 0.01$, **** $p < 0.0001$ by 2-way ANOVA.

3.3 Discussion

Previously, T cell immunity has not been studied in age de-escalation clinical vaccine trials of ChAd63.MVA ME-TRAP, or indeed of any adenovirus prime – MVA boost vaccine. Analysis of initial immunogenicity data indicated a reduction in TRAP-specific T cells when the ChAd63.MVA ME-TRAP regimen was administered across age de-escalating groups, ranging from adults to young infants. However, such direct comparisons of immunogenicity in different age groups are complex. In addition to the differences between paediatric and adult T cell immunity, infants and young children have very different haematological parameters to older children and adults: total blood volume is substantially lower and numbers of circulating lymphocytes per millilitre significantly higher [410]. There are also distinct differences in body mass and therefore variation in vaccine dose per kilogram of body weight when a standard or similar dose is administered to all age groups. The inverse correlation between absolute numbers of lymphocytes circulating per millilitre of blood and age is a key consideration when reporting cellular immunogenicity across age groups and highlights the potential utility for an alternative analysis of *ex vivo* IFN- γ ELISpot data that reports the antigen-specific response as a frequency of circulating whole blood (i.e. per millilitre of blood), as opposed to per defined number of PBMC.

Our novel approach incorporated the lymphocyte differential from the full blood count performed at the same time as the immunological assays. This was used to standardise the denominator of the units used to report ELISpot responses, taking into account the higher lymphocyte frequencies measured in young children and infants, and giving a concentration of antigen-specific cells circulating per millilitre of blood. This approach permits a fairer and more consistent comparison of vaccine-induced responses across different age groups. The frequency of antigen-specific cells per millilitre of blood is also an arguably more relevant readout *per se*, as an increased rate of antigen-specific cells trafficking through the lymph nodes and spleen would increase the likelihood of antigen recognition from antigen-presenting cells following vaccination, giving rise to a greater cellular immune response. Furthermore, the level of antigen-specific T cells per millilitre of blood is more likely to correlate with vaccine efficacy than T cells per million PBMC, as

the former relates to the rate at which antigen specific T cells enter the liver: the relevant target organ for T cells in malaria-infected individuals.

Using this approach, responses measured in paediatric vaccinees are comparable to that of malaria-naïve or malaria-exposed adults. In the case of the youngest age group of 10-week old infants, responses were significantly higher by almost 3-fold compared with adults. This indicates that ChAd63.MVA ME-TRAP T cell immunogenicity is not reduced with administration to younger participants in age de-escalation vaccine trials, despite the reduction in MVA ME-TRAP dose. This preliminary observation demonstrates that replication-deficient viral vectors can elicit immune responses in infants that are comparable or indeed superior to those in adult populations. The wider implication is delivery of antigens by viral vectors may overcome the limitations of the immature infant immune system, which have been shown to limit seroconversion and cellular responses to EPI vaccines [372, 388, 391, 392, 411, 412]. This is consistent with strong CTL function previously measured following congenital infection with cytomegalovirus and *Trypanosoma cruzi*, which together demonstrate that under certain conditions, the immature human immune system is indeed capable of potent CTL activity [398, 413].

Whilst T cell responses were comparable between the 5-12 month old paediatric vaccinees in Burkina Faso and The Gambia, responses measured these two age groups in Burkina Faso were the lowest of all trials compared, and significantly lower than that of 10 week old vaccinees in The Gambia. This is likely due to a combined effect of age and differing malaria exposure by field site [405]. Malaria-related immunosuppression could lead to reduced vaccine immunogenicity or differences in innate immunity in the early weeks of life in these children. Congruently, malaria transmission in the Gambia is markedly lower than in Burkina Faso [68, 414] thus these combined effects would likely be seen in older Gambian children who have had more years of lower level malaria exposure; and in younger infants in Burkina Faso where transmission is higher [415, 416].

One limitation of this novel reporting method is the assumption of a comparable monocyte count across populations. In the populations tested, UK adults had a higher monocyte to lymphocyte ratio than infants and older children both pre- and post- vaccination. Monocytes are antigen-presenting cells that form part of the PBMC compartment that is separated from whole blood and

used in the *ex vivo* IFN- γ ELISpot. They are unlikely to produce significant amounts of IFN- γ in the assay, but can skew and amplify the Th1 response measured, in addition to providing antigen presentation via MHC class I and II. In the novel ELISpot analysis, the response per million PBMC is multiplied by the number of lymphocytes per millilitre of blood. The ELISpot response per mL therefore has scope to be higher in volunteers with a higher proportion of lymphocytes in the assay, with a high monocyte to lymphocyte ratio giving an underestimate of antigen-specific cells per millilitre of blood. To explore the effects of different M:L in these populations, a correction factor was applied to groups where both monocyte and lymphocyte count were available at peak immunogenicity time point. The same trends were observed between UK adults and Burkinabe infants, supporting the validity of the SFC/mL blood readout, and its corresponding findings.

Supporting data from multiparameter flow cytometry with intracellular cytokine staining, where frequency of antigen-specific cells is the readout, was key in order to corroborate this finding of comparable vaccine-induced responses in infant vaccinees. With paediatric clinical vaccine trials carried out in the field, sites are often under-resourced to perform such a complex assay on fresh PBMC samples in real time. Published ICS data from thawed PBMC samples from ChAd63.MVA ME-TRAP paediatric vaccinees are lower than those measured in UK adults, however the results are not directly comparable, due to the UK adult samples being performed on fresh PBMC samples and African infants on frozen PBMC [287, 417], and it is known from previous ChAd63.MVA ME-TRAP adult vaccinees in the UK, that CD4⁺ T cell responses are particularly reduced when ICS is performed on frozen [418].

One solution to the difficulties in obtaining robust ICS data from field sites was to use a whole blood ICS protocol, whereby the samples are stimulated and subsequently frozen down at the field site, for batched thawing, staining, acquisition and analysis at a later date. Furthermore, this assay permits sample shipment to a single laboratory for central analysis. Whole blood ICS has many other advantages over PBMC ICS, including a standardised stimulation procedure with no requirement for PBMC separation or counting, reduced blood volume requirements, the option to freeze samples post-stimulation for batched staining and analysis and the evaluation of T cell responses in the presence of autologous cellular and serum components. The main drawback with

switching to a whole blood ICS platform is the omission of live/dead staining in all published protocols, due to the early fixation of samples required by such as assay. Despite this, such whole blood ICS assays are routinely used in adult and paediatric clinical vaccine testing, including by GSK in the evaluation of RTS,S, and in several TB vaccine trials [304, 406, 419-421].

Dead cells can skew responses in several ways, predominantly through changes in intracellular constituents such as flavoproteins and porphyrins, resulting in autofluorescence, usually of shorter wavelength and therefore increasing background responses of certain fluorochromes. This was apparent in the dataset gated with and without dead cell exclusion, whereby significantly higher background was measured in the FITC (IFN- γ) channel. Dead cells can form cell clumps and also bind non-specifically to the fluorochrome conjugated antibodies used in ICS, resulting in increased background staining, which in turn can skew or dilute the reported antigen-specific response. Identifying these cells is especially important at a field site that has previously suffered with poor *ex vivo* cell viability. These factors prompted the development and validation of a more robust whole blood assay based on already established whole blood ICS protocols.

Adaptations were made to a current whole blood protocol of *Hanekom et al* [406]. The red cell lysis and PBMC fixation step was split into 2 separate procedures, with a dead cell staining step added in between. The subsequent staining of leukocytes with an amine-reactive dead cell dye survived fixation and a freeze-thaw step, thus enabling the freezing step to be retained in the protocol. Validation of the adapted frozen whole blood ICS protocol was achieved through correlation with comparative assays with the equivalent fresh PBMC on identical vaccinee samples. The standard deviation of cell numbers obtained using the whole blood ICS were considerably lower compared with the PBMC assay, despite a freeze-thaw step, indicating robustness of the whole blood assay to consistently yield sufficient cell numbers to pass data QC. Comparison of cytokine responses from these samples revealed assay type accounted for only 1-3% of response variation across all CD4 and CD8 cytokines, with measured responses either comparable between assays or higher in the whole blood assay. Most disagreement in cytokine response was measured with responses less than 0.1% and approaching the lower limit of detection. Further validation was achieved through correlation of each vaccinees' *ex vivo* IFN- γ ELISpot response with IFN- γ responses measured in the

frozen whole blood and fresh PBMC ICS assays. Both assays positively correlated with ELISpot response, driven by both the CD4⁺ IFN- γ ⁺ and CD8⁺ IFN- γ ⁺ T cell response.

Interestingly, the whole blood ICS assay appears more sensitive than the *ex vivo* IFN- γ ELISpot assay in Vac 58. This is likely due to the fact that the ELISpot response to a single peptide pool was used for correlation with ICS data, and ELISpot responses were higher when smaller peptide pools were used to sum the TRAP-specific response. This finding highlights the increased sensitivity of the whole blood ICS assay to a single pool of peptide, over that of the *ex vivo* IFN- γ ELISpot, which is an important implication if future clinical trial designs have lower blood volumes taken from infants at each time point, resulting in insufficient PBMC available for testing of multiple peptide pools by ELISpot. This is especially important, as an impediment to basic immunologic investigations of infants and young children has been limited blood volume available for testing [422], which obviously is required to be within safe limits [423].

The whole blood ICS assay was tailored to the requirements of a Phase I paediatric clinical vaccine trial in The Gambia, and used as one of the main immunological readouts in 3 groups of infants, primed at aged 1 week, 8 weeks and 16 weeks of age, and boosted 8 weeks later. This trial was not powered to detect interference of EPI vaccines on the vaccine-induced response by ChAd63.MVA ME-TRAP regimen, rather the immunological objective was to assess immunogenicity between the age groups. These data conclude that there are not significant differences between these 3 age groups, however there is a trend towards reduced immunogenicity in the 1 week old vaccinees compared with the 16 week olds, driven by both response rate and magnitude. This could be due to the immune system at 1 week of age being more immature, which is consistent with reduced pro-inflammatory responses in neonates in previous studies (reviewed in [373, 374]), but by 8 and 16 weeks of age has matured sufficiently to mount more robust pro-inflammatory cellular responses. The co-administration of the MVA ME-TRAP boost with other EPI vaccines may also play a role. The 1-week old infants received MVA ME-TRAP alongside oral polio vaccine, pneumococcal conjugate vaccine and DTP/Hib/hepatitis B at 2 months of age, whereas the 16 week olds received their MVA ME-TRAP boost alongside a vitamin A supplement only. However, these 16 and 8 week old infant vaccinees have a peak ELISpot response that is 200-300 SFCx10⁶ PBMC less than the very

similar age category in Vac 42 (10 week olds) where no vaccinations were co-administered with EPI vaccinations (Vac 58 ELISpot data not shown). This emphasises the need to thoroughly test these candidate vaccines alongside EPI, in a larger scale interference study that is powered to detect such differences. Importantly, responses to EPI vaccines in this study were comparable between infant ChAd63.MVA ME-TRAP vaccinees and controls, which is encouraging with respect to further clinical evaluation of ChAd63.MVA ME-TRAP in infants.

Finally, comparison of infant whole blood ICS data is currently only possible with that of adults from the UK, who were administered the malaria vectored vaccines staggered alongside leading vaccine candidate RTS,S. Encouragingly, many responses are statistically comparable between the UK adults and Gambian infants. Differences in certain cytokine responses could be indicative of reduced vaccine-immunogenicity in infants, although the adjuvanting effects of AS01 in the adult vaccinees, in addition to the possible interference of EPI vaccinations and the different post-MVA ME-TRAP time points measured in these 2 studies are all possible reasons why reduced responses in Vac 58 infants were measured.

Overall, these data demonstrate that immunisation of children and infants with the candidate prime-boost regimen induces robust T cell responses to a pre-erythrocytic malaria antigen that has previously demonstrated efficacy against CHMI in malaria naïve adults and against natural infection in semi-immune adults [285, 287, 295]. These data also indicate that vaccination using viral vectors may overcome the limitations of the immature, infant immune system, as demonstrated by levels of TRAP-specific T cells being highest in 10-week old infants.

Chapter 4

Scheduling of *Plasmodium*
falciparum malaria vaccines:
ChAd63 ME-TRAP and
MVA ME-TRAP

4 Scheduling of *Plasmodium falciparum* malaria vaccines: ChAd63

ME-TRAP and MVA ME-TRAP

4.1 Introduction

Vaccination strategies using pre-erythrocytic malaria antigen TRAP fused to a multi-epitope string, ME, have developed over the last decade. Initially, ME-TRAP DNA plasmid vaccination and MVA ME-TRAP were assessed alone and in heterologous prime-boost regimens, with a double DNA prime followed by an MVA ME-TRAP boost the most optimal regimen. This regimen induced a mean T cell response of 450 SFC/10⁶ PBMC to ME-TRAP and exhibited sterile protection against CHMI in 12.5% (1/8) of vaccinees [252]. Regimens using a double priming vaccination of recombinant fowlpox virus (strain FP9) encoding ME-TRAP (FP9 ME-TRAP) and MVA ME-TRAP induced a slightly higher antigen-specific T cell response of 475 SFC/10⁶ PBMC and protected 12.5% (2/16) of vaccinees [282]. In field studies of FP9.MVA ME-TRAP, T cell responses following vaccination were lower, and were associated with reduced vaccine efficacy [424, 425].

Heterologous prime-boost vaccination against malaria with the viral vectors ChAd63 and MVA, induces potent T-cell responses in humans. The ChAd63.MVA ME-TRAP prime-boost regimen has been evaluated in Phase I safety and immunogenicity studies in both malaria-naïve [266] and malaria-endemic populations [283, 294, 426, 427], in addition to Phase II controlled human malaria infection studies [285, 287] in the UK and efficacy studies in West and East Africa [295, 426]. In all studies, the vaccines were tested in a standard 8 week prime-boost regimen (denoted A_M), with immunogenicity routinely measured as 14-28 days following vaccination with ChAd63 ME-TRAP and 7 days following boosting with MVA ME-TRAP.

A stark increase in the antigen-specific T cell response following ChAd63.MVA.ME-TRAP, compared with previous prime-boost regimens, led to an increase in sterile protection in *P. falciparum* challenged vaccinees. The 8 week regimen induced a peak median *ex vivo* IFN- γ ELISpot response of over 2400 SFC/10⁶ PBMC and demonstrated 21% (3/14 vaccinees) sterile efficacy and a delay to parasitaemia in a further 36% of vaccinees [287]. Protective efficacy positively correlated

with the frequency of monofunctional CD8⁺ IFN- γ ⁺ T cells measured one day pre-challenge and CD8⁺CD107a⁺ T cells 150 days post challenge. In a field efficacy study of ChAd63.MVA ME-TRAP, a 67% reduction in malaria infection was measured in adults in the malaria endemic region of coastal Kenya. In this setting, T cell responses directed towards the mid-section of the TRAP insert correlated with protection [295].

With strong evidence that increased cellular immune responses against ME-TRAP are associated with increased protection against *P. falciparum* challenge, greater efficacy vaccine might be achieved by inducing even higher frequencies of antigen-specific CD8⁺ T cells through administration of ChAd63 ME-TRAP and MVA ME-TRAP via already established doses and routes, but according to novel schedules. Such schedules were evaluated in a single Phase I clinical vaccine trial (Vac 43) and included homologous administration of the ChAd63 ME-TRAP prime and/or MVA ME-TRAP boost, the use of alternating priming and boosting vectors and comparative schedules with 4- and 8-week intervals between vaccinations (Table 12).

Table 12 Summary of ChAd63 ME-TRAP and MVA ME-TRAP vaccine regimens

“A” denotes intramuscular administration of 5x10¹⁰ virus particles of ChAd63 MVA ME-TRAP, “M” denotes intramuscular administration of 2x10⁸ plaque forming units of MVA ME-TRAP, “_” denotes an 8 week interval, no underscore denotes a 4 week interval.

Group (n=6)	Time point of vaccine administration						Schedule abbreviation
	Day 0	4 weeks	8 weeks	12 weeks	16 weeks	24 weeks	
1	ChAd63 ME-TRAP	ChAd63 ME-TRAP	ChAd63 ME-TRAP		MVA ME- TRAP		AAA_M
2	ChAd63 ME-TRAP		ChAd63 ME-TRAP		MVA ME- TRAP	MVA ME- TRAP	A_A_M_M
3	ChAd63 ME-TRAP	ChAd63 ME-TRAP	MVA ME- TRAP	MVA ME- TRAP			AAMM
4	ChAd63 ME-TRAP		MVA ME- TRAP		MVA ME- TRAP	MVA ME- TRAP	A_M_M_M
5	ChAd63 ME-TRAP	MVA ME- TRAP	ChAd63 ME-TRAP		MVA ME- TRAP		AMA_M
6	ChAd63 ME-TRAP	MVA ME- TRAP	ChAd63 ME-TRAP	MVA ME- TRAP			AMAM
7	ChAd63 ME-TRAP		MVA ME- TRAP		ChAd63 ME-TRAP	MVA ME- TRAP	A_M_A_M
Standard Regime (G4+7)	ChAd63 ME-TRAP		MVA ME- TRAP				A_M
Shortened Regime (G5+6)	ChAd63 ME-TRAP	MVA ME- TRAP					AM

It has been previously demonstrated in mice that the phenotype of T cell memory may differ depending on the number of vaccinations given: in the absence of anti-vector immunity, secondary and tertiary antigen-specific responses to vaccination may result in the generation of memory CD8⁺ T cells that retain effector-like properties, as opposed to central memory T cell phenotypes. Furthermore, these cells accumulate preferentially in non-lymphoid tissues [428]. In mice primed with adenovirus and boosted with MVA both encoding ME-TRAP, blood CD8⁺ T cells of an effector memory phenotype correlate with protection against malaria liver-stage infection following *P. berghei* challenge [274].

Flexibility in the 8 week prime-boost interval of ChAd63.MVA ME-TRAP is currently untested in human vaccinees. In the murine challenge model, AdC9 ME-TRAP (another simian adenovirus serotype) and MVA ME-TRAP were assessed alone and in prime-boost combination by sporozoite challenge at 2 and 8 weeks post boost. The highest degree of sterile protection was achieved when mice were primed with AdC9 ME-TRAP and boosted with MVA ME-TRAP with an 8 week interval (96% sterile protection when challenged 2 weeks post boost and 59% sterile protection when challenged 8 weeks post boost), when compared with MVA-AdC9, MVA alone and AdC9 alone. However shorter prime boost intervals of 1, 2 and 4 week intervals using this regimen were also efficacious [274].

The use of multiple homologous ChAd63 ME-TRAP priming vaccinations have not previously been tested in humans and it is unknown whether multiple administrations will enhance regimen immunogenicity or impair it through boosting pre-existing anti-vector immunity. Preliminary data, whereby a small number of subjects administered the ChAd63.MVA ME-TRAP regimen were re-boosted with either ChAd63 ME-TRAP or MVA ME-TRAP after a long interval averaging 8 months, show that T cell responses can indeed be re-boosted by both a delayed homologous MVA administration (A_M__M) and a delayed ChAd63 ME-TRAP administration (A_M__A) [266].

The first Phase I clinical vaccine study of ChAd63.MVA ME-TRAP saw >90% of vaccinees seroconvert to the ChAd63 vector following vaccination, but measured no correlation between baseline ChAd63 antibody titres and peak *ex vivo* IFN- γ ELISpot response to the vaccine insert post MVA ME-TRAP boost [266]. Subsequent trials have seen a trend towards a higher peak T cell

responses to TRAP in volunteers with pre-existing anti-vector antibodies [287], and a negative correlation between pre-vaccination ChAd63 titre and post-ChAd63 T cell response to TRAP, which disappeared after boosting [294]. In a hepatitis C (HCV) clinical vaccine trial using adenovirus vectors, a positive correlation was measured between boosting of adenovirus-specific T cells and that of the HCV-specific T cell response, following administration of ChAd3 and human Ad6 encoding non-structural HCV proteins [429].

Data from macaques indicates that vaccination schedules containing more than one administration of an adenoviral-vectored vaccine component generate enhanced cell mediated immunogenicity, when using viral vectors expressing Gag of HIV-1 [430]. A similar macaque study found that boosting with MVA ME-TRAP after a ChAd63 ME-TRAP prime, increased both the magnitude and breadth of the immune response to the vaccine insert [431], however subsequent MVA boosts did not broaden the responses compared with a single MVA boost [431]. Further MVA ME-TRAP vaccinations boosted the magnitude of the T cell and antibody responses, however the peak frequencies obtained after the second and third MVA immunisations did not surpass levels achieved by a single MVA ME-TRAP administration.

In this study, T cell immunogenicity was measured for each novel vaccination regimen by *ex vivo* IFN- γ ELISpot and by flow cytometry with ICS. Responses were compared with the internal A_M control within the study, corresponding to the current standard 8 week regimen of ChAd63.MVA ME-TRAP. This was achieved by combining groups 4 and 7 following the first 2 vaccinations and using the immunogenicity data up to week 16, when a third vaccination was administered. Responses were also compared with other clinical trials using the ChAd63.MVA ME-TRAP regimen. Finally, anti-vector responses were measured, including T cell responses to the ChAd63 hexon and antibody responses to MVA, and correlated with TRAP-specific T cell responses, in addition to TRAP antibody responses.

4.2 Results

4.2.1 Time course kinetics and peak response by *ex vivo* IFN- γ ELISpot

Following a single ChAd63 ME-TRAP prime, 96% of vaccinees had a positive response to vaccination, as measured by *ex vivo* IFN- γ ELISpot. Of the 2 initial non-responders, both responded positively after either a second ChAd63 ME-TRAP vaccination at day 56 (group 2), or MVA ME-TRAP vaccination at day 28 (group 5). Response time course by *ex vivo* IFN- γ ELISpot shows the response kinetics per group (Figure 4.1).

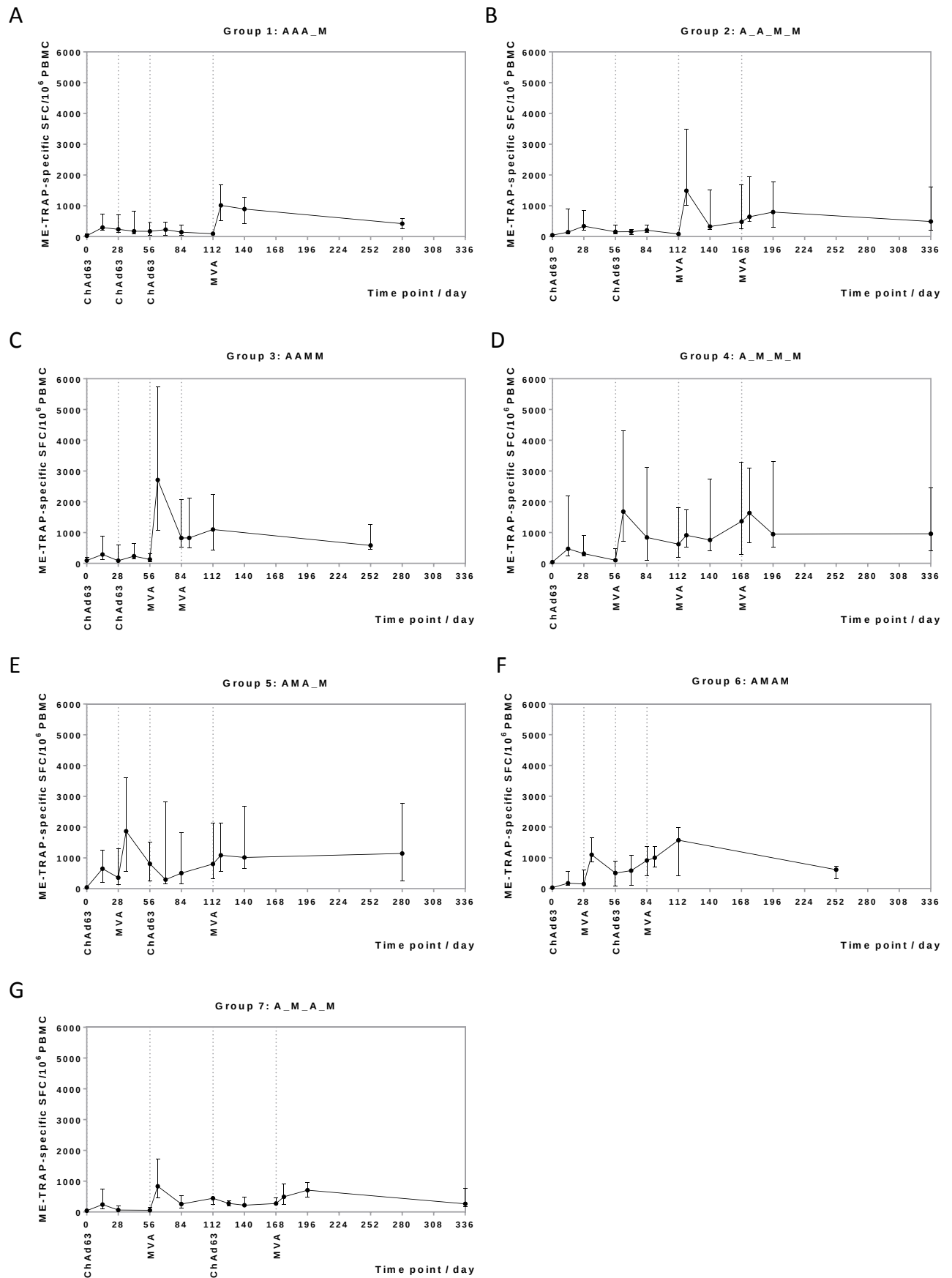


Figure 4.1 Time course of *ex vivo* IFN- γ ELISpot response by group.

Summed *ex vivo* IFN- γ ELISpot response to ME and TRAP pools. Autologous background subtracted. Median with IQR displayed.

Direct comparison of peak immunogenicity was used to assess the effect of a 4- or 8-week prime-boost interval with ChAd63 ME-TRAP priming and MVA ME-TRAP boosting. ELISpot responses for the 12 volunteers boosted after a 4 week (group 5 + 6) compared with an 8 week interval (group 4 + 7) were not significantly different at 1 or 4 weeks post MVA ME-TRAP vaccination (Figure 4.2 A, $p=0.7103$ and 0.6949 respectively, Mann Whitney test).

The administration of 2 or 3 ChAd63 ME-TRAP vaccinations did not enhance the post-MVA ME-TRAP ELISpot response at 1 or 4 weeks post first MVA ME-TRAP vaccination (Figure 4.2 B + C, $p=0.3722$ and 0.5233 respectively, Kruskal-Wallis test) or the response measured 1 and 4 weeks post final MVA ME-TRAP vaccination (Figure 4.2 D + E, $p=0.4284$ and 0.4519 respectively, Kruskal-Wallis test). For group 1, this is the response to the single MVA ME-TRAP vaccination.

The kinetics of ME-TRAP-specific responses were similar across all groups following ChAd63ME-TRAP prime, peaking 14-28 days post-vaccination. The ELISpot response to the first ChAd63 ME-TRAP vaccination was significantly higher than that measured after the second vaccination, with no difference measured after the third vaccination (Figure 4.2 F, ChAd63 ME-TRAP 1 and ChAd63 ME-TRAP 2 $p=0.0305$, ChAd63 ME-TRAP 1 and ChAd63 ME-TRAP 3 $p=0.0625$, ChAd63 ME-TRAP 2 and ChAd63 ME-TRAP 3 $p=0.6875$, Wilcoxon matched pairs signed rank test). In groups with multiple MVA ME-TRAP vaccinations, the peak response to the first MVA ME-TRAP was at 7 days, but following the second MVA, responses peaked after 28 days or later (data not shown). The highest peak in ELISpot response was measured after the first dose of MVA ME-TRAP in 61% of volunteers. Paired analysis of peak responses after consecutive MVA ME-TRAP vaccination in group 2 (A_A_M_M), group 3 (AAMM) and group 4 (A_M_M_M) show no significant boosting in peak response with subsequent MVA ME-TRAP vaccinations (Figure 4.2 G, MVA ME-TRAP 1 and MVA ME-TRAP 2 $p=0.0737$, MVA ME-TRAP 1 and MVA ME-TRAP 3 $p=0.1563$ or MVA ME-TRAP 2 and MVA ME-TRAP 3 $p=0.3125$, Wilcoxon matched pairs signed rank test).

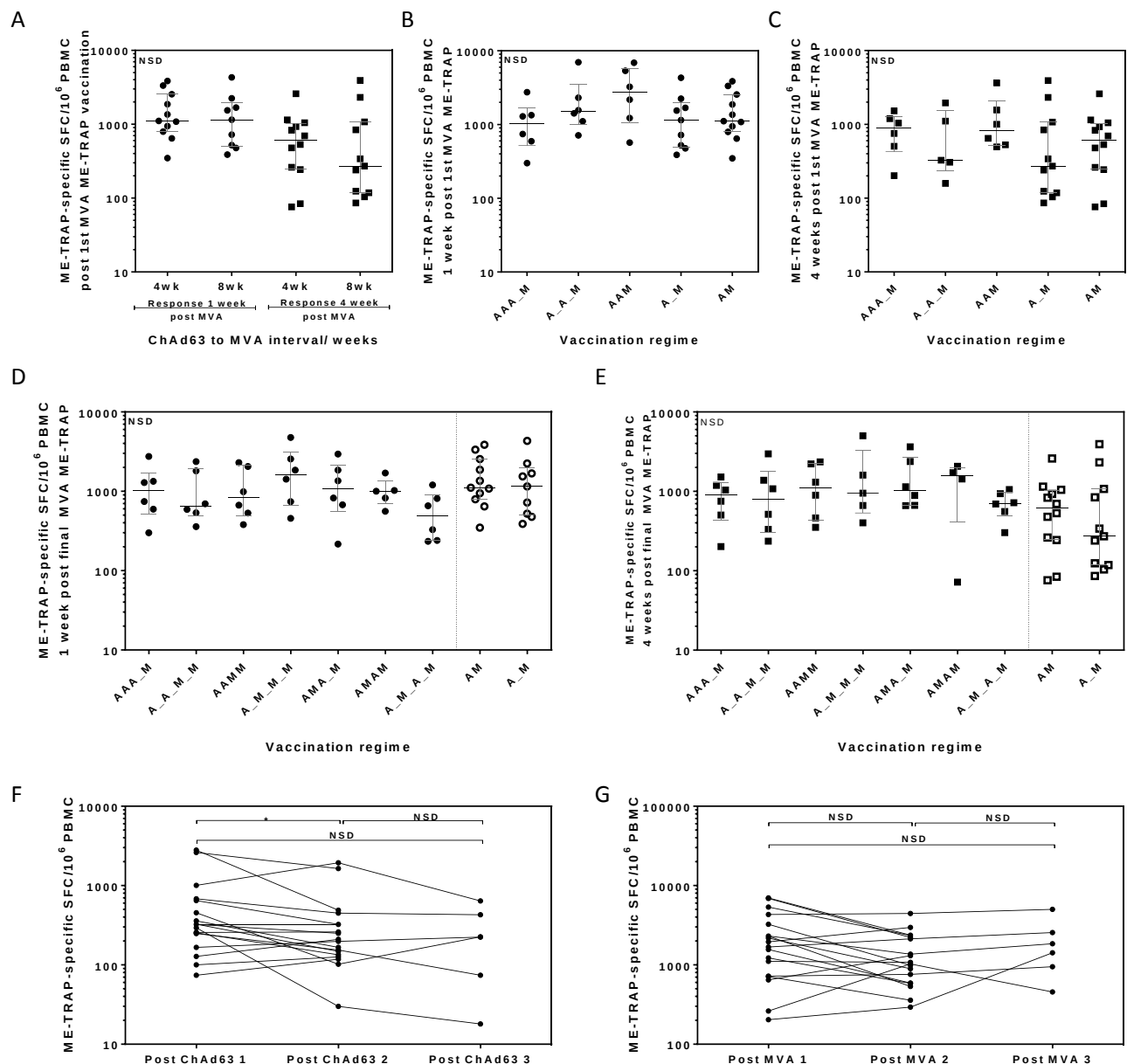


Figure 4.2 Summary of ELISpot responses across the 7 vaccination regimens

Ex vivo IFN- γ ELISpot response A) 1 week and 4 weeks post-MVA ME-TRAP responses for volunteers boosted at 4 weeks compared to 8 weeks post ChAd63 ME-TRAP. No significant difference at 1 or 4 weeks post boost, Mann Whitney test. Ex vivo IFN- γ ELISpot response B) 1 week and C) 4 weeks post-MVA ME-TRAP response following single, double and triple ChAd63 ME-TRAP priming vaccination(s), with single MVA ME-TRAP boost. No significant difference between groups by Kruskal-Wallis test with Dunn's correction for multiple comparisons between all groups. Ex vivo IFN- γ ELISpot response D) 1 week and E) 4 weeks post final MVA ME-TRAP vaccination in each group, with prime-boost regimens of 4 and 8 weeks displayed for reference only. No significant difference between groups by Kruskal-Wallis test with Dunn's correction for multiple comparisons between all groups. F) Peak ex vivo IFN- γ ELISpot response following each ChAd63 ME-TRAP response per volunteer. Wilcoxon matched pairs signed rank test ChAd63 ME-TRAP 1 and ChAd63 ME-TRAP 2, $p=0.0305$. G) Peak ex vivo IFN- γ ELISpot response following each MVA ME-TRAP response per volunteer. No significant difference by Wilcoxon matched pairs signed rank test. Median with IQR displayed.

With no significant boosting detected with multiple MVA ME-TRAP vaccinations, ELISpot response in all groups was assessed at 1 week and 4 weeks post first MVA ME-TRAP boost (Figure 4.3, Kruskal-Wallis tests). All groups showed significant boosting of their pre-vaccination MVA ME-TRAP response at 1 week post boost. Group 4+7 and group 1 showed boosting 4 weeks post MVA ME-TRAP boost (Figure 4.3, Kruskal-Wallis tests).

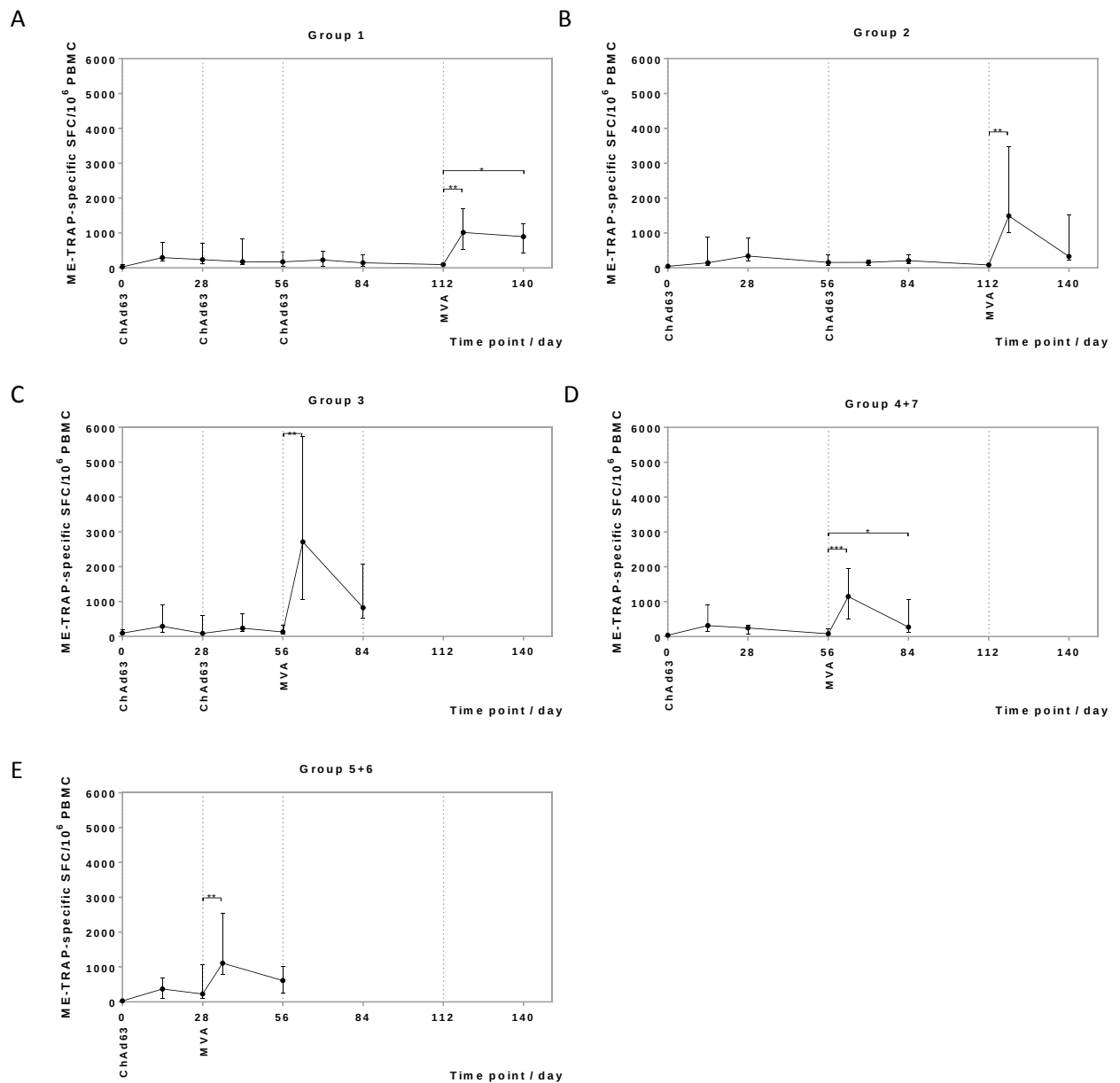


Figure 4.3 Summary of boosting time course by priming regimen

Responses 1 week and 4 weeks post MVA ME-TRAP boost. Kruskal-Wallis ANOVA with Dunn's correction for multiple comparisons between time points, *p < 0.05, **p < 0.01, ***p < 0.001. Median with IQR displayed.

4.2.2 Area-under-curve analysis by *ex vivo* IFN- γ ELISpot

A more comprehensive analysis of these vaccination regimens involves an area-under-curve (AUC) analysis. The AUC value is calculated per volunteer and represented as a proportion of the regimen length (AUC/days) from day 0 to 4 weeks post first or final MVA ME-TRAP vaccination. It is representative of both peak IFN- γ responses to ME-TRAP and maintenance of this response over the duration of the defined time course, as determined by *ex vivo* IFN- γ ELISpot. This scaled AUC analysis showed no differences in immunogenicity with the different priming schedules up to 4 weeks after the first MVA ME-TRAP dose (Figure 4.4 A, $p=0.3675$, Kruskal-Wallis test), and demonstrated no improvement of any 4 dose-vaccination regimen or over either 2 dose-vaccination prime boost regimens. To specifically evaluate response durability, AUC was calculated for all groups from the point of final MVA-ME-TRAP vaccination for a fixed follow up period of 4 weeks. Durability was comparable between all groups (Figure 4.4 C, Kruskal-Wallis test). Despite no significant differences between groups measured by this analysis, group 4 (A_M_M_M) consistently shows the largest response by AUC (Figure 4.4 B+C), whereby the response magnitude is maintained by the subsequent MVA ME-TRAP boosts.

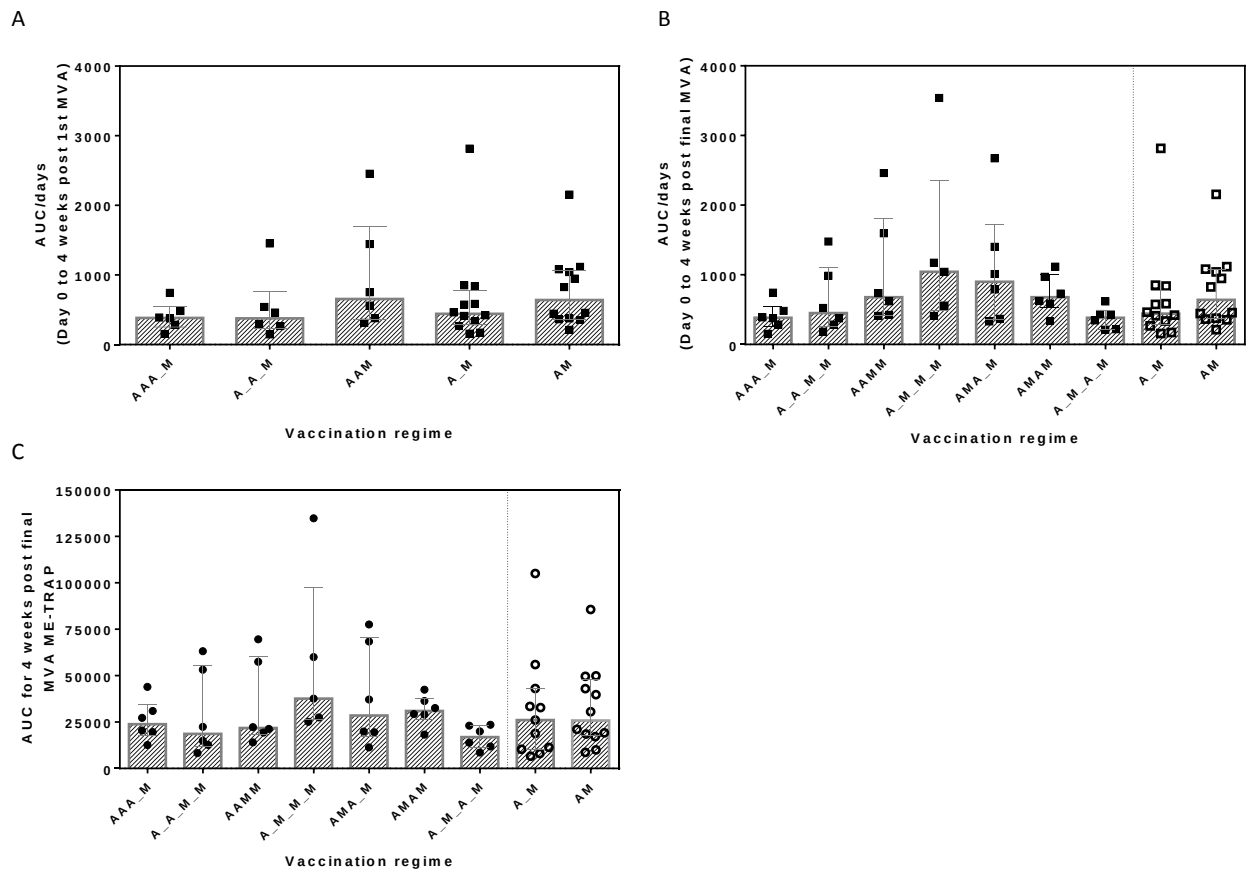


Figure 4.4 Area-under-curve immunogenicity by *ex vivo* IFN- γ ELISpot

A) Individual AUC response for day 0 to 4 weeks post 1st MVA ME-TRAP vaccination. No significant difference between groups by Kruskal-Wallis test with Dunn's correction for multiple comparisons between all groups. B) Individual AUC response for day 0 to 4 weeks post final MVA ME-TRAP vaccination. No significant difference between the 2- or 4-vaccination regimens. C) Individual AUC response for day of final MVA ME-TRAP boost to 4 weeks later. No significant difference between groups by Kruskal-Wallis test with Dunn's correction for multiple comparisons between all groups. Median with IQR displayed.

The number of positive responses detected to the different TRAP peptide pools was then analysed to determine whether vaccination regimen affected the breadth of the immune response. Responses induced by ChAd63 ME-TRAP priming were of moderate frequency and breadth, with positive responses to a median of 2 of the 7 ELISpot peptide pools corresponding to the ME-TRAP vaccine insert. Homologous ChAd63 ME-TRAP immunisations did not further broaden this response (Figure 4.5 A). MVA ME-TRAP broadened the response to a median of 5 positive pools, with a significant expansion in breadth over ChAd63 ME-TRAP at the second and third vaccination (Figure 4.5 B, $p=0.0002$, Mann Whitney test and Figure 4.5 C, $p<0.0001$, Mann Whitney test). To assess multiple-prime, multiple-boost and alternating vector regimens, the groups were combined and compared against single prime-boost responses. The distribution of positive responses was similar between regimens using multiple-prime, alternating vectors and single prime-boost, with much of the response generated against TRAP T9/96 (TT) pool 1 and 2. The multiple-MVA boost approach resulted in a slightly larger proportion of the response directed at the C terminus of the vaccine insert (Figure 4.5 D).

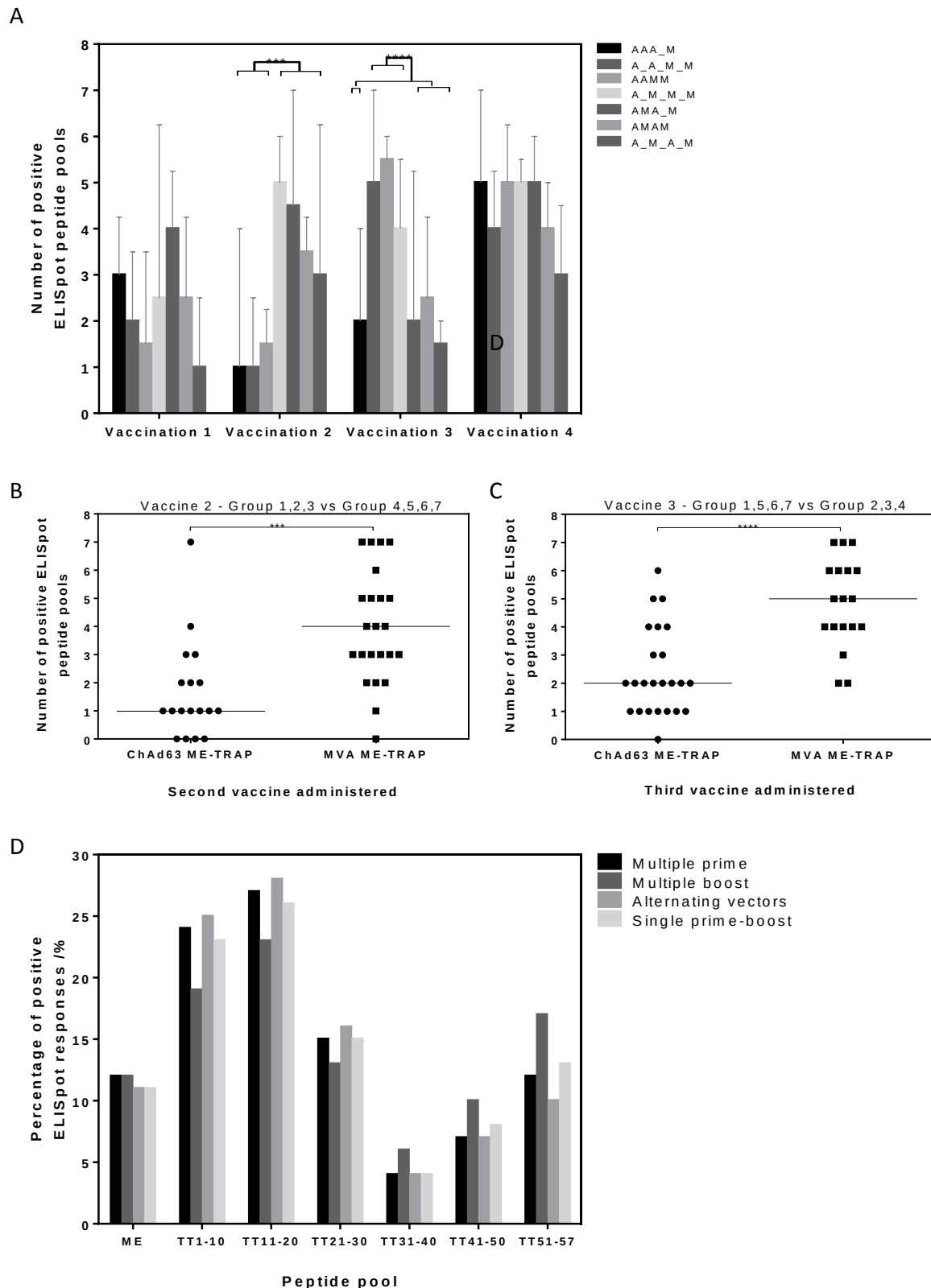


Figure 4.5 Summary of response breadth by *ex vivo* IFN- γ ELISpot

A) Response breadth by number of positive ELISpot pools per group at the peak post vaccination (14 - 28 days post-ChAd63 ME-TRAP and 7-28 days post-MVA ME-TRAP) to TRAP T9/96 (TT) pool 1-6 and multiple epitope pool. Median plus interquartile range displayed. Response breadth to B) ChAd63 ME-TRAP and C) MVA ME-TRAP by Mann-Whitney test at vaccination 2 and 3, *** $p < 0.001$, **** $p < 0.0001$, with group data pooled depending on vector used at 2nd and 3rd vaccine. Medians displayed. 3C) Distribution of positive responses to each ELISpot peptide pool, as a proportion of total positive response. Groups combined to assess multiple prime (group 1 AAA_M, group 2 A_A_M, group 3 AAM), multiple boost (group 4 A_M_M_M), alternating vectors (group 5 AMA_M, group 6 AMAM, group 7 A_M_A_M) and single prime boost (group 5+6 AM, group 4+7 A_M).

4.2.3 Immunogenicity by flow cytometry with ICS

4.2.3.1 *Schedules with a double ChAd63 ME-TRAP prime are more immunogenic than the standard regime*

To determine whether vaccination regimen had a different effect on CD4⁺ or CD8⁺ T cell responses, flow cytometry with intracellular cytokine staining was performed 1 week and 4 weeks post MVA ME-TRAP vaccination, measuring IFN- γ , IL-2 and TNF- α , in addition to CD107a (also known as LAMP-1), which is used to identify CD8⁺ T cells with cytotoxic potential. No difference between individual cytokine production by TRAP-specific CD4⁺ or CD8⁺ T cells were measured 1 week after first MVA ME-TRAP boost, when comparing a 4 and 8 week prime boost interval (Figure 4.6 A+B, Kruskal-Wallis test). Any-of-3 ICS analysis (IFN- γ ⁺, IL-2⁺ or TNF α ⁺) also revealed no significant difference between the 5 priming strategies for CD4⁺ and CD8⁺ T cells (Figure 4.6 C+D, Kruskal-Wallis test).

Responses 4 weeks following MVA ME-TRAP vaccination showed significantly higher frequencies of cytotoxic CD8⁺ CD107a⁺ T cells and CD8⁺ TNF α ⁺ T cells in volunteers immunised with a 4 week ChAd63 ME-TRAP to MVA ME-TRAP interval, compared with an 8 week interval (Figure 4.6 F, $p < 0.001$, Kruskal-Wallis test). A similar but non-significant trend was also measured in TNF- α secreting CD4⁺ T cells (Figure 4.6 E). Any-of-3 cytokine analysis of the 5 different priming regimens showed a significantly higher magnitude of TRAP-specific CD8⁺ T cells in the 2 regimens with a double ChAd63 prime (group 2 A_A_M and group 3 AAM) compared with the standard 8 week prime-boost regimen (group 4 and 7 combined, A_M), (Figure 4.6 H, $p = 0.0034$, Kruskal-Wallis test). Again, a similar but non-significant trend was seen with TRAP-specific CD4⁺ T cells (Figure 4.6 G).

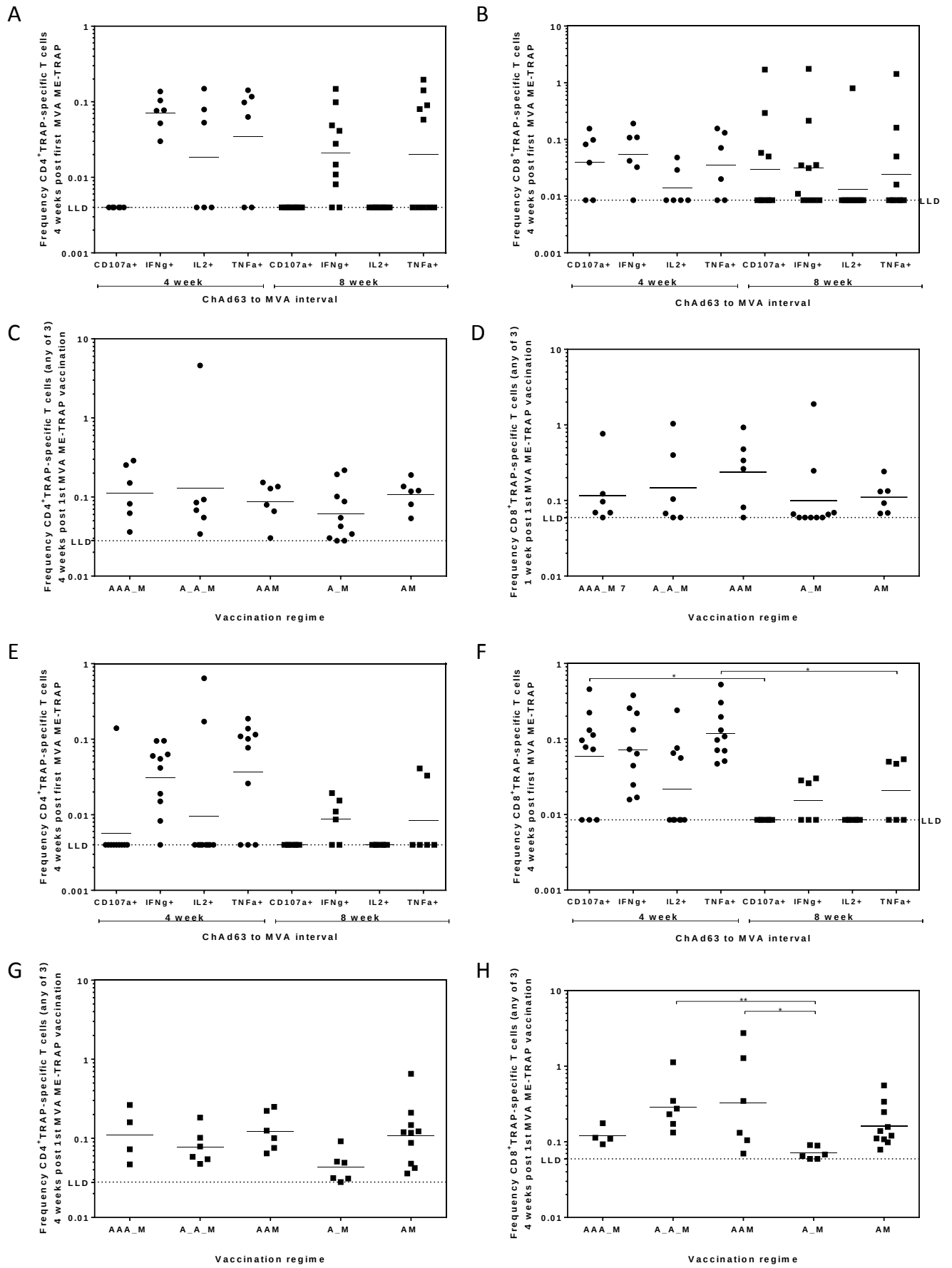


Figure 4.6 Summary of ICS responses at 1 week and 4 weeks post 1st MVA ME-TRAP across the 5 different priming schedules

A+B) Frequency of TRAP-specific CD4⁺ T cells and CD8⁺ T cells producing IFN- γ /IL-2/TNF α at 1 week post-MVA ME-TRAP for volunteers boosted at 4 weeks compared to 8 weeks post ChAd63 ME-TRAP. No significant difference by Kruskal-Wallis ANOVA with Dunn's correction of paired cytokines at 4 vs 8 weeks. C+D) Frequency of CD4⁺ T cells and CD8⁺ T cells producing any-of-3 cytokines from IFN- γ , IL-2 and TNF α 1 week post first MVA ME-TRAP vaccination. No significant difference by Kruskal-Wallis ANOVA between all groups with Dunn's correction. E+F) Frequency of TRAP-specific CD4⁺ T cells and CD8⁺ T cells producing IFN- γ /IL-2/TNF α at 4 weeks post-MVA ME-TRAP for volunteers boosted at 4 weeks compared to 8 weeks post ChAd63 ME-TRAP. Kruskal-Wallis ANOVA with Dunn's correction of paired cytokines at 4 vs 8 weeks. G+H) Frequency of CD4⁺ T cells and CD8⁺ T cells producing any-of-3 cytokines from IFN- γ , IL-2 and TNF α 4 week post first MVA ME-TRAP vaccination. Kruskal-Wallis ANOVA between all groups with Dunn's correction. *p < 0.05, **p < 0.01. See appendix Figure S1 for sample getting strategy. Geometric means displayed. Minimum of 44,600 live CD3⁺ cells acquired per sample.

4.2.3.2 Correlates of protection

The correlates of protection previously identified with the A_M regimen, CD107a expression and monofunctional CD8⁺ T cell expression of IFN- γ (IFN- γ ⁺, IL-2⁻, TNF α ⁻ phenotype), were compared with each of the vaccination groups. One week post first MVA ME-TRAP vaccination, monofunctional IFN- γ was comparable between all priming regimens, as was CD107a expression (data not shown). By 4 weeks post first MVA ME-TRAP vaccination, the 2 regimens with a double ChAd63 prime (group 2 A_A_M and group 3 AAM) had significantly higher TRAP-specific CD8⁺ CD107a⁺ T cells compared with the standard A_M regimen (Figure 4.7 A, p=0.0198, Kruskal-Wallis test). Significantly higher monofunctional IFN- γ ⁺ CD8⁺ T cells were also measured in group 3 AAM compared with the standard A_M regimen (Figure 4.7 C, p=0.0444, Kruskal-Wallis test). These differences were not measured following the final MVA ME-TRAP vaccination (Figure 4.7 B+D).

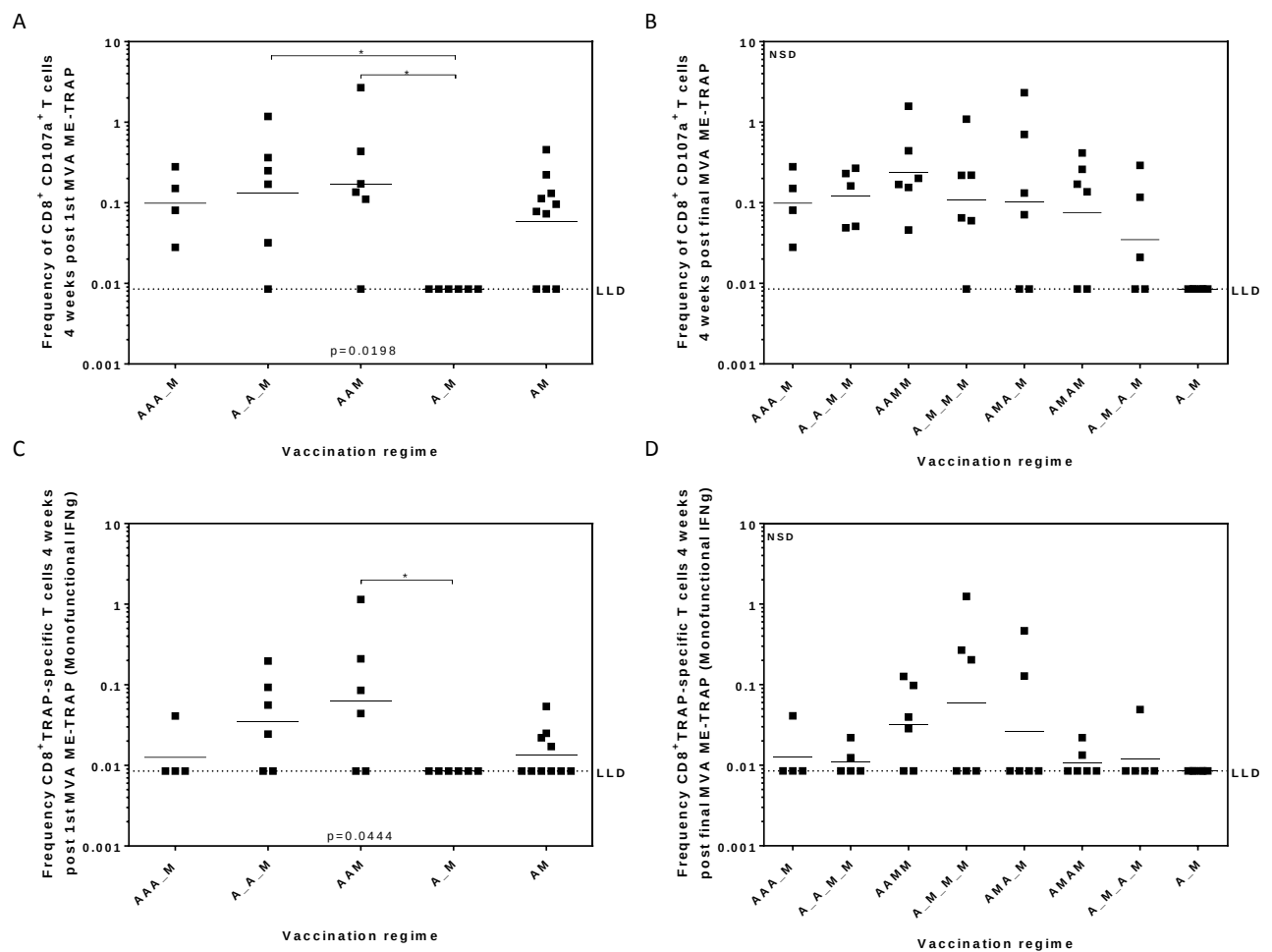


Figure 4.7 Frequencies of CD8⁺ CD107a⁺ T cells and CD8⁺ IFN-γ⁺ monofunctional T cells

A) Frequency of antigen-specific CD8⁺ CD107a⁺ T cells 4 weeks post first MVA ME-TRAP vaccination. Kruskal-Wallis ANOVA with Dunn's correction against A_M group, $p=0.0198$. B) Frequency of antigen-specific CD8⁺ CD107a⁺ T cells 4 weeks post final MVA ME-TRAP vaccination. No significant difference by Kruskal-Wallis ANOVA between all 4-vaccination groups with Dunn's correction $p=0.7699$, and against A_M control group $p=0.0605$. C) Frequency of antigen-specific monofunctional CD8⁺ IFN-γ⁺ T cells 4 weeks post 1st MVA ME-TRAP vaccination. Kruskal-Wallis ANOVA with Dunn's correction against A_M group, $p=0.0444$. D) Frequency of antigen-specific monofunctional CD8⁺ IFN-γ⁺ T cells 4 weeks post final MVA ME-TRAP vaccination. No significant difference by Kruskal-Wallis ANOVA between all 4-vaccination groups with Dunn's correction p -value 0.5846, and against A_M control group $p=0.3261$. Geometric means displayed.

4.2.3.3 Polyfunctional cytokine analyses

Polyfunctional T cells have correlated with protection against parasitic *Leishmaniasis major* infection in mice [432] and have been shown to be induced by vaccination in humans populations (reviewed in [433]). However, monofunctional CD8⁺ IFN- γ ⁺ responses have previously correlated with protection following administration of the standard A_M regimen [287]. In order to compare the different functional profiles induced by the 7 novel vaccination regimens, polyfunctional analyses were performed for all combination of IFN- γ , TNF- α and IL-2 production by CD4⁺ and CD8⁺ T cells.

Polyfunctional analysis revealed similar patterns in the ICS responses at different points along the different vaccination time courses. TRAP-specific CD8⁺ T cell responses were generally higher than CD4⁺ T cell response at all time points measured. TRAP-specific CD4⁺ T cells responses were largely polyfunctional and consisted of 2 major phenotypes (IFN- γ ⁺ IL-2⁺ TNF α ⁺ or IFN- γ ⁻ IL-2⁺ TNF α ⁺), (Figure 4.8 A, C +E). More variation was seen in the phenotype of TRAP-specific CD8⁺ T cells, with monofunctional cytokine responses alongside the polyfunctional profile. By 4 weeks post final MVA ME-TRAP vaccination, the dominant CD8⁺ T cell subsets were IFN- γ ⁺ IL-2⁻ TNF α ⁺ and monofunctional TNF α ⁺, with de-granulating TRAP-specific CD8⁺ T cells also highest at this time point (Figure 4.8 B, D + F).

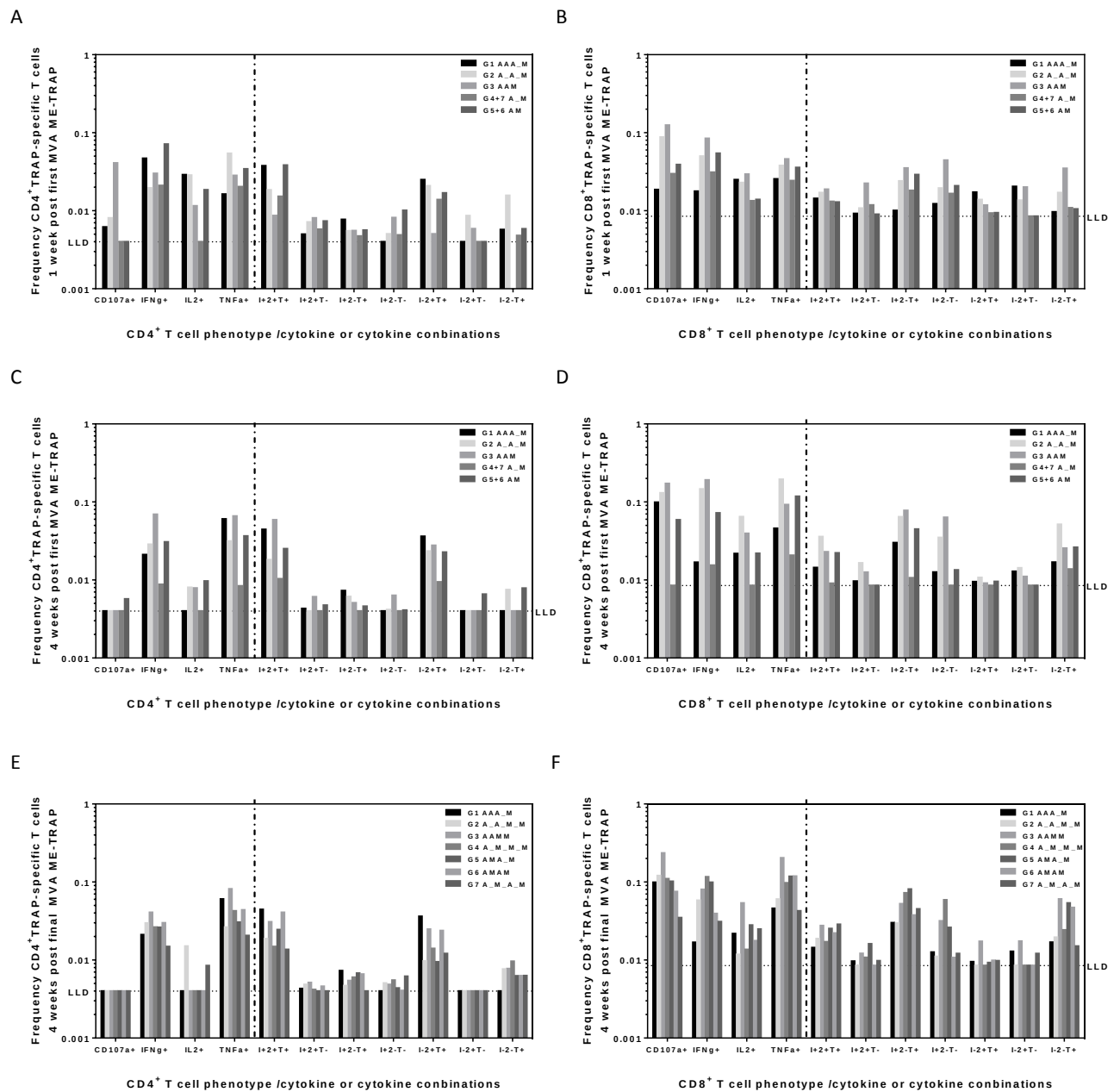


Figure 4.8 Summary of TRAP-specific T cell phenotype following MVA ME-TRAP vaccination.

Frequency of TRAP-specific CD4⁺ T cells and CD8⁺ T cells A+B) 1 week post first MVA ME-TRAP vaccination, C+D) 4 weeks post first MVA ME-TRAP vaccination and E+F) 4 weeks post final MVA ME-TRAP vaccination. Individual cytokines analyses separated from polyfunctional analyses by dotted line. Geometric mean displayed.

4.2.4 Summary of T cell responses to vaccine insert

T cell immunogenicity to TRAP/ME-TRAP are summarised in Table 13, with magnitude of response ranked by colour for each parameter. By assessing all parameters, the 4 week AM regimen consistently outperforms the 8 week A_M regimen. Of all 2- and 4- regimens, group 3 (AAM(M)) is very often the most immunogenic.

Table 13 Summary of *ex vivo* IFN- γ ELISpot responses to ME-TRAP and ICS responses to TRAP
Response colour coded by magnitude. ELISpot median response. ICS geometric mean response. Statistics combined for analyses where regimens are identical.

		Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 4 + 7	Group 5 + 6
Readout	Parameter analysed	AAA_M	A_A_M_M	AAMM	A_M_M_M	AMA_M	AMAM	A_M_A_M	A_M	AM
ELISpot	1 week post 1st MVA ME-TRAP	1017	1489	2711					1150	1110
ELISpot	4 week post 1st MVA ME-TRAP	895	326	825					272	614
ELISpot	1 week post final MVA ME-TRAP	1017	643	828	1635	1088	1002	492	1150	1110
ELISpot	4 week post final MVA ME-TRAP	895	796	1102	948	1015	1573	707	272	614
ELISpot	AUC/days day 0 to 4 weeks post 1st MVA	382.3	377	655.9					441.7	641.2
ELISpot	AUCdays day 0 to 4weeks post final	382.3	449.9	677.9	1043	900.5	677.3	383.6	441.7	641.2
ELISpot	AUC 4 week period post final MVA ME-	23867	18662	21795	37625	28522	30940	16909	26089	25802
ELISpot	Number of +ve ELISpot pools following 1st MVA ME-	5	5	6	5	5	4	3	3	4
ELISpot	Number of +ve ELISpot pools following final MVA	5	4	5	5	6	4	3	3	4
ICS	CD4 any-of-3 response 4 weeks post 1st MVA ME-	0.1125	0.1292	0.08663					0.06091	0.108
ICS	CD8 any-of-3 response 4 weeks post 1st MVA ME-	0.1171	0.1474	0.2395					0.1004	0.1103
ICS	CD4 any-of-3 response 4 weeks post 1st MVA ME-	0.1095	0.07785	0.1228					0.04296	0.1083
ICS	CD8 any-of-3 response 4 weeks post 1st MVA ME-	0.1195	0.2883	0.3253					0.07074	0.1607
ICS	CD8+ CD107a+ 4 weeks post 1st MVA	0.09896	0.1306	0.1719					0.0085	0.05893
ICS	CD8+ CD107a+ 4 weeks post final MVA	0.09896	0.1203	0.2352	0.1097	0.1017	0.07525	0.03488	0.0085	0.05893
ICS	Monofunctional CD8+ CD107a+ 4 weeks post 1st MVA ME-TRAP	0.0126	0.03491	0.06345					0.0085	0.01344
ICS	Monofunctional CD8+ CD107a+ 4 weeks post final MVA ME-TRAP	0.0126	0.01109	0.03169	0.05893	0.02606	0.01074	0.01208	0.0085	0.01344

4.2.5 Anti-vector immunity

4.2.5.1 *T cell responses to the ChAd63 hexon do not affect vaccine immunogenicity*

Pre-vaccination T cell responses to the ChAd63 hexon were positive for 67% (8/12) of volunteers by *ex vivo* IFN- γ ELISpot, were generally low in magnitude (median 133 SFC per 10^6 PBMC) and were comparable between groups 1 and 2. Post-vaccination responses were also comparable between groups. Individual hexon ELISpot responses increased significantly in group 1 after 2 doses of ChAd63 administered with a 4 week interval (Figure 4.9 A, $p=0.0130$ by Friedman test within group, $p=0.0760$ Kruskal-Wallis test between groups). The overall anti-vector fold change increase from day 0 was no greater in the triple ChAd63 ME-TRAP group, compared with the group receiving two priming doses in the same 8 week period (Figure 4.9 B, Kruskal-Wallis test).

There was no correlation between pre-vaccination T cell response to the ChAd63 hexon and the peak post-ChAd63 ME-TRAP T cell response to the vaccine insert (Figure 4.9 C, Spearman rank test with group 1 and 2 combined). However a negative correlation was measured between pre-existing T cell responses to the ChAd63 hexon and peak TRAP antibody titre post-ChAd63 ME-TRAP vaccination(s) (Figure 4.9 D, Spearman $r = -0.7832$, $p = 0.0038$). Boosting with MVA ME-TRAP overcomes this effect, with no correlation measured between pre-existing T cell responses to the ChAd63 hexon and post-MVA ME-TRAP TRAP antibody titres. (data not shown). This negative correlation was not enhanced with vaccine-induced ChAd63 hexon-specific T cells, with no correlation between the hexon T cell response following multiple ChAd63 ME-TRAP vaccinations at day 70 and TRAP antibody titre at day 84 (data not shown).

Any-of-3 (IFN- γ^+ , TNF- α^+ and/or IL-2 $^+$) ICS analysis of ChAd63 hexon stimulated PBMC revealed that both CD4 $^+$ and CD8 $^+$ cytokine-secreting T cells contribute to the ChAd63-hexon specific T cell response, with significant boosting of ChAd63 hexon-specific CD4 $^+$ T cells in groups 1 and 2 after 2 doses of ChAd63 ME-TRAP (Figure 4.9 E, group 1 $p=0.0167$, group 2 $p=0.0001$, Friedman test) and ChAd63 hexon-specific CD8 $^+$ T cells in group 1 only (Figure 4.9 F, group 1 $p=0.0065$, group 2 $p=0.3086$, Friedman test). Anti-vector CD4 $^+$ and CD8 $^+$ T cell responses between groups were comparable at each time point (data not shown). No correlations were measured between anti vector CD4 $^+$ or

CD8⁺ T cell response and the T cell response to the vaccine insert. A negative correlation was measured between pre-vaccination CD4⁺ ChAd63-specific T cells and TRAP antibody titre 8 weeks post final ChAd63 ME-TRAP vaccination (Figure 4.9 G, Spearman $r = -0.6901$, $p\text{-value} = 0.0234$). TRAP antibody data produced by C. Snudden and G. Bowyer.

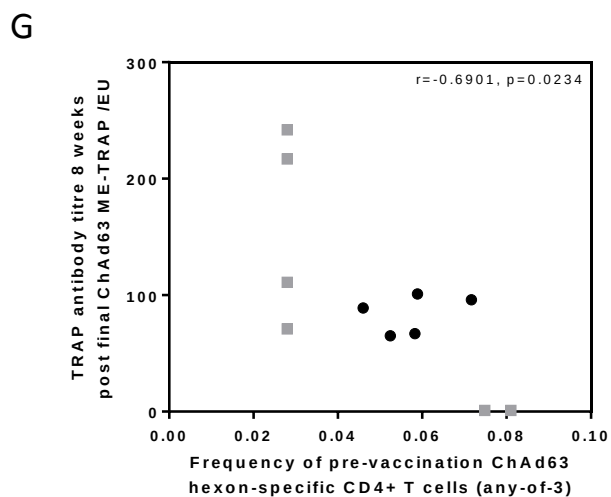
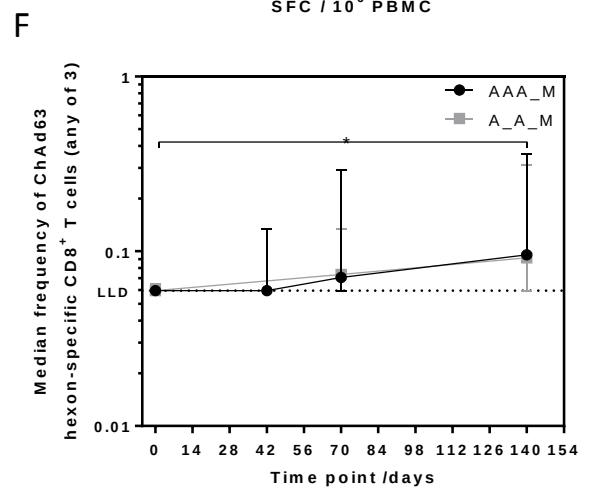
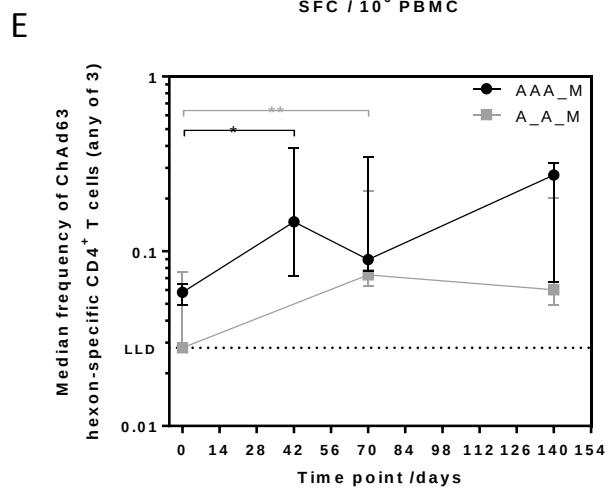
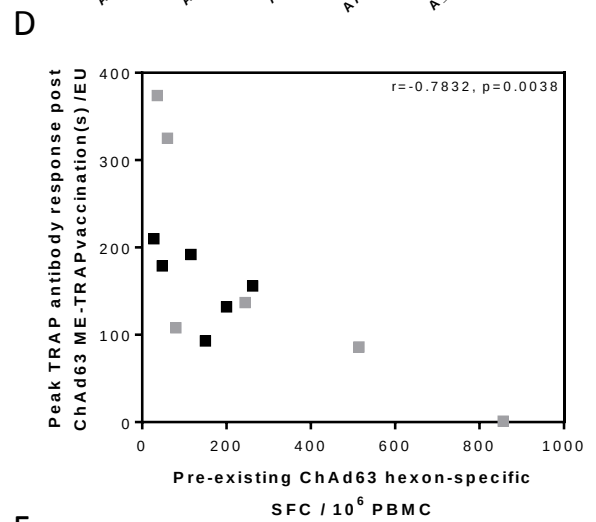
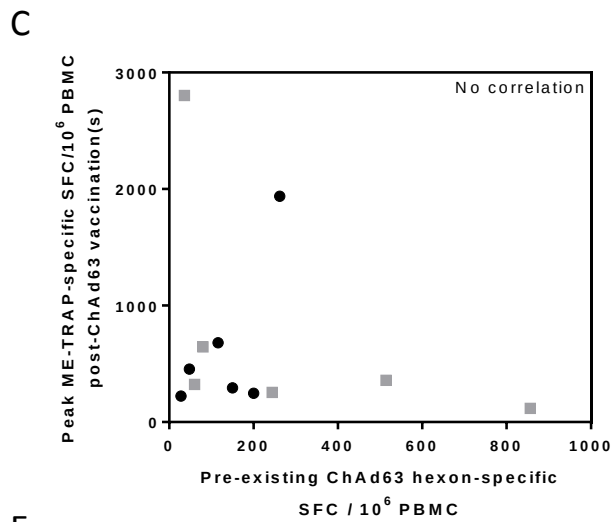
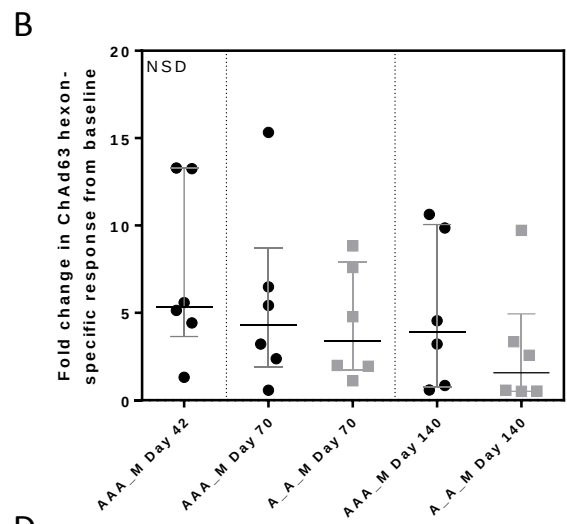
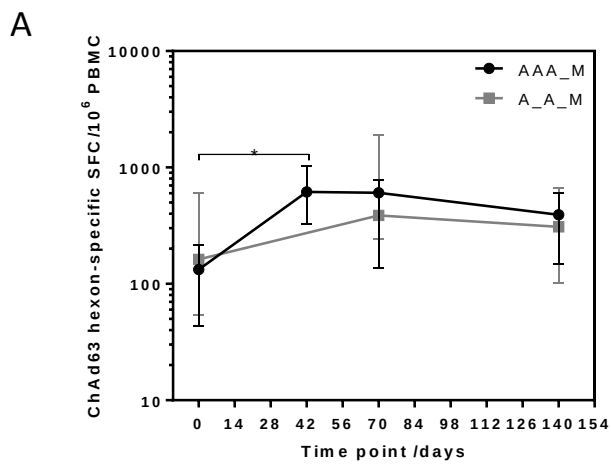


Figure 4.9 Summary of T cell responses to the ChAd63 hexon

A) ChAd63 hexon-specific response time course by *ex vivo* IFN- γ ELISpot following triple ChAd63 ME-TRAP priming and double ChAd63 ME-TRAP priming. Friedman test with Dunn's correction for time course within group $p=0.0130$, Kruskal-Wallis test with Dunn's correction for between group analysis $p=0.0760$. B) Fold change in anti-ChAd63 hexon response by *ex vivo* IFN- γ ELISpot. No significant difference between groups or time points by Kruskal-Wallis AVOVA with Dunn's correction, $p\text{-value} = 0.3463$. C) Correlation between pre-vaccination ChAd63 hexon *ex vivo* IFN- γ ELISpot responses and peak post-ChAd63 *ex vivo* IFN- γ ELISpot responses to ME-TRAP by Spearman r . $r = -0.2378$, $p\text{-value} = 0.4573$. D) Correlation between pre-vaccination ChAd63 hexon *ex vivo* IFN- γ ELISpot responses and peak post-ChAd63 TRAP antibody titre by Spearman r . $r = -0.7832$, $p\text{-value} = 0.0038$. E) Frequency time course of ChAd63-specific (any-of-3) CD4⁺ T cells, Friedman test with Dunn's correction against pre-vaccination within group 1 $p=0.0167$, group 2 $p=0.0001$, no significant difference between groups Kruskal-Wallis test with Dunn's correction between time points $p=0.0160$. F) Frequency time course of ChAd63-specific (any-of-3) CD8⁺ T cells, Friedman test with Dunn's correction against pre-vaccination within group 1 $p=0.0065$, group 2 $p=0.3086$, no significant difference between groups Kruskal-Wallis test with Dunn's correction between time points $p=0.0391$. G) Correlation between pre-vaccination ChAd63 hexon-specific CD4⁺ T cells and post-ChAd63 TRAP antibody titre at day 112 by Spearman r . $r = -0.6901$, $p\text{-value} = 0.0234$. Black = group 1, grey = group 2. * $p < 0.05$, ** $p < 0.01$. Minimum of 71,500 live CD3⁺ cells acquired per sample.

4.2.5.2 Antibody responses to the MVA vector do not affect vaccine immunogenicity

Anti-MVA antibody levels were measured using an ELISA to the MVA WR113/D8L protein [434], following each MVA ME-TRAP vaccination in groups 2-7. Analysis of combined groups saw a significant increase in anti-MVA antibodies after the first MVA ME-TRAP vaccination, and again after the second vaccination (Figure 4.10 A, $p < 0.0001$, Friedman test). The fold change from day 0 to post second MVA ME-TRAP was not affected by the interval between MVA ME-TRAP vaccinations (Figure 4.10 B, Kruskal-Wallis test). A positive correlation was measured between anti-MVA antibodies 4 weeks post first MVA ME-TRAP vaccination and ME-TRAP-specific T cells 1 week post first MVA ME-TRAP vaccination (Figure 4.10 C, Spearman $r = 0.3649$, $p\text{-value} = 0.0435$), with no correlation following the second MVA ME-TRAP vaccination (Figure 4.10 D, Spearman rank test). No correlation was measured between MVA antibodies 4 weeks post first MVA ME-TRAP vaccination and TRAP antibody titre following the first and second MVA ME-TRAP vaccination (Figure 4.10 E+F, respectively, Spearman rank test). No correlations were measured between anti-MVA antibodies and TRAP T cells or antibodies following the second or third MVA ME-TRAP vaccination (data not shown). MVA antibody data produced by A. Grobbelaar and G. Bowyer.

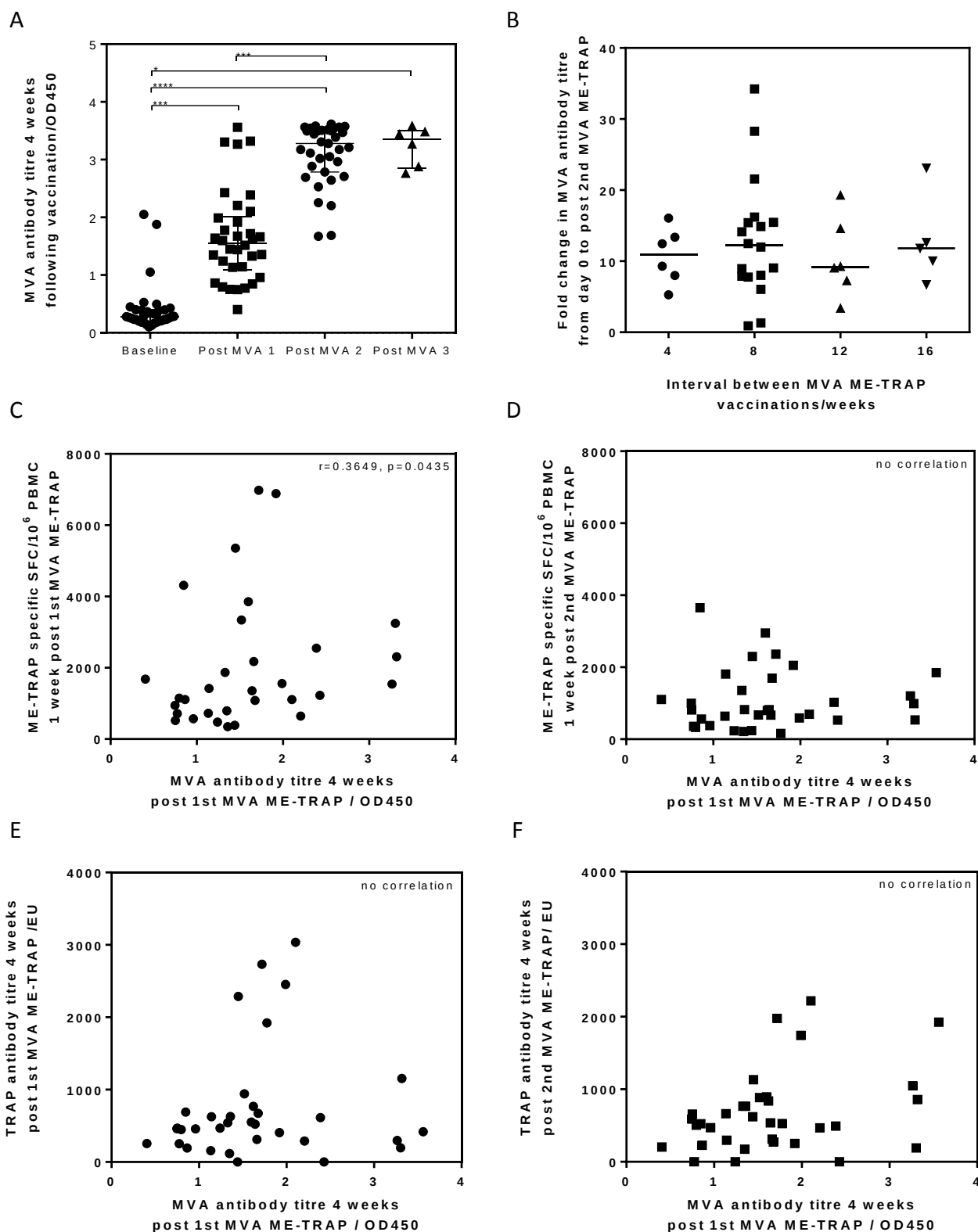


Figure 4.10 Summary of antibody responses to MVA

A) Anti-MVA ELISA OD 28 days post MVA-ME-TRAP for groups 2-7. Friedman test with Dunn's comparison between multiple time points. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$. B) Fold change in anti-MVA ELISA OD stratified by interval between MVA ME-TRAP vaccinations. No significant difference in fold change with different interval length by Kruskal-Wallis ANOVA between all groups with Dunn's correction p -value = 0.9099. C) Correlation anti-MVA antibody titre 4 weeks post 1st MVA ME-TRAP and T cell response to ME-TRAP 1 week post- 1st MVA ME-TRAP (Spearman $r = 0.3649$, p -value = 0.0435). D) Correlation anti-MVA antibody titre 4 weeks post 1st MVA ME-TRAP and T cell response to ME-TRAP 1 week post- 2nd MVA ME-TRAP (Spearman $r = 0.1260$, p -value = 0.4847). E) Correlation anti-MVA antibody titre 4 weeks post 1st MVA ME-TRAP and TRAP antibody titre 4 weeks post 1st MVA ME-TRAP vaccination (Spearman $r = 0.1754$, p -value = 0.3210). F) Correlation anti-MVA antibody titre 4 weeks post 1st MVA ME-TRAP and TRAP antibody titre 4 weeks post 2nd MVA ME-TRAP vaccination (Spearman $r = 0.2796$, p -value = 0.1093).

4.3 Discussion

One of the primary goals of vaccination is to achieve high and durable immune response. Vac 43 was designed to identify the most immunogenic vaccination regimen, in terms of vaccine sequence and interval. The work described in this chapter analysed the effect of vaccination regimen on the cell-mediated immune response to the vaccine antigen, in addition to the vaccine vector, with the aim of identifying the most suitable, immunogenic regimen going forward. The degree of flexibility of the prime-boost approach, which is a key consideration in the advancement of this vaccine platform, was also assessed.

Although the groups sizes in this Phase I clinical vaccine trial were small, these data do not suggest that reducing the prime-boost interval from 8 to 4 weeks has a negative impact on induction of TRAP-specific antibodies or T cells. This is consistent with published using ChAd63 and MVA viral vectors at shorter intervals for vaccination against Ebola virus disease [260], in a trial conducted in quick succession to this trial. In fact, higher frequencies of antigen-specific CD8⁺ T cells producing TNF- α and antigen-specific cells capable of de-granulation in response to TRAP peptide stimulation were detected using the shortened prime-boost regimen, AM.

CD107a and b are markers of CD8⁺ T cell de-granulation that have been shown to mediate cytolytic activity in an antigen-specific manner [435]. Following an 8 week ChAd63 ME-TRAP and MVA ME-TRAP prime-boost regimen, TRAP-specific CD8⁺ CD107a⁺ T cells measured 24 weeks post boost and monofunctional TRAP-specific CD8⁺ T cells producing IFN- γ measured 14-21 days post boost correlated with a higher degree of protection against CHMI [287]. In this study, both of these parameters were similar across all 2-vaccination and 4-vaccination groups at 1 week post first MVA and at 4 weeks post final MVA. However, at a previous unreported time point of 4 weeks post first MVA ME-TRAP vaccination, significantly higher frequencies of TRAP-specific de-granulating CD8⁺ T cells were measured in the 2 groups with a double ChAd63 prime (group 2 and 3) compared with the standard 8 week prime-boost regimen. This improvement over the 8 week prime-boost regimen was also measured in monofunctional CD8⁺ IFN- γ ⁺ T cells in group 3. These two findings suggest greater vaccine efficacy could be achieved by including an additional ChAd63 ME-TRAP vaccination, with a 4

or 8 week vaccination interval (AAM or A_A_M), than with the 8 week ChAd63.MVA ME-TRAP prime-boost schedule (A_M). Interestingly, a 4 week prime-boost interval schedule (AAM) with three vaccine doses could fit well with the standard 6, 10, 14 week EPI schedule used for infant vaccination in most countries where malaria is highly endemic.

Monofunctional IFN- γ -producing CD8⁺ T cells have a proven association with protection and is therefore a key CD8⁺ T cell phenotype to measure in these novel vaccination schedules. CD8⁺ IFN- γ ⁺ TNF- α ⁻ IL-2⁻ cells correlated with protection in human clinical testing of ChAd63.MVA ME-TRAP [287] and were associated with protection in a triple DNA prime, AdHu5 boost vaccination regimen of CS and AMA1 in humans [292]. CD8⁺ IFN- γ ⁺ T effector memory cells also correlated with protection in mice vaccinated with chimpanzee adenovirus 9 (AdC9) and MVA, both encoding ME-TRAP [274]. Furthermore, this is a physiological relevant correlate, as IFN- γ has been shown to have a role in clearance of several parasitic, bacterial, viral and fungal infections [338, 436-438]. Furthermore, IFN- γ production by NKT cells in mice inhibits intrahepatic *P. yoelii* development in a partially IFN- γ dependent manner [439]. TNF- α has similar effector functions to IFN- γ , especially related to its role in the sustained production of the inducible nitric oxide synthase gene and resulting nitric oxide [440, 441]. In murine and *in vitro* models of *L. major* and *Mycobacterium tuberculosis*, presence of both IFN- γ and TNF- α has shown to be a requirement for protection [442-444]. Furthermore, in an Ebola virus disease challenge model of non-human primates, vaccine-induced glycoprotein-specific CD8⁺ IFN- γ ⁺ TNF- α ⁺ T effector cells were associated with protection [445]. Therefore, alongside the monofunctional CD8⁺ IFN- γ ⁺ profile, it is encouraging that dominant T cell phenotypes were also measured with dual function IFN- γ ⁺ TNF- α ⁺ in the case of CD8⁺ T cells, and triple function IFN- γ ⁺ TNF- α ⁺ IL-2⁺ in the case of CD4⁺ T cells, in all of the novel vaccination regimens tested.

Consistent with previous macaque data using the same viral vectors, MVA ME-TRAP vaccination further broadens the response induced by ChAd63 ME-TRAP vaccination, and multiple MVA ME-TRAP boosts do not significantly enhance cellular immunogenicity beyond the magnitude of the first MVA ME-TRAP vaccination [431]. However additional dose(s) of MVA boosted individual peak T cell immunogenicity in over 1/3 of volunteers, which may be an important consideration

when tested in larger numbers volunteers, or in populations where co-infection with malaria or other factors can reduce cellular responses to vaccination [446-449]. Vaccinees in group 4 (A_M_M_M) also consistently produce the highest AUC ELISpot response, indicative of response durability. This is consistent with a DNA.MVA ME-TRAP and FP9.MVA ME-TRAP study in Gambian adults, where an additional MVA ME-TRAP boost 12-13 months after the initial MVA ME-TRAP boosted the response to a similar or higher magnitude to the first MVA ME-TRAP boost, and furthermore maintained good response durability following this re-boosted response [450].

The breadth of T cell response to the vaccine insert is comparable with previous non-human primate studies with respect to ChAd63 ME-TRAP and MVA ME-TRAP vaccinations and multiple MVA ME-TRAP boosting vaccinations [431]. The reported response breadth of 2 pools measured after ChAd63 ME-TRAP vaccination and 5 pools after MVA ME-TRAP vaccination is comparable or broader than previously reported in vaccinated populations in the UK and Africa [266, 294]. With no single immunodominant epitope in ME-TRAP, T-cell responses directed towards a wider variety of epitopes may confer enhanced protection [451].

A previous clinical vaccine trial assessing vaccine efficacy of ChAd63.MVA ME-TRAP against natural malaria infection in Kenyan adults demonstrated that a large proportion of the ELISpot response is directed towards TRAP T9/96 pools 2 (TT 11-20) and 3 (TT 21-30) (table S1). T cell responses to pool 3 were significantly associated with protection against natural malaria infection [295], and all regimens tested in this study directed 10-20% of positive responses to this region of the TRAP antigen.

ICS data suggests that this negative correlation between pre-existing ChAd63-hexon specific T cells measured by ELISpot and post prime TRAP antibody titre was driven by a ChAd63 hexon-specific CD4⁺ T cell response. Because prior exposure to ChAd63 itself is extremely unlikely in UK volunteers, it is assumed that the pre-existing T cells represent cross-reactivity with and prior exposure to some human adenovirus. After three homologous ChAd63 ME-TRAP vaccinations, adenovirus-specific CD4⁺ T cells were of a similar order-of-magnitude to those measured in the HIV vaccine STEP trial with triple homologous administration of human adenovirus 5 vaccinations (median 0.09-0.3% and 0.2-0.3%, respectively) [452], however no correlation with ME-TRAP-specific T cells was detected at

any time point post vaccination. While the first dose of ChAd63 ME-TRAP increases the frequency of ChAd63 hexon-specific T cells, these vector-specific T cells apparently are not detrimental to vaccine-induced T cell or antibody immunogenicity towards the vaccine insert. Furthermore, post-vaccination ChAd63 hexon-specific T cells frequencies were comparable between the 2 groups at each time point, suggesting that additional doses of ChAd63 ME-TRAP after the initial priming dose does not increase the magnitude of the T cell response against the vector, however a 3rd dose may prolong the persistence of these cells.

One possible explanation for this is that pre-existing, naturally-acquired T cell anti-vector immunity is of a different quality to that of vaccination-acquired anti-vector T cell immunity, as demonstrated by negative correlations between the TRAP antibody response and day 0 anti-vector T cell responses, followed by loss of this correlation once ChAd63 is administered as a vaccine. Firstly, pre-existing ChAd63 hexon-specific T cell responses are likely due to cross reactivity with human adenovirus exposure, and not pre-existing ChAd63 exposure. Secondly, wild-type adenovirus and genetically modified vaccine vectors differ in their replication competency. The implications in this setting are that multiple homologous administrations of ChAd63 do not impair immunogenicity to ME-TRAP following vaccination, but that pre-existing T cells to the adenoviral vector raised by natural infection may impact upon vaccine immunogenicity, particularly on the antibody response to the vaccine insert.

The positive correlation between anti-MVA antibodies and TRAP-specific T cell responses following the first MVA vaccination is analogous to the trend previously measured towards higher peak T cell responses to the TRAP insert in those volunteers with anti-vector antibodies [287]. TRAP-specific T cells were re-boosted by subsequent homologous MVA ME-TRAP vaccinations to levels comparable to that of the first boost, which is similar to observations in a previous macaque study [431]. It was unknown whether multiple administrations would enhance overall vaccine regimen immunogenicity or impair it through induced anti-vector immunity: with no negative correlations measured between anti-MVA antibodies and T cell or TRAP antibody response following double and triple administration of MVA ME-TRAP, it is concluded that multiple MVA vaccinations do not induce detrimental anti-vector immunity.

In summary, CD8⁺ T cell responses following vaccination are of particular importance, due to their proven association with protection at the liver stage of infection in mice [207, 208, 453] and humans [287]. Improved CD8⁺ T cell immunogenicity was measured over the previously reported 8 week prime-boost regimen (A_M) with a reduced interval of 4 weeks (AM), and with the incorporation of an additional priming vaccination of homologous ChAd63 ME-TRAP at a 4 or 8 week interval (AAM or A_A_M). Furthermore, these double ChAd63 ME-TRAP priming regimens do not induce detrimental anti-vector T cell immunity. The flexibility demonstrated in these vaccination regimens would facilitate incorporation into the WHO's standard EPI strategy (Expanded Programme on Immunization), whereby childhood immunisations are administered at intervals ranging from 4 to 12 weeks. Such flexibility also permits combining this approach with other partially protective malaria vaccine regimens using protein-based subunit vaccines plus adjuvant. It also suggests the prime-boost interval could be further reduced to 1 or 2 weeks.

Chapter 5

Optimisation of an *in vitro* T cell parasite inhibition assay

5 Chapter 5 – Optimisation of an *in vitro* T cell parasite inhibition assay

5.1 Introduction

Understanding the mechanism of protection in malaria vaccinees is key to improving the design of sub-optimally protective vaccine regimens. For T cell vaccines that function at the liver stage of infection, controlled human malaria infection is the primary method by which to measure vaccine-induced cell-mediated parasite inhibition. Such an *in vivo* system does not permit exploratory immunology beyond that of the peripheral blood. An *in vitro* assay aims to measure cellular immunity and direct parasite inhibition using T cells from vaccinated volunteers in a parasite infection model of human hepatocytes. An assay of this nature would permit the protective mechanism(s) of vaccine-induced T cells to be explored, and potentially provide a platform to efficiently down-select potential pre-erythrocytic candidate antigens, before the initiation of expensive CHMI studies and large scale efficacy studies in malaria endemic regions. Furthermore, the WHO's Malaria Vaccine Technology Roadmap identifies the need for standardised, functional assays in the characterisation of vaccine-induced responses [99].

The first published report of an *in vitro* sporozoite infection producing convincing numbers of exoerythrocytic forms was that of avian malaria, *Plasmodium fallax*, in embryonic turkey brain cells in 1966 [454]. In 1979, sporozoites from rodent malaria species, *P. berghei*, were first cultured *in vitro* in embryonic rat brain cells, with exoerythrocytic development reported to immature schizonts [455]. Other host cell types, including human liver (Chang), human lung (L 132) and embryonic liver cells from many species including rat, mouse, hamster, turkey and humans, were also used in preliminary experiments [456], until complete development of *P. berghei* sporozoites through to merozoite release was published by Hollingdale *et al.* in 1981, using human embryonic lung fibroblast (W138) cells. Infection and culture within hepatocytes, the natural host tissue for *Plasmodium* sporozoite infection, was achieved in the early 1980s with *P. yoelii* and *P. berghei* infection of rat hepatocytes [457, 458], and merozoite release was reported for *P. yoelii* [459]. *P. berghei* infection

of human hepatoma line HepG2-A16 in 1983 showed good rates of infection, plus generation of infectious merozoites [460]. The field has since developed with the identification of several more human hepatoma cell lines supporting *P. berghei* infection, including Huh1, 2, 6 and 7, PLC/PRF/5, HepG3 and NCH2 [461]. Finally, *P. berghei* cultivation in primary mouse hepatocytes was achieved in 1989 [462].

Development of *in vitro* *P. falciparum* culture was first achieved in primary human hepatocytes in 1985 [463], although infectivity remains low at ~0.1%. Primary human hepatocytes require specialised isolation and culture protocols, and human hepatoma cell lines provide a viable alternative for *P. falciparum* infection, with full development within human hepatoma lines reported for the HSS-102 [464] and HC-04 [465] cell lines. Infectivity is lower compared with primary human hepatocytes [184], with infectivity at 0.0009% in HSS-102 [464] and 0.066% in HC-04 [465]. Partial liver stage development is reported for *P. falciparum* infection of the Huh1 and Huh2 human hepatoma cell lines and human embryonic lung fibroblast W138 cells [466], however these are unsuitable for studying liver stage parasite development, due to parasite developmental arrest following invasion.

The advances in *Plasmodium* sporozoite culture highlight that *in vitro* infection is not species- or tissue- specific, and rodent malaria infection of human hepatocytes is a viable method to partially model human liver stage infection. Coupled with the use of novel transgenic *P. berghei* expressing *P. falciparum* antigens, there is scope to expand the field and study human immune responses to malaria-infected human hepatocytes, without the associated risks of using *P. falciparum* as the infectious agent and its associated low levels of infectivity. Such transgenic *P. berghei* parasites express *P. falciparum* gene(s) in addition to, or in replacement of their *P. berghei* homologue. They also express a reporter cassette, of mCherry, GFP and/or luciferase, permitting live infection readouts by microscopy and FACS, without the need to fix and stain parasites [360]. Assay optimisation in this chapter uses *PbPfTRAP* parasites, which express an additional copy of *P. falciparum* TRAP under the UIS4 promotor, plus the GFP/luciferase cassette.

Unlike *in vitro* assays of blood stage malaria, where parasites can be maintained in continuous culture [467], liver-stage parasites undergo a single transformation from sporozoites to

merozoites. Sporozoites therefore cannot be sustained in continuous culture. The continuous culture of merozoites made possible the blood stage *in vitro* parasite growth inhibition assay (GIA), which acts as a useful pre-clinical tool with a correlation between antibody levels to some antigens and parasite growth inhibition [468, 469].

For a liver stage parasite *in vitro* assay, each experiment requires generation of sporozoites in the mosquito vector, followed by subsequent salivary gland dissection and immediate use of sporozoites. Current human, pre-erythrocytic *in vitro* assays measure antibody inhibition of sporozoite invasion (ISI) [181] and of liver stage development (ILSDA) [470]. Reduced sensitivity, standardisation and reproducibility of these assays and the reliance on a microscopy readout with immunostaining have limited their use (reviewed in [471]). These assays use *P. falciparum* sporozoites, so high numbers of sporozoites are required for each assay replicate. The risk assessment of this pathogen requires handling under Containment Level 3 conditions, adding a further level of complexity to the dissection, infection and culture protocols. Finally, the isolation and culture of primary hepatocytes requires extensive expertise, or significant costs if purchased commercially. Thus the adaptation of the human ISI or ILSDA protocols into one which assesses the inhibitory effects of T cells, requires much development and extension, with the careful consideration of several key factors.

Several murine models are reported to assess *in vitro* lymphocyte activity against hepatocytes infected with *Plasmodium* species. Two groups in the late 1980's and early 1990's developed protocols that used splenocytes from mice vaccinated with irradiated sporozoites and with synthetic *P. yoelii* CS peptide [133, 204, 472]. One such protocol cultured mouse splenocytes with peptide and IL-2, to expand the antigen-specific population of T cells for evaluation [473]. All studies measured positive inhibition of liver-stage parasites following fixation, staining and manual counting. More recently splenic and intrahepatic CD8⁺ T cells from mice vaccinated using *P. yoelii* genetically attenuated parasites were evaluated by *in vitro* live cell imaging, apoptosis assays and indirect immunofluorescence staining. The study concluded sterile protection in mice was completely dependent on CD8⁺ T lymphocytes [474].

A recent model developed at the Jenner Institute uses a murine hepatoma cell line Hepa1-6 and GFP-expressing *P. berghei* sporozoites to study liver stage T cell immunity in mice. Percentage reduction of infected hepatoma cells was measured after addition of CD8⁺ splenocytes from ChAd63.MVA *P. berghei* TRAP vaccinated or control (ChAd63.MVA luciferase) vaccinated mice. Liver stage parasites were inhibited in an E:T- (effector to target cell ratio) dependent manner [363]. The nature of this assay permitted MHC-matching of C57BL/6 mouse splenocytes with that of the C57L hepatoma Hepa1-6 cells. Unfortunately, development of an equivalent *in vitro* assay using human T cells will not support the same degree of HLA-matching between T cells and hepatocytes, due to the vast polymorphism in human HLA and the relatively restricted primary hepatocytes and human hepatoma cell lines available. T cells respond to foreign, mis-matched MHC proteins in a similar way to self MHC proteins that are bound to foreign peptides, and therefore matching of HLA, and degree of HLA-mismatch, is a key consideration for the development of a human parasite inhibition assay.

The development of an *in vitro* assay to assess human vaccine-induced, cellular responses relies on the processing and presentation of sporozoite antigen MHC class I epitopes being presented on the surface of infected hepatocytes [188]. Two-6 hours following Hep-G2 infection by *P. berghei* sporozoites, CSP is measured in the hepatocyte cytoplasm and subsequently becomes associated with the outer rim of the hepatocyte nuclear envelope. By 12-24 hours post-infection, this perinuclear distribution of CSP is highly pronounced [475]. While CSP is detected in the host cytoplasm, it is not known how it traverses the parasitophorous vacuole membrane, as it is understood that this membrane is impermeable to macromolecules the size of CSP [476]. CSP translocates to the hepatocyte nucleus, where it is responsible for downregulated expression of the NF- κ B transcription factor, thus downregulating several genes that would be involved in the hepatocyte response to infection [106]. During this translocation via the hepatocyte cytoplasm, the CSP is susceptible to degradation by proteasomes, and can be presented on the hepatocyte surface. Following *in vitro* infection with *P. berghei*, mouse primary hepatocytes have been shown to process and present CSP peptides in a proteasome dependent manner. Furthermore, sporozoite- traversed in addition to sporozoite- infected cells were able present antigen in this manner [189].

It is widely believed that during human liver stage infection, parasite proteins are degraded by host proteasomes, and loaded into the endoplasmic reticulum lumen by TAP1 and TAP2. These parasite peptides then associate with MHC class I molecules, and are transported to the hepatocyte surface [477]. Similarly to CSP, TRAP is expressed during the sporozoite and early liver stage of infection [478], and is shed during the process of sporozoite gliding [479]. It is therefore hypothesised that TRAP peptides can also be presented via MHC class I on the infected hepatocyte surface.

The aim here is to develop a protocol that can be used to quantify liver stage parasite inhibition by human T cells, using human hepatoma cells, T cells from ChAd63.MVA ME-TRAP vaccinated volunteers, and transgenic *P. berghei* parasites expressing the *P. falciparum* TRAP antigen, in addition to the *P.berghei* homologue. The assay requires a medium- to high-throughput readout that can accurately measure antigen-specific parasite inhibition, for assessment of multiple volunteers with sufficient technical replicates. This chapter reports the series of experiments that led to the development of a finalised assay protocol for the evaluation of vaccinee T cells, described in Chapter 6.

5.2 Results

5.2.1 Optimisation of hepatoma cell infection.

5.2.1.1 Hepatocellular carcinoma cell lines

Human hepatocellular carcinoma lines, more commonly termed human hepatoma cell lines, Huh7 (kindly provided by Shahid Khan, University of Leiden), PLC/PRF/5 (MR4, BEI Resources Depository, NIAID, NIH), HC-04 (MR4, BEI Resources Depository, NIAID, NIH) and HepG2 (European Collection of Cell Cultures (ECACC)) were HLA typed to confirm the HLA serotypes previously reported [480, 481]. DNA was extracted from the hepatoma cell lines and HLA typed by PCR. HepG2 and HC-04 cells are serologically identical to one another at all HLA loci, perhaps because the latter was derived accidentally from the former by passage. Huh7 cells are homozygous at all HLA loci (Table 14).

Table 14 Summary of HLA serotype of commercially available hepatoma cell lines

HLA serotype inferred by PCR analysis of hepatoma cell line DNA. Hepatoma DNA extraction performed by C. Bliss, and PCR analysis performed by Transplant Immunology and Immunogenetics laboratory, Churchill Hospital, Oxford.

Cell Line	HLA Class I						HLA Class II					
	A	A	B	B	Cw	Cw	DR	DR	DR	DR	DQ	DQ
Huh7	11	11	54	54	1	1	8	8			6	6
PLC/PRF/5	33	3	42	53	4	17	8	13	52	52	7	6
HC04	24	2	51	35	16	4	13	16	51	52	7	6
HepG2	2	24	51	35	16	4	13	16	51	52	7	6

For optimal infection, it is generally accepted that cell lines need to be approximately 60-70% confluent at the point of sporozoite infection [482], however division of hepatoma cells continues post-infection, impacting the infection percentage measured at different time points following infection. In order to assess how quickly these cell lines divide, HC-04 and Huh7 cells were plated in standard 96 well plates at 3 seeding densities and cell numbers measured at several time points following plating. For the first 8-12 hours, cell division was slow, due to the hepatoma cells recovering from the trypsin treatment and adhering to the plate. By 24 hours the cells were dividing, and continued dividing at a similar rate for the duration of the time course, with cell numbers

doubling in the 24-40 hour period for HC-04, and 24-56 hours for Huh7 (Figure 5.1). At 72 hours post plating, HC-04 cells seeded at 50,000 and 70,000 cells per well had similar total cell numbers, indicating that once 100% confluency is achieved, division rate may begin to slow due to spatial restrictions.

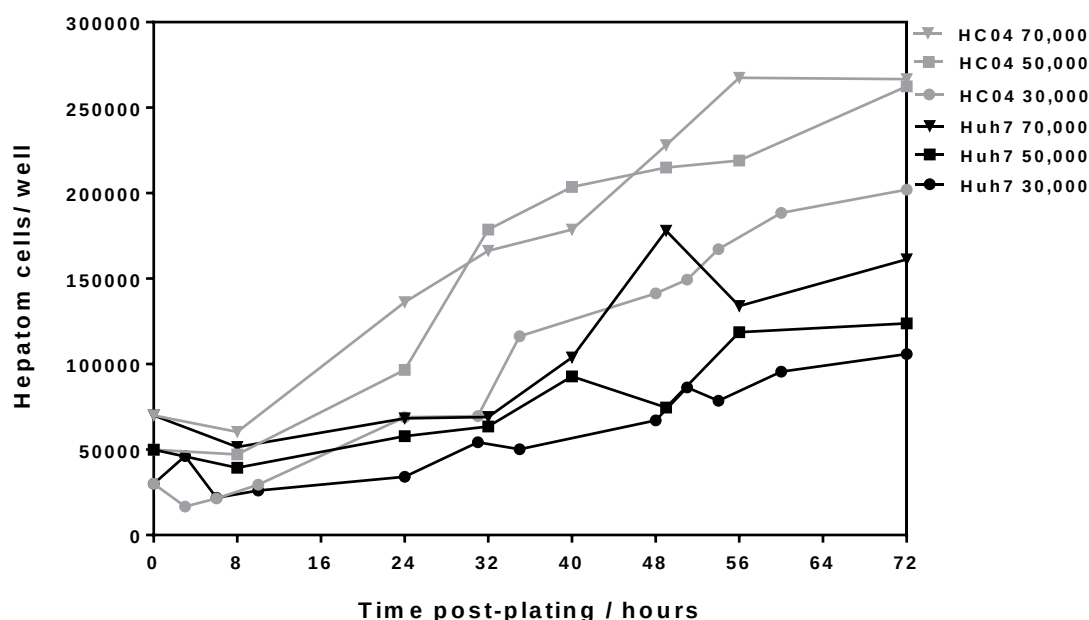


Figure 5.1 Time course of HC-04 and Huh7 cell division rate.

HC-04 and Huh7 cells were plated at 3 different seeding densities in 96 well plates. Cells were detached at multiple time points up to 72 hours using trypsin digest and counted immediately using Trypan blue exclusion dye. Mean live cell number from 6 wells plotted per time point.

In later experiments, the use of half surface area plates was explored as an alternative to standard 96 wells plates. This enabled fewer hepatoma cells to be plated to achieve the correct level of confluency and required the addition of fewer sporozoites per well to achieve the same percentage infection. This therefore increased the throughput of the assay and allowed for higher E:T values to be achieved upon the addition of vaccinee T cells.

5.2.2 Hepatoma cell infection with transgenic *P. berghei*

In order to be able to assess TRAP-specific parasite inhibition from ChAd63.MVA ME-TRAP vaccinees, a transgenic *P. berghei* expressing an addition copy of *P. falciparum* TRAP (*PbPfTRAP*) was

identified as suitable for use within the assay. Infectivity of this parasite was measured in 4 human hepatoma lines (HC-04, HepG2, Huh7 and PLC/PRF/5) and 1 mouse hepatoma line (Hepa1-6). Hepatoma cells were plated in half surface area 96 wells plates and infected with the *PbPf*TRAP sporozoites 25 hours later, once the hepatoma cells had fully attached to the plate. Reduced monolayer detachment had previously been observed when hepatoma cells were infected at a slightly higher confluency than advised, at ~80-90%. This also reduced the special capacity for further cell line division in the period following infection, which affects the percentage infection calculated.

Intactness of the monolayer was evaluated 24 hours post infection (HPI). The HepG2 monolayer was poorly maintained, with the formation of clumps particularly apparent. A degree of monolayer loss was also seen in the Huh7 line. HC-04 and PLC/PRF/5 cells maintained an intact monolayer following infection. The mouse Hepa1-6 cells appeared healthy and were simply seeded too sparsely to form a complete monolayer (Figure 5.2).

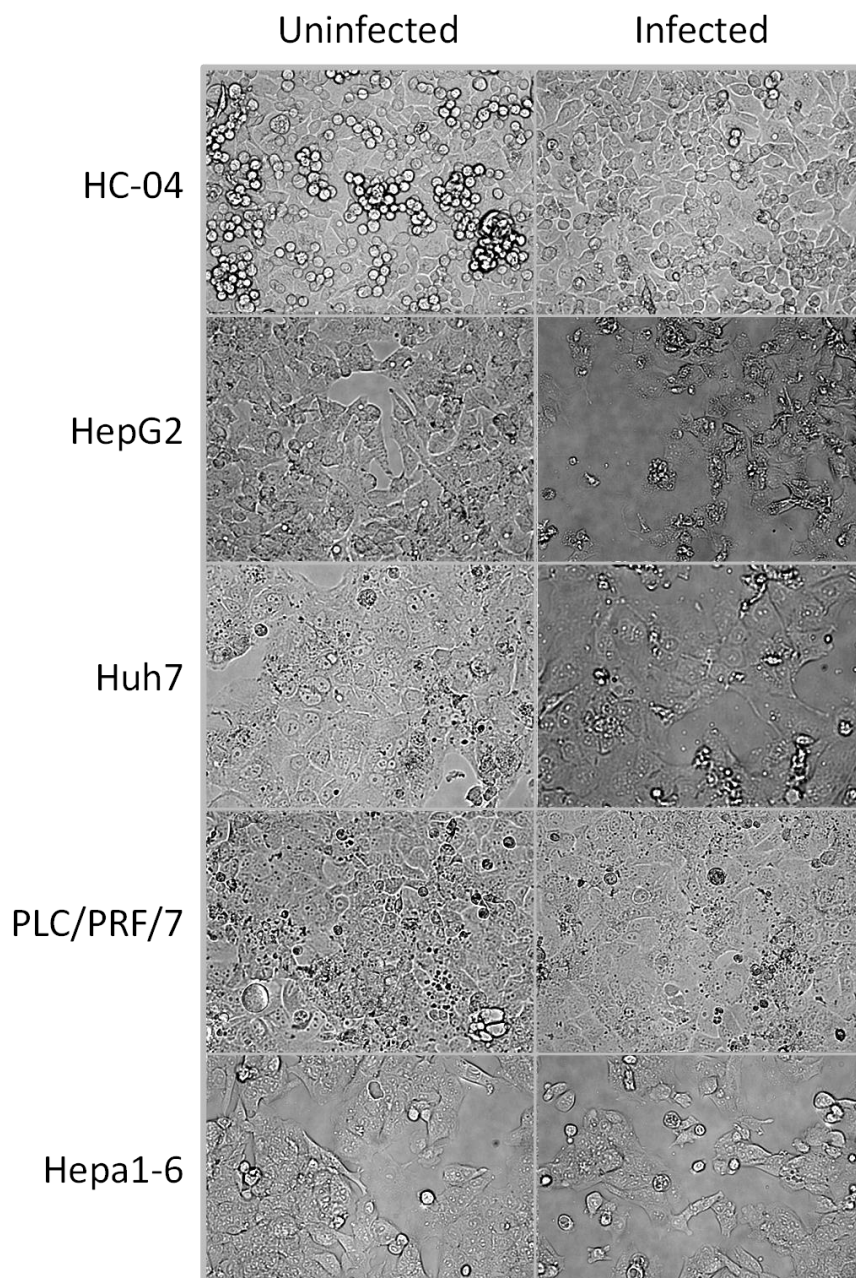


Figure 5.2 Representative images of cell line monolayers.

Cell lines plated at 20,000/well, 25 hours prior to infection with 11,000 *PbPf*TRAP sporozoites. Images taken 24 hours following infection with *PbPf*TRAP sporozoites. Uninfected well images taken at a comparable time point. x20 magnification.

As each cell line had different reported rates of infectivity (in addition to sourcing from different groups and suppliers), it was important to compare the infectivity parameters for the 5 cell lines. An optimal sporozoite infection ratio of 1:3 to 1:5 is reported in the literature [482], which with an estimated 30,000 hepatoma cells at the point of infection, would equate to 6,000-10,000 sporozoites per well. Pilot testing indicated better results at the upper end of this ratio, so sporozoite inoculation numbers were tested at 11,000 and 22,000 sporozoites per well, in order to

explore the effects of increasing the ratio. Clearly the more sporozoites used in the infection, the more liver cells become infected. This is true for both percentage infection and number of infected cells measured (Figure 5.3 A+B, 2-way ANOVA). While the HepG2 line appears to support the highest level of infection, the counts data revealed that a lower number of cells were infected compared to the HC-04 and PLC/PRF/5 lines. However, this frequency is artificially elevated by the loss of hepatoma cell monolayer and subsequent low number of cells acquired overall (Figure 5.3 D, 2-way ANOVA). The Huh7 cells maintain a monolayer, but have a low infection frequency and low cell recovery, indicating that cells may specifically die off once infected.

As previously described with GFP-expressing parasites, GFP fluorescence intensity is a measure of the extent of parasite development within liver cells [483], and is an effective measure of parasite copy number, particularly at later time points [482, 484]. Despite the *PbPf*TRAP parasite species being native to rodents, the mean fluorescence intensity of the parasite growth at 24 hours post infection was lowest in the mouse Hepa1-6 cell line (Figure 5.3 G, $p=0.0017$ Kruskal-Wallis test).

These data indicate the use of HC-04 or PLC/PRF/5 cells for the preferred infection protocol, as these lines support significantly higher numbers of infected cells which would be targets for vaccinee T cells (Figure 5.3 F, $p=0.0020$ Kruskal-Wallis test). The data suggests inoculation using 11,000 sporozoites per well is preferable over 22,000, as this achieves good infectivity without high hepatoma cell loss through cell death or monolayer detachment, particularly in the 2 identified preferred cell lines (Figure 5.3 H). This is consistent with published data, which suggests that an increased sporozoite to hepatocyte ratio results in greater cell death from increased cell traversal, and the inability of some cells to reseal their membrane after they have been traversed [36].

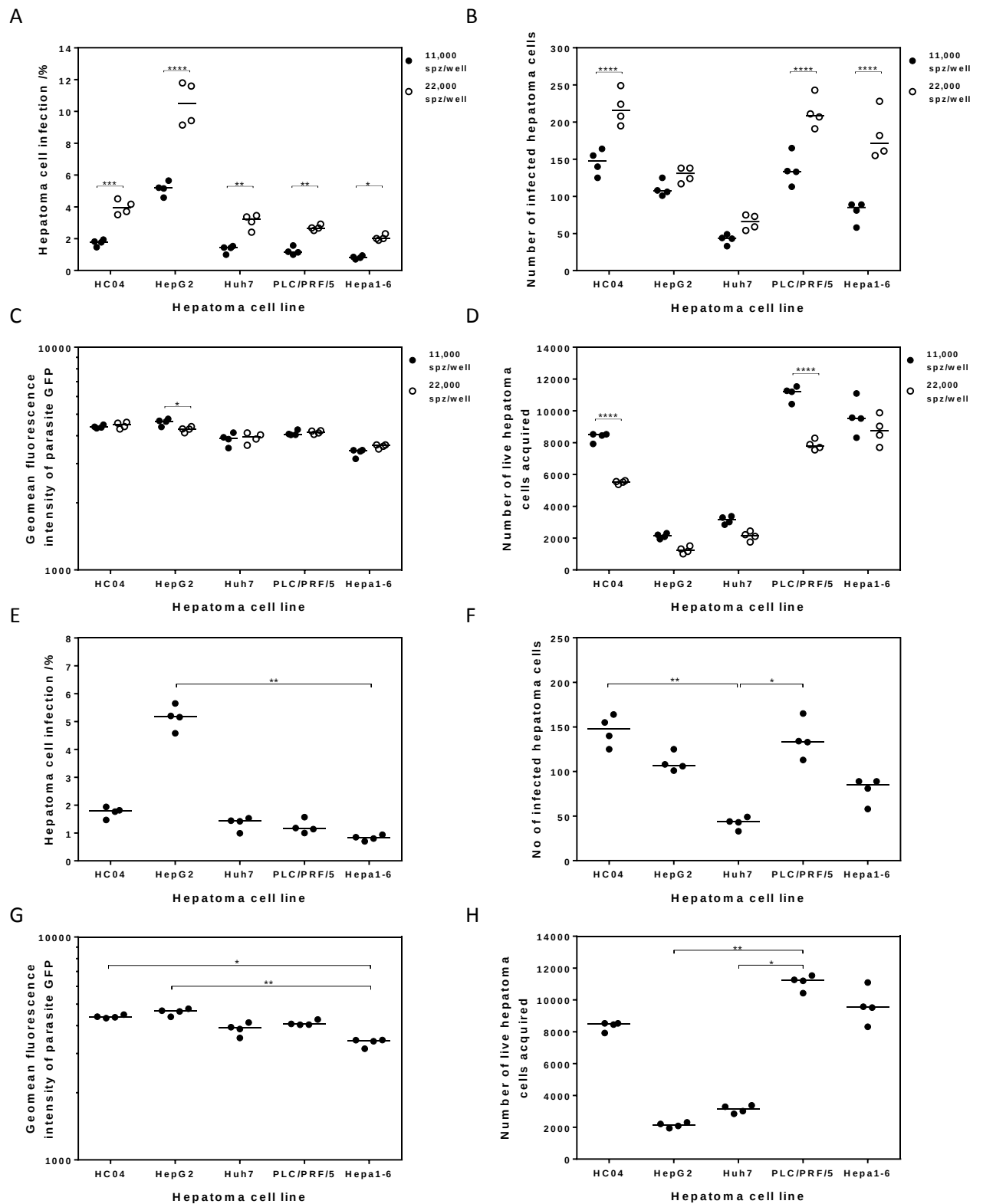


Figure 5.3 Infection summary for 5 hepatoma cell lines

20,000/well hepatoma cells were plated in half surface area 96 well plates. 25 hours later, 11,000 or 22,000 *PbPf*TRAP sporozoites were added per well and incubated for 30 minutes at room temperature and 2.5 hours at 37°C. Sporozoites were removed from the plates, and hepatoma cells washed in fresh media. Plates were left to incubate at 37°C for a further 22 hours, before removal of hepatoma cells by trypsin digest and acquisition by FACS. A+E) Percentage infectivity. B+F) Number of infected hepatoma cells. C+G) Geometric mean fluorescence intensity of parasite GFP. D+H) Number of live hepatoma cells acquired. A-D) 2-way ANOVA of 11,000 vs 22,000 sporozoites per well. E-H) Kruskal-Wallis test with Dunn's correction for multiple comparisons between all cell lines infected with 100,000 sporozoites per well. * $p < 0.05$. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Medians displayed.

5.2.2.1 *Infection parameters*

Previously published protocols advise a 3 hour infection period [363], with a brief incubation at room temperature prior to at 37°C. This is believed to mimic the inoculation of sporozoites into the dermis, whereby the ambient temperature around the sporozoites may not instantaneously rise to the core body temperature of 37°C.

Briefly, sporozoites were added to the plated hepatoma cells and centrifuged to increase sporozoite contact with the hepatoma cell monolayer. This is followed by a 20-30 minute incubation at room temperature and 2.5 hour incubation at 37°C prior to changing cell media and thus removing any excess sporozoites and potential bacterial contaminants coming from the mosquito dissection. Reducing the sporozoite infectivity time would permit an extended duration for T cell-mediated parasite inhibition, due to the necessity to add T cells separately to sporozoites, the finite liver stage period of *P. berghei* and the associated time restrictions of the assay readout. Reducing the infection time to under 3 hours was therefore assessed in HC-04 cells, whereby the incubation at room temperature was reduced to 20 minutes and incubation at 37°C was reduced to 40 minutes, totalling a 1 hour infection period before sporozoite removal. A trend towards reduced infectivity was measured with a 1 hour infection period compared to three hours (Figure 5.4 A-C, Mann-Whitney test). Conversely, to determine whether infectivity could be improved by increased sporozoite-hepatoma cell contact time, the 37°C incubation duration was increased to 26 hours, but this did not increase the frequency or number of infected hepatoma cells (Figure 5.4 D-F Mann-Whitney test). This extended infection strategy does not permit the additional washing steps that reduces bacterial contamination or removal of exogenous DiD membrane dye, described later. Furthermore, free-floating sporozoites that have failed to infect may induce innate activation or antigen non-specific activation of T cells. Therefore the 3 hour infection, consisting 30 minutes at room temperature and 2.5 hours at 37°C, was chosen as the optimal infection period for this assay, and was used in all subsequent experiments.

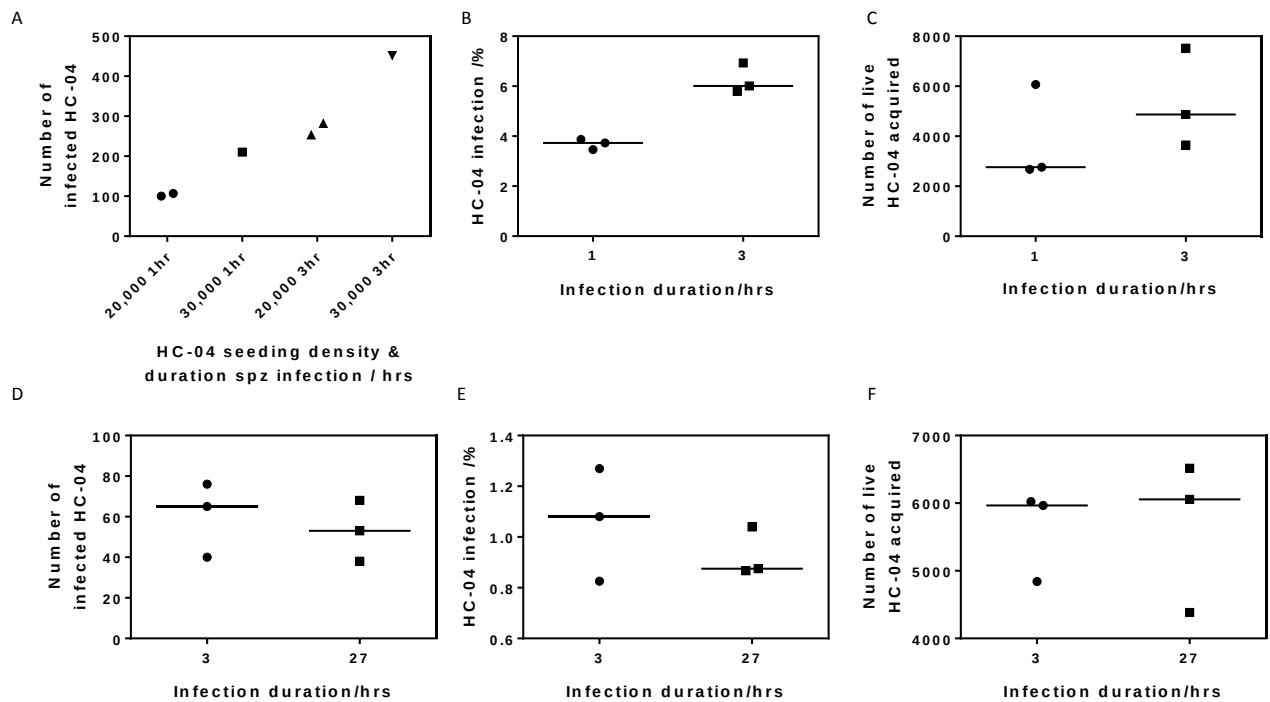


Figure 5.4 Sporozoite infection time summary

A-C) 20,000 or 30,000/well hepatoma cells were plated in 96 well plates. 15 hours later, 20,000 *PbPf*TRAP sporozoites were added per well and incubated according to 1 hour or 3 hour infection protocol, before sporozoites were removed from the plates, and hepatoma cells washed in fresh media. Plates were left to incubate at 37°C to 24 hours post initial infection, before removal of hepatoma cells by trypsin digest and acquisition by FACS. D-F) 30,000/well hepatoma cells were plated in 96 well plates. 12 hours later, 20,000 *PbPf*TRAP sporozoites were added per well and incubated for 30 minutes at room temperature and 2.5 hours at 37°C, when the “3 hour” sporozoites were removed from the plates, and hepatoma cells washed in fresh media. Plates were left to incubate at 37°C for a further 24 hours, before removal of hepatoma cells by trypsin digest and acquisition by FACS. All comparisons tested by Mann-Whitney test were non-significant.

Seeding density of hepatoma cells and timing of infection post-hepatoma cell plating were also assessed. Cells plated one day before infection (day -1) had a more intact cell monolayer post-infection than those plated on the day of infection (day 0), improving hepatoma cell recovery after 24 hour incubation. Percentage infection was higher when hepatoma cells were plated at lower seeding densities (Figure 5.5 A+D, Kruskal-Wallis test), although a higher seeding density supported a larger number of infected cells, despite the same number of infectious sporozoites added per well (Figure 5.5 B+E, Kruskal-Wallis test). When infected with 2×10^4 sporozoites, the most optimal hepatoma cell seeding densities were 20,000-30,000 HC-04 plated at day -1, with live HC-04 recovery poor when plated at day 0 (Figure 5.5 C+F, Kruskal-Wallis test).

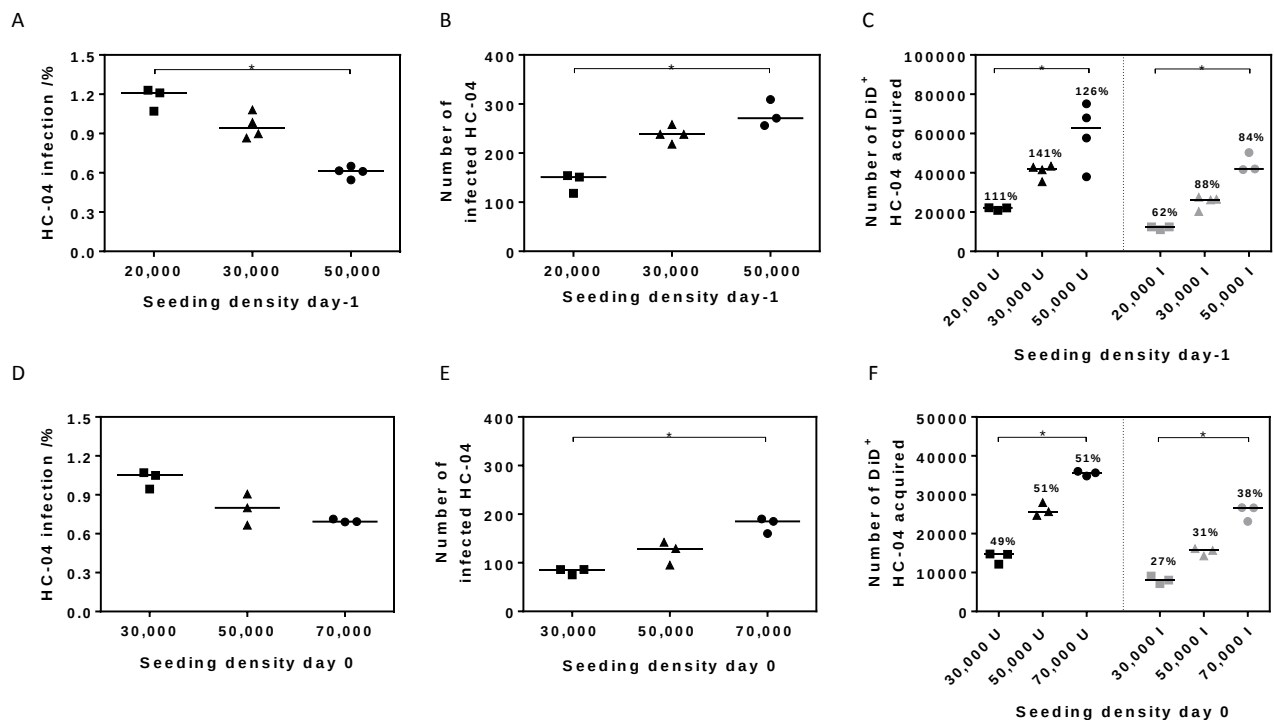


Figure 5.5 HC-04 seeding density

A-C) Day -1 and D-F) day 0 plating of 3 HC-04 cell seeding densities in standard 96 well plates was assessed by percentage infectivity, number of infected cells and recovery of live HC-04 cells at 24 hours post-infection with 20,000 *PbPf*TRAP sporozoites per well. Percentages represent recovery of original number of HC-04 cells plated. U = uninfected, I = infected. Kruskal-Wallis test with Dunn's correction for multiple comparisons. * $p < 0.05$.

5.2.2.2 Antibiotics

Sporozoite preparations dissected from the salivary glands of infected mosquitoes are prone to bacterial contamination. While this can be overcome by the addition of antibiotics to the dissection and culture media, it was important to determine the anti-parasitic effects of their use. Increasing concentrations of penicillin and streptomycin in the dissection media resulted in a reduced parasite infection at 24 hours post-infection (Figure 5.6, $p < 0.0003$, Kruskal-Wallis test). 1% penicillin and streptomycin were sufficient to eliminate bacterial contamination during the assay incubation period of multiple independent assays. This was tested by inoculation of lysogeny broth with 20 μ L of supernatant from hepatoma cells cultured with and without sporozoites and PBMCs. Inoculated broths were left to culture at 37°C for 72 hours in a shaking incubator. Initial sporozoite dissection

suspension and the 3 hour post-infection supernatant induced bacterial growth when in lysogeny broth (Table 15). Bacterial populations were identified by Gram staining as Gram negative (data not shown) and therefore the choice of antibiotic cocktail of penicillin and streptomycin was deemed suitable for preventing bacterial contamination.

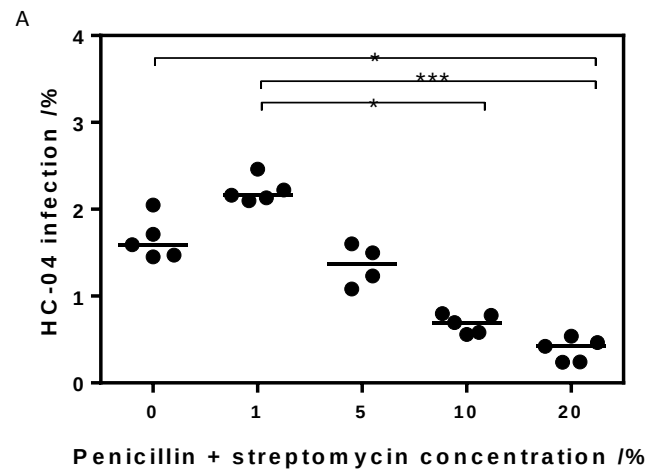


Figure 5.6 Summary of sporozoite infection with increasing titrations of antibiotic cocktail in dissection media.

Dissected *PbPf*TRAP sporozoites were incubated in antibiotic-containing dissection media for 20 minutes prior to infection of HC-04 cells. After a 3 hour infection, dissection media was removed and RPMI media containing 1% penicillin and streptomycin plus 10% foetal calf serum (FCS) used to wash the hepatoma cells, then incubate the hepatoma cells for a further 21 hours. Kruskal-Wallis test with Dunn's correction for comparisons between multiple concentrations, * $p < 0.05$, *** $p < 0.001$. Medians displayed.

Table 15 Summary of antibiotic effectiveness

Detection of bacterial growth in lysogeny broth, following broth inoculation with sporozoite suspension and hepatoma cell supernatant taken at 3 and 24 hours post-infection.

Supernatant	Bacterial culture (-/+)
Lysogeny broth + 1% penicillin and streptomycin	-
Lysogeny broth+ sporozoites in 1% penicillin and streptomycin	+
Lysogeny broth + supernatant at 3 hours from uninfected hepatoma cells	-
Lysogeny broth + supernatant at 3 hours from transgenic <i>P. berghei</i> -infected hepatoma cells	+
Lysogeny broth + supernatant at 24 hours from uninfected hepatoma cells	-
Lysogeny broth + supernatant at 24 hours from transgenic <i>P. berghei</i> -infected hepatoma cells	-
Lysogeny broth + supernatant at 24 hours from transgenic <i>P. berghei</i> -infected hepatoma cells + vaccinee PBMC	-

5.2.2.3 Dissection media

Use of different dissection media has previously been reported to affect sporozoite gliding motility and longevity of sporozoites post-dissection [485]. To achieve sufficient numbers of sporozoites for the assay, large numbers of mosquitoes need to be dissected, and therefore sporozoites are kept on ice for a number of hours prior to hepatocyte infection. Three dissection buffers were tested to determine the most appropriate buffer for use during these long mosquito dissection periods. PBS, RPMI, DMEM and heat-inactivated human and mouse serum have all been used for salivary gland dissection [181, 184, 460, 461, 463, 482], however in recent years, the use of insect media has become more popular [485]. In this assay, Grace's insect media with a 1% BSA supplement gave rise to significantly higher sporozoite infectivity (Figure 5.7 A+B, $p < 0.0001$ Kruskal-Wallis test) and had the least measurable effect on parasite fitness, as measured by schizont size inferred from parasite geometric mean fluorescence intensity (gMFI) (Figure 5.7 C, $p < 0.05$, Kruskal-Wallis test), when compared with RPMI and PBS. Despite this being an insect media, the infection

preparations appear to be sufficiently diluted out in cell culture media (RMPI + 1% penicillin and streptomycin and 10% FCS) upon dilution before infection, so as not to significantly affect hepatoma cell recovery following infection (Figure 5.7 D, $p < 0.01$, Kruskal-Wallis test).

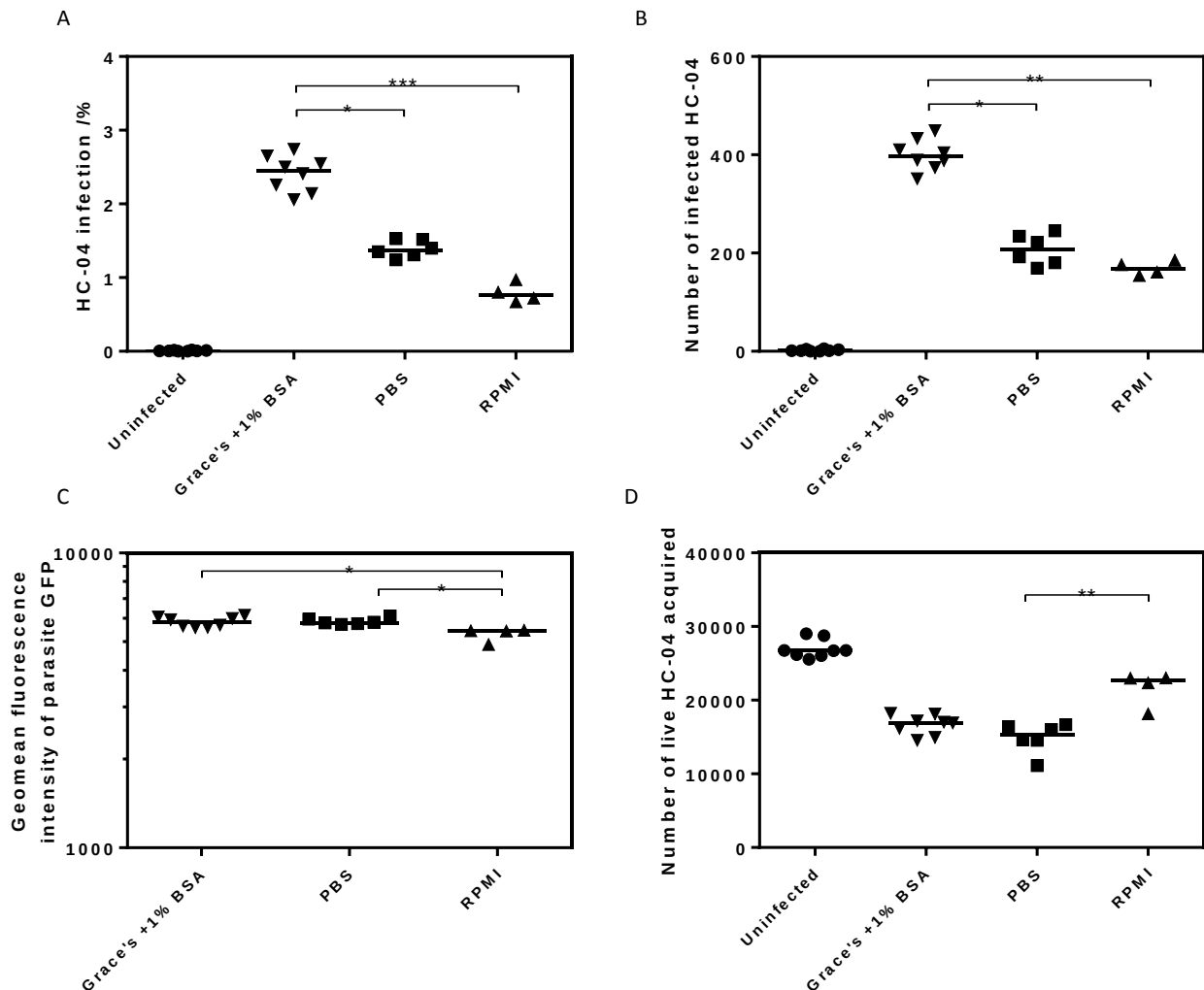


Figure 5.7 Effect of dissection media of sporozoite infectivity.

20,000/well of HC-04 cells were plated in half surface area 96 well plates and infected 26 hours later with 12,000 *PbPf*TRAP sporozoites dissected into either Grace's media + 1% BSA, RPMI media or PBS, all containing 1% penicillin and streptomycin. 25 hours later, hepatoma cells were removed from plates using trypsin digest and acquired by FACS. A) Percentage infection, B) number of infected hepatoma cells, C) geometric mean fluorescence intensity of parasite GFP and D) live hepatoma cell recovery was measured. Kruskal-Wallis test with Dunn's correction for multiple comparisons between conditions. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Medians displayed.

5.2.2.4 Fluorescence-Activated Cell Sorting experimental readout

5.2.2.5 Staining of hepatoma cells

For an accurate readout of percentage parasite infection, it is important to accurately discriminate between hepatocytes and lymphocytes. Lipid-binding dye, Vybrant DiD (Life Technologies) was used to stain the phospholipid bilayer of HC-04 cells, and visualised by FACS over time. As hepatoma cells continue to divide in cultures and DiD is a membrane bound, it was important to establish if the dye would be detectable on hepatoma cells after culture. When used on HC-04 cells, stained and non-stained cells could be easily differentiated by flow cytometry, detecting DiD fluorescence in the Allophycocyanin (APC) cytometer channel. In a time course experiment, the geometric mean fluorescence intensity reduced by a log during the 48 hour period following staining and plating of 30,000 HC-04 cells, however this did not affect the identification of the DiD⁺ population (Figure 5.8). This rate of DiD loss is consistent with the measured division rate of the HC-04 cell line (Figure 5.1).

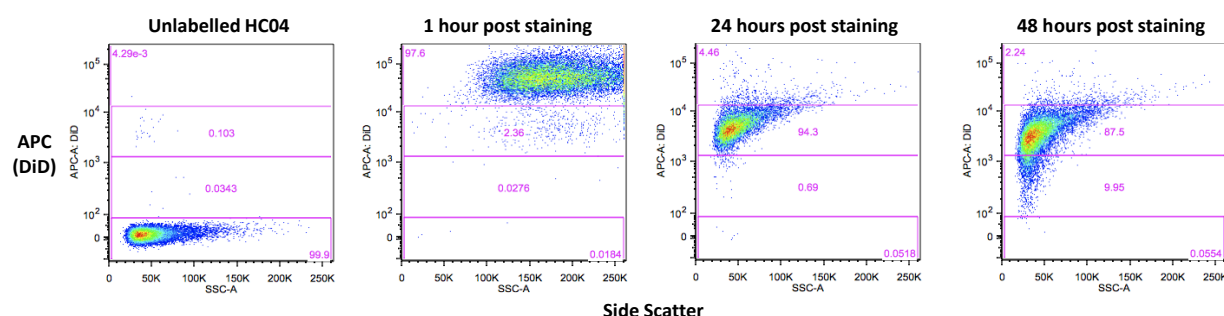


Figure 5.8 DiD hepatoma cell staining time course

HC-04 cells were counted and stained with DiD. Excess dye was washed off and cells plated in 96 well plates. At 1 hour-, 24 hours- and 48 hours- post plating, HC-04 cells were acquired by FACS, and fluorescence intensity of the DiD signal measured.

The inclusion of a GFP-luciferase reporter cassette in the transgenic *PbPfTRAP* parasites permits experimental readout by both fluorescence microscopy and FACS. Autofluorescence is more easily differentiated from GFP positivity when visualised by microscopy, as the autofluorescent signal is considerably weaker than that of GFP⁺ parasites. FACS permits a more quantitative and higher throughput analysis, but the autofluorescent threshold is more difficult to determine. To overcome

this issue, FACS data was acquired with an additional empty channel on the cytometer. Since the autofluorescence signal from hepatoma cells is equally detected in both the GFP channel and the empty channel, in this case phycoerythrin (PE), infected hepatoma cells that are truly GFP⁺ express a higher proportion of GFP to PE signal. A hierarchical gating strategy was used to measure infectivity of DiD stained HC-04 cells, with infectivity determined in the final gate (Figure 5.9).

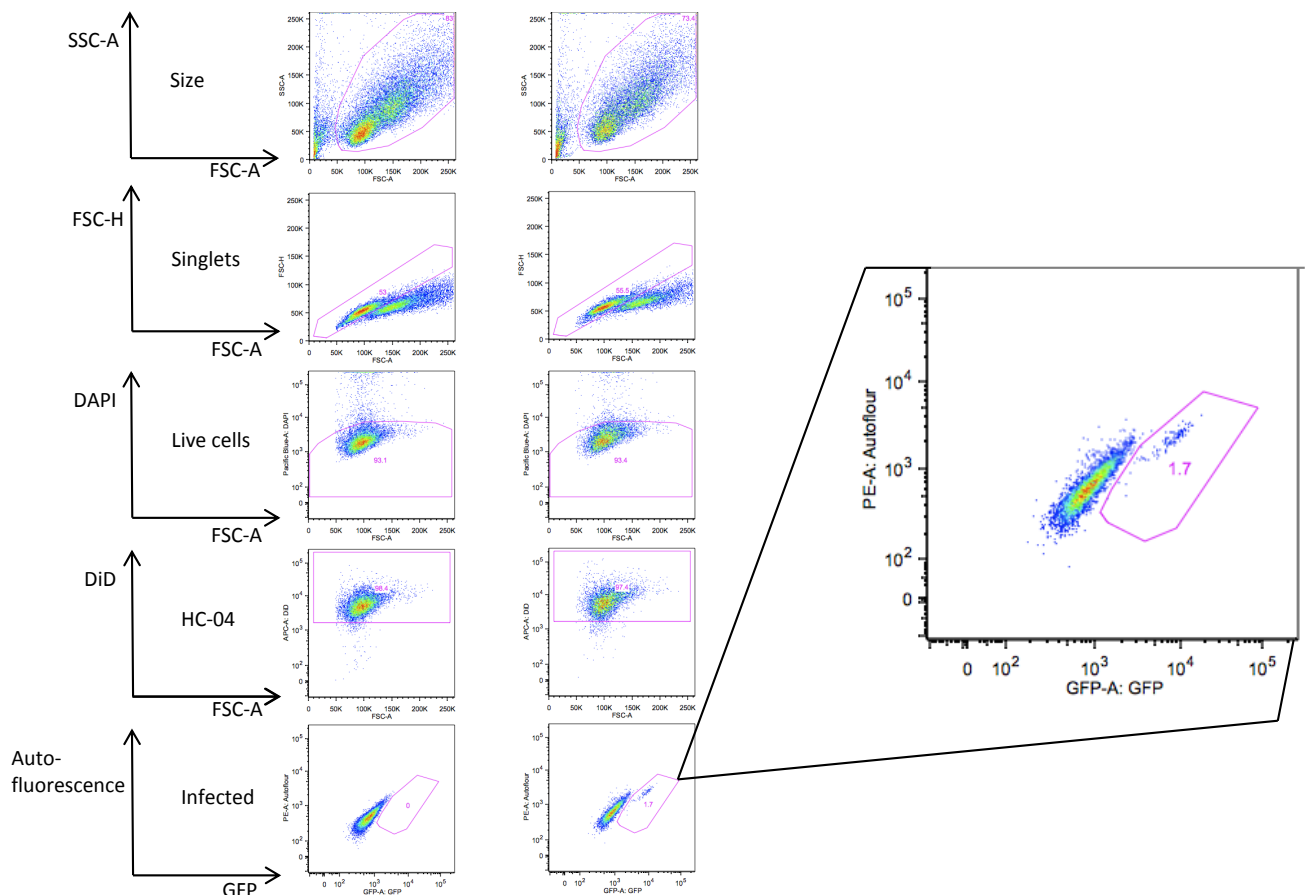


Figure 5.9 Infected hepatoma cell gating strategy using DiD

Hierarchical gating strategy: size and granularity gating of hepatoma cells, positive gating of singlets, dead cell exclusion using DAPI, selection of DiD⁺ HC-04 cells and identification of GFP⁺ (infected) HC-04 cells.

The use of DiD staining in the hepatoma cell infection model was evaluated to ensure the staining did not affect the ability to detect a GFP signal from the parasites. A higher number of HC-04 cells were recovered per well, when unstained HC-04 cells were used, compared with DiD stained cells (Figure 5.10 A, $p=0.0043$ Mann-Whitney test). Infected HC-04 cells have higher DiD fluorescence intensity than uninfected cells from the same well (Figure 5.10 B, $p=0.0286$ Mann-

Whitney test), indicating the hepatoma cells may cease to divide once infected. Importantly DiD did not interfere with the precise gating of infected cells. DiD staining of HC-04 and HepG2 cells did not affect the capacity of sporozoites to infect (data not shown), however Huh7 cells could not be infected with transgenic *PbPfTRAP* parasites when the DiD membrane dye was used prior to sporozoite infection (Figure 5.10 C, $p=0.0034$ Kruskal-Wallis test).

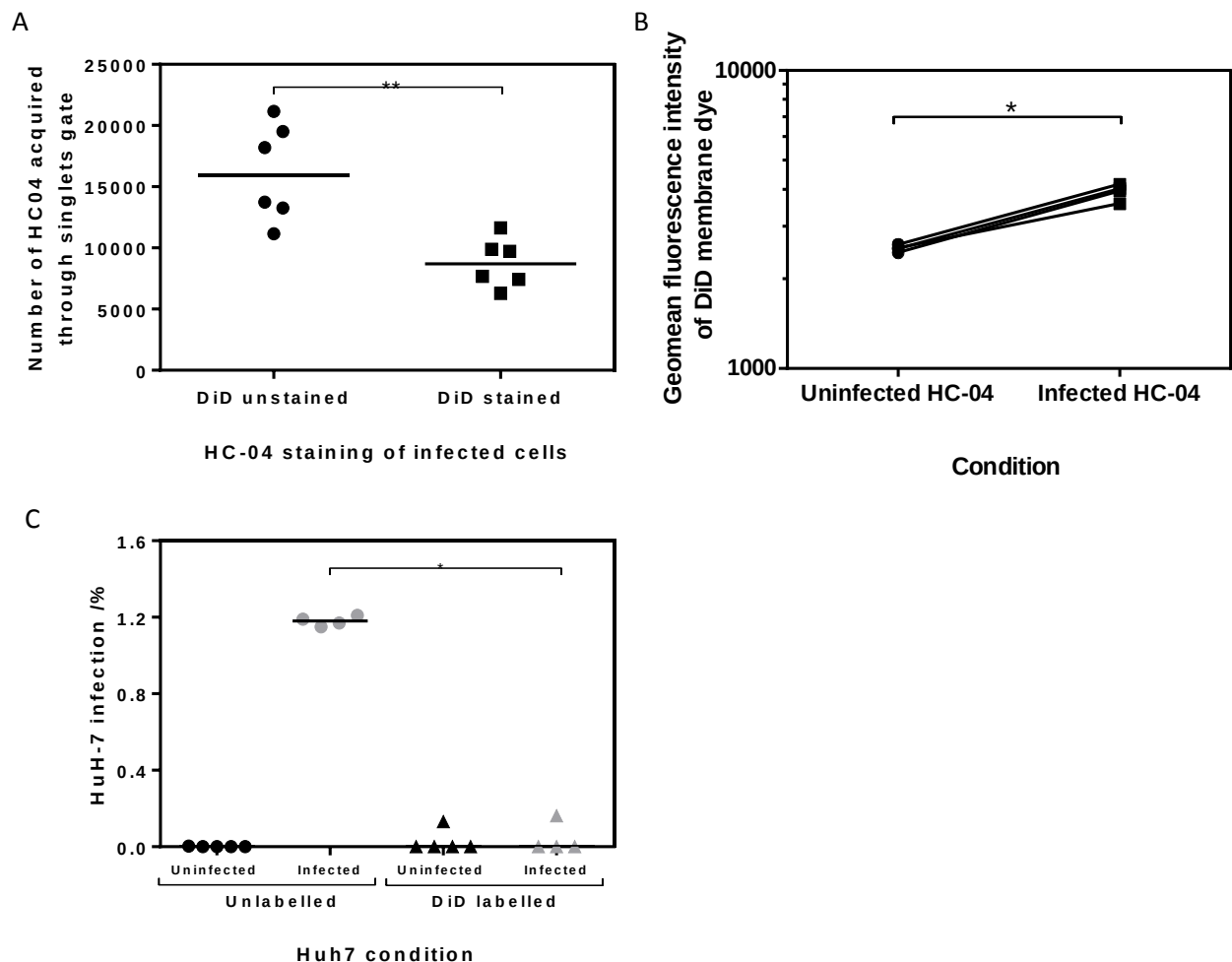


Figure 5.10 Infection summary using DiD membrane dye

A) Number of HC-04 cells acquired and B) geometric mean fluorescence intensity of infected and uninfected HC-04 cells using DiD membrane dye. 30,000/well HC-04 cells were plated in 96 well plates and 20,000 *PbPfTRAP* sporozoites added 5 hours later. Plates were left to incubate at 37°C for 24 hours, before removal of hepatoma cells by trypsin digest and acquisition by FACS. Mann-Whitney tests, * $p<0.05$, ** $p<0.01$. C) Percentage infection following infection of DiD⁻ and DiD⁺ Huh7 cells. 50,000/well Huh7 were plated in 96 well plates and 20,000 *PbPfTRAP* sporozoites added 10 hours later. Plates were left to incubate at 37°C for 24 hours, before removal of hepatoma cells by trypsin digest and acquisition by FACS. * $p<0.05$, Kruskal-Wallis test with Dunn's correction for comparison between multiple groups.

5.2.2.6 Staining of T cells

To circumvent the reduced infectivity and cell recovery associated with hepatoma cell DiD staining prior to infection, a protocol to stain the T cell component of the assay was investigated. In order to accurately differentiate T cells from liver cells in the FACS analysis, a marker present on T cells and not hepatoma cells was required. CD45 antibody binding both the RA and RO isoforms resulted in >99% of cultured T cells identified as CD45⁺ and >99% of HC-04 identified as CD45⁻ (Figure 5.11 A+B). These findings were reproducible across 2 independent experiments. When cultured CD4⁺, CD8⁺ pentamer⁻ and CD8⁺ pentamer⁺ cells and non-cultured NK cells were not gated out through the size, singlet or live gate, the CD45⁻ gate removed these cells from the analysis. (Figure 5.11 C-F). This allowed for the accurate calculation of percentage infection based on total numbers of HC-04 cells only. The CD45 gate was not required for identifying the number of infected HC-04.

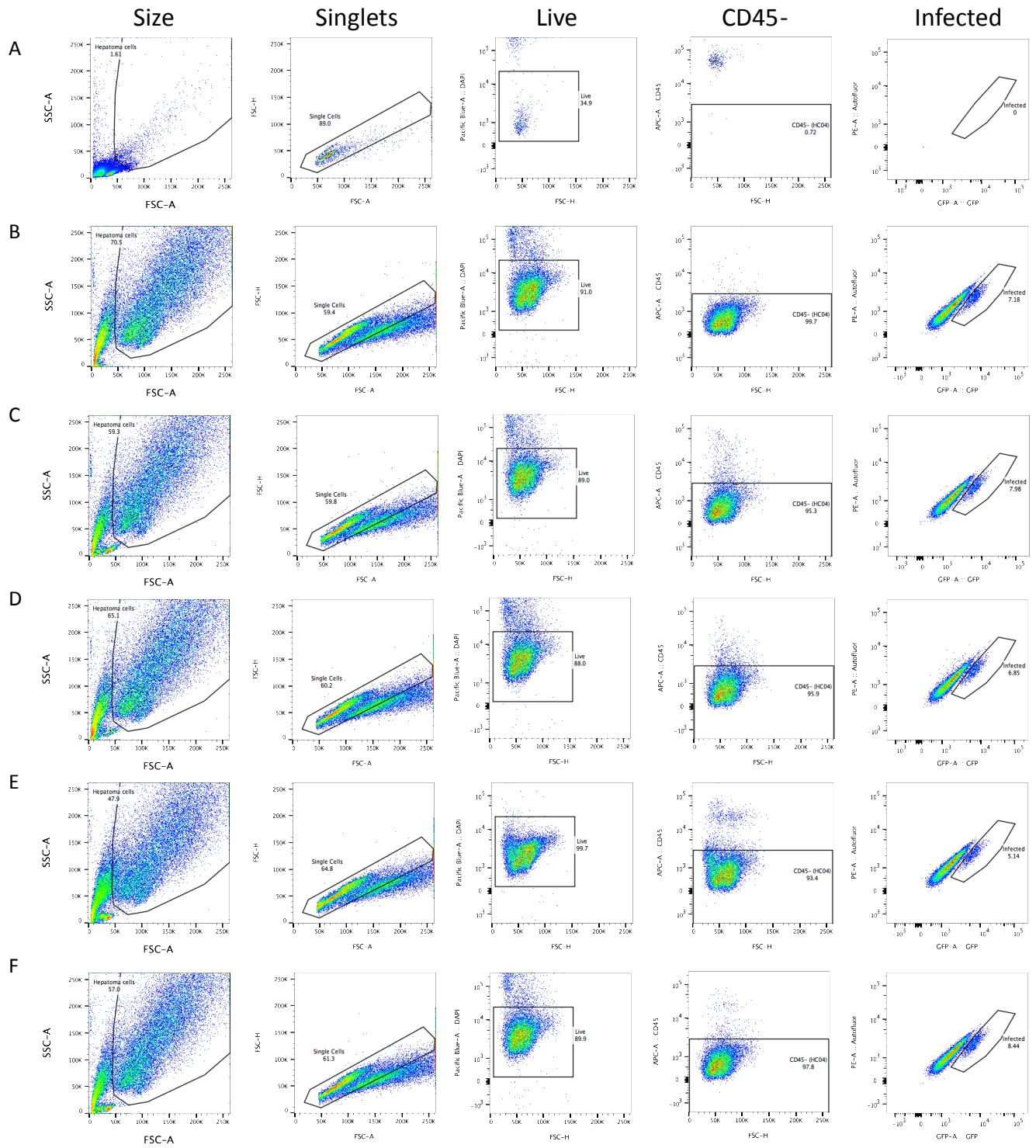


Figure 5.11 Summary of CD45 staining profile

A) CD45 stained CD4⁺ T cells only. B) CD45 stained infected HC-04 only. C) CD45 stained infected HC-04 & cultured CD4⁺ T cells. D) CD45 stained infected HC-04 & cultured CD8⁺ pentamer⁺ T cells. E) CD45 stained infected HC-04 & fresh NK cells. F) CD45 stained infected HC-04 & cultured CD8⁺ pentamer⁻ T cells.

5.2.2.7 Infection time course

In order to determine the optimal time point post-infection for assay readout, an infectivity time course was performed. HC-04 cells were infected for 3 hours with transgenic *PbPfTRAP* and infectivity measured by FACS at 4 time points post-infection. Infectivity peaked between 25 and 31 hours post-infection, followed by a reduction in the frequency of GFP⁺ cells. This reduction in frequency was not mirrored by a reduction in number of infected cells, indicating that the frequency reduces due to the continued cell division of the HC-04 cells, and not due to infected cell death or rupture (Figure 5.12 B). Geometric mean fluorescence intensity of the GFP signal increased with time, consistent with increasing schizont size within liver cells (Figure 5.12 A+C). Previous testing of transgenic *PbPfTRAP* identified that dead parasites do not express GFP (data not shown).

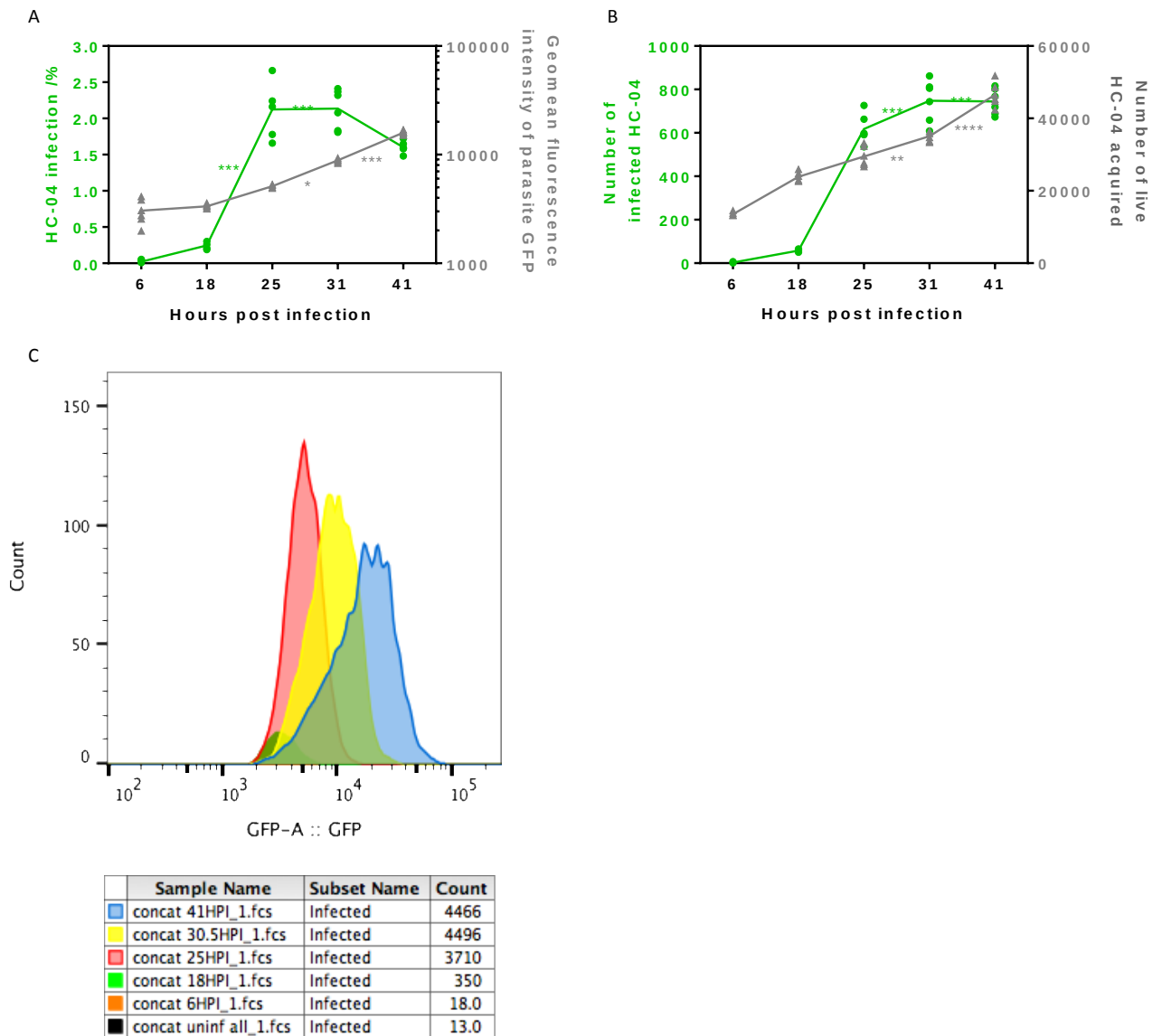


Figure 5.12 Time course of *PbPf*TRAP infection of HC-04 cells.

20,000/well of HC-04 cells were plated in half surface area 96 well plates and infected 22 hours later with 14,500 *PbPf*TRAP sporozoites. At 6, 18, 25, 31 and 41 hours post infection, hepatoma cells were removed from plates using trypsin digest and acquired by FACS. A) Percentage infection (green) and geometric mean fluorescence intensity of GFP signal (grey) and B) number of infected HC-04 (green) and number of live HC-04 (grey) measured at 6-12 hour intervals following sporozoite infection. Kruskal-Wallis tests with Dunn's correction for multiple comparisons between time points, * $p < 0.05$. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. C) Histogram of geometric mean fluorescence intensity of parasite GFP, overlaid by time point.

5.2.3 HLA class I and II

5.2.3.1 HLA class I and II surface expression on hepatoma cells

As surrogates of HLA class I and II, expression of HLA-A2 and HLA-DR were measured on the cell surface of uninfected and infected HC-04 cells, using a fluorochrome-conjugated mouse anti-human HLA-A2 and anti-human HLA-DR antibodies. Isotype controls were used alongside the HLA-specific antibodies, to measure background, non-specific binding to hepatoma cells. Over 99.7% of uninfected and infected HC-04 cells were positive for HLA-A2 expression, with no HLA-DR staining measured over that of the isotype control (Figure 5.13 A). Geometric mean fluorescence intensity was unchanged by hepatoma cell infection, indicating that surface expression of HLA-A2 on a per cell basis was not reduced by *P. berghei* infection. In fact, when compared to uninfected, sporozoite exposed HC-04, HLA-A2 gMFI was significantly higher, indicating upregulation of surface HLA-A2 expression may occur following parasite infection. HLA-A2 results were replicated in a separate experiment using a different HLA-A2 antibody conjugate of the same clone, lacking isotype controls. Similar frequencies of uninfected HepG2 and HC-04 cells were positive for HLA-A2 expression, and <2% of HLA-A11 homozygous Huh7 cells were identified as partially positive, with a HLA-A2 dim phenotype (data not shown). This was consistent with non-specific, background antibody binding, and validated the previous findings of HLA-A2 expression on HC-04 cells.

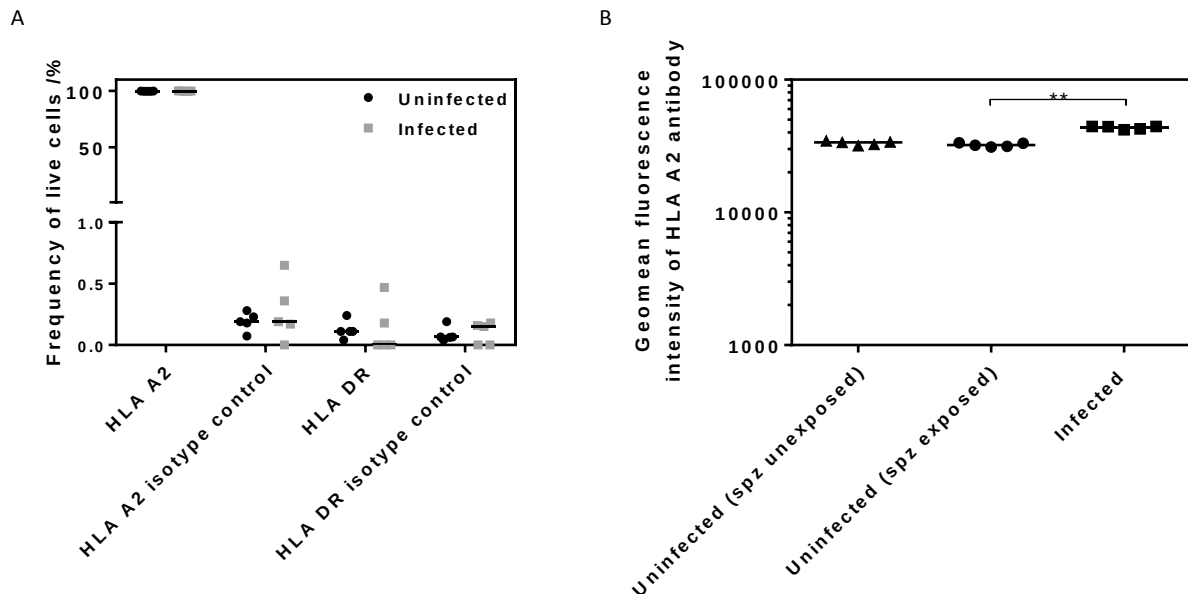


Figure 5.13 HLA-A2 and HLA-DR surface staining of uninfected and infected HC-04.

20,000/well of HC-04 cells were plated in half surface area 96 well plates and infected 22 hours later with 14,500 *PbPf*TRAP sporozoites. At 26 hours post infection, hepatoma cells were removed from plates using trypsin digest and acquired by FACS. A) Frequency of HLA-A2 and HLA-DR positive cells (black) and the matched antibody isotype controls (grey). B) Geometric mean fluorescence intensity of HLA-A2 APC signal. Kruskal-Wallis test with Dunn's correction for multiple comparisons, ** $p < 0.01$

5.2.3.2 Effects of HLA mismatch between human hepatoma cells and human CD8⁺ T cells

Given the inability to match vaccine volunteer to all hepatoma HLA loci, it was important to determine whether level of HLA mismatching had an effect on the assay readout. All clinical trials' volunteers are HLA typed by PCR upon enrolment in the Jenner Institute's malaria vaccine trials. To identify partially HLA-matched unvaccinated controls for assay optimisation, an internal HLA database was set up at the Jenner Institute. Volunteers from within the Institute consented to HLA typing via University of Oxford Occupational Health Services' policy document 1/03, and were subsequently invited to donate blood for use in assay optimisation experiments. All volunteer numbers from this database are prefixed 'int_'. HLA-A2 was the most common HLA-A serotype in both the clinical trials' and the internal typing HLA databases, present in over 60% of volunteers. HLA-A1, -A3, -A11 and -A24 were also prevalent. Thus a partial-HLA match could be made with all 4 human hepatoma lines at a single HLA-A locus by most volunteers. HLA-B*44 was the most common

HLA-B serotype with a frequency of ~30%, however this matched none of the human hepatoma lines available.

The assay relies on MHC class I presentation of parasite antigens, so it is logical to select vaccinee PBMC for testing based on a HLA-A and/or -B and/or -C serotype match with the hepatoma cells. The HLA loci considered most important in organ transplantation are HLA-A, -B and -DR [486], and are usually matched only to the serotype level. Thus, in this setting, HLA-A and -B loci were considered most important loci to match to vaccinee T cells at the serotype level. Furthermore, with more lab reagents commercially available for exploiting HLA-A loci, and with the consideration that HLA-A2 is the most common HLA-A serotype in European populations [487], which matches the HC-04 cell line, partial HLA-matching on HLA-A loci was done in the first instance, with HLA-B loci considered secondarily.

Non-specific killing of uninfected and infected hepatoma cells was assessed with single and double serotype matches at the HLA-A locus from non-vaccinated individuals. A general trend towards reduced HC-04 cell recovery and lower numbers of infected cells was measured when more PBMC were added, however this was no more pronounced in the single-HLA loci match compared to the double (Figure 5.14 A-H, M-P), indicating this is not tightly linked to number of matched HLA loci, at least at the seeding densities measured. This trend was not seen in the percentage infection, likely due to similar losses of both infected and uninfected hepatoma cells. This indicates this HC-04 loss through addition of PBMC is not targeted towards infected cells, with the measured effects largely non-specific. The exception was when very high numbers of PBMC were added per well, with 5×10^5 PBMC causing a significant reduction in recovered HC-04 (Figure 5.14 D).

With 100,000 PBMC determined as the maximum number of cells that could be added per well without inducing high levels of non-specific HC-04 cell death, the problem arose of achieving physiologically relevant effector to target cell ratios for the assay. At the peak time point following ChAd63.MVA ME-TRAP vaccination, the number of antigen-specific cells in peripheral blood is ~0.05-0.4% CD8⁺ T cells, as measured in chapter 4 any-of-3 ICS responses and in previous studies of ChAd63.MVA ME-TRAP [287]. Thus only ~150 CD8⁺ effector cells would be present per well of

100,000 PBMC. With 3% infectivity consistently being achieved in HC-04 cells, this gives an E:T of 0.1-0.2. Through MACS enrichment of CD8⁺ T cells, this could be increased to an E:T of 0.5 at best.

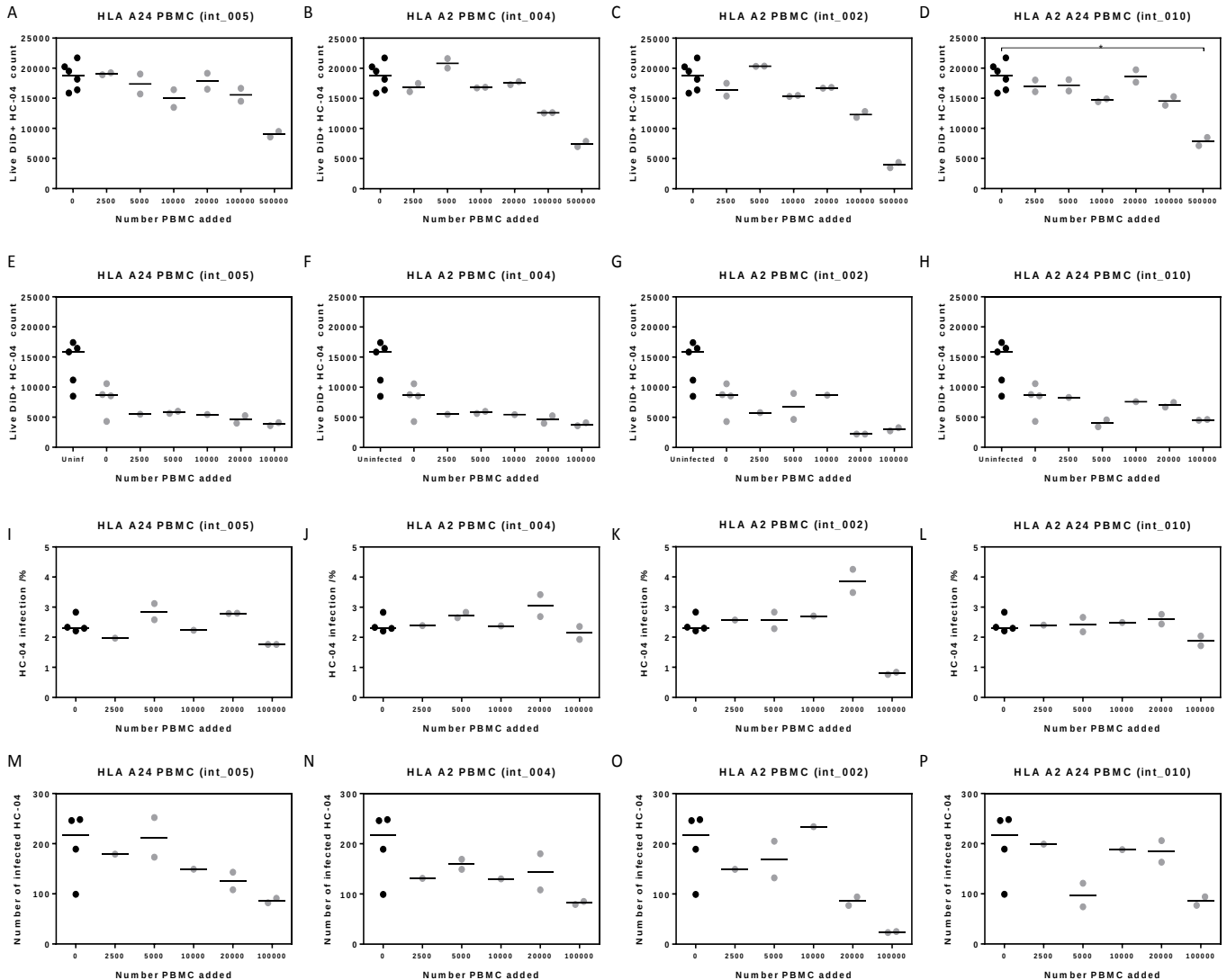


Figure 5.14 HLA mismatch of PBMC and hepatoma cells

20,000/well of HC-04 cells were plated in 96 well plates and infected 21 hours later with 20,000 *PbGFP* sporozoites. 3 hours later, sporozoites were removed, hepatoma cells washed with culture media and PBMC added from volunteers with different degrees of HLA mismatch to the HLA A loci. At 25 hours post infection, hepatoma cells were removed from plates using trypsin digest and acquired by FACS. A-D) Live HC-04 cell recovery from uninfected wells, E-H) live HC-04 cell recovery from infected wells, I-L) percentage infection and M-P) number of infected HC-04 cells, with addition of increasing numbers of PBMC from single (HLA-A2 or HLA-A24) and double (HLA-A2 and HLA-A24) HLA-A serotype-matched volunteers. Four volunteers tested (int_005, int_004, int_002, int_010). Kruskal-Wallis test with Dunn's correction for multiple comparisons performed only for datasets with duplicate wells, * $p < 0.05$. Medians displayed.

5.2.4 Enrichment of TRAP-specific T cells using T cell culture

5.2.4.1 *Parasite inhibition assay with T cells cultured with peptides covering full length of TRAP antigen – part I*

With the limit of ~100,000 PBMC per well, the frequency of antigen-specific T cells needed enriching further to achieve the physiologically relevant E:T >30, as identified in the murine *in vitro* model [363]. A previously published 10 day T cell culture protocol was adapted to expand PBMC from 2 ChAd63.MVA ME-TRAP vaccinees, using recombinant human IL-2 and a megapool of TRAP 3D7 or CTL peptides [488]. Volunteer 039 (HLA-A2) and 041 (HLA-A2) from Mal 34 group 4 were selected due to their partial HLA-match with the HC-04 hepatoma cells. A 6 hour stimulation protocol followed by flow cytometry with ICS was performed at day 13 to calculate numbers of effector CD8⁺ T cells. Any-of-2 (IFN- γ ⁺ and/or TNF- α ⁺) numbers were calculated and antigen-specific CD8⁺ T cells identified as 11.5% and 6.3% for TRAP 3D7 and 12.1% and 35% for CTL, in volunteer 039 and 041 respectively. The inhibition assay was performed 4 days following culture, and of the 4 cultured T cell preparations, only the CTL culture from volunteer 041 was still viable. These T cells were plated into the assay to assess the non-specific effects of cultured T cells on parasite infected cells. In a similar fashion to fresh PBMC (Figure 5.14), parasite inhibition was measured when increasing numbers of cells were added. This reduction was significant when 3.7×10^5 T cells were added, although the trend was apparent when 9.3×10^4 T cells were added (Figure 5.15). For similar cell numbers, higher levels of non-specific inhibition were measured with these cultured cells, compared to fresh cells in Figure 5.14.

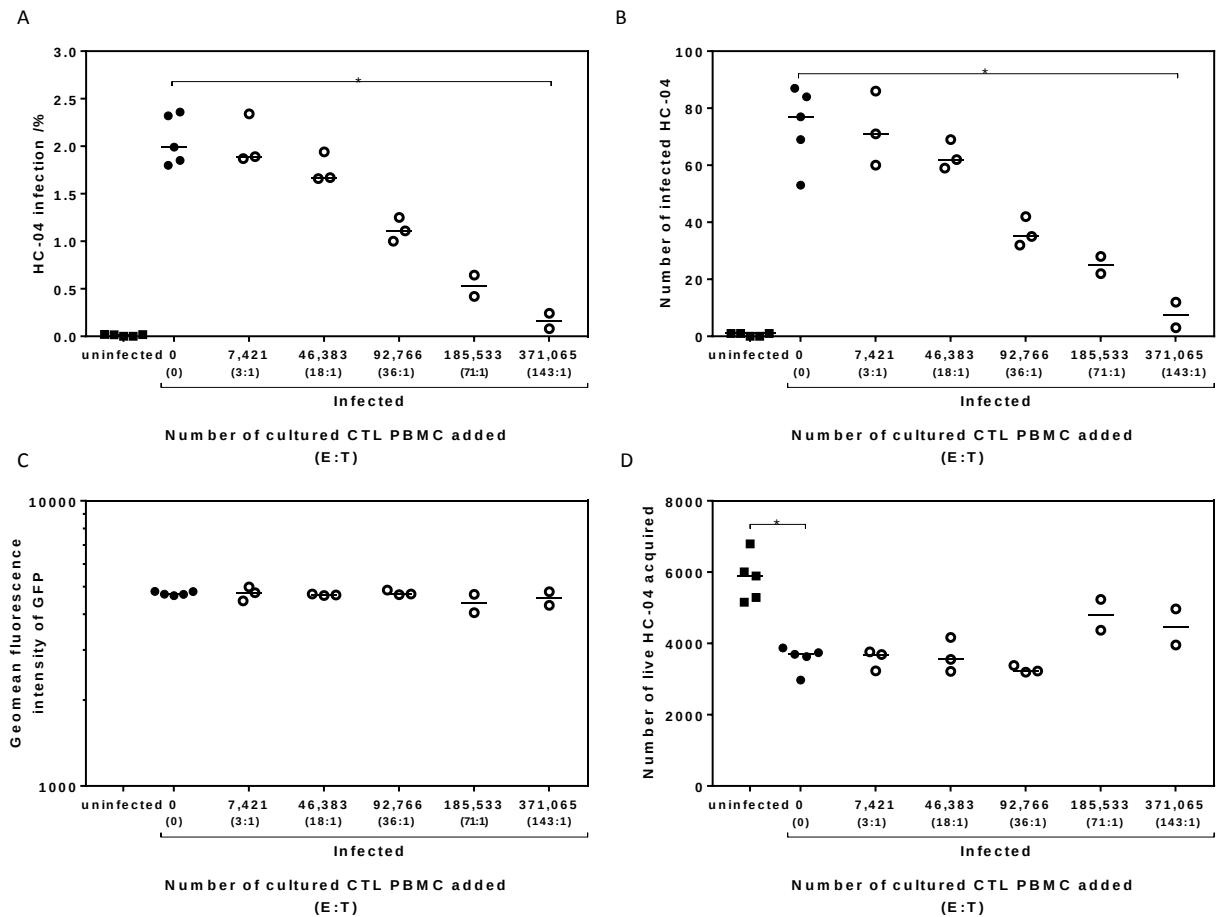


Figure 5.15 Non-specific parasite inhibition using cultured PBMC

20,000/well of HC-04 cells were plated in 96 well plates and infected 21 hours later with 11,500 *PbPf*TRAP sporozoites. 3 hours later, sporozoites were removed, hepatoma cells washed with culture media and cultured PBMC added. At 26 hours post infection, hepatoma cells were removed from plates using trypsin digest and acquired by FACS. A) Percentage infection, B) number of infected HC-04, C) parasite geometric mean fluorescence intensity and D) number of live DiD⁺ HC-04 cells recovered when increasing number of CTL-specific T cells were added per well. Cell numbers assayed in duplicate or triplicate wells. Kruskal-Wallis test with Dunn's correction for multiple comparisons against a single control (A-C 0 PBMC infected control, D uninfected control), * p<0.05. Medians displayed.

5.2.4.2 T cell culture with peptide and recombinant human IL-2 yields highest numbers of live, antigen-specific CD8⁺ T cells after an 8 day culture.

Given the high level of non-specific killing observed with cultured cells, it was important to establish an optimised culture protocol that sufficiently expanded antigen-specific cells with minimised levels of non-specific killing. Protocols for T cell culture provided a basis for this optimisation, with peptide plus rhIL-2 a standard method to expand antigen-specific T cells. Such protocols are usually used for performing cultured ELISpot analyses of vaccinee T cells, whereby background activation following stimulation can be subtracted from the response. However, in a functional assay, such activation would likely cause background parasite inhibition. Additionally, high levels of rhIL-2 in PBMC culture can activate NK cells to acquire enhanced cytotoxic function (known as lymphokine-activated killing (LAK)), where they recognise a broader range of targets and killing with increased lytic potential [489]. Cells with LAK function particularly target carcinoma cells, which posed an issue for this assay. Several test cultures were set up with a pool of CTL peptides, in order to find the optimal culture conditions relating to rhIL-2 concentration, and timing of cellular activation and rest.

Eight and 10 day CTL peptide cultures were set up in 4 unvaccinated volunteers from the internal HLA typing database, according to the schedule outlined in Table 16. Cells were rested in RPMI containing 10% FCS and 1% penicillin and streptomycin for 2 days following their removal from culture, then stimulated for 6 hours with the peptides matching those used in their expansion. Brefeldin A and monensin were added for the final 5.5 hours. Flow cytometry with ICS determined the number of antigen-specific cells following culture. As predicted, the CTL peptide pool expanded the CD8⁺ T cell population to CMV, EBV and influenza peptides (Figure 5.16 A+B), with CD4⁺ T cell responses below 0.1% (Figure 5.16 C+D). With little difference measured between the antigen-specific CD8⁺ T cells measured after 8 and 10 day culture, other parameters were assessed. Two different concentrations of IL-2 were tested in volunteer int_008. Antigen-specific CD8⁺ T cells were of no higher frequency when a higher concentration of rhIL-2 was added to the T cell culture (Figure 5.16 E). CD56⁺ cells, indicative of NK cells with presumed LAK function acquired [490], were of

significantly higher frequencies when 50U/mL of rhIL-2 was used compared with 10U/mL (Figure 5.16 F, $p=0.0313$ Wilcoxon matched-pairs signed rank test).

Table 16 Culture summary for 8 and 10 day T cell expansion

Day	8 day culture	10 day culture
0	Thaw PBMC + 4ug/mL peptide	Thaw PBMC + 4ug/mL peptide
1		
2		
3	+IL-2 10U/ml	+IL-2 10U/ml
4		
5		
6		
7	+IL-2 10U/ml	+IL-2 10U/ml
8	Remove cells from culture	
9		
10	Quantification by ICS	Remove cells from culture
11		
12		Quantification by ICS

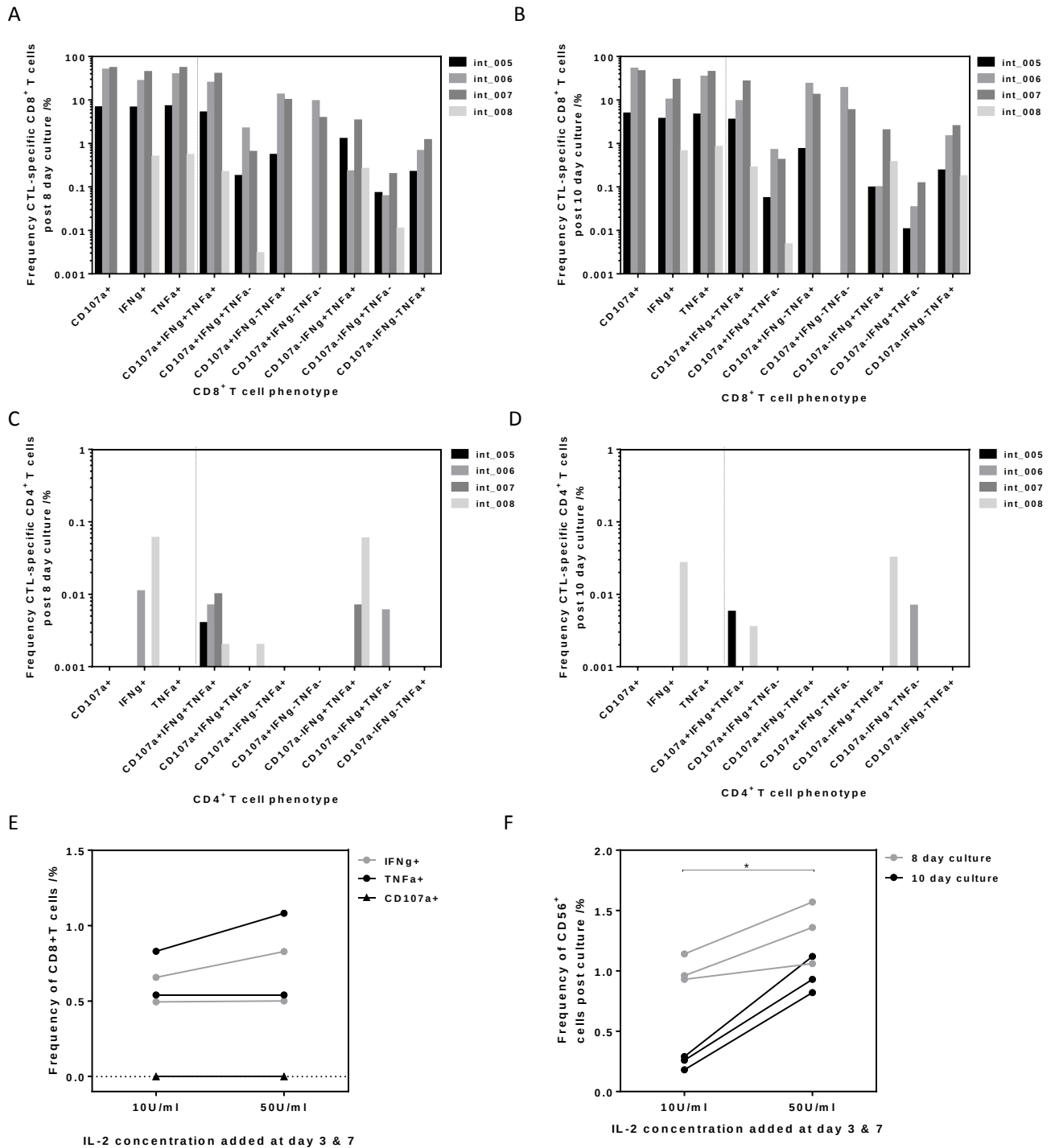


Figure 5.16 ICS summary of 8 and 10 day T cell culture protocol

Frequency of antigen-specific CD8⁺ T cells at A) day 10 following an 8 day culture and B) day 12 following a 10 day culture. Frequency of antigen-specific CD4⁺ T cells at C) day 10 following an 8 day culture and D) day 12 following a 10 day culture. E) Frequency of antigen-specific CD8⁺ T cells following culture with 10U/mL and 50U/mL of recombinant human IL-2 in volunteer int_008. F) Frequencies of CD56⁺ cells following culture with 10U/mL and 50U/mL in volunteer int_008. $p=0.0313$, Wilcoxon matched-pairs signed rank test.

Time course data from IFN- γ ELISpot analysis revealed that both culture protocols expanded the antigen-specific response compared to baseline in all volunteers except for int_008, however the reported T cell response for all volunteers is markedly lower by ELISpot than by ICS (Figure 5.17 C+F). This could be due to issues with cell viability. The resting period following T cell culture reduced the background activation of the cells (Figure 5.17 B+E), however coupled with this was an increased proportion of dead cells. Dead cells increased from ~20% at the end of the culture period, to 30-80% following 2 days of rest. A 1 day rest period was therefore deemed optimal.

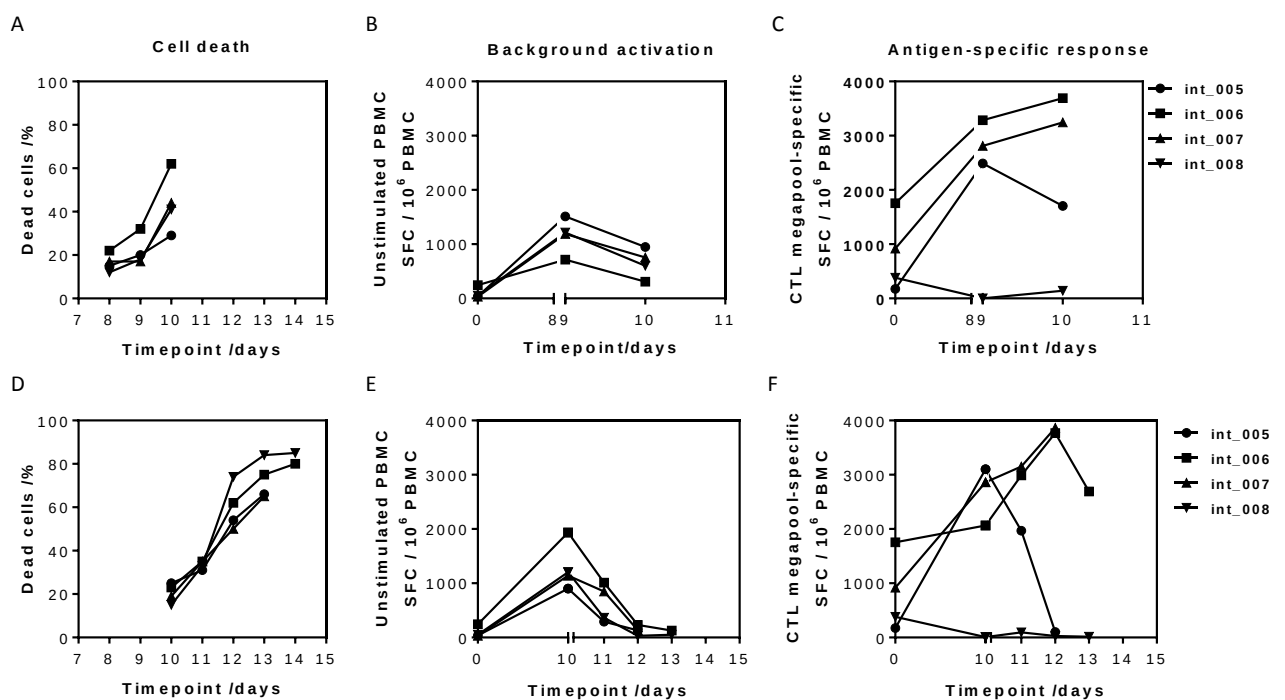


Figure 5.17 ELISpot time course summary for 8 and 10 day culture

A+D) Time course of dead cell percentage, measured by manual Trypan Blue counting. B+E) Time course of background T cell activation following culture, as measured by *ex vivo* IFN-g ELISpot. C+F) Time course of antigen-specific T cell response following culture, as measured by *ex vivo* IFN-g ELISpot to CTL peptide megapool (background subtracted).

Further testing of the culture protocol explored a shortened culture period of 6 and 7 days (Table 17). Antigen-specific T cell responses were up to 100-fold lower by ICS, compared with the 8 and 10 day cultures. Cell death followed a similar pattern to the longer culture protocols following removal from culture, with over 20% cell death by the second rest day (Figure 5.18). Therefore an 8 day T cell culture protocol was taken forward for use in the *in vitro* parasite inhibition assay. This protocol expanded 4-5 million PBMC per well in 12 well tissue culture plates, with peptide added at day 0, 10U/mL rhIL-2 added at day 3 and day 7 and cells rested for one day before subsequent addition in to *in vitro* assay.

Table 17 Culture summary for 6 and 7 day T cell expansion

Day	6 day culture	7 day culture
0	Thaw PBMC + 4ug/mL peptide	Thaw PBMC + 4ug/mL peptide
1		
2	+IL-2 10U/ml	+IL-2 10U/ml
3		
4		
5	+IL-2 10U/ml	+IL-2 10U/ml
6	Remove cells from culture	
7	Quantification by ICS	Remove cells from culture
8		Quantification by ICS

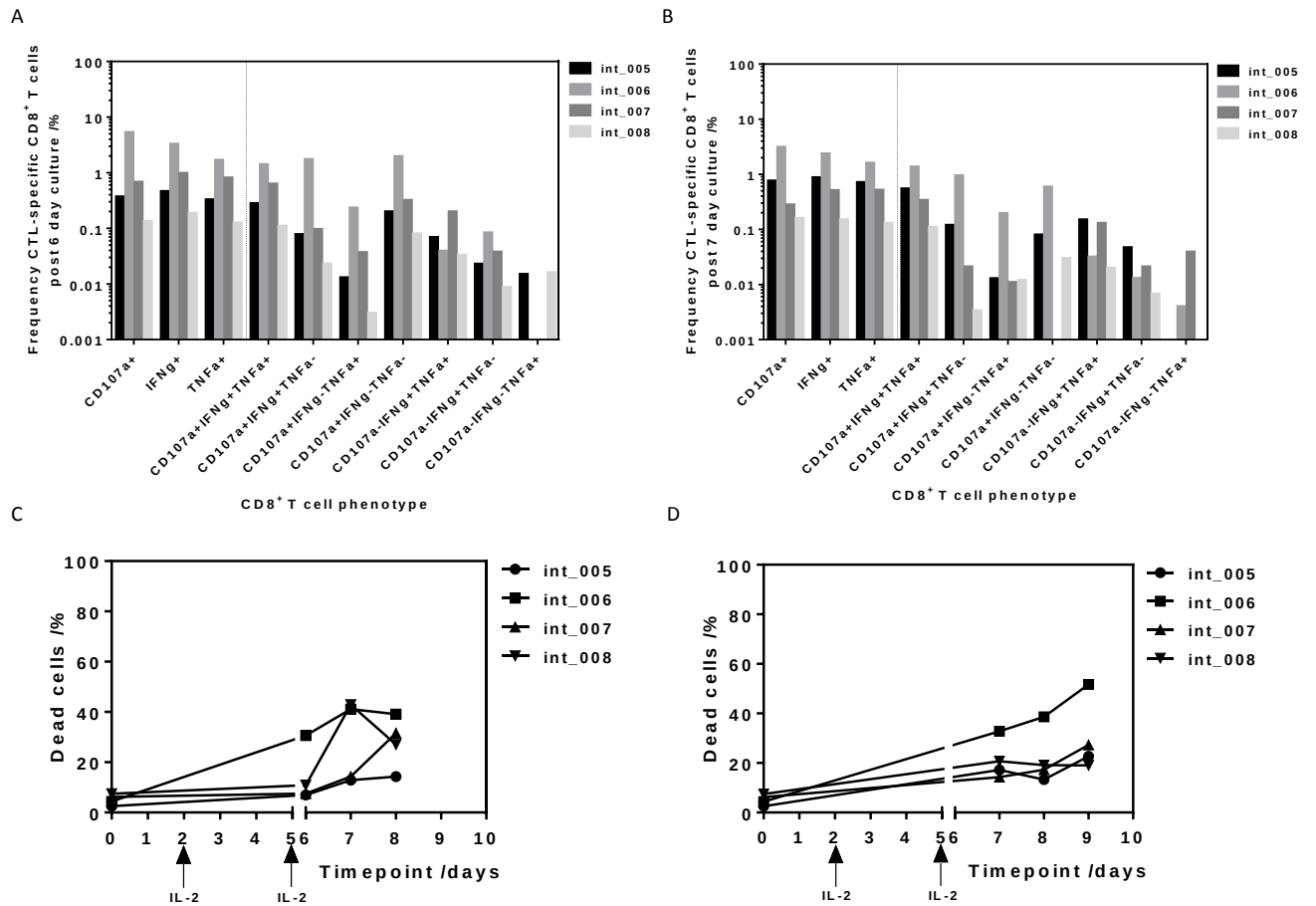


Figure 5.18 ICS and cell death summary for 6 and 7 day T cell culture protocol

Frequency of antigen-specific CD8⁺ T cells by ICS at A) day 7 following a 6 day culture and B) day 8 following a 7 day culture. C+D) Time course of dead cell percentage, measured by manual Trypan Blue counting.

5.2.4.3 Inhibition assay with T cells cultured with TRAP peptides covering full length of antigen – part II

Having optimised the culture conditions to achieve the highest frequency of effector T cells, 8 day T cell cultures with peptide and rhIL-2 were used to expand PBMC from 3 ChAd63.MVA ME-TRAP vaccinees, using TRAP 3D7 or CTL peptides. Expanded effector T cells were then tested in the inhibition assay. The volunteers selected were partial HLA matches to the HC-04 hepatoma cells: volunteer 1282 (HLA-A2), 1285 (HLA-A2 homozygous) and 1290 (HLA-A24), from Vac 48 group 3. A 6 hour stimulation protocol followed by flow cytometry with ICS was performed at day 9 to calculate numbers of effector CD8⁺ T cells (Figure 5.19). Responses are summarised as any-of-2 (IFN- γ ⁺ and/or TNF- α ⁺) for use in calculating matching E:T or TRAP and CTL (Table 18). In 2 of the 3 vaccinees, the frequency of antigen-specific cells was higher following expansion with CTL peptides, compared with TRAP peptides. CD8⁺ T cell phenotypes were similar across the 2 cultures, with most IFN- γ ⁺ or TNF- α ⁺ cells of a de-granulating phenotype (CD107a⁺).

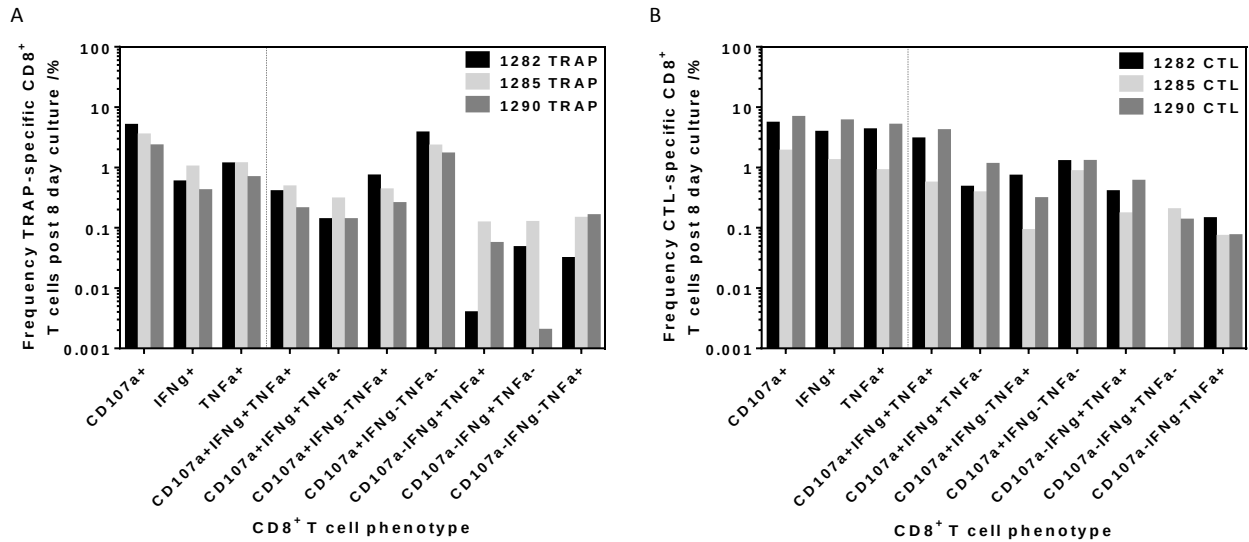


Figure 5.19 ICS summary of antigen-specific CD8⁺ T cells following 8 day culture of vaccinee PBMC with TRAP or CTL peptide megapool
Frequency of A) TRAP 3D7-specific CD8⁺ T cells and B) CTL-specific CD8⁺ T cells by short stimulation ICS at day 9, following an 8 day T cell culture with peptide and rhIL-2.

Table 18 Summary of any-of-2 antigen-specific CD8⁺ T cells following 8 day peptide and IL-2 culture.

Volunteer/ culture	Any-of-2 antigen- specific CD8 ⁺ T cells
1282 3D7 TRAP	1.3
1282 CTL	4.7
1285 3D7 TRAP	1.6
1285 CTL	1.5
1290 3D7 TRAP	0.8
1290 CTL	6.3

T cell populations were then further enriched by MACS, to obtain a CD8⁺ and CD8⁻ fraction for each volunteer. TRAP-specific and CTL-specific cells were matched on their effector cell frequencies, and added into the parasite inhibition assay 4 hours following infection with *PbPf*TRAP. The assay was left to incubate for 20 hours before collection of supernatants, staining of CD45 (to identify T cells), prior to HC-04 removal from the plate using trypsin digest and acquisition by FACS.

At CD8⁺ T cell E:T of 5:1 and 6:1, only volunteer 1285 exhibited parasite inhibition over that of the control wells. This effect was not TRAP-specific, as a similar percentage infection and number of infected wells were measured when autologous CTL-specific CD8⁺ T cells were added at the same E:T (Figure 5.20 A+C, Figure 5.21 A+C, Kruskal-Wallis test). At higher E:T of 68:1 and 88:1, parasite inhibition was measured in all volunteers. Volunteer 1290 showed a marked statistical difference between the inhibition measured with TRAP-specific CD8⁺ T cells over that of the infected-only control, however a similar, yet non-statistically significant trend, was measured with autologous CTL-specific CD8⁺ T cells (Figure 5.20 B+D, Figure 5.21 B+D, Kruskal-Wallis test). For all volunteers, gMFI of parasite GFP was unaffected by T cell addition at both high and low E:T (Figure 5.20 E+F, Figure 5.21 E+F). Recovery of HC-04 cells was only affected at the high E:T, particularly when cell numbers above the 100,000/well threshold were plated (Figure 5.20 H, Kruskal-Wallis test). No significant TRAP-specific inhibition was measured with the addition of CD8⁻ T cells (Figure 5.22 + Figure 5.23).

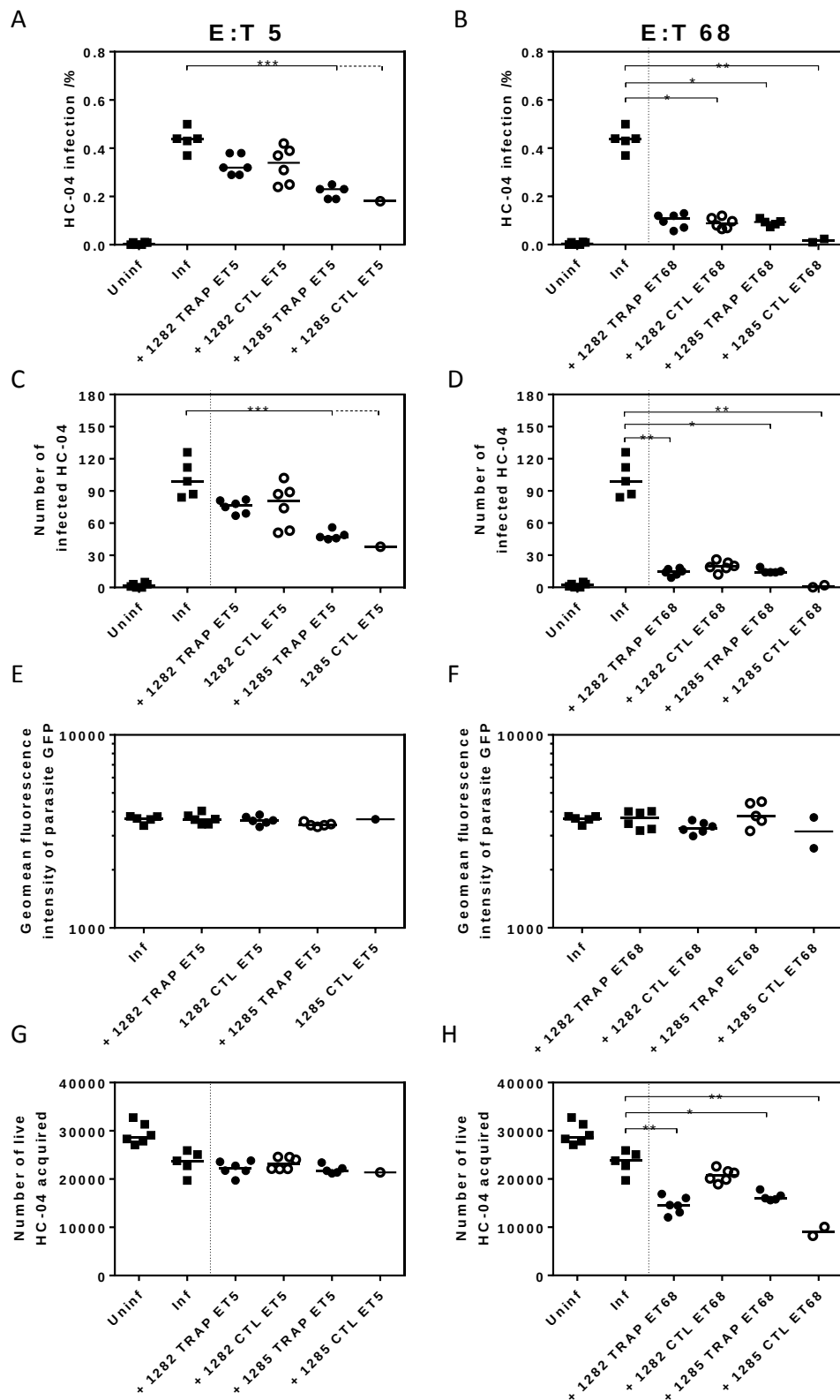


Figure 5.20 Parasite inhibition using TRAP- and CTL-specific CD8⁺ T cells (plate 1)

20,000/well of HC-04 cells were plated in half surface area 96 well plates and infected 26 hours later with 10,000 *PbPf*TRAP sporozoites. 3 hours later, sporozoites were removed, hepatoma cells washed with culture media and cultured PBMC added. At 26 hours post infection, hepatoma cells were removed from plates using trypsin digest and acquired by FACS A+B) Percentage infection, C+D) number of infected hepatoma cells, E+F) geometric mean fluorescence intensity of parasite GFP and G+H) live hepatoma cell recovery following HC-04 infection with *PbPf*TRAP sporozoites and addition of E:T matched CD8⁺ T cells from TRAP or CTL culture. E:T of 5:1 and 68:1 measured. Kruskal-Wallis test with Dunn's correction for multiple comparisons against a single control (infected only). Uninfected control wells excluded from statistical analyses. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Medians displayed. Statistical analysis not performed on single replicates.

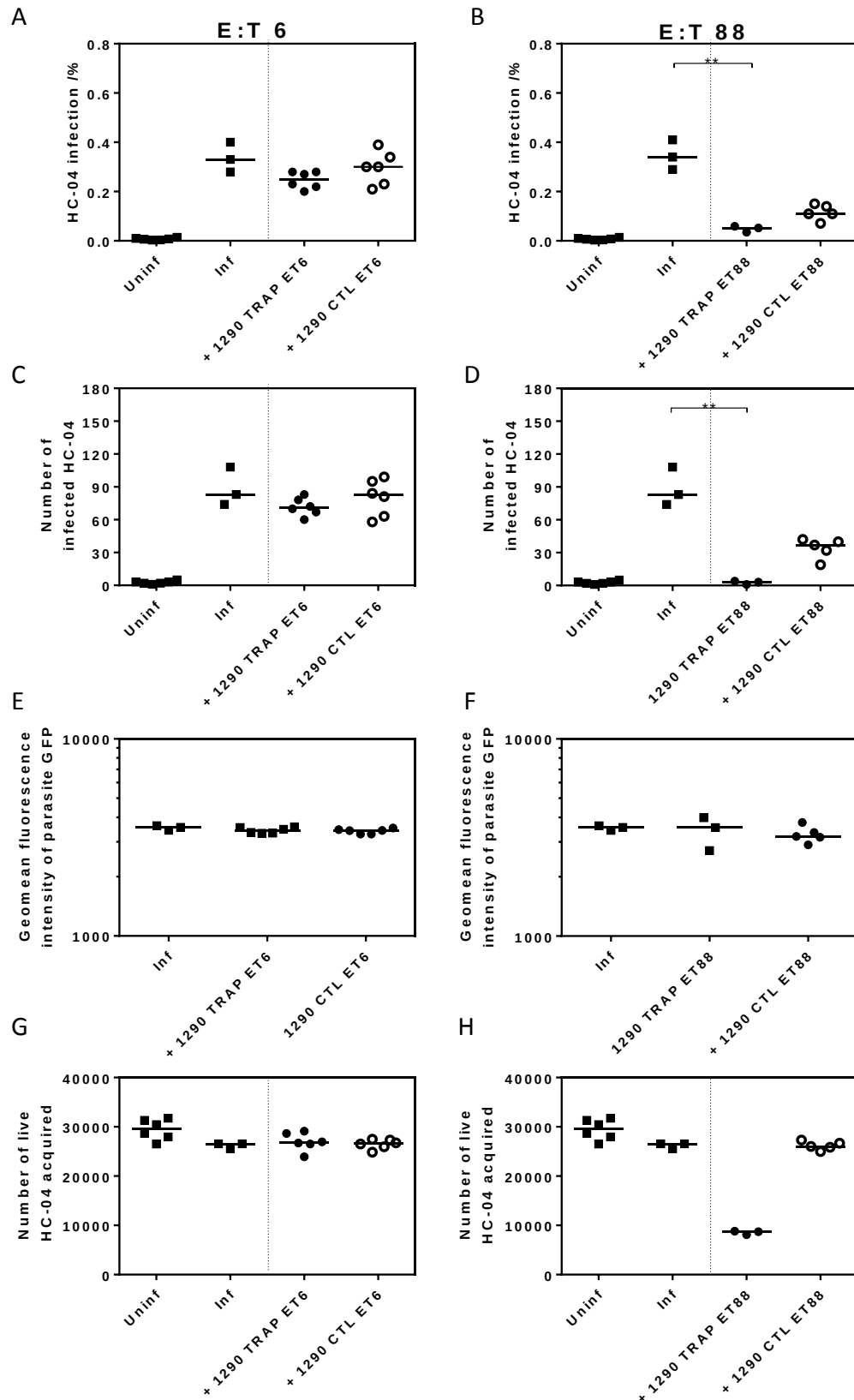


Figure 5.21 Parasite inhibition using TRAP- and CTL-specific CD8⁺ T cells (plate 2)

Identical protocol to Figure 5.20 used. A+B) Percentage infection, C+D) number of infected hepatoma cells, E+F) geometric mean fluorescence intensity of parasite GFP and G+H) live hepatoma cell recovery following HC-04 infection with *PbPf*TRAP sporozoites and addition of E:T matched CD8⁺ T cells from TRAP or CTL culture. E:T of 6:1 and 88:1 measured. Kruskal-Wallis test with Dunn's correction for multiple comparisons against a single control (infected only). Uninfected control wells excluded from statistical analyses. ** p<0.01. Medians displayed. Statistical analysis not performed on single replicates.

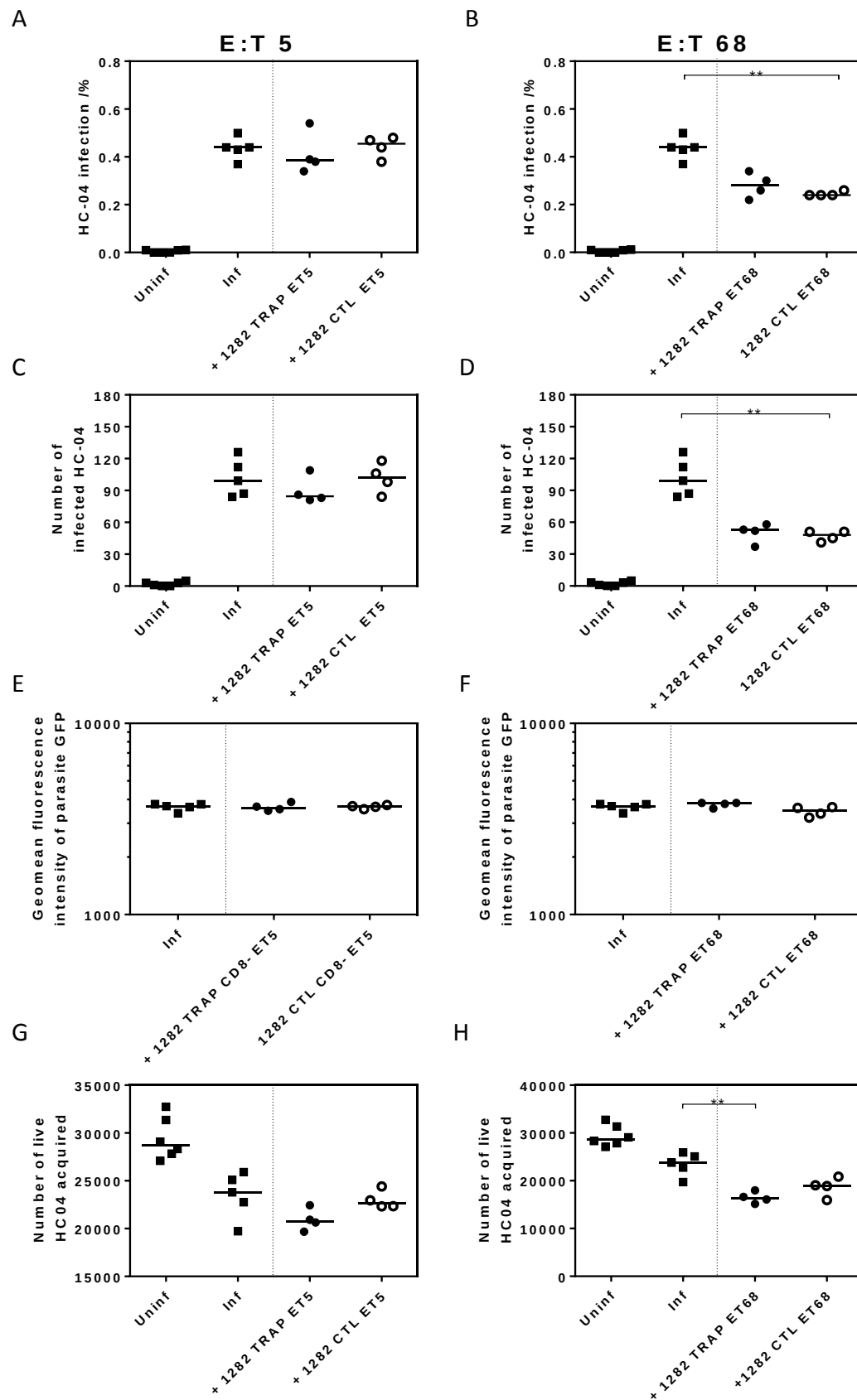


Figure 5.22 Parasite inhibition using TRAP- and CTL-specific CD8⁺ T cells (plate 1)

Identical protocol to Figure 5.20 used. A+B) Percentage infection, C+D) number of infected hepatoma cells, E+F) geometric mean fluorescence intensity of parasite GFP and G+H) live hepatoma cell recovery following HC-04 infection with *PbPf*TRAP sporozoites and addition of E:T matched CD8⁺ T cells from TRAP or CTL culture. E:T of 6:1 and 88:1 measured. Kruskal-Wallis test with Dunn's correction for multiple comparisons against a single control (infected only). Uninfected control wells excluded from statistical analyses. ** p<0.01. Medians displayed.

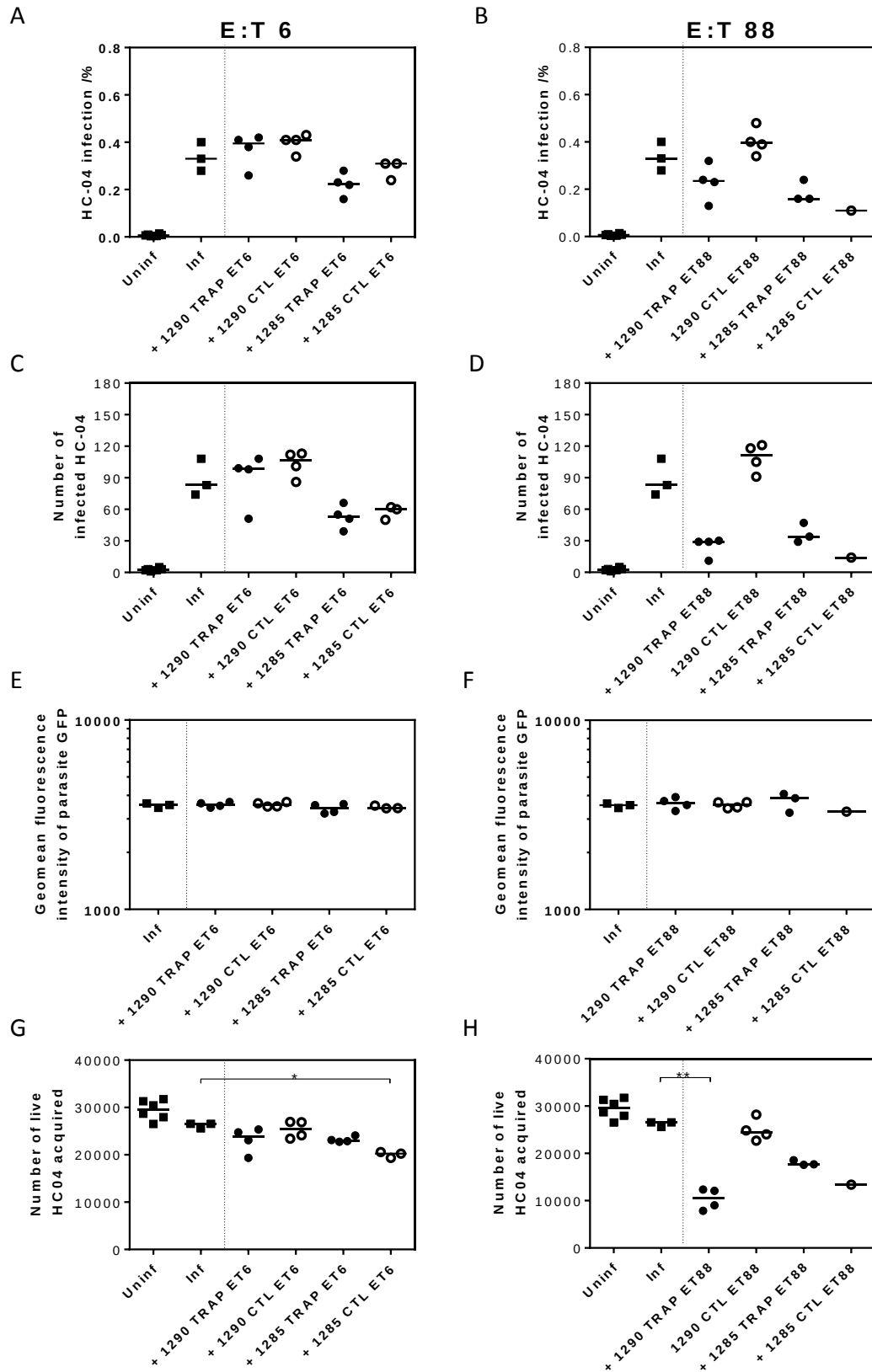


Figure 5.23 Parasite inhibition using TRAP- and CTL-specific CD8⁺ T cells (plate 2)

Identical protocol to Figure 5.20 used. A+B) Percentage infection, C+D) number of infected hepatoma cells, E+F) geometric mean fluorescence intensity of parasite GFP and G+H) live hepatoma cell recovery following HC-04 infection with *PbPf*TRAP sporozoites and addition of E:T matched CD8⁺ T cells from TRAP or CTL culture. E:T of 6:1 and 88:1 measured. Kruskal-Wallis test with Dunn's correction for multiple comparisons against a single control (infected only). Uninfected control wells excluded from statistical analyses. * $p < 0.05$. ** $p < 0.01$. Medians displayed. Statistical analysis not performed on single replicates.

These preliminary data highlight some key areas required for the development of the assay. Firstly, infectivity of ~0.4% in these experiments was markedly lower than in the optimised infectivity model, where 1-4% infection was consistently achieved. This is likely due to the assay plates spending an extended period of time at room temperature as opposed to 37°C, while the T cell component was added. Additionally, the disparity between TRAP and CTL CD8⁺ T cell expansion in peptide and rhIL-2 culture means a higher number cells are required per well in order for the TRAP E:T to match that of CTL. As previously demonstrated, even these non-TRAP-specific cells contribute to non-specific parasite inhibition, therefore it could be argued that parasite inhibition measured in the TRAP wells is not necessarily antigen-specific. Controlling for this would require additional wells, whereby total cell number is also controlled for. To circumvent this, an alternative approach was adopted to include antigen-specific CD8⁺ T cell sorting.

5.2.5 Enrichment of TRAP-specific CD8⁺ T cells using MHC class I pentamers

5.2.5.1 Selecting an epitope for customised pentamer manufacture

Further enrichment of antigen-specific CD8⁺ T cells was required, beyond that of T cell culture plus MACS enrichment of CD8⁺ T cells. MHC class I pentamers permit cell sorting of epitope-specific CD8⁺ T cells using FACS. In order to select the most appropriate customised MHC class I pentamer, 2 previously identified immunodominant HLA-A2 epitopes (Table 19 and Table 20) were tested by *ex vivo* IFN- γ ELISpot in ChAd63.MVA ME-TRAP vaccinees and unvaccinated controls at the C-1 time point of Vac 55. Vaccinee responses to 10-mer peptide FLIFFDLFLV were significantly higher than that of controls, however the magnitude of the response ranged from undetectable to >1000 SFC/10⁶ PBMC (Figure 5.24 A, $p=0.0235$ Mann-Whitney test). Comparatively, responses to 9-mer peptide NLTDLALLQV were lower, and did not significantly differ between vaccinees and controls (Figure 5.24 B). All pentamer cell sorting performed by D. Worth of the Jenner Institute's FACS core facility.

Table 19 Summary of dominant minimal epitopes identified in Thrombospondin-related adhesion protein

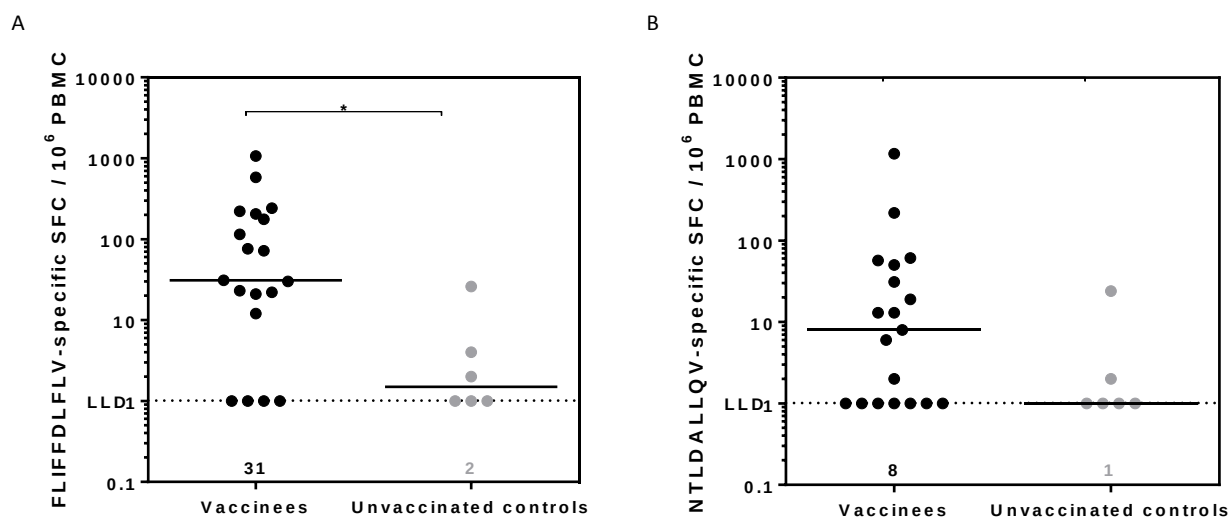
Dominant minimal epitopes identified using fresh and frozen PBMCs from volunteers vaccinated with ChAd63-MVA ME-TRAP, thesis by R. Longley. Not all epitopes confirmed in multiple volunteers.

Peptide	Minimal epitope
2 (aa11-30)	FLIFFDLF
3 (aa21-40)	FLVNGRDVQNNIVDEIKYSE
6 (aa51-70)	CSGSIRRHN/SIRRHWN
9 (aa81-100)	LNDNAIHLYVNVFSNNAKEI
12 (aa111-130)	KALIIIRSL
13 (aa121-140)	NLTDLALLQV
15 (aa141-160)	NANQLVV
16 (aa151-170)	VILTDGIPD
24 (aa231-250)	VEKTASCGV/EKTASCGVW

Table 20 Summary of putative epitopes

Independently identified epitopes for TRAP T9/96 and corresponding responses in HLA A2 matched and non-matched vaccinees. Work completed by D. Wright, Jenner Institute laboratories.

20mer amino acid sequence	Putative epitope	HLA-matched vaccinees with positive ELISpot response /%	HLA non-matched vaccinees with positive ELISpot response /%
VIVFLIFFDLFLVNGRDVQN	FLIFFDLFLV (A2)	80	0
	FLIFFDLFL (A2)		
LNDNAIHLYVNVFSNNAKEI	LNDNAIHLY (A1)	80	25
	NVFSNNAK (A3)	100	0
LLSTNLPYGRNTLDALLQV	NLTDALLQV (A2)	80	0
ENVKNVIGPFMKAVCVEVEK	FMKAVCVEV (A2)	80	0

**Figure 5.24 ex vivo IFN- γ ELISpot response to individual TRAP peptides**

Responses using fresh PBMC from 19 ChAd63.MVA ME-TRAP vaccinees and 6 controls to A) single FLIFFDLFLV 10-mer peptide, $p=0.0235$ Mann-Whitney test, and B) single NLTDALLQV 9-mer peptide, NSD by Mann-Whitney test. Medians displayed.

5.2.5.2 Testing FLIFFDLFL-MHC class I pentamer

With frequencies of FLIFFDLFLV-specific cells ranging from 0-1%, in order to obtain sufficient numbers of FLIFFDLFL-specific CD8⁺ T cells for evaluation in the parasite inhibition assay, it was predicted that peptide and rhIL-2 culture would be required prior to cell sorting using the pentamer. Eight day T cell cultures were set up using thawed PBMC from 2 ChAd63.MVA ME-TRAP vaccinees 1426 (HLA-A2) and 1431 (HLA-A2 homozygous) from Vac 45 group 2. Their frozen ELISpot responses to FLIFFDLFLV were 481 SFC/10⁶ and 226 SFC/10⁶, respectively (data not shown). T cell cultures to an irrelevant 9-mer control peptide from human cytomegalovirus pp65 (495-503, NLVPMVATV, see supplementary Table S5 CTL5) were set up in parallel to act as a negative control.

The FLIFFDLFL-MHC class I pentamer was tested at 3 different titrations, with gMFI used to select the optimal titration of 10µL (Figure 5.25 A+B). Following 8 day culture with FLIFFDLFLV peptide and rhIL-2, frequencies of pentamer-positive CD8⁺ T cells were 10% and 6.1% (Figure 5.25 C+D). Frequencies of CD8⁺ pentamer⁺ T cells from non-cultured cells, and from cells cultured with an irrelevant peptide and rhIL-2, were comparable and less than 0.1% (Figure 5.25 C+D). Cross reactive binding of the MHC class I pentamer to CD4⁺ T cells was not detected, with 0.2% the highest CD4⁺ pentamer⁺ frequency detected (Figure 5.25 E+F).

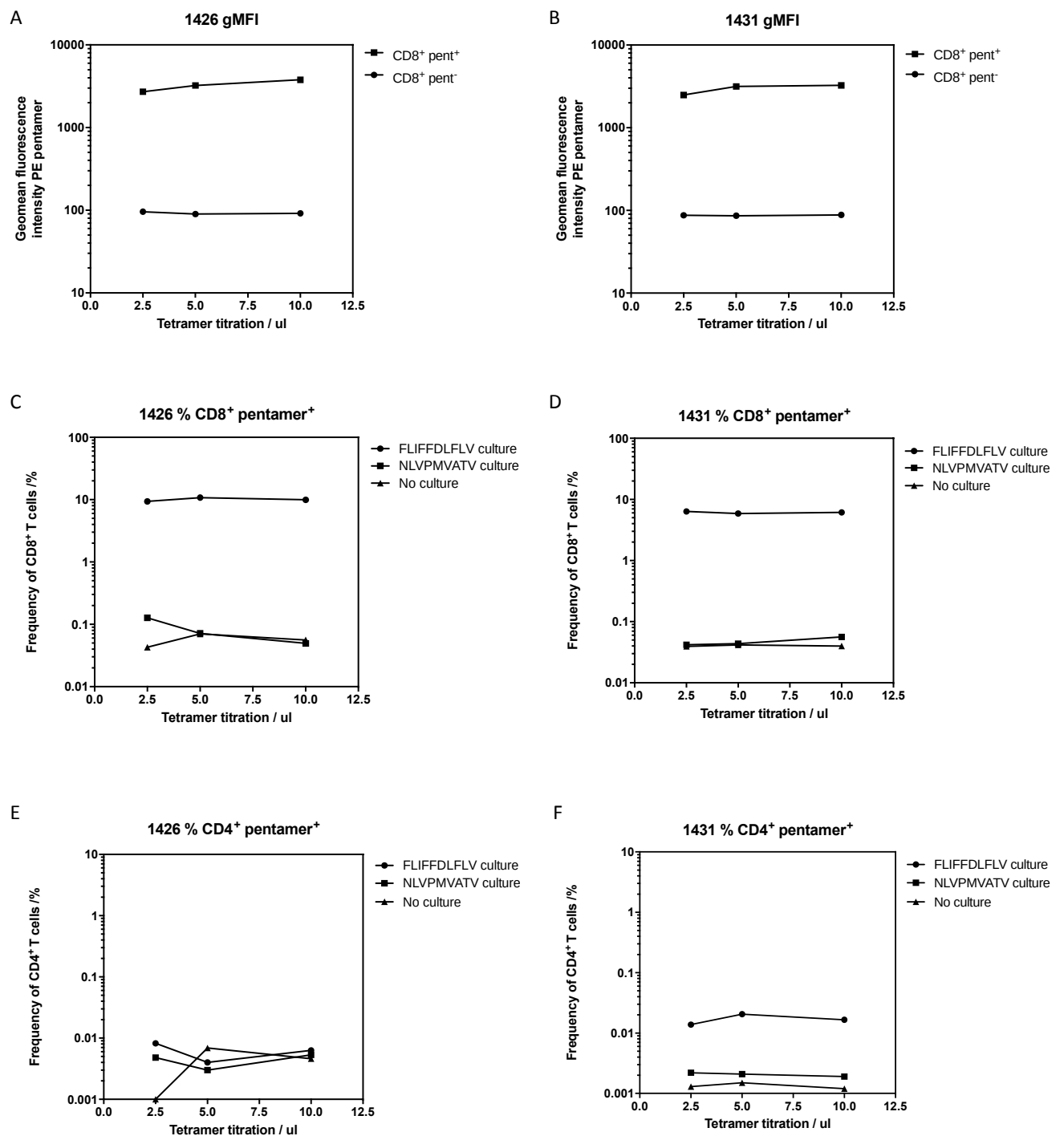


Figure 5.25 Titration of FLIFFDLFL-MHC class I pentamer

Evaluation of MHC class I pentamer at 3 titrations, in 2 ChAd63.MVA ME-TRAP vaccinees, using 3 cell culture conditions: no culture, homologous peptide (FLIFFDLFLV) culture and irrelevant peptide culture NLVPMTATV). A+B) Raw geometric mean fluorescence intensity of pentamer PE signal. C+D) Raw frequency of CD8⁺ pentamer⁺ cells. E+F) Raw frequency of CD4⁺ pentamer⁺ cells.

5.2.5.3 *Pentamer⁺ cells kill cells expressing their cognate antigen*

To ensure that cultured and pentamer sorted cells were able to kill hepatocytes, the functionality of these CD8⁺ T cells, sorted CD8⁺ pentamer⁺ cells were tested in a small proof-of-concept experiment. Volunteer 004 (HLA-A2 homozygous) from Vac 55 group 1 was tested alongside volunteers 1426 and 1431. Briefly, HC-04 cells were pulsed overnight with FLIFFDLFLV peptide and labelled with DiD membrane dye. Unpulsed HC-04 cells were labelled with CFSE. The pulsed DiD⁺ and unpulsed CFSE⁺ cells were plated in a 50:50 ratio and left to settle for 6 hours. Meanwhile 8 day T cell cultures with FLIFFDLFLV peptide and rhIL-2 were allowed to rest for 2 days, and were subsequently stained with a FACS panel identifying live CD56⁻ CD8⁺ pentamer^{+/-} populations. A control culture to a peptide from influenza PB1 protein region (591-599, VSDGGPNLY, see supplementary Table S5 CTL1) was recovered and remained unsorted. Matched numbers of CD56⁻ CD8⁺ pentamer⁺ cells, CD56⁻ CD8⁺ pentamer⁻ cells and cells from the unsorted, irrelevant PBMC culture were added to the 50:50 cultures of pulsed and unpulsed HC-04 cells and left to incubate for 18 hours. HC-04 cells were then removed from the plate using trypsin digest, and the proportion of DiD⁺ FLIFFDLFLV-pulsed to CFSE⁺ unpulsed cells measured by FACS. Reduction of DiD⁺ and CFSE⁺ cells was calculated, based on the ratios measured in control cells, where no T cells were added.

A reduction in DiD⁺ cells was measured when vaccinee CD8⁺ pentamer⁺ T cells were added. This ranged from 5.5 – 26.6%. In one volunteer with a high number of cells obtained from the cell sort, 3 different E:T were tested. The magnitude in DiD⁺ cell reduction was greater with increasing E:T ratio of CD8⁺ pentamer⁺ cells added (Figure 5.26 A). Addition of CD8⁺ pentamer⁻ cells and PBMC from irrelevant peptide culture gave an indication of baseline reduction in DiD⁺ cells, which ranged from 0.7-1.7% with CD8⁺ pentamer⁻ cells and 0.7%-3.1% with irrelevant PBMC cultures (Figure 5.26 B+C). Correspondingly, negligible changes in CFSE⁺ unpulsed cells were measured with the addition of all 3 cell types (Figure 5.26 D-F).

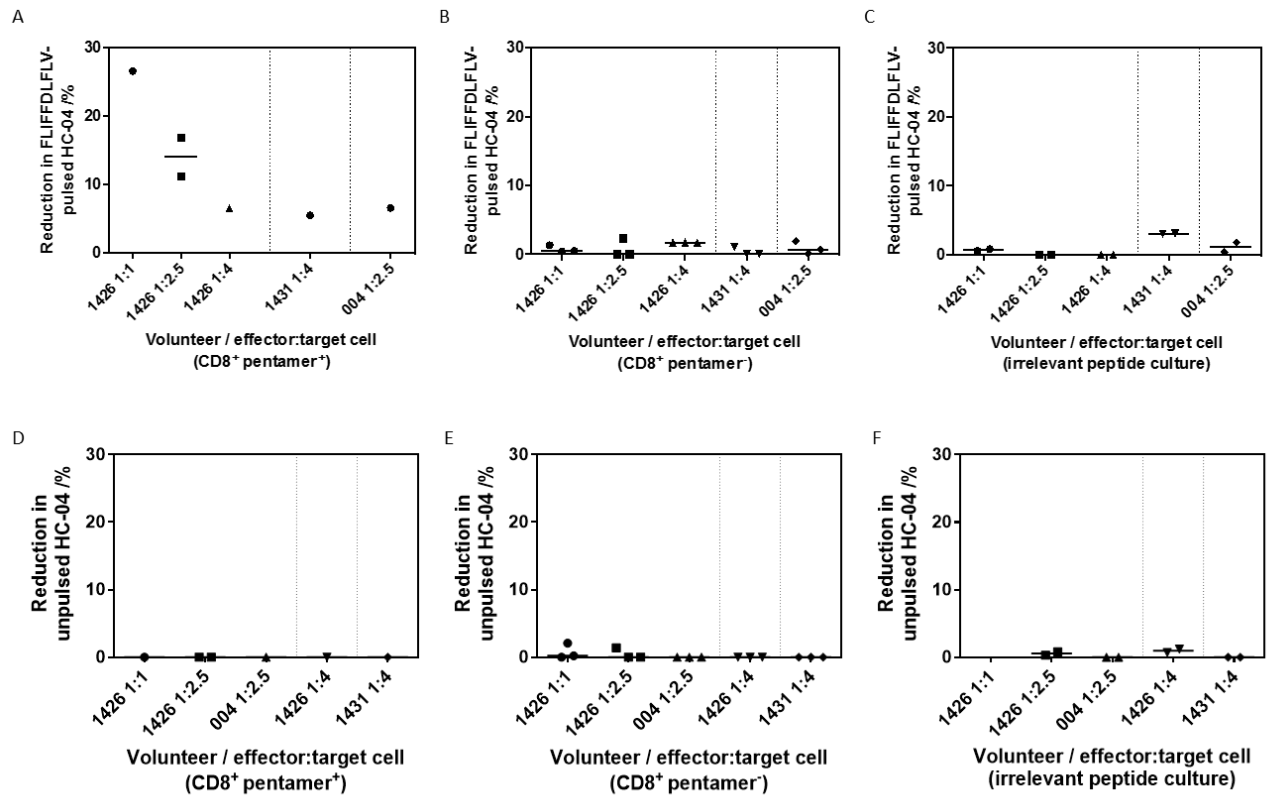


Figure 5.26 Proof-of-concept peptide pulse assay

A-C) Percentage reduction in DiD⁺ FLIFFDLFLV-pulsed HC-04 cells and D-F) percentage reduction in DiD⁺ FLIFFDLFLV-unpulsed HC-04 cells, when CD8⁺ pentamer⁺ cells T cells, CD8⁺ pentamer⁺ T cells and PBMC from irrelevant peptide culture were co-incubated for 18 hours.

5.2.5.4 Identification of a suitable control pentamer

Following the successful culture and enrichment of CD8⁺ T cells from vaccinated volunteers, a control pentamer was required for use alongside that of the pentamer for TRAP-specific epitope FLIFFDLFLV, in order to differentiate background, non-specific parasite inhibition from antigen-specific parasite inhibition. An influenza epitope present in the CTL megapool, against which many clinical trials' vaccinees are ELISpot tested was selected. This 9-mer peptide from matrix 1 protein of influenza A (58-66, GILGFVFTL, see supplementary Table S5 CTL4) was selected based its HLA-A2 restriction, the reported human memory CD8⁺ T cell frequencies specific to this epitope following natural exposure to influenza and the routine use of this epitope in tetramer immunoassays [491-493]. A small PBMC subset from 8 HLA-A2 ChAd63.MVA ME-TRAP vaccinees was cultured in either

FLIFFDLFLV peptide or GILGFVFTL peptide and rhIL-2 for 10 days, followed by 2 days of rest. Post-culture samples were then sorted using the corresponding MHC class I pentamer, to assess post culture frequencies (Table 21).

Table 21 Summary of pentamer sort in 8 vaccinees

PBMC cultured in FLIFFDLFLV or GILGFVFTL peptide plus rhIL-2 for 10 days, rested for 2 day, then stained and sorted by MoFlo FACS. Frequency of pentamer⁺ cells reported from live CD4⁺CD8⁺CD56⁻ populations.

Vaccine study	Volunteer tested	Percentage of FLIFFDLFL pentamer ⁺ CD8 ⁺ T cells	Percentage of GILGFVFTL pentamer ⁺ CD8 ⁺ T cells
Vac 45 G2	1407	1.69	5.33
Vac 45 G2	1408	6.63	2.89
Vac 45 G2	1426	37.58	10.95
Vac 45 G2	1431	6.89	8.45
Vac 45 G2	1451	6.76	8.64
Vac 45 G2	1454	30.58	23.13
Vac 52 G1	1371	2.31	4.05
Vac 55 G1	011	0.79	0.97

5.2.5.5 Pentamer dissociation from CD8⁺ T cells

Identification of a control pentamer permitted further testing of the kinetics of the pentamer staining. To determine the rate at which the pentamer detached or is internalised from the CD8⁺ T cells, a FACS time course was performed post pentamer sort. The aim was to determine the frequency of CD8⁺ T cells that remained bound to the pentamer and to what intensity, as high level pentamer binding could interfere with TCR-MHC class I signalling between the T cells and hepatoma cells in the assay. After 9 hours at 37°C, the frequency of pentamer stained cells had reduced from 2-3% down to 0.5%. The geometric mean fluorescence of the pentamer PE signal had reduced after 9 hours, with intensity at 9 hours similar to that measured at 24 hours, indicating that prolonging the addition of the cells into the inhibition assay beyond 9 hours was unlikely to result in any further benefits relating to loss of pentamer-binding (Figure 5.27). Re-identification of CD8⁺ pentamer⁺ cells was possible by re-staining with the pentamer, although lower frequencies and gMFI were measured. This indicates that the epitope-specific TCR were still present on the T cells, but had likely been down regulated.

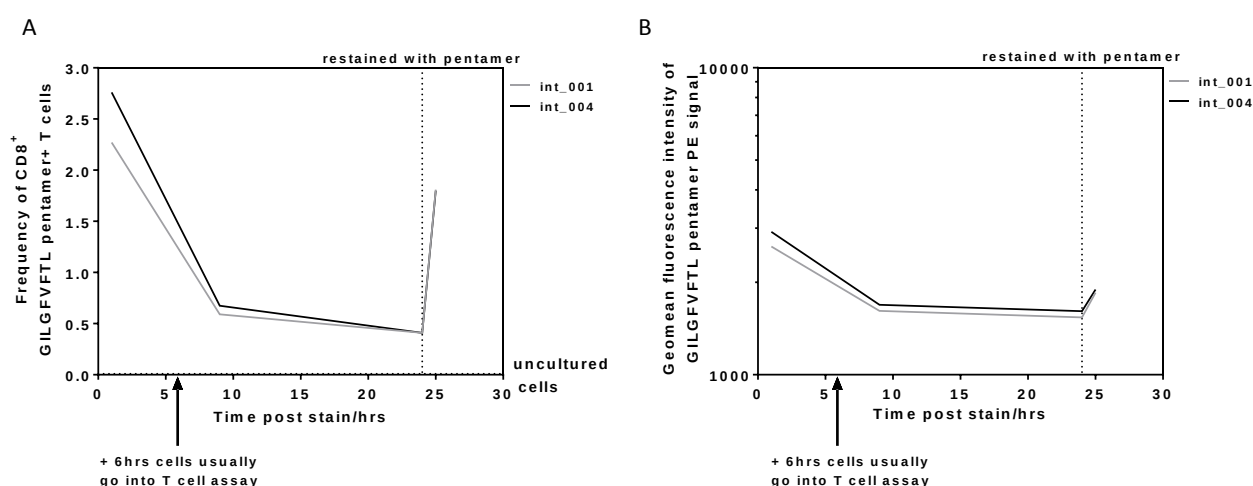


Figure 5.27 Summary of pentamer dissociation at 37°C

GILGFVFTL pentamer⁺ cells were sorted as per the standard staining protocol. CD8⁺ pentamer⁺ cells were then incubated at 37°C, re-stained for surface markers and re-acquired by FACS at 1, 9 and 24 hours post-cell sort. At 24 hours, an aliquot of cells were re-stained with the pentamer and acquired at 25 hours. A) Time course of CD8⁺ pentamer⁺ cell frequency. B) Time course of geometric mean fluorescence intensity of pentamer PE signal.

5.2.6 Finalised model for evaluation of TRAP-specific parasite inhibition by vaccinee T cells.

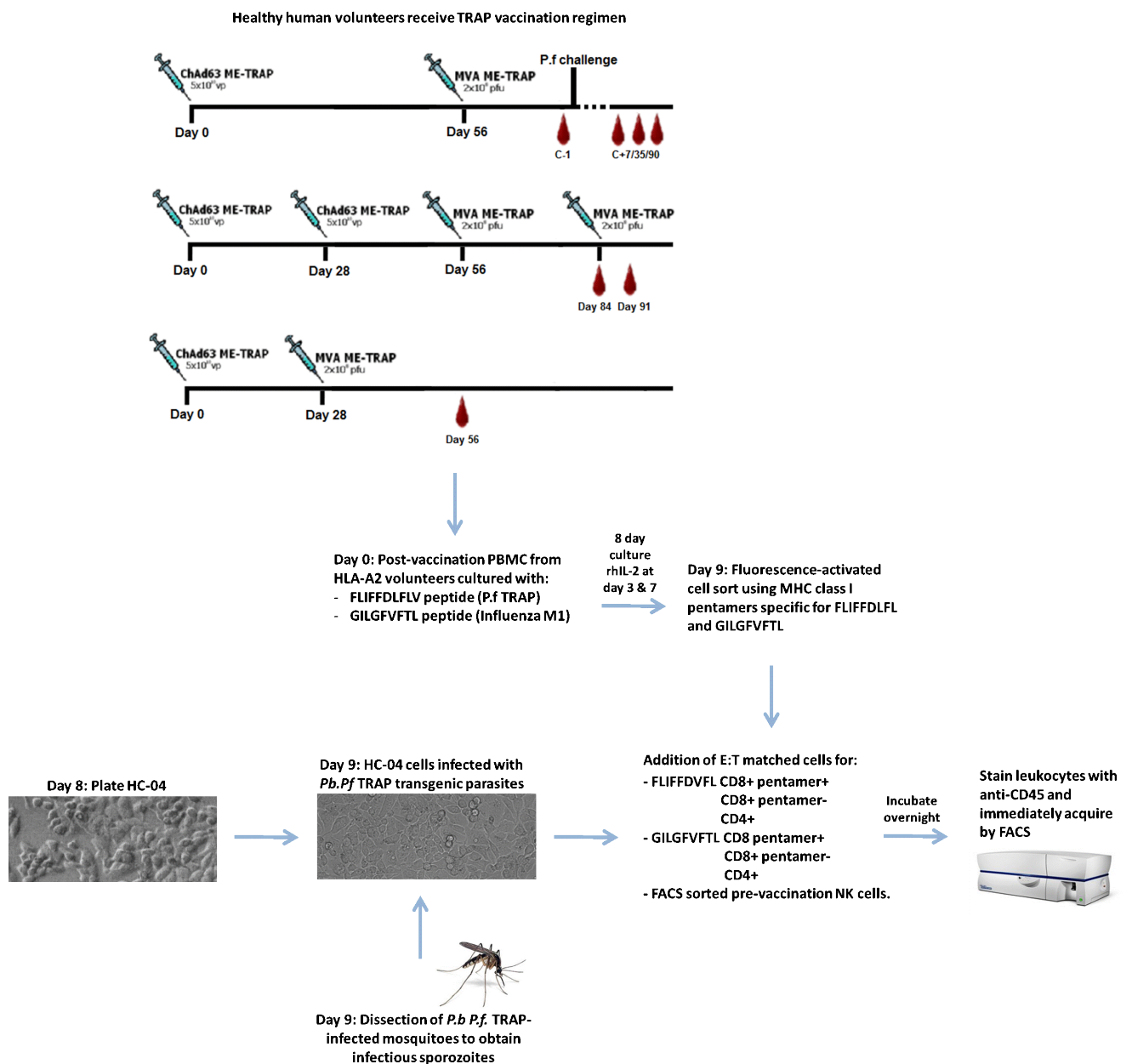


Figure 5.28 Assay schematic for evaluation of TRAP-specific parasite inhibition by vaccinee T cells.

5.3 Discussion

The development of a novel *in vitro* T cell assay to evaluate inhibition of liver stage malaria parasites required extensive optimisation of each aspect of the assay. Preliminary experiments suggested that the assay could not utilise a modified version of the *in vitro* assay used to measure mouse T cell inhibition of parasites due to a need to switch to a HLA compatible hepatoma cell line and because of the low frequencies of TRAP specific cells in peripheral blood post-vaccination.

The initial optimisation steps required the infectivity protocol of *PbPfTRAP* in human hepatoma cells to be as effective as possible. Preliminary experiments measured ~1% infectivity, which left little scope to detect significant parasite inhibition. Selection of cell line, plate type, hepatoma cell seeding density, timing of infection and sporozoite inoculation number were straightforward parameters to optimise, and resulted in 2-5% infection in the HC-04, PLC/PRF/5 and Huh7 lines, which can be reliably measured through a flow cytometry based readout of GFP-positive hepatoma cells. This shows improvement over or similarity to previously published infectivity of *berghei* species in human hepatoma cells lines, of 0.15%-4% in Huh7 and 0.4% in PLC/PRF/5 cells [461] and 0.2-4% in HC-04 cells [482, 494, 495]. The HC-04 and PLC/PRF/5 cell lines were quickly established as preferred lines, as they sustained a good level of infection, showed robustness in their monolayer and were of a matching HLA-A2 or HLA-A3 serotype to the vast majority of volunteers enrolled in the Jenner Institute's UK-based clinical vaccine trials.

The generation of transgenic *P. berghei* sporozoites via the mosquito host is not an aseptic technique, and bacterial contamination would likely affect both the liver and T cells in the assay, with lipopolysaccharide likely to induce a strong pro-inflammatory response from monocytes or dendritic cells present in the assay. Several antibacterial drugs have demonstrated anti-parasitic effects *in vitro*, including erythromycin, tetracycline, chloramphenicol, clindamycin and rifampin [496]. Antibiotic use was required but could not interfere with sporozoite infectivity and liver stage development. Fortunately, the same concentration of penicillin and streptomycin, as used in standard PBMC and cell line culture was sufficient to eliminate bacterial growth in the assay. However, determining the optimal assay media required more experimentation, as the hepatoma cells require culture in MEM, sporozoites in insect media and T cell in RPMI media. Results of a 2010

study on *P. berghei* sporozoites found that sodium bicarbonate was a critical component in cell culture media that initiated sporozoite transformation – a process that is irreversible and therefore would reduce infectivity. Additionally, serum albumin prolongs sporozoite viability [497]. To maintain all 3 cell types in a single culture required hepatoma cells to switched to the same cell culture media as PBMC (RPMI + 10% FCS + 1% penicillin and streptomycin). Sporozoites were dissected into Grace's insect media with 1% penicillin and streptomycin and 1% BSA, and only diluted into the sodium bicarbonate-containing RPMI media immediately prior to liver cell infection, in order to avoid premature transformation.

Hepatoma cells must be distinguishable from T cells if the assay is to have a robust readout. In addition, true GFP signal must be differentiated from hepatoma cell background autofluorescence for infectivity to be accurately measured. The use of DiD membrane dye to identify hepatoma cells, as used in the published murine *in vitro* assay, was not suitable in this setting. Not only did the dye render some cell lines uninfected by sporozoites, it also appeared to affect the ability of the hepatoma cells to retain a viable monolayer. For these reasons, the protocol was reversed to positively identify the T cells in the assay, using an anti-human pan CD45 antibody.

The addition of T cells into the assay required the effects of HLA mismatch between vaccinee T cells and the hepatoma cell lines to be explored. It is known from the mixed lymphocyte reaction that T cells in an individual will respond to stimulation by cells from another, unrelated, member of the same species, due to recognition of polymorphisms in allogeneic MHC molecules. In this reaction, activated T cells rapidly acquire the expression of both Fas and Fas ligand on their surface [498], resulting in cell death. Cross-reactivity of CD8⁺ T cells towards allogeneic class I MHC molecules is well documented for viral antigens [499-501]. NK cells have been reported to aid T cell proliferation in response to non-self MHC in this setting [502]. Furthermore, NK cells are regulated in part by inhibitory receptors that recognize MHC class I molecules. Inhibitory receptors that recognise self HLA class I molecules belong to the KIR (killer cell immunoglobulin-like receptor) family, and when matching HLA-class I molecules are not detected, this inhibitory effect is lost and results in targeted cell death by NK cells [256, 503]. Such responses by CD8⁺ T cells and NK cells have the potential to occur in the *in vitro* parasite killing assay, however such effects should be minimised by

NK cell removal during the pentamer sort, and CD8⁺ T cells corresponding to only a single epitope being present in the assay.

Culture techniques were optimised to produce high numbers of antigen-specific T cells. These cells required sufficient resting before addition into the inhibition assay in order to circumvent high levels of non-specific cytotoxicity. Preliminary experiments showed that TRAP-expanded CD8⁺ T cells could reduce parasite numbers in the *in vitro* model, however these effects were often matched by the autologous control CTL CD8⁺ T cells. This type of result was also seen in some of the early murine *in vitro* T cell parasite inhibition assays, whereby cells from control mice also exhibited a substantial level of inhibition [473] [133].

The effects of cell number in E:T matched experiments could not be elucidated, nor were the antigen-specific cells of high enough frequency to differentiate specific from non-specific parasite inhibition. This approach was successful in the murine model of *in vitro* *P. yoelii* parasite killing by Weiss *et al*, whereby splenocytes from sporozoite immunised mice were expanded in peptide and rat Concanamycin A culture supernatant, as a source of IL-2. Up to 90% parasite inhibition was measured, in a MHC-restricted manner. One can assume that the numbers of antigen-specific cells from vaccinated mice that have also undergone peptide culture were of a high frequency. Peptide plus rhIL-2 cultures of ChAd63.MVA ME-TRAP vaccinee PBMC produced TRAP-specific T cells in the region of just 0.8-1.6%. This was the rationale for further enrichment of antigen-specific cells by pentamer sorting. The finalised assay protocol uses sorted TRAP-epitope and influenza-epitope specific CD8⁺ T cells matched at E:T and cell number, following an 8 day culture, 1 day rest period. These cells are then added into the fully optimised protocol for *PbPft*TRAP infection of human hepatoma cells, and results determined by FACS readout.

The extensive optimisation of this assay has been required in order to generate a protocol with defined parameters. The lack of routine use of the ILSDA and ISI assays for evaluation of vaccine-induced antibodies in human vaccinees has been due to the lack of standardisation between laboratories, lack of assay sensitivity and reproducibility, lack of defined protocol boundaries for publication, and by the non-specific inhibition measured in negative controls. Furthermore, the

subjectivity associated with relying on microscopy and manual counting limited the throughput of these assays [362].

Encouragingly, the standardisation of the *P. falciparum* ILSDA assay was performed by Zou and colleagues in 2013 [184]. This covered many of the aspects of the assay, including the use of primary human hepatocytes over that of HC-04 for *P. falciparum* infection, the use of centrifugation to aid sporozoite infection, evidence of liver stage parasite burden saturation with increasing sporozoite number and the concentration-dependent inhibitory effects of antibodies on parasite development. The importance of such standardisation is highlighted by the MIATA (Minimal Information about T Cell Assays) guidelines for T cell immunoassays, which provide key guidelines for publication regarding samples tested, reagents used, assay conditions, data acquisition and analysis, and laboratory conditions under which the experiments were undertaken [504]. These guidelines are being used by several journals to aid T cell assay standardisation, and with such a complex assay as the human *in vitro* T cell assay of parasite inhibition discussed in this chapter, such rigorous optimisation is a sensible and necessary approach to aiding *in vitro* assay development across the field.

Chapter 6

Functional *in vitro* assay to measure T cell parasite inhibition

6 Functional *in vitro* assay to measure T cell parasite inhibition

6.1 Introduction

Decades of research using rodent models of *Plasmodium* infection have helped guide malaria vaccine development, including the identification of CD8⁺ T cells as critical mediators of liver stage immunity. Despite this, our understanding of the precise mechanism of protective memory CD8⁺ T cell responses against liver stage *Plasmodium* infection is still somewhat unclear. Mouse models have been extensively used to explore the protective mechanisms at the liver stage of malaria, although often having conflicting results, with some mechanisms redundant when tested more stringently. Furthermore, in human studies, direct mechanisms cannot currently be studied, only inferred from correlates of protection.

Sterile liver stage protection in mice and humans can be induced through vaccination with radiation attenuated sporozoites (RAS) [196, 197], and provides a platform for exploring protective mechanisms and correlates of protection. Many studies identify a pivotal role for CD8⁺ T cell in pre-erythrocytic malaria protection [104, 178, 204, 206, 208, 211, 477, 505]. This CD8⁺ T cell role has been demonstrated through the *in vivo* depletion of CD8⁺ T cells in mice leading to abrogation of protection, while adoptive transfer of CD8⁺ T cells to naive mice has also been shown to confer protection. Furthermore, beta-2 microglobulin knockout (-/-) mice are not protected by active immunization against malaria or by adoptive transfer of wild-type, splenic T cells from immune mice [209].

Obvious CD8⁺ T cell effector mechanisms include the release of cytolytic perforin granules alongside Granzymes, in addition to Fas/Fas ligand interactions between the T cells and hepatocytes. However, in mouse models where one or both of these mechanisms are deficient, liver stage *Plasmodium* species can still be eliminated [506, 507]. The mechanistic findings can often depend on the model used. For protection against *P. yoelii* and *P. berghei* in a DC-prime, *Listeria monocytogenes*-boost regimen against CSP epitopes, IFN- γ and TNF- α have been identified as important components of the non-cytolytic pathways that underpin memory CD8⁺ T cell-mediated immunity against liver stage *Plasmodium* infection. Perforin production was also essential for *P.*

yoelii protection [508]. Using a genetically attenuated *P. yoelii* vaccination approach in mice, only a partial requirement for perforin and minimal requirement for IFN- γ for liver stage protection was revealed [474]. Systemic inhibition of IFN- γ and TNF- α in mice have shown these cytokines are not essential for protection, as *P. yoelii* challenged mice were still protected by adoptively transferred protective CD8⁺ T cell clones against CSP [206, 509]. Similar results challenging the importance of IFN- γ include protection against *P. yoelii* challenge of mice receiving adoptively transferred IFN- γ deficient CS-specific CD8⁺ T cells [29].

Several studies using models ranging from irradiated and genetically attenuated parasite vaccination, to DNA vaccination and synthetic peptide vaccination with and without adjuvant, do identify a critical role for IFN- γ involvement in CD8⁺ T cell mediated protection [104, 211, 510, 511]. The requirement for both CD8⁺ T cells and IFN- γ in the effector mechanism of sporozoite-induced protective immunity suggests that the IFN- γ is secreted by CD8⁺ T cells, as a result of the interaction of CD8⁺ T cells with the peptide-MHC complex on the surface of the infected hepatocyte [204, 512].

Additionally, a role for nitric oxide was common, and it is hypothesised that IFN- γ from malaria-specific CD8⁺ T cells, induces cells of the liver and/or Kupffer cells to produce nitric oxide (NO) for the destruction of infected hepatocytes or the parasite within these cells [104, 509, 513]. Thus CD8⁺ T cells in liver stage protection may not necessarily be that of cytotoxicity by direct CD8⁺ T cell Fas/Fas ligand interactions or exocytosis of lytic granules and Granzymes. Further to this, unpublished data also elucidates a role for CD4⁺ T cells and IFN- γ , mediating protection in a non-cytotoxic manner.

Roles of IL-1, IL-6 and TNF- α have also been shown *in vivo* and *in vitro* using the rat and mouse *P. berghei* model [514-516]. Furthermore, infection of primary mouse cells indicated addition of IFN- γ consistently reduced parasite infection when added 3 hours after infection, and that this effect was lost upon competitive inhibition or depletion of L-arginine, the substrate for production of nitric oxide by hepatocytes. Furthermore, the effects of recombinant IFN- γ were not enhanced with addition of TNF- α , nor did recombinant TNF- α inhibit parasite liver stage infection when used alone [509].

Studies regarding mechanisms of protection in human hepatocytes are less numerous. *In vitro* assays using *P. yoelii* infection of HepG2-CD81 (modified human HepG2 hepatoma cells) and *P. yoelii* infection of primary human hepatocytes showed a reduction in parasite infection when hepatocytes were pre-incubated with TNF- α (24-48 before parasite infection), with no additional cellular component within the system, indicating either direct anti-parasitic effects of TNF- α or an inducible effect upon infected hepatocytes. However, this model gives little indication of vaccine-induced protection, as the effects were only seen with pre-incubation of TNF- α , although the mechanisms indicated that this was not NO dependent, suggesting other mechanisms are induced by TNF- α [517]. A more detailed mechanism related to TNF- α had previously been elucidated through a rodent malaria model, whereby the inhibitory effect was mediated by IL-6 secreted by non-parenchymal liver cells in response to TNF- α stimulation [516].

A second study of *P. berghei* infection in HepG2 cells showed that recombinant human IFN- γ reduced parasite burden by up to 75% when added 3-6 hours following completion of sporozoite infection, or by 100% when added 6 hours prior to invasion [518]. This has more relevant implications with regard to vaccine-induced protective mechanisms, as the effector molecule could be supplemented after the time of infection.

Radiation attenuated sporozoite vaccination of human subjects has been successful in providing protection from sporozoite challenge [197, 198, 214]. Immunogenicity data from these studies shows that RAS vaccination of human volunteers elicits responses consisting of IFN- γ , TNF- α , and IL-2, following direct *ex vivo* sporozoite stimulation of PBMC [214, 215]. A similar but lower frequency cytokine profile was measured in human subjects following vaccination with virulent sporozoites concurrent with the administration of anti-malarial drug chloroquine [217, 218]. More detailed examination of the RAS vaccination immunogenicity data revealed a dose-dependent increase in the frequency of *Plasmodium*-specific IFN- γ ⁺ CD8 T cells that was associated with protection of human subjects from challenge [16]. This finding is consistent with clinical vaccine trials of ChAd63.MVA ME-TRAP, where monofunctional TRAP-specific CD8⁺ IFN- γ producing T cells correlate with protection following CHMI [287].

The aims of this chapter were to run the newly developed *in vitro* parasite inhibition assay to quantify TRAP-specific protection from vaccinee lymphocytes, and use the supernatants from these assays to explore correlates of protection, that may elucidate the protective mechanism at play. Many of the previously identified correlates from rodent models, *in vitro* models using human hepatocytes and human clinical vaccine trials guided analyte choice for such analysis of assay supernatants. Finally, the *in vitro* inhibition assay was extended to a *P. falciparum* model in primary human hepatocytes, to explore whether similar results were measured with wildtype parasites in non-carcinoma tissues, better mimicking natural human infection.

6.2 Results

6.2.1 Measuring *in vitro* parasite inhibition by anti-malarial drugs

Currently, Primaquine and Atovaquone are the only licensed and commercially available antimalarial drugs that target liver stage *Plasmodium* parasites. Primaquine is active against both gametocytes, which are responsible for mosquito to human transmission, and all exoerythrocytic forms of *Plasmodium*, including the latent hypnozoites of *P. ovale* and *P. vivax* [78, 483]. Atovaquone has inhibitory effects on *P. berghei* in cultured hepatocytes [519] and in rats [520] and *P. falciparum* in humans [521], in addition to against erythrocytic falciparum parasites [522].

To assess the sensitivity of the assay to measure targeted parasite inhibition, increasing titrations of these drugs were supplemented into the assay following sporozoite infection, using titrations in published studies as a guide [523, 524]. Both Primaquine and Atovaquone reduced the percentage infection of *PbPf*TRAP in HC-04 cells, in addition to the number of infected cells (Figure 6.1 A+B, Figure 6.2 A+B, Kruskal-Wallis test). At higher titrations Primaquine was also toxic to the HC-04 cells, which resulted in significantly fewer live cells recovered 22 HPI (hours post infection) when 50 μ M Primaquine was used (Figure 6.1 D, $p=0.0018$. Kruskal-Wallis test). While the number of cells detected as GFP positive were reduced with drug treatment, when the drug titration was suboptimal, the remaining infected cells had significantly lower geometric mean fluorescence intensity of parasite GFP. As previously described, parasite GFP fluorescence intensity is a measure of the extent of parasite development [483], thus the reduced intrahepatic growth measured here is indicative of reduced fitness of the remaining parasites (Figure 6.1 C, Figure 6.2 C, Kruskal-Wallis test).

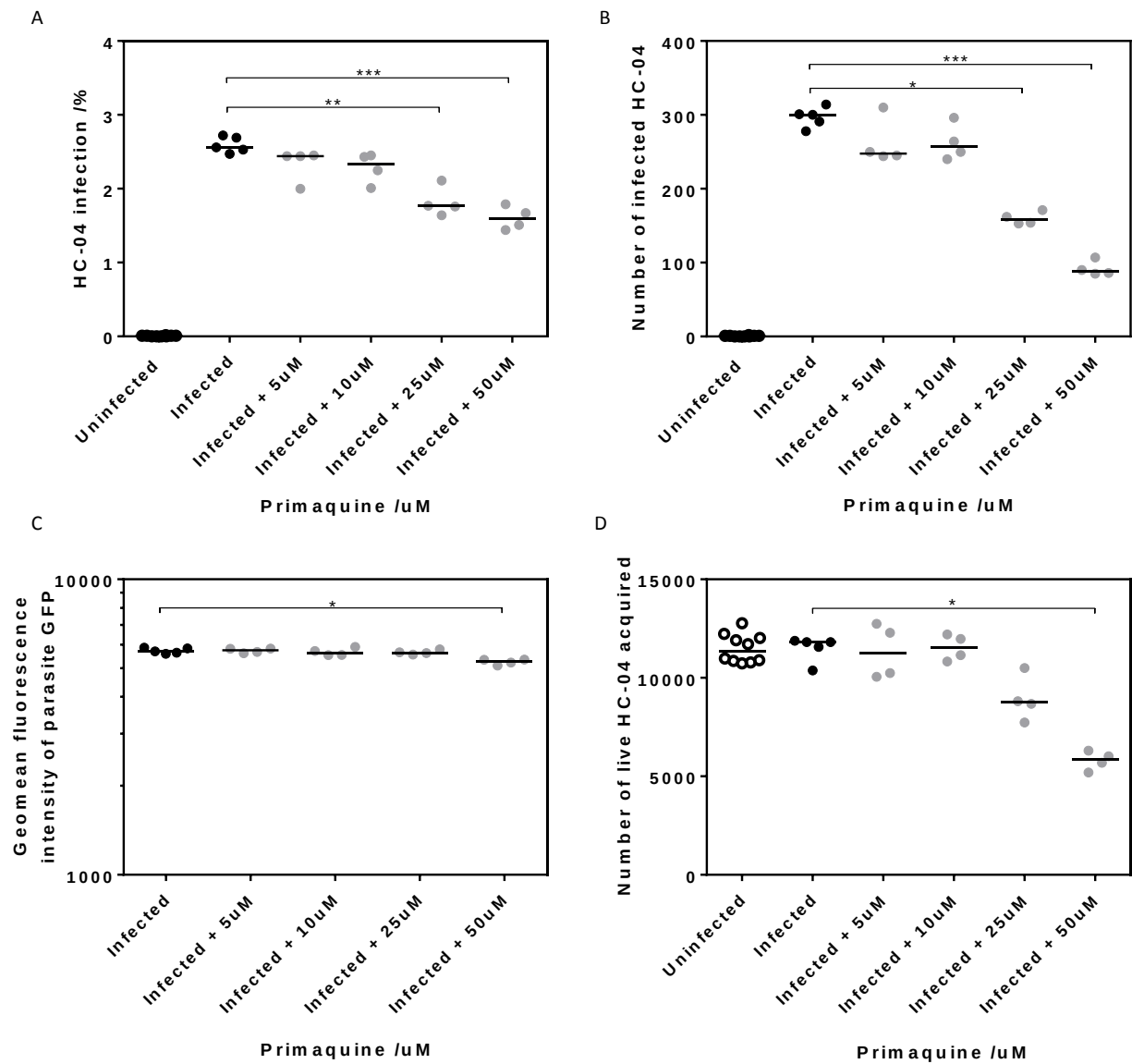


Figure 6.1 Effects of Primaquine on *in vitro* *P. berghei* infection

20,000/well HC-04 cells were plated in half surface area 96 well plates. 24 hours later, 14,000 *PbPf*TRAP sporozoites were added per well according to the standard infection protocol. 3 hours later, sporozoites were removed and HC-04 washed in fresh media, before the addition of Primaquine. Cultures were acquired at 22 HPI, according to the standard acquisition protocol. Kruskal-Wallis tests with Dunn's correction for multiple comparisons against infected control group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Medians displayed.

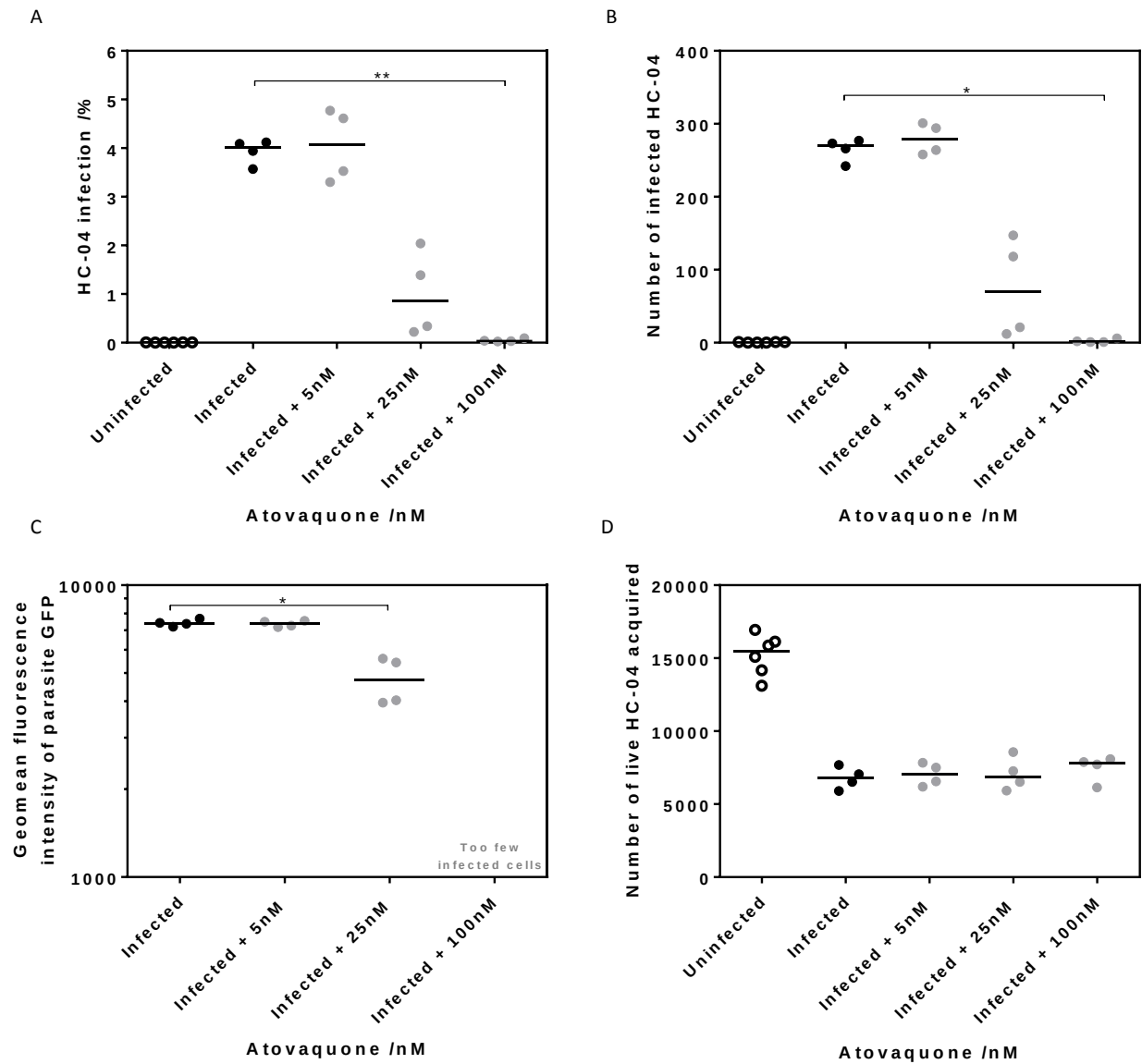


Figure 6.2 Effects of Atovaquone on *in vitro* *P. berghei* infection

20,000/well HC-04 cells were plated in half surface area 96 well plates. 24 hours later, 17,200 *PbPf*TRAP sporozoites were added per well according to the standard infection protocol. 3 hours later, sporozoites were removed and HC-04 washed in fresh media, before the addition of Atovaquone. Cultures were acquired at 23 HPI, according to the standard acquisition protocol. Kruskal-Wallis tests with Dunn's correction for multiple comparisons against infected control group, * $p < 0.05$. ** $p < 0.01$. Medians displayed.

Time course analysis of drug-mediated parasite inhibition was explored, to ascertain whether the effects measured were time-dependent following sporozoite infection. Significant parasite inhibition was measured only when Atovaquone was added in the first 8 hours of infection. Addition of Atovaquone at 20 HPI resulted in no parasite inhibition when measured 2 hours later, at 22 HPI (Figure 6.3 A+C, Kruskal-Wallis test) and non-significant parasite inhibition 10 hours later at 32 HPI (Figure 6.3 B+D, Kruskal-Wallis test). These results emphasise the requirement of early initiation/addition of anti-parasitic effector molecules for optimal parasite inhibition. Therefore, in the *in vitro* inhibition assay, CD8⁺ T cells will be added within the first 8 hours of parasite infection.

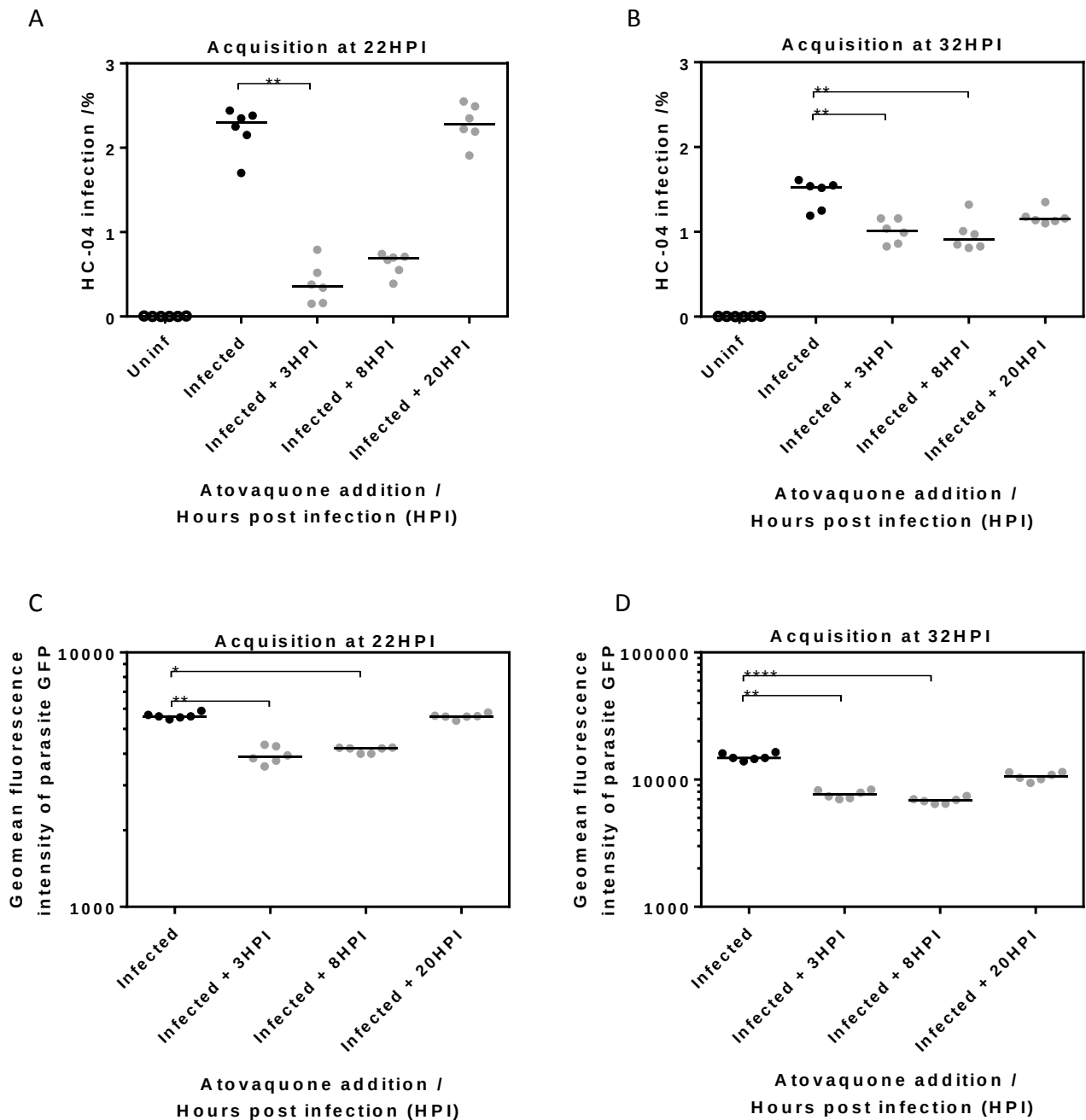


Figure 6.3 Summary of Atovaquone time course

20,000/well HC-04 cells were plated in half surface area 96 well plates. 24 hours later, 14,600 *PbPf*TRAP sporozoites were added per well according to the standard infection protocol. 3 hours later, sporozoites were removed and HC-04 washed in fresh media, before the addition of Atovaquone at 3, 12 and 20 HPI. Cultures were acquired at 22 and 32 HPI, according to the standard acquisition protocol. Kruskal-Wallis tests with Dunn's correction for multiple comparisons against infected control group, * $p < 0.05$. ** $p < 0.01$, **** $p < 0.0001$. Medians displayed.

6.2.2 CD4⁺ and CD8⁺ T cells inhibit *PbPf*TRAP parasites in a TRAP-specific manner

With the assay capable of detecting both a reduction in infection and reduced parasite growth, cultured T cells from 9 ChAd63.MVA ME-TRAP vaccinees were tested according to the newly developed protocol. Briefly, thawed PBMC from post MVA ME-TRAP time points were rested then cultured with either *P. falciparum* TRAP epitope FLIFFDLFLV or influenza matrix protein 1 epitope GILGFVFTL, with recombinant human IL-2 added at day 3 and day 7. Cultures were recovered on day 8 and rested overnight. Cell recovery was 38-80% (median = 60%). Rested cells were then stained using an MHC class I pentamer to the epitope homologous to the culture condition, alongside a basic surface marker panel and propidium iodide for live cell identification. Cells were sorted under sterile conditions on a FACS Aria III into 3 separate populations: live CD4⁺ CD8⁻ CD56⁻, live CD4⁻ CD8⁺ CD56⁻ pentamer⁺, live CD4⁻ CD8⁺ CD56⁻ pentamer⁻. Cell fractions were left to incubate at 37°C for a minimum of 7 hours. Depending on the number of cells recovered from the sort, matched cell numbers of each population were added into the HC-04 model, 4-6 hours following *PbPf*TRAP infection. 23-24 hours post infection, assay supernatants were removed and stored at -20°C. The HC-04 and T cell co-cultures were stained with anti-CD45 APC, then removed from the plate using trypsin digest, followed by immediate acquisition by FACS.

Percentage inhibition for cell fractions from each volunteer from TRAP FLIFFDLFLV culture (denoted F) and influenza GILGFVFTL (denoted G) was calculated by the reduction in number of infected HC-04, as compared to the infected control wells internal to each assayed plate. Infectivity in these control wells across the 9 experiments was 1.6-7.5%. A significant effect of antigen on parasite inhibition was measured with CD8⁺ pentamer⁺ T cells, with TRAP-specific cells exhibiting a mean of 7.3% parasite inhibition (range -0.3-17.2%) over that of autologous influenza-specific CD8⁺ pentamer⁺ T cells (Figure 6.4, 2-way-ANOVA, $p=0.0015$). As expected, the individual vaccinees tested (denoted volunteer) were responsible for a substantial proportion of variation measured in parasite inhibition, in this case 33.6%. (Figure 6.4, $p=0.0001$, 2-way ANOVA).

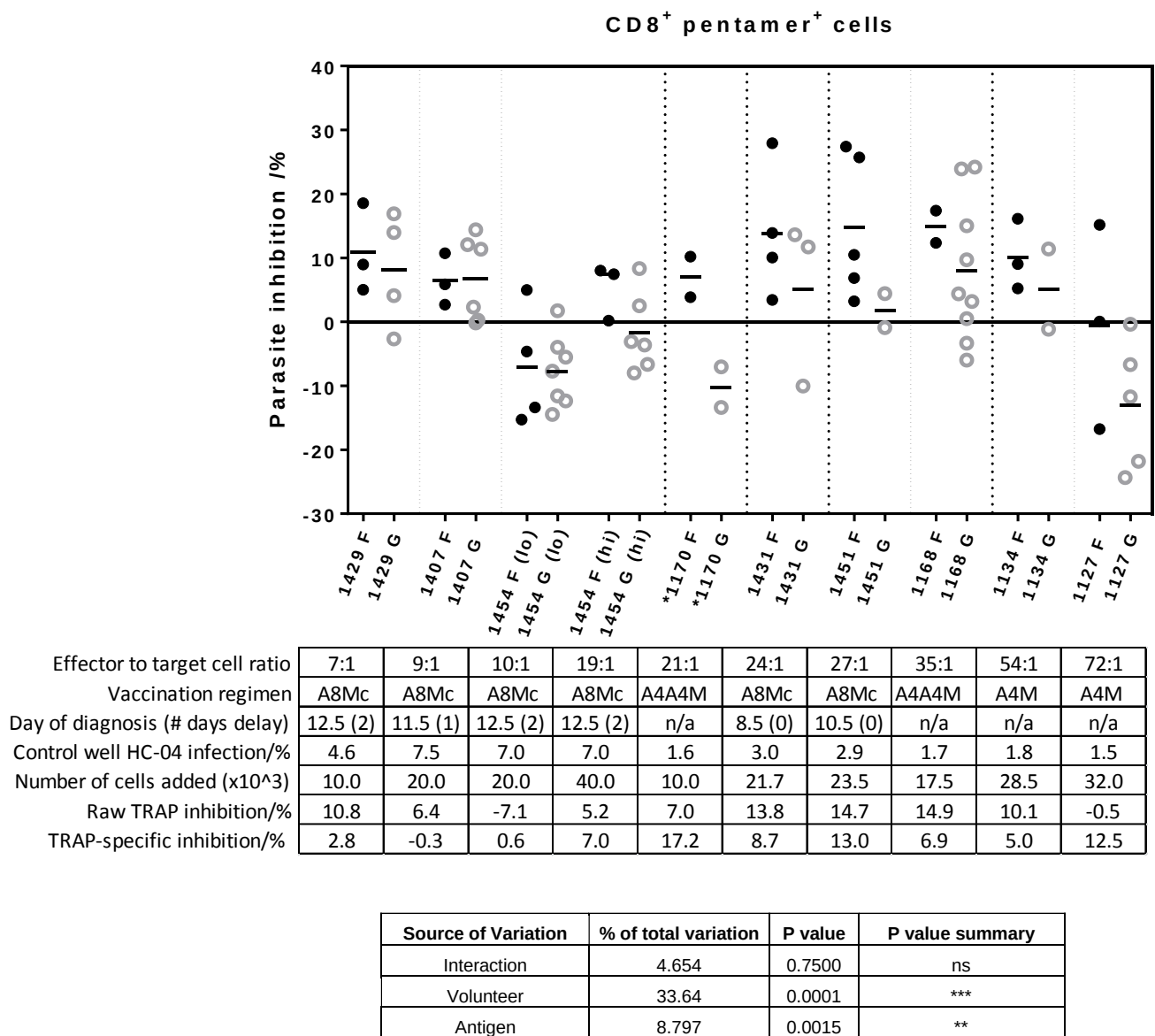


Figure 6.4 Summary of parasite inhibition assay with E:T-matched FLIFFDLFL-specific (F) and GILGFVFTL-specific (G) CD8⁺ pentamer⁺ T cells.

20,000/well HC-04 cells were plated in half surface area 96 well plates. 24 hours later, a mean of 14,700 (range 13,500-17,000) *PbPf*TRAP sporozoites were added per well according to the standard infection protocol. 3 hours later, sporozoites were removed and HC-04 washed in fresh media, before the addition of CD8⁺ pentamer⁺ T cells 1-3 hours later. Cultures were acquired at 25-30 HPI, according to the standard acquisition protocol. 2-way ANOVA with Sidak post-test for comparisons between FLIFFDLFL and GILGFVFTL cell populations, ** p<0.01, *** p<0.001. Mean inhibition displayed.

*'1170' = gating strategy altered based on laser availability.

TRAP-specific parasite inhibition did not correlate with extent of T cell and hepatoma cell serotype matching at HLA class I or class II loci, or with numbers of CD8⁺ pentamer⁺ cells added into the assay (Figure 6.5 A-C, Spearman rank). Percentage infection negatively correlated with TRAP-specific parasite inhibition measured (Figure 6.5 E, Spearman $r = -0.6991$, $p=0.0299$, significance is lost when p-value is corrected for the 5 correlations performed). This was consistent with a positive, yet non-significant correlation of parasite inhibition with E:T of CD8⁺ pentamer⁺ cells used (Figure 6.5 D, Spearman $r = 0.5273$, $p=0.1231$). Furthermore, in the single volunteer with sufficient CD8⁺ pentamer⁺ T cells to measure 2 separate E:T, TRAP-specific parasite inhibition increased from 0.6% to 7.0% with an E:T increase from 10:1 and 19:1 respectively (Figure 6.4, volunteer 1454 lo and 1454 hi). This indicates that the number of T cells in proportion to the number of infected cell targets and subsequent the interaction between these cells, may be key in determining cell-mediated parasite inhibition, as opposed to simply the number of CD8⁺ T cells *per se*.

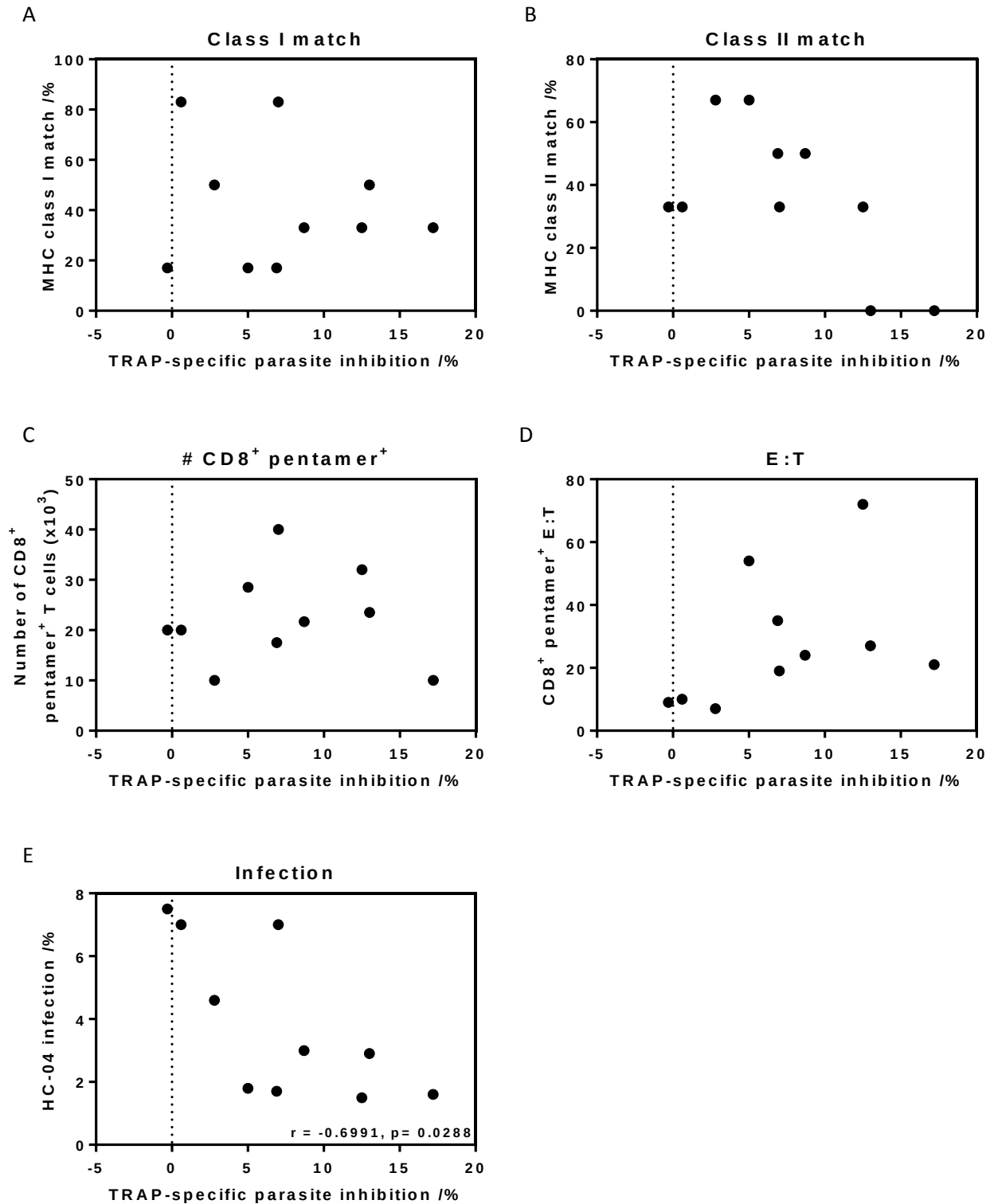


Figure 6.5 Correlation of CD8⁺ pentamer⁺ TRAP-specific parasite inhibition with assay parameters

Correlation between TRAP-specific parasite inhibition and A) MHC class I matching between T cells and HC-04 cells ($r = 0.1063$, $p=0.7702$), B) MHC class II matching between T cells and HC-04 cells ($r = -0.5178$, $p=0.1304$), C) numbers of CD8⁺ pentamer⁺ T cells added per well ($r = 0.1768$, $p=0.6236$), D) effector to target cell ratio ($r = 0.5273$, $p=0.1231$) and E) percentage infection for each assay ($r = -0.6991$, $p=0.0288$). Correlations measured by Spearman rank test.

The same assay was performed concurrently with the CD8⁺ pentamer⁻ and CD4⁺ T cell fractions from the FLIFFDLFLV and GILGFVFTL cultures. Unlike the CD8⁺ pentamer⁺ cells, significant antigen-specific parasite inhibition was not measured using CD8⁺ T cells that were not specific to the peptide culture i.e. pentamer⁻ cells (Figure 6.6, 2-way ANOVA). This supports the notion that CD8⁺ pentamer⁺ parasite inhibition was indeed TRAP-specific. If the effects were non-specific, parasite inhibition would also be measured with non-TRAP specific cells from the TRAP epitope culture. Variation in parasite inhibition due to volunteer measured with CD8⁺ pentamer⁻ cells was similar to that of the pentamer⁺ cells, at 33.3%. This suggests some volunteer-specific effects that are unrelated to antigen in the assay, as indicated by lack of significant interaction between volunteer and antigen. Such volunteer-specific effects could be related to number of cells added into the assay, non-specific cellular effects from rhIL-2 culture or variation in HLA class I or other human genes.

A significant antigen-specific effect was measured with CD4⁺ T cells, with CD4⁺ T cells sorted from TRAP culture exhibiting a mean of 7.0% parasite inhibition (range -2.6 -21.9%) over that of autologous CD4⁺ T cells from influenza culture (Figure 6.7, ***p<0.001, 2-way ANOVA). Furthermore, TRAP-specific parasite inhibition achieved significance within an individual vaccinee, where 21.9% inhibition over the influenza cultured CD4⁺ T cells was measured (Figure 6.7, *p<0.05, 2-way ANOVA). The effect of volunteer was slightly lower than measured with the other cell fractions, at 27%. A larger and more significant effect of antigen was measured with CD4⁺ T cells, compared with CD8⁺ pentamer⁺ cells (9.4% and 8.8%, respectively). Similarly to CD8⁺ pentamer⁺ inhibition, CD4⁺ parasite inhibition was unrelated to number of CD4⁺ T cells added into the assay, or to level of HLA class I or II matching (Figure 6.8 A-C, Spearman rank test). However, unlike the associations seen with CD8⁺ pentamer⁺ parasite inhibition, CD4⁺ E:T ratio and percentage infection of HC-04 cells did not correlate with CD4⁺ T cell TRAP-specific parasite inhibition (Figure 6.8 D-E, Spearman rank).

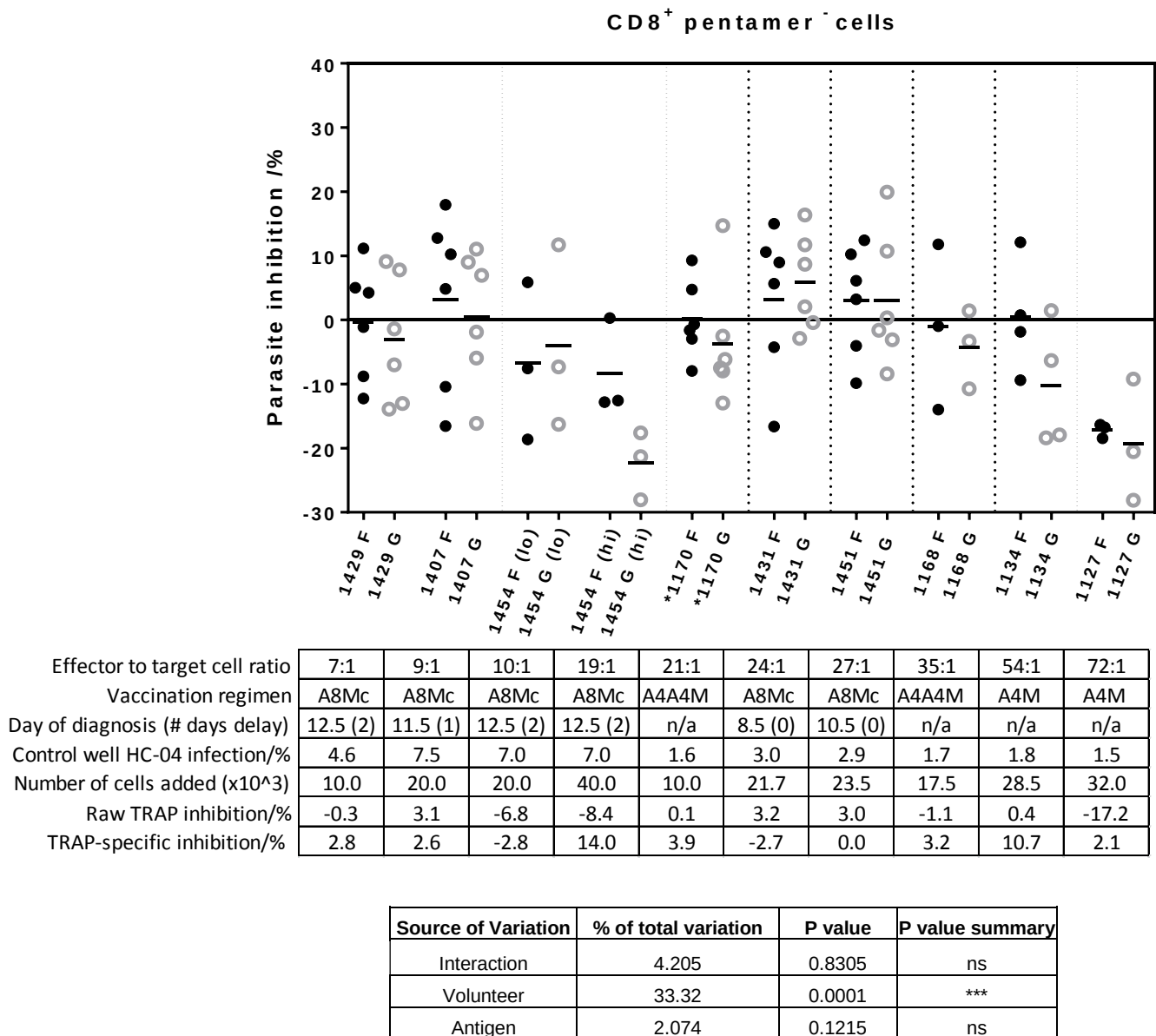


Figure 6.6 Summary of parasite inhibition assay with E:T-matched CD8⁺ pentamer⁻ T cells from FLIFFDLFLV (F) and GILGFVFTL (G) cultures

20,000/well HC-04 cells were plated in half surface area 96 well plates. 24 hours later, a mean of 14,700 (range 13,500-17,000) *PbPf*TRAP sporozoites were added per well according to the standard infection protocol. 3 hours later, sporozoites were removed and HC-04 washed in fresh media, before the addition of CD8⁺ pentamer⁻ T cells 1-3 hours later. Cultures were acquired at 25-30 HPI, according to the standard acquisition protocol. 2-way ANOVA with Sidak post-test for comparisons between FLIFFDLFL and GILGFVFTL cell populations, *** p<0.001. Mean inhibition displayed. ‘*1170’ = gating strategy altered based on laser availability.

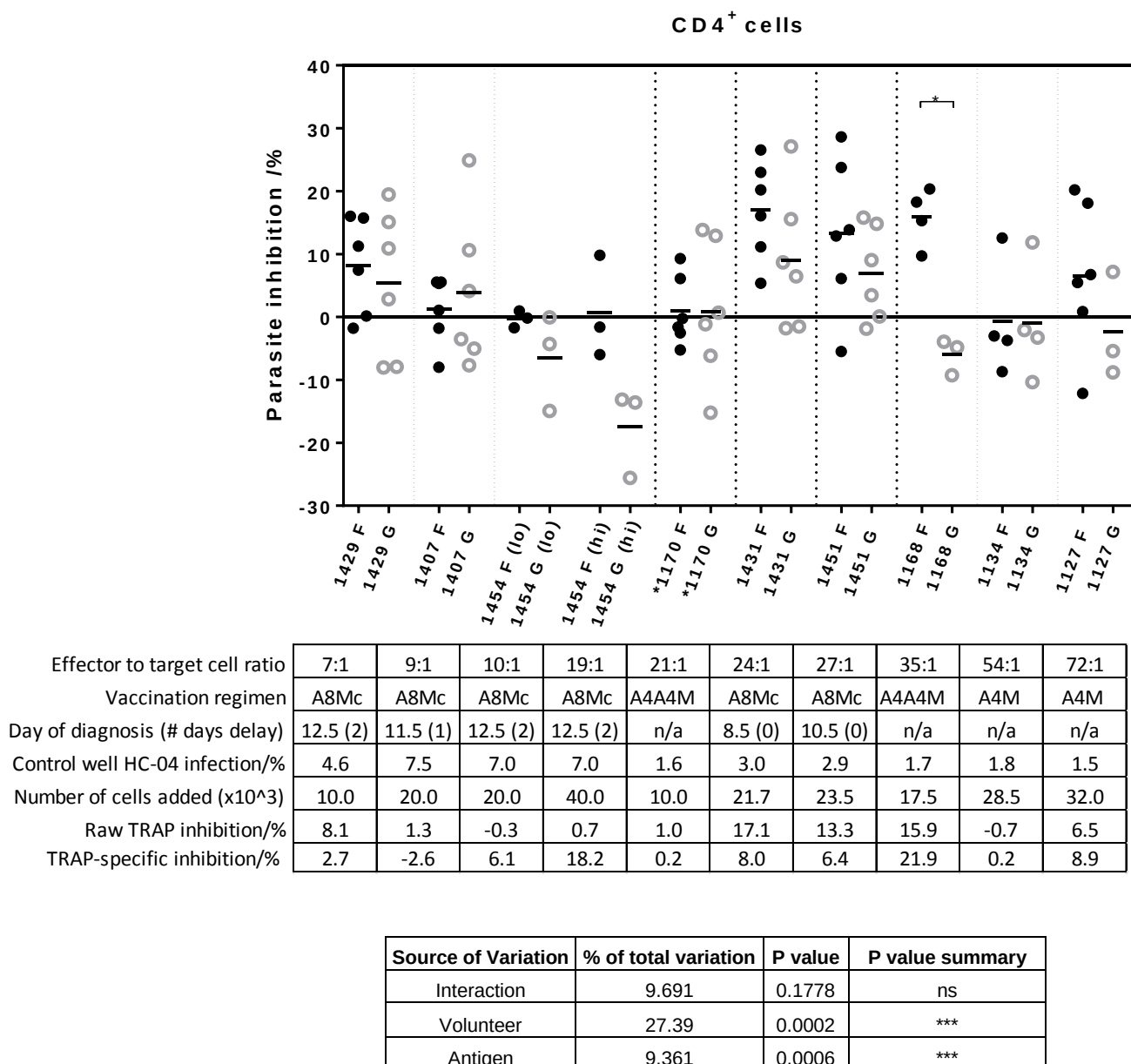


Figure 6.7 Summary of parasite inhibition assay with E:T- matched CD4⁺ T cells from FLIFFDLFLV (F) and GILGFVFTL (G) cultures

20,000/well HC-04 cells were plated in half surface area 96 well plates. 24 hours later, a mean of 14,700 (range 13,500-17,000) *PbPf*TRAP sporozoites were added per well according to the standard infection protocol. 3 hours later, sporozoites were removed and HC-04 washed in fresh media, before the addition of CD4⁺ T cells 1-3 hours later. Cultures were acquired at 25-30 HPI, according to the standard acquisition protocol. 2-way ANOVA with Sidak post-test for comparisons between FLIFFDLFL and GILGFVFTL cell populations. * p<0.05, *** p<0.001. Mean inhibition displayed. ‘*1170’ = gating strategy altered based on laser availability.

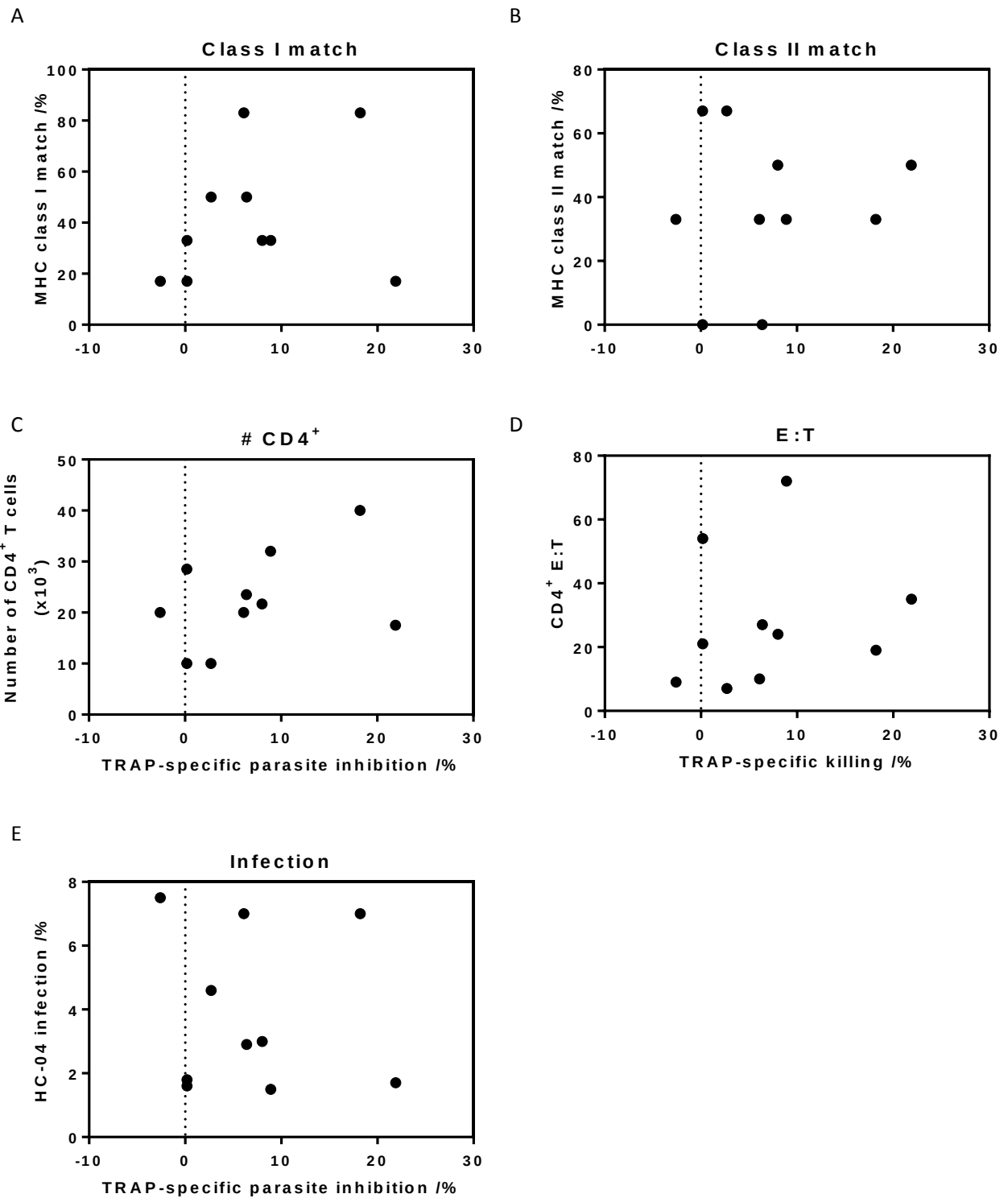


Figure 6.8 Correlation of CD4⁺ TRAP-specific parasite inhibition with assay parameters

Correlation between TRAP-specific parasite inhibition and A) MHC class I matching between T cells and HC-04 cells ($r = 0.2509$, $p = 0.4775$), B) MHC class II matching between T cells and HC-04 cells ($r = 0.0380$, $p = 0.9214$), C) numbers of CD4⁺ T cells added per well ($r = 0.3700$, $p = 0.2884$), D) effector to target cell ratio ($r = 0.4073$, $p = 0.2414$) and E) percentage infection for each assay ($r = -0.2500$, $p = 0.4813$). Correlation measured by Spearman rank test.

6.2.3 Innate responses of pre-vaccination NK cells to *P. berghei* infection

NK cells can provide innate effects against liver stage malaria. Recently, it was shown that *Plasmodium* liver-stage infection induces a typical type I IFN signature in the liver of infected mice, with hepatocytes being the primary source of the response [101]. With this in mind, innate immune responses to liver stage infection are likely triggered by this hepatic response. As hepatocytes are not substantial producers of IFN- γ [525], it is hypothesised that the type I IFN response likely recruits myeloid cells, in particular NK and NK-T cells, which are known to secrete large amounts of IFN- γ , to the infected hepatocytes [526]. Furthermore, NK themselves possess diverse PRRs which do not only recognize PAMPs, but also damage-associated molecular patterns (DAMPs), which could be directly stimulated by infected hepatocytes. Liver-resident lymphocytes, consisting of NK cells, NKT cells, T cells, and B cells, account for about 25% of the non-parenchymal cells present in the liver [527] with NK cells representing about 35–50% of this population [528]. NK cells were therefore tested in the parasite inhibition assay, with the hypothesis that IFN- γ alone could produce anti-parasitic effects, with or without the addition of NK cytotoxic activities. Furthermore, NK cells and interleukin-12 (IL-12) have reported roles in supporting the priming of CD8⁺ T cells in the *P. yoelli* mouse model [529].

With no parasite-specific, adaptive cellular response from pre-vaccination cells, innate responses of NK cells were explored within the *in vitro* parasite inhibition assay. Pre-vaccination PBMC from the same 9 volunteers who were subsequently vaccinated with ChAd63.MVA ME-TRAP were simply thawed, stained and sorted on live CD4⁺ CD8⁺ CD56⁺. Without a direct control for the non-specific effects of NK cells on HC-04 cells, percentage infection was used as the assay readout, as opposed to number of infected cells. Non-specific effects of NK cells would target uninfected and infected HC-04 equally, thus maintaining the same percentage infection. Pre-vaccination CD56⁺ NK cells from 7/9 volunteers inhibited the parasite development (Figure 6.9). Inhibition ranged from -1.3 - 19.3%, and was not associated with level of HLA-mismatch at class I or II loci or total number of NK cells added into the assay (Figure 6.10 A-C, Spearman rank). Conversely to the inhibition measured by CD8⁺ pentamer⁺ T cells, a positive correlation was measured between inhibition by NK cells and percentage infection of HC-04 cells (Figure 6.10 E, Spearman $r = 0.7699$, $p=0.0193$). This

finding could be linked to the direct activation of NK cells by infected hepatocytes, and thus a larger percentage infection with more infected hepatocytes per well, resulted in increased NK activation and effector functions in the vicinity of infected cells.

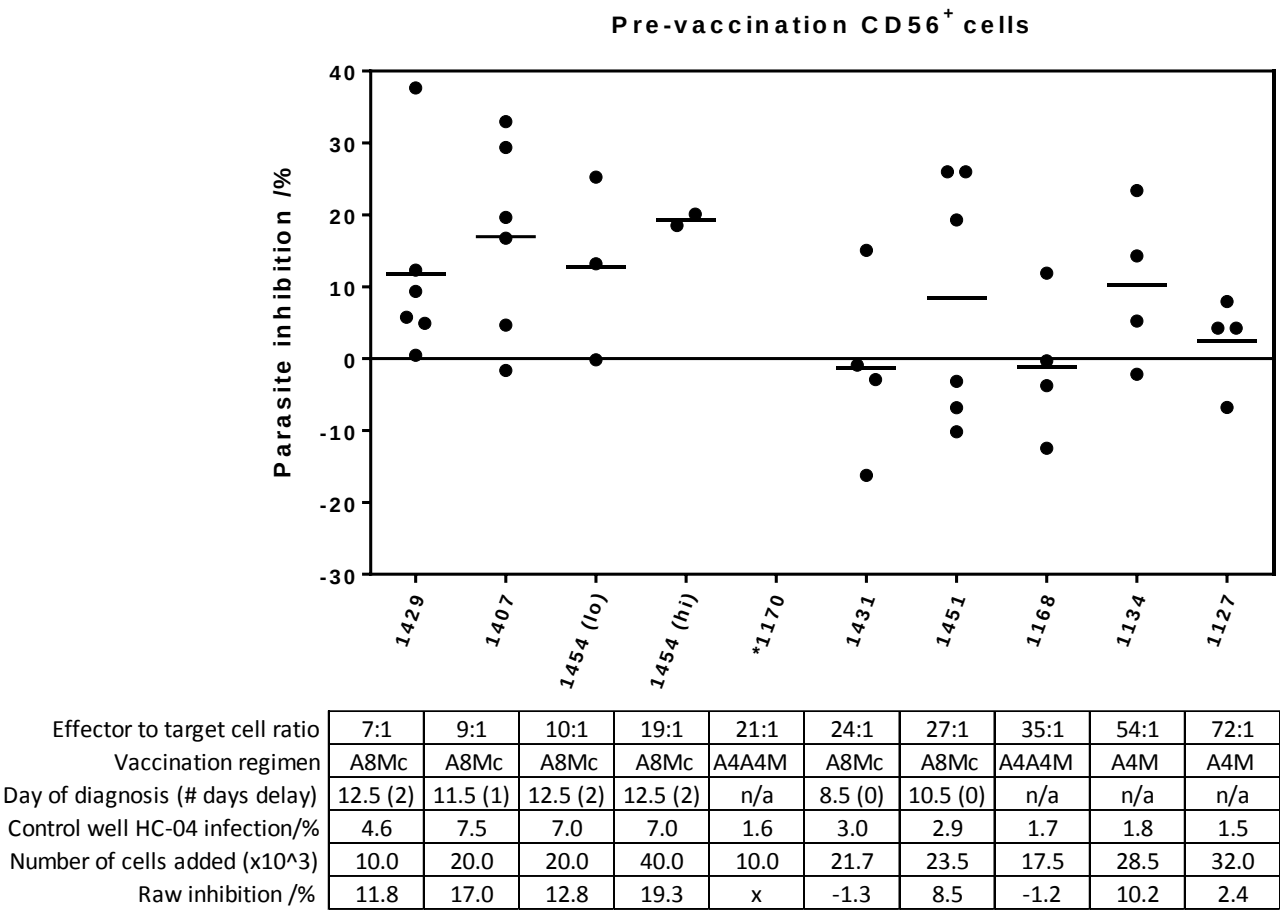


Figure 6.9 Summary of parasite inhibition assay with pre-vaccination CD56⁺ NK cells

20,000/well HC-04 cells were plated in half surface area 96 well plates. 24 hours later, a mean of 14,700 (range 13,500-17,000) *PbPf*TRAP sporozoites were added per well according to the standard infection protocol. 3 hours later, sporozoites were removed and HC-04 washed in fresh media, before the addition of CD56⁺ NK cells 1-3 hours later. Cultures were acquired at 25-30 HPI, according to the standard acquisition protocol. Mean inhibition displayed. ‘*1170’ percentage infection data not available, due to altered FACS gating strategy.

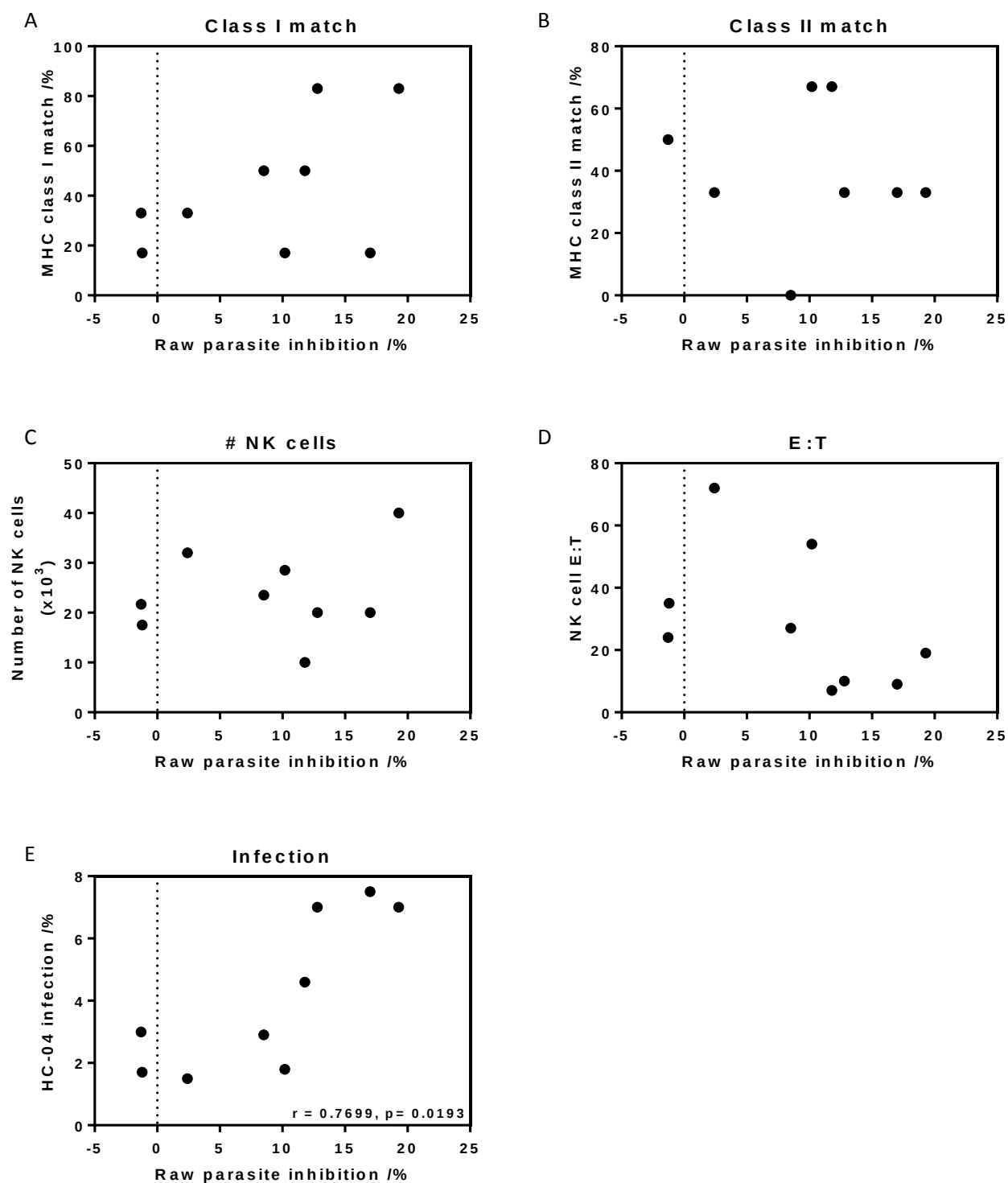


Figure 6.10 Correlation of CD56⁺ NK cell-mediated parasite inhibition with assay parameters

Correlation between TRAP-specific parasite inhibition and A) MHC class I matching between T cells and HC-04 cells ($r = 0.4122$, $p = 0.2683$), B) MHC class II matching between T cells and HC-04 cells ($r = -0.2372$, $p = 0.5407$), C) numbers of CD56⁺ NK cells added per well ($r = 0.1088$, $p = 0.7838$), D) effector to target cell ratio ($r = -0.6000$, $p = 0.0968$) and E) percentage infection for each assay ($r = 0.7699$, $p = 0.0193$). Correlation measured by Spearman rank test.

6.2.4 Exploring mechanisms of parasite inhibition

6.2.4.1 Analysis of supernatants from parasite inhibition assay

Supernatants from the CD8⁺ pentamer⁺, CD4⁺ T cell and CD56⁺ NK aspects of the *in vitro* parasite inhibition assay were assessed by Luminex, to determine whether any key soluble analytes correlated with the parasite inhibition measured. Twenty analytes were measured (Table 22). These were selected based on published associations with protection in human, animal and *in vitro* models of liver stage malaria infection, as well as positive analytes from previous pilot testing of supernatants from this model (data not shown). Analytes included key pro-inflammatory cytokines such as IFN- γ , TNF- α and IL-12, effector molecules such as Fas/Fas ligand and Granzyme A and B, as well as MIP-1 α and CXCL9 chemokines. Analysis was performed on raw data in uninfected and infected HC-04 wells, and for CD8⁺ pentamer⁺ T cells, CD4⁺ T cells or CD56⁺ NK cells by subtraction of baseline analyte level from infected HC-04 cells from that of the analyte level in the presence of leukocytes. A threshold of >10pg/mL was applied to determine a positive response, which in the original 100 μ L inhibition assay, equated to >1pg. All negative control wells containing only cell culture media measured undetectable levels of each analyte. (For raw Luminex data, see Appendix 3).

Table 22 Luminex panel summary

Analytes measured by R&D Luminex Assay® and the associated data QC using internal standard curve.

Analyte name	QC pass
CCL3/MIP-1 alpha	Yes
CXCL8/IL-8	No, many values not on linear section of standard curve
CXCL9/MIG	Yes
Fas	Yes
Fas Ligand	Yes
GM-CSF	Yes
Granzyme A	Yes
Granzyme B	Yes
IFN-gamma	Yes
IL-1 alpha	Yes
IL-1 beta	Yes
IL-10	Yes
IL-12 p70	Yes
IL-17A	Yes
IL-18	Yes
IL-2	Yes
IL-21	Yes
IL-3	Yes
IL-6	Yes
TNF-alpha	Yes

6.2.4.2 Baseline analyse expression from HC-04 cells

Expression of analytes was comparable between uninfected and infected cells, across the 9 *in vitro* experiments, with infection status not a significant factor in determining the analyte expression profile (Figure 6.11, $p=0.8953$, 2-way ANOVA). Most analytes fell under the 10pg/mL cut off, but substantial expression of IL-2, CXCL9 and Fas was measured. CXCL9 is reported to be expressed by hepatocytes and hepatic non-paranchymal cells during Concanavalin A-induced hepatic injury in mice [530], by mouse hepatoma cell line Hepa 1-6 in response IFN- γ and/or TNF- α . *in vitro* [530] and in tissue samples from human patients with hepatocellular carcinoma [531]. Thus CXCL9 detection in all HC-04 wells acts as a positive control within the assay. Detection of Fas receptor expression was encouraging, as carcinoma cells often downregulate Fas receptor expression as a means of immune escape [532], which could be detrimental to the mechanisms being explored using this assay. However, it is known that level of Fas receptor expression is not correlated with susceptibility of the cells to Fas ligand induced apoptosis [533] and thus remains a possible limitation of the readout.

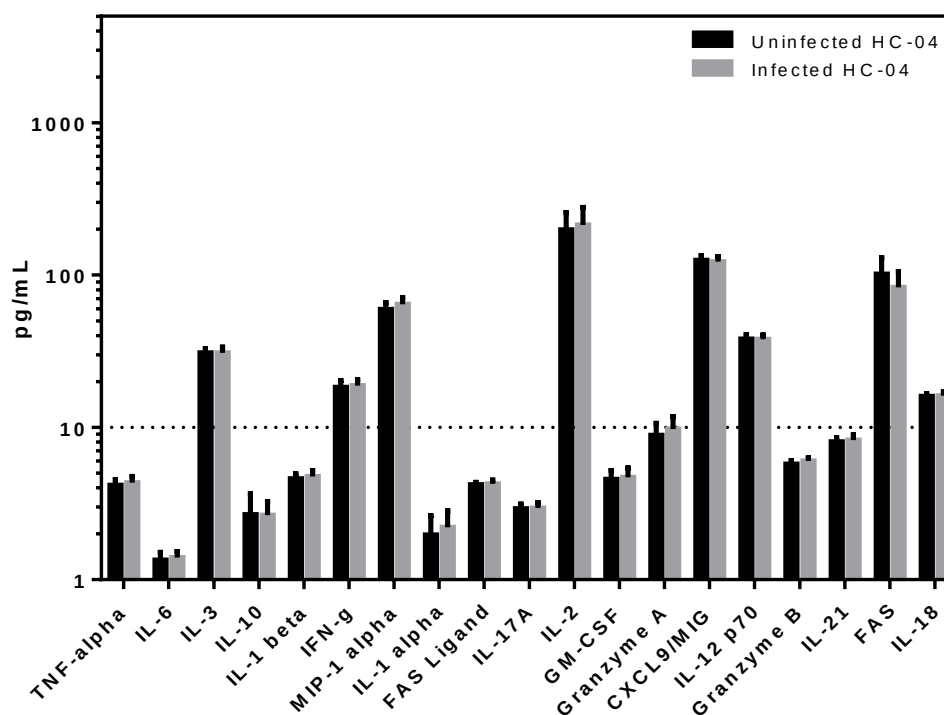


Figure 6.11 Supernatant responses of uninfected and infected HC-04 cells

Uninfected and infected wells combined from each of the 9 experimental plates, and measured by Luminex in 9 sets of duplicates. 2-way ANOVA with Sidak post test performed to assess the effects of analyte and infection on variation measured. 92.34% of variation due to analytes measured ($p < 0.0001$). No significant effect of infection status. Mean plus standard deviation displayed.

6.2.4.3 Positive supernatant responses from infected HC-04 cells

In order to focus the analysis, analytes from vaccinee test wells without a positive response over that of the infected control wells were removed from further analysis. Remaining positive responses included MIP-1 α , IL-2, Granzyme A (GrA), CXCL9, Granzyme B (GrB) and Fas from CD8⁺ pentamer⁺ T cells; IL-2, GrA, CXCL9, GrB and Fas from CD4⁺ T cells; and TNF- α , IL-6, IL-10, IL-1 β , IFN- γ , MIP-1 α , Fas ligand, IL-2, GM-CSF, GrA and CXCL9 from CD56⁺ NK cells (Figure 6.12 A-C).

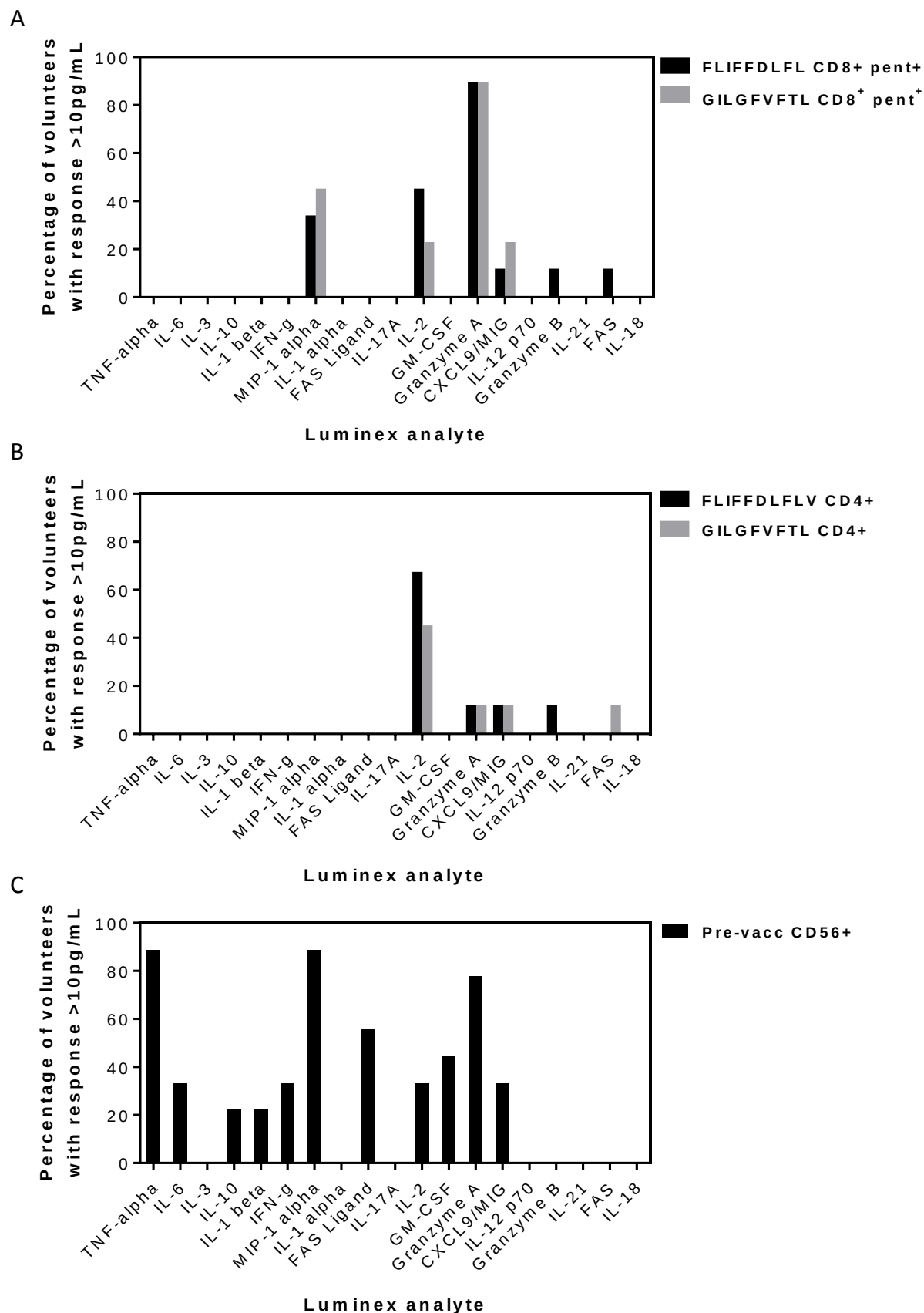


Figure 6.12 Summary of positive responses to the full Luminex panel

Percentage of positive responses by each cell type was determined across the 9 vaccinees tested. A response was deemed positive if the test well response minus the corresponding infected control well was >10pg/mL.

6.2.4.4 Cytokines and chemokines produced by vaccinee T cells

Analyte concentrations from supernatants were plotted on a per volunteer basis, in order to directly compare the levels from TRAP-specific cells to the responses from autologous influenza-specific control cells. Expression of many analytes was of similar magnitude for CD8⁺ pentamer⁺ cells regardless of the antigen culture, indicating a substantial level of background cellular activation was present from the rhIL-2 culture. When listed in order of increasing TRAP-specific parasite inhibition, the vaccinees with greater TRAP-specific parasite inhibition generally had higher Granzyme A expression in the supernatants from CD8⁺ FLIFFDLFL⁺ cells compared with the influenza control cells, although both were of a similar order of magnitude (Figure 6.13).

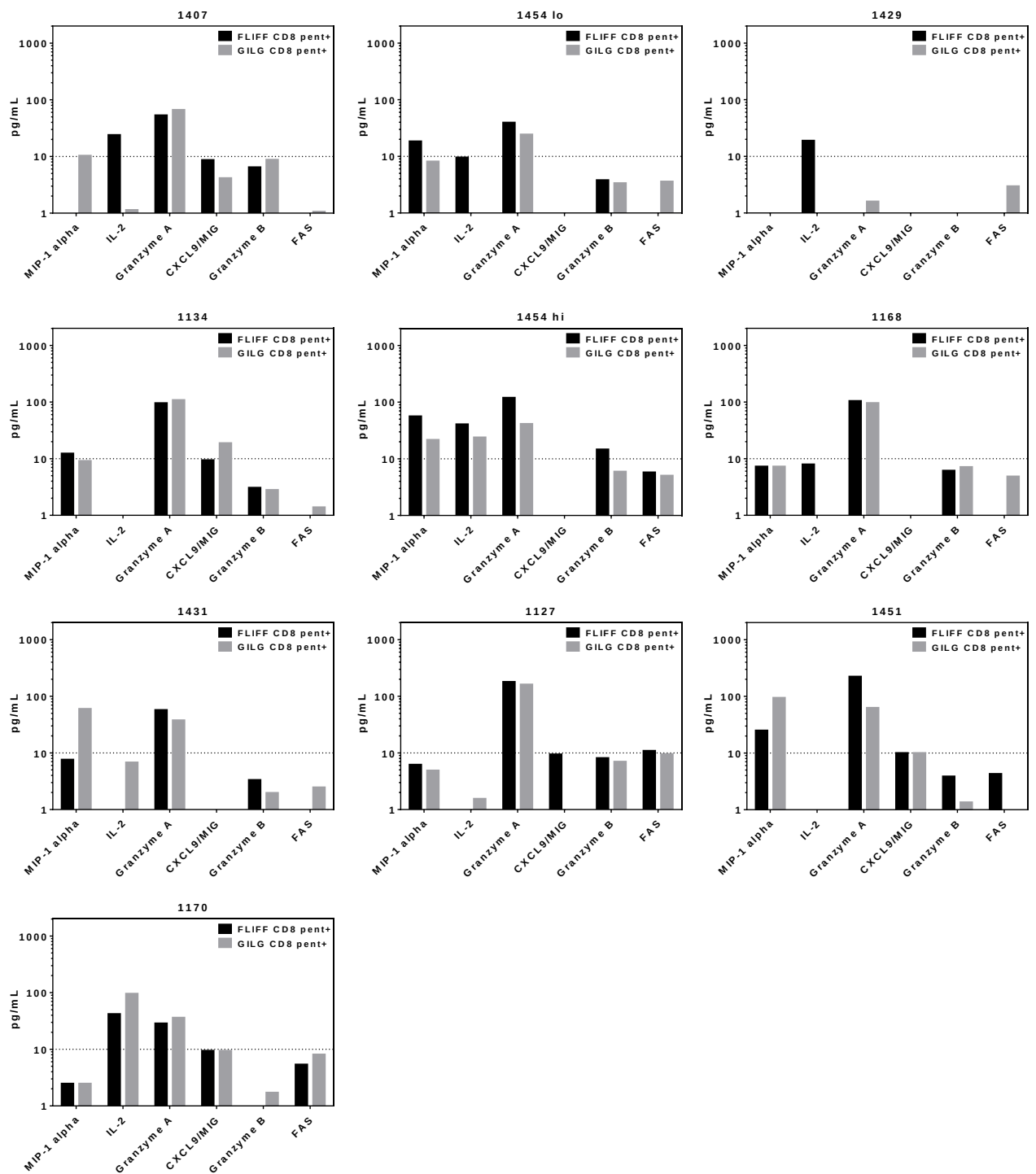


Figure 6.13 Supernatant analysis for CD8⁺ pentamer⁺ T cells

Supernatants were combined from all replicate wells in the *in vitro* parasite inhibition assay, and split across 2 duplicate wells in the Luminex assay, where possible. Responses from infected-control wells were subtracted. Dotted line represents 10pg/mL cut off. Volunteers listed in order of lowest (1407) to highest (1170) TRAP-specific parasite inhibition by CD8⁺ pentamer⁺ T cells.

When correlated with TRAP-specific parasite inhibition, only Fas expression from CD8⁺ FLIFFDLFL⁺ T cell wells showed a significant, positive correlation. Most Fas expression was below the threshold of 10pg/mL, although this measure is of soluble Fas receptor, which is just an indication of the total level of Fas receptor expression in the system. It is assumed that the HC-04 cells are the source of this soluble Fas receptor, due to the constitutive expression of the receptor across many tissues [534], including the liver. However this correlation is with the fractionated additional Fas measured when CD8⁺ FLIFFDLFL⁺ cells are added to infected cells, not with Fas expression on infected liver cells *per se* (Figure 6.14, Spearman $r=0.7952$, $p=0.0099$). There is no correlation between TRAP-specific parasite inhibition and Fas receptor expression in wells with CD8⁺ GILGFVFTL⁺ T cells added.

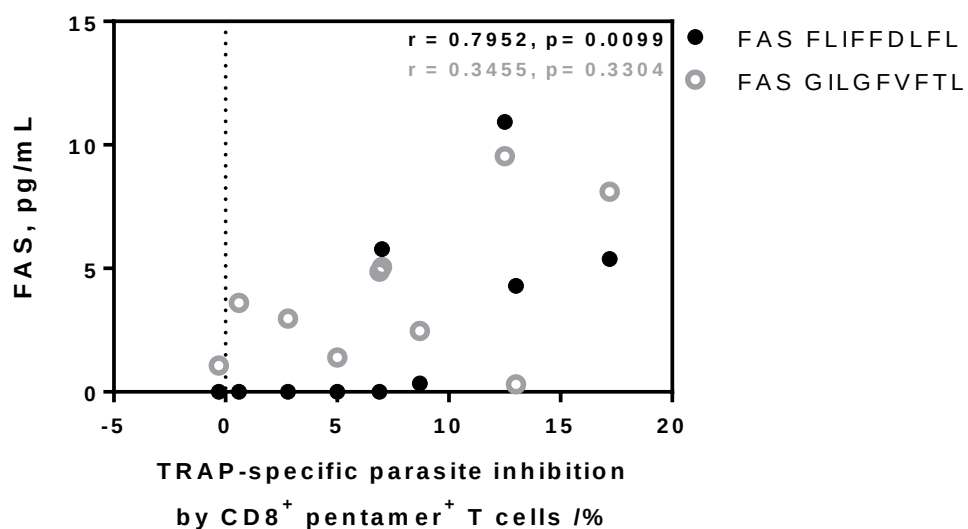


Figure 6.14 Correlation between Fas expression in assay wells containing the two CD8⁺ pentamer⁺ T cell subsets and CD8⁺ pentamer⁺ TRAP-specific parasite inhibition
Responses with background Fas receptor expression of infected control wells subtracted. Spearman $r=0.7952$, $p=0.0099$ for CD8⁺ FLIFFDLFL⁺ wells and $r=0.3456$, $p=0.3304$ for CD8⁺ GILGFVFTL⁺ wells.

Analyte expression from CD4⁺ T cells in the assay did not elucidate a mechanism by which these cells are detected to inhibit the parasite. Similarly to cytotoxic CD8⁺ T cells, cytotoxic CD4⁺ T cells generally exert their effector functions through exocytosis of lytic granules [535], with Fas/Fas ligand interactions also reported [536], as well as TRAIL-DR4/5 pathway [537]. However the only analyte to be upregulated in the CD4⁺ T cell cultures was IL-2, which itself does not have reported anti-parasitic effector functions (Figure 6.15). In fact, IL-2 is reported to exacerbate parasitic infections such as Leishmaniasis [538].

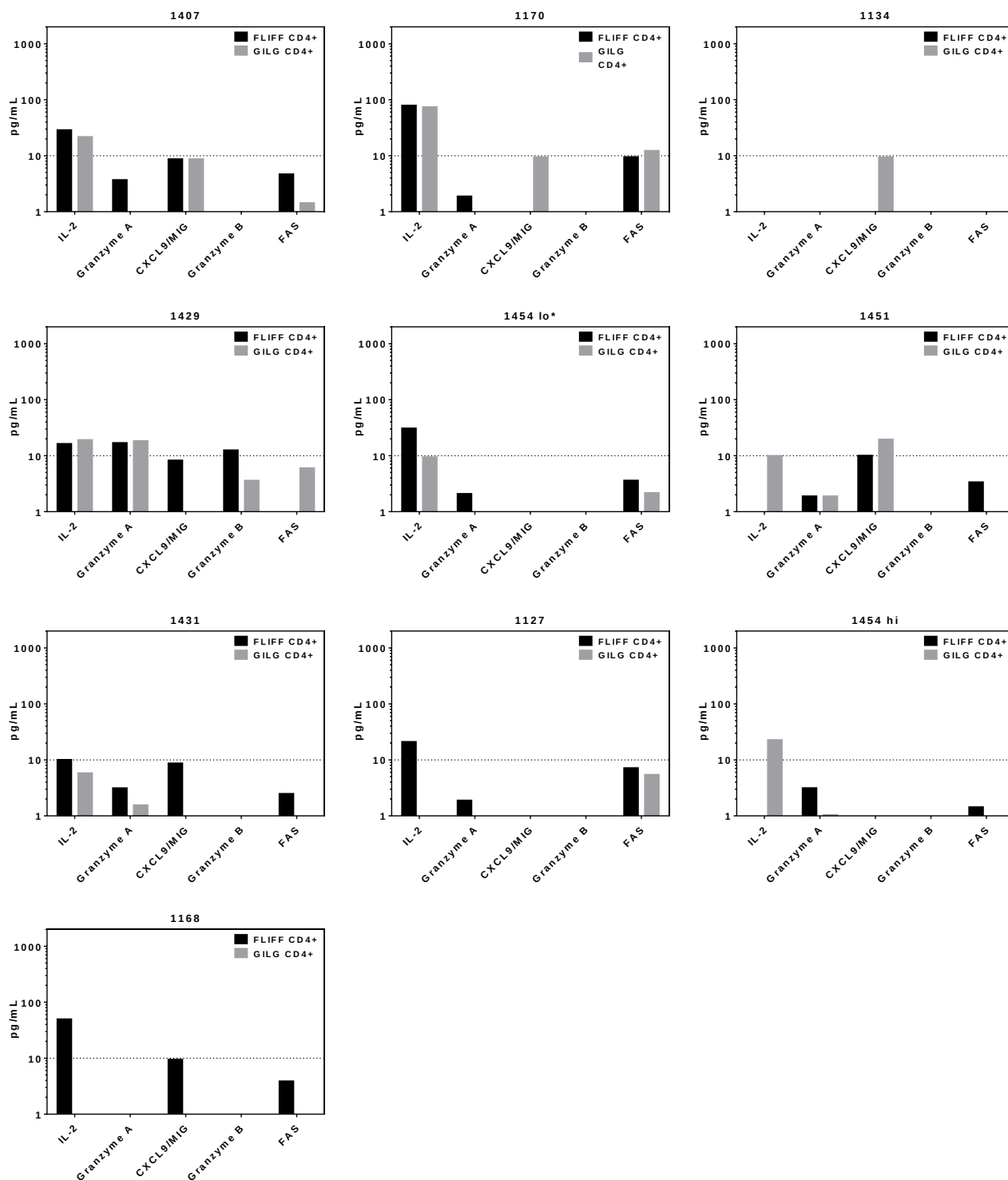


Figure 6.15 Supernatant analysis for CD4⁺ T cells

Supernatants were combined from all replicate wells in the *in vitro* parasite inhibition assay, and split across 2 duplicates wells in the Luminex assay, where possible. Responses from infected-control wells were subtracted. Dotted line represents 10pg/mL cut off. Volunteers listed in order of lowest (1407) to highest (1168) TRAP-specific parasite inhibition by CD4⁺ T cells.

6.2.4.5 Cytokines and chemokines produced by pre-vaccination NK cells

Analysis of supernatants from wells with CD56⁺ NK cells revealed high levels of MIP 1 α in 8/9 volunteers, in addition to elevated levels of TNF- α and Granzyme A in several volunteers (Figure 6.16). With a positive correlation previously measured between NK-induced parasite inhibition and percentage parasite infection in the assay, it is likely that inhibition is linked to level of NK activation. A possible source of their activation is the IL-2 produced by both uninfected and infected HC-04 cells, as an accessory cytokine for NK cell activation via Toll-like and nucleotide oligomerization domain (NOD)-like receptors [539, 540]. Activated NK cells produce large amounts of MIP-1 α [541], and can have cytotoxic effects via exocytosis of perforin and Granzyme, however neither MIP-1 α or Granzyme A correlated with the percentage parasite inhibition measured as a result of NK cells (data not shown), although this does not rule out their potential role in parasite inhibition.

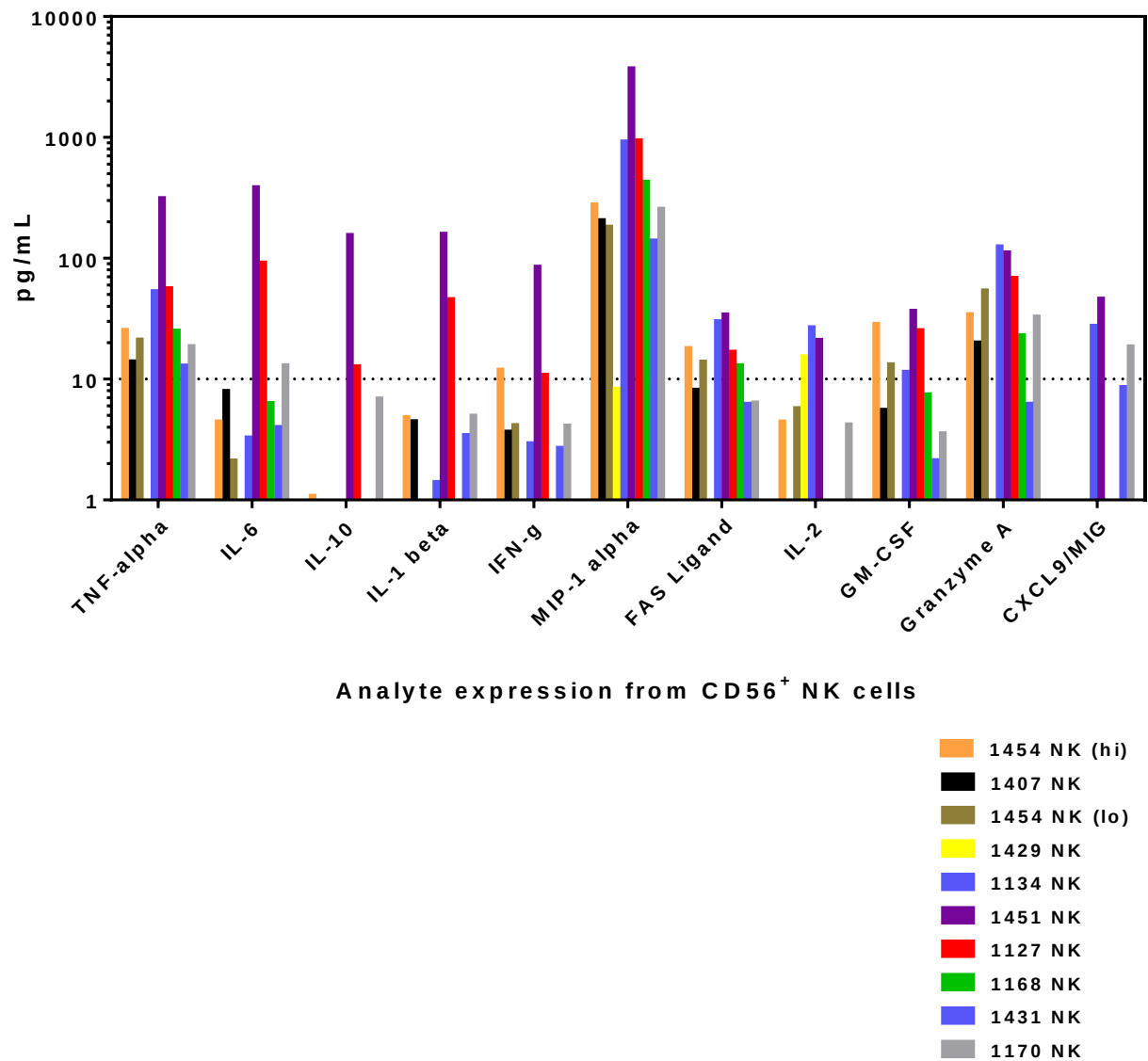


Figure 6.16 Supernatant analysis for CD56⁺ NK cells

Supernatants were combined from all replicate wells in the *in vitro* parasite inhibition assay, and split across 2 duplicates wells in the Luminex assay, where possible. Responses from infected-control wells were subtracted. Dotted line represents 10pg/mL cut off. Volunteers listed in order of highest (1454 hi) to lowest (1170) parasite inhibition by NK cells.

6.2.4.6 Cytokines and chemokines produced by vaccinee cells in response to FLIFFDLFLV peptide

Alongside the *in vitro* parasite inhibition assay, FLIFFDLFL-specific CD8⁺ T cells, GILGFVFTL-specific CD8⁺ T cells and CD56⁺ NK cells were added in matching E:T to HC-04 cells pulsed with FLIFFDLFLV peptide. Pulsing results in MHC class I presentation of FLIFFDLFLV, as indicated by the proof-of-concept assay in Chapter 5 (Figure 5.26). Supernatant analysis of a subset of vaccinees revealed that in the presence of their cognate antigen (FLIFFDLFLV), FLIFFDLFL-specific CD8⁺ T cells produce pro-inflammatory cytokine TNF- α and IFN- γ , in addition to MIP-1 α and GM-CSF. Granzyme A production was also higher in FLIFFDLFLV-specific CD8⁺ T cells, compared to GILGFVFTL-specific CD8⁺ T cells and NK cells, in all 3 volunteers tested in the FLIFFDLFLV-pulsed system (Figure 6.17).

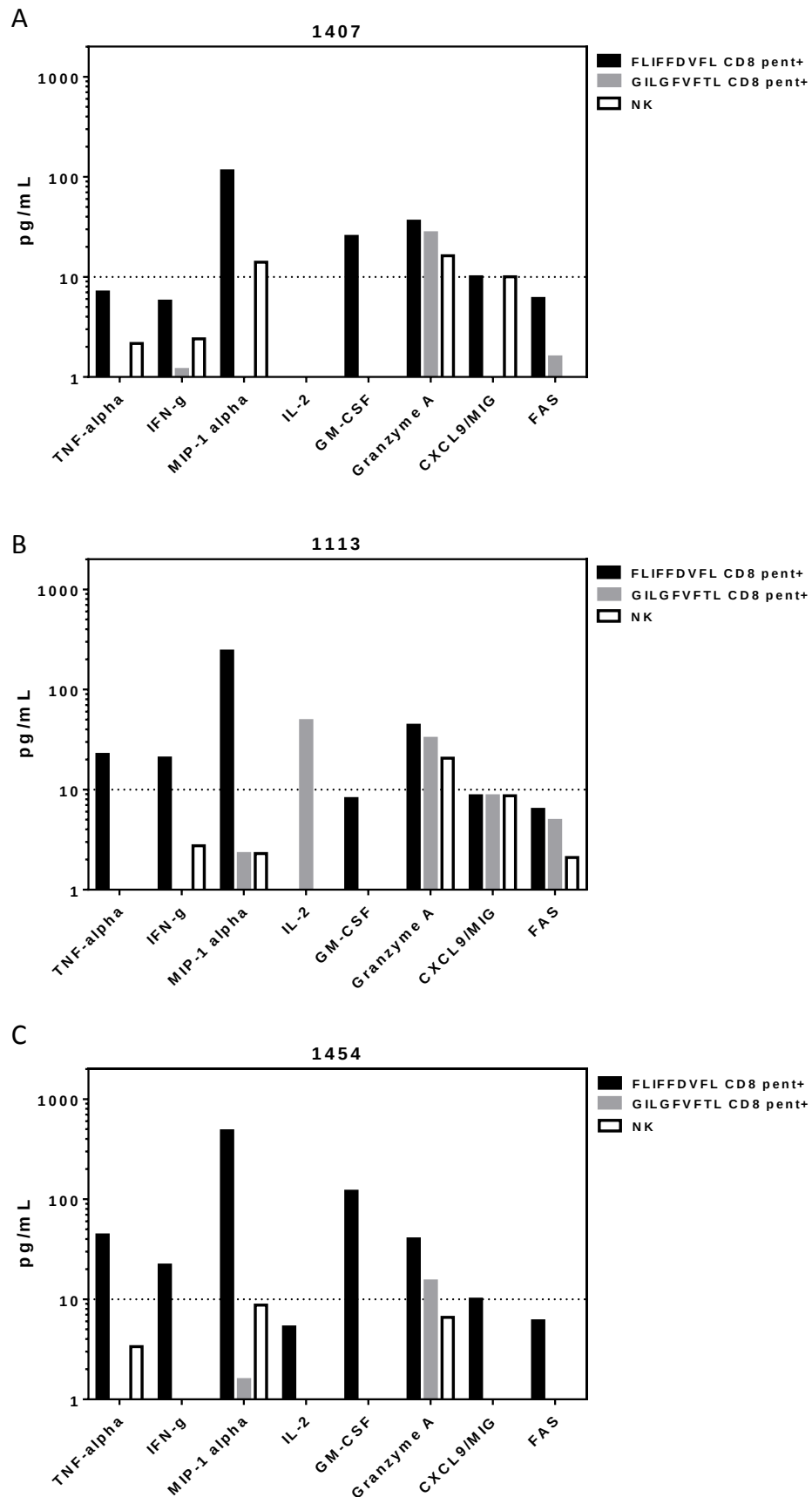


Figure 6.17 Supernatant analysis for cell fractions added to FLIFFDLFLV-pulsed HC-04 cells
 Supernatants were combined from all replicate wells from peptide-pulsed HC-04 cells, and split across 2 duplicate wells in the Luminex assay, where possible. Responses from peptide pulsed control wells were subtracted. Dotted line represents 10pg/mL cut off.

6.2.5 Summary of findings from *in vitro* PbPfTRAP parasite inhibition assay

The data from the *in vitro* parasite inhibition assay were collated with assay parameters, vaccinee information and Luminex data, to provide a comprehensive summary (Figure 6.18).

Volunteer	1429	1407	1454 lo	1454 hi	1170	1431	1451	1168	1134	1127
Effector to target cell ratio	7:1	9:1	10:1	19:1	21:1	24:1	27:1	35:1	54:1	72:1
Vaccination regimen	A8Mc	A8Mc	A8Mc	A8Mc	A4A4M	A8Mc	A8Mc	A4A4M	A4M	A4M
Day of diagnosis (# days delay)	12.5 (2)	11.5 (1)	12.5 (2)	12.5 (2)	n/a	8.5 (0)	10.5 (0)	n/a	n/a	n/a
Control well HC-04 infection/%	4.6	7.5	7.0	7.0	1.6	3.0	2.9	1.7	1.8	1.5
Number of cells added (x10 ³)	10.0	20.0	20.0	40.0	10.0	21.7	23.5	17.5	28.5	32.0
TRAP-specific parasite inhibition CD8 ⁺ pentamer ⁺	2.8	-0.3	0.6	7.0	17.2	8.7	13.0	6.9	5.0	12.5
Luminex analytes CD8 ⁺ pentamer ⁺ where F>G	IL-2	IL-2	MIP-1 α, IL-2, GrA	MIP-1 α, IL-2, GrA, GrB	/	GrA	GrA	GrA	MIP-1 α	GrA, FAS
TRAP-specific parasite inhibition CD4 ⁺	2.7	-2.6	6.1	18.2	0.2	8.0	6.4	21.9	0.2	8.9
Luminex analytes CD4 ⁺ where F>G	GrB	IL-2	IL-2	/	IL-2	IL-2	/	IL-2	/	IL-2
Inhibition by pre-vaccination NK cells	11.8	17.0	12.8	19.3	x	-1.3	8.5	-1.2	10.2	2.4
Luminex analytes from NK cells	IL-2	TNF-α, MIP-1 α, GrA	TNF-α, MIP-1 α, FAS-L, GM-CSF, GrA	TNF-α, IFN-γ, MIP-1 α, FAS-L, GM-CSF, GrA	TNF-α, IL-6, MIP-1 α, GrA, CXCL9	TNF-α, MIP-1 α	TNF-α, IL-6, IL-10, IL-1 β, IFN-γ, MIP-1 α, FAS-L, IL-2 GM-CSF, GrA, CXCL9	TNF-α, MIP-1 α, FAS-L, GrA	TNF-α, MIP-1 α, FAS-L, IL-2, GM-CSF, GrA, CXCL9	11.8
TRAP-specific parasite inhibition CD8 ⁺ pentamer ⁻	2.8	2.6	-2.8	14.0	3.9	-2.7	0.0	3.2	10.7	2.1
HLA match to HC-04 class I /%	50	17	83	83	33	33	50	17	17	33
HLA match to HC-04 class II /%	67	33	33	33	0	50	0	50	67	33
HC-04 HLA	24									
	2									
	51									
	35									
	16									
	4									
	13									
	16									
	51									
	52									
	7									
	6									
HLA class I	A	3	2	2	2	2	3	2	3	24
	A	2	1	11	11	29	2	2	1	2
	B	35	44	51	51	44	60	35	60	7
	B	60	55	51	51	44	60	7	8	7
	C	4	5	4	4	5	10	4	7	7
	C	10	9	16	16	16	10	7	10	7
	DR	1	4	17	17	4	1	1	17	15
	DR	13	14	4	4	7	15	4	15	15
	DR	52	52	52	52	53	51	53	51	51
HLA class II	DR	52	53	53	53	53	51	53	52	51
	DQ	5	5	2	2	2	5	8	2	6
	DQ	6	7	7	7	8	6	5	6	6

Figure 6.18 Combined summary of *in vitro* PbPfTRAP parasite inhibition assay

Summary includes volunteer identification number, E:T, details of vaccination regimens received (c denotes CHMI), day of diagnosis following CHMI, assay parameters, parasite inhibition measured (colour coded by magnitude), raw HLA inferred serotype data of each volunteer and of HC-04 cells and degree of HLA matching between volunteers and cell line (colour coded by magnitude). Supernatant analysis summary lists analytes with a response >10pg/mL where the response in TRAP wells (denoted F) exceeds that of influenza matrix protein 1 wells (denoted G).

6.2.5.1 *Role of Fas/Fas ligand interaction on parasite inhibition*

With supernatant analysis by Luminex highlighting a possible role for the Fas/Fas ligand pathway in parasite inhibition, this was explored further in the infection model. Addition of anti-Fas antibody or soluble Fas ligand results in destruction of all cells expressing Fas receptor [542-544], with the liver particularly susceptible [545]. Supernatant analyses did not detect an increase in soluble Fas receptor specifically in infected HC-04 wells, but with infectivity at <10% and only soluble Fas receptor measured, this could just be a sensitivity issue with uninfected cells diluting out the response. Even without an increase Fas receptor expression, the responsiveness of infected cells to Fas ligand induced apoptosis could be altered by sporozoite infection. This was explored through addition of multimeric Fas ligand into the infection model, to assess whether effects were exerted specifically on infected cells, indicating that they are more susceptible to Fas ligand induced apoptosis.

HC-04 cells were highly responsive to Fas ligand induced apoptosis, with over 90% loss of live HC-04 cells (Figure 6.19 G, $p < 0.0001$, Kruskal-Wallis test). PLC/PRF/5 cells, which previously showed a high level of monolayer robustness in the infection model, were also tested. Conversely, infected PLC/PRF/5 cells were unaffected by addition of multimeric Fas ligand, with no reduction in percentage parasite infection, number of infected cells or intrahepatic parasite growth measured by parasite GFP fluorescence intensity (Figure 6.19 B, D + F). This indicated no parasite-specific effects of Fas ligand induced apoptosis. There was also no significant reduction in total cell number of PLC/PRF/5 cells acquired (Figure 6.19 H). These results would suggest that Fas ligand induced apoptosis does not occur in a manner specific to parasite infected cells, however the data from PLC/PRF/5 cells could be explained by total lack of Fas receptor expression or a refractory response to Fas ligand in this cell line.

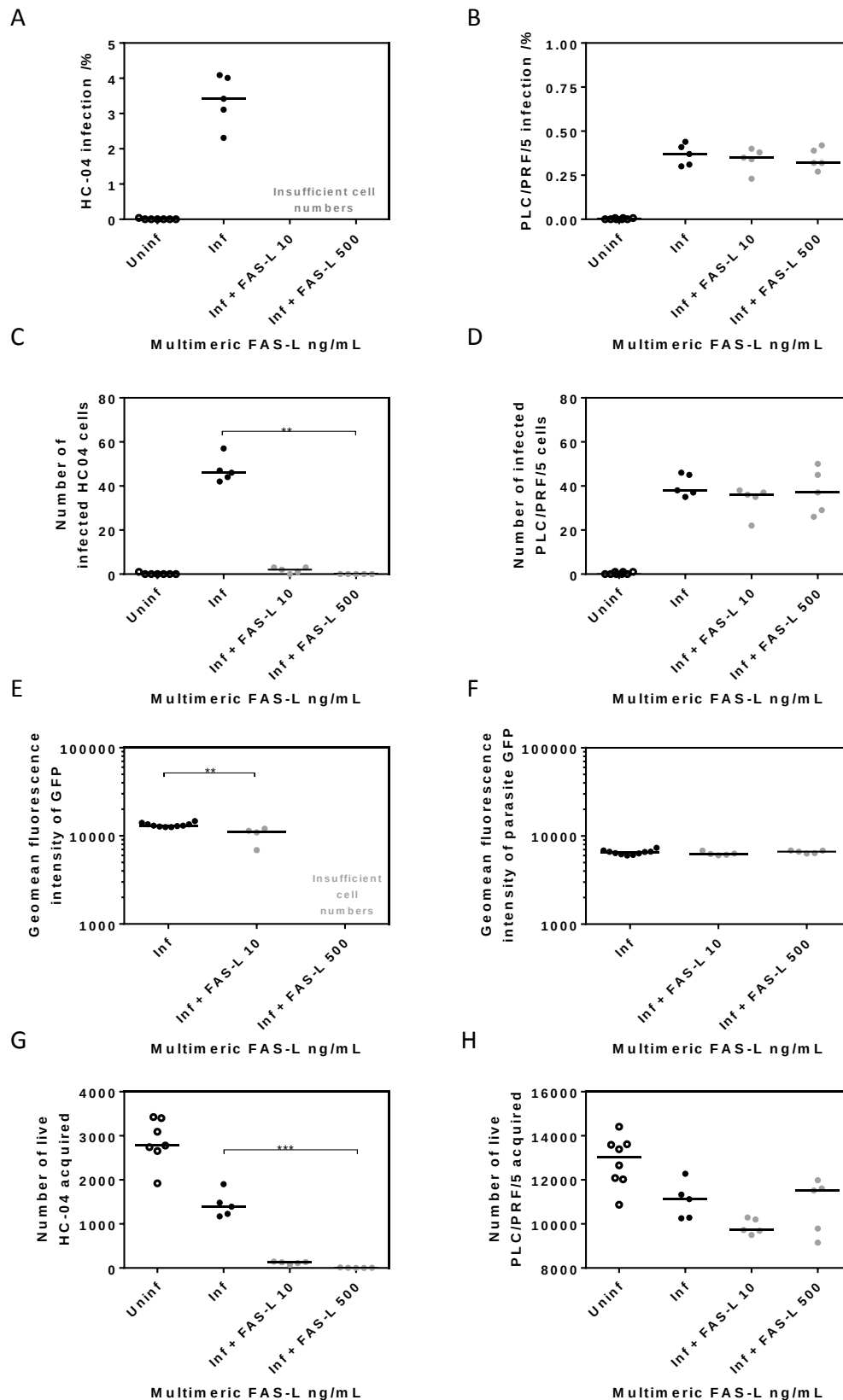


Figure 6.19 Effects of multimeric FAS ligand on infected hepatoma cell cultures

20,000/well HC-04 and PLC/PRF/5 cells were plated in half surface area 96 well plates. 24 hours later, 15,000 *PbPf*TRAP sporozoites were added per well according to the standard infection protocol. 2.5 hours later, sporozoites were removed and HC-04 washed in fresh media, before the addition of multimeric FAS ligand at 10ng/mL and 500ng/mL. Cultures were acquired at 22 HPI, according to the standard acquisition protocol. A-D) Readout of HC-04 cell line and E-H) readout of PLC/PRF/5 cell line, using infection percentage, number of infected cells, geometric mean fluorescence intensity and total live cell number readouts. B-D, F-H) Kruskal-Wallis tests with Dunn's correction for multiple comparisons against infected control group. E) Mann-Whitney test, ** p<0.01, *** p<0.001. Medians displayed.

6.2.5.2 Effects of pro-inflammatory cytokines on parasite growth

When FLIFFDLFL-specific CD8⁺ T cells were incubated with HC-04 cells presenting FLIFFDLFLV peptide, supernatant analyses by Luminex identified production of both IFN- γ and TNF- α , with little or no expression from autologous GILGFVFTL-specific CD8⁺ T cells. A small panel of pro-inflammatory cytokines were therefore evaluated for anti-parasitic effects within the *in vitro* assay infection model. Recombinant human IFN- γ (rhIFN- γ) was initially tested, due to its protective role identified in CHMI trials of ChAd63.MVA ME-TRAP, in addition to several animal and *in vitro* models. No reduction in the percentage of infected HC-04 was measured with rhIFN- γ added at 3 or 8 hours post infection, and the cytokine appeared to promote cell survival, with significantly higher numbers of live HC-04 cells acquired when high titrations of rhIFN- γ were used (Figure 6.20 A-F, Kruskal-Wallis test). While cell survival was promoted, parasite fitness was impaired, with consistently reduced parasite GFP fluorescence intensity measured when 500-5000U/ml of rhIFN- γ was supplemented into the assay (Figure 6.20 G, Kruskal-Wallis test). Addition of rhIFN- γ was more effective at lower concentrations when added 3 hours post sporozoite infection, compared to 8 hours, even when given the same period of incubation time with the cytokine. This result is similar to that of Atovaquone, whereby better parasite inhibition was measured when the drug was added into the assay at early time points following infection.

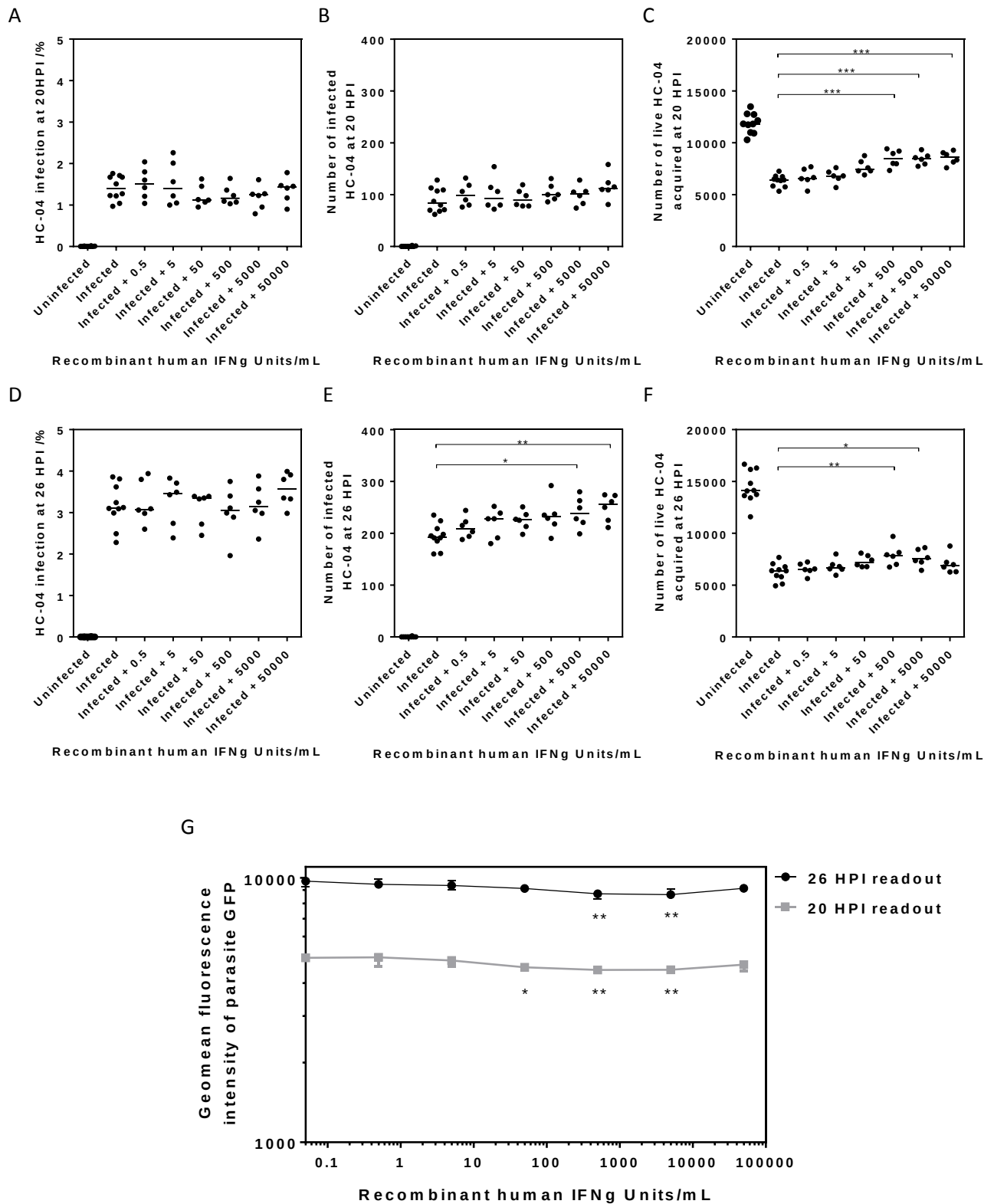


Figure 6.20 Titration and time course summary of recombinant human IFN- γ in HC-04 infection model 20,000/well HC-04 cells were plated in half surface area 96 well plates. 24 hours later, 13,000 *PbPf*TRAP sporozoites were added per well according to the standard infection protocol. 3 hours later, sporozoites were removed and HC-04 washed in fresh media, before the addition of rh IFN- γ at A_C) 3 HPI with acquisition at 20 HPI, and D-F) rh IFN- γ at 8 HPI with acquisition at 26 HP, according to the standard acquisition protocol. Medians displayed. G) Time course of geometric mean fluorescence intensity of parasite GFP. Median + IQR displayed. Kruskal-Wallis test with Dunn's correction for multiple comparisons against infected control group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Using a titration of 500U/mL of recombinant cytokine, rhTNF- α and rhIL-6 were tested in the infection model alone and in combination with IFN- γ across 2 independent experiments (Figure 6.21, Figure 6.22). No individual cytokine or cytokine cocktail specifically reduced percentage infection (Figure 6.21 A, Figure 6.22 A, Kruskal-Wallis test). The combination of rhIL-6 + rhTNF- α significantly reduced the number of infected cells (Figure 6.22 B, $p < 0.0001$, Kruskal-Wallis test,), however this was accompanied by a similar but non-significant reduction in overall numbers of live HC-04 cells acquired, so may be linked to the level of cytotoxicity of the cocktail. Consistently across both experiments, all cytokine cocktails containing rhIFN- γ significantly reduced the fluorescence intensity of parasite GFP (Figure 6.21 C, Figure 6.22 C $P < 0.0001$, Kruskal-Wallis test), with rhIFN- γ and rhTNF- α the most effective cocktail tested.

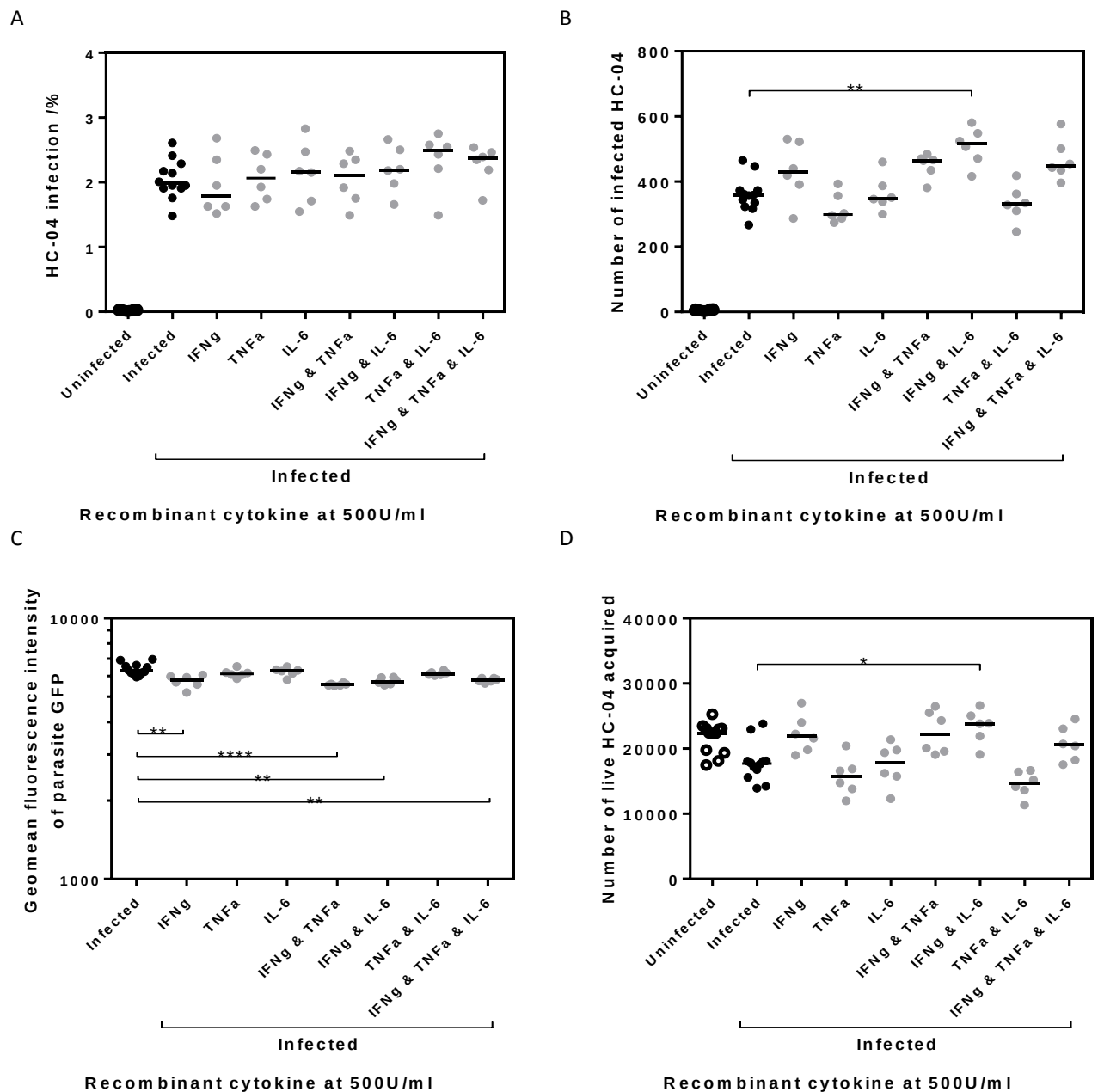


Figure 6.21 Anti-parasitic effects of recombinant human cytokine cocktails in HC-04 infection model – part 1

20,000/well HC-04 cells were plated in half surface area 96 well plates. 24 hours later, 13,000 *PbPf*TRAP sporozoites were added per well according to the standard infection protocol. 3 hours later, sporozoites were removed and HC-04 washed in fresh media, before the addition of recombinant human cytokine cocktails. Cells were acquired at 23 HPI, according to the standard acquisition protocol. Kruskal-Wallis test with Dunn's correction for multiple comparisons against infected control group, * $p < 0.05$. ** $p < 0.01$, **** $p < 0.0001$. Medians displayed.

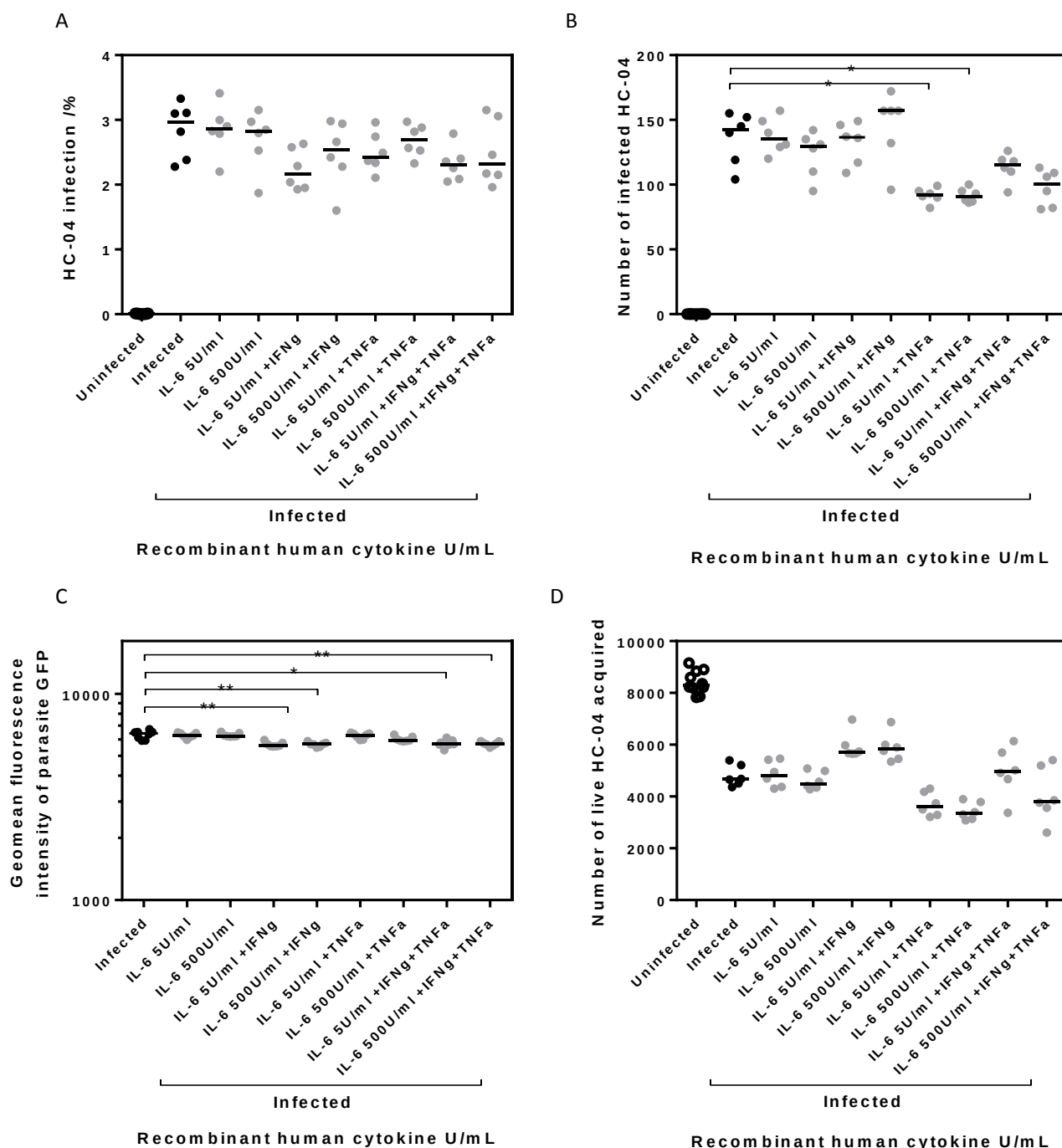


Figure 6.22 Anti-parasitic effects of recombinant human cytokine cocktails in HC-04 infection model – part 2

20,000/well HC-04 cells were plated in half surface area 96 well plates. 24 hours later, 17,000 *PbPf*TRAP sporozoites were added per well according to the standard infection protocol. 3 hours later, sporozoites were removed and HC-04 washed in fresh media, before the addition of recombinant human cytokine cocktails. RhIFN- γ and rhTNF- α concentrations set at 500U/mL in all cytokine cocktails. Cells were acquired at 22 HPI, according to the standard acquisition protocol. Kruskal-Wallis test with Dunn's correction for multiple comparisons against infected control group, * $p < 0.05$. ** $p < 0.01$. Medians displayed.

Additional testing of the rhIFN- γ + rhTNF- α cytokine pairing could further reduce parasite GFP, under conditions where the cocktail was added at multiple time points following infection. Increased inhibition of parasite growth identified by reduced GFP fluorescence intensity was measured when the cytokine cocktail was supplemented into the assay at 3, 12 and 21 hours post infection, compared with addition at 3 hours only (Figure 6.23 A, $p > 0.0001$, Kruskal-Wallis test). This level of inhibition was lost by 40 hours post infection (Figure 6.23 B, Kruskal-Wallis test). Furthermore, growth kinetics were of a similar order of magnitude both with and without the addition of cytokine (Figure 6.23 C). Therefore early production, or in this case addition, of rhIFN- γ +/- TNF- α may have anti-parasitic effects and adversely affect parasite fitness in the short term, but these effects are not sufficient to fully inhibit parasite growth during liver stage infection.

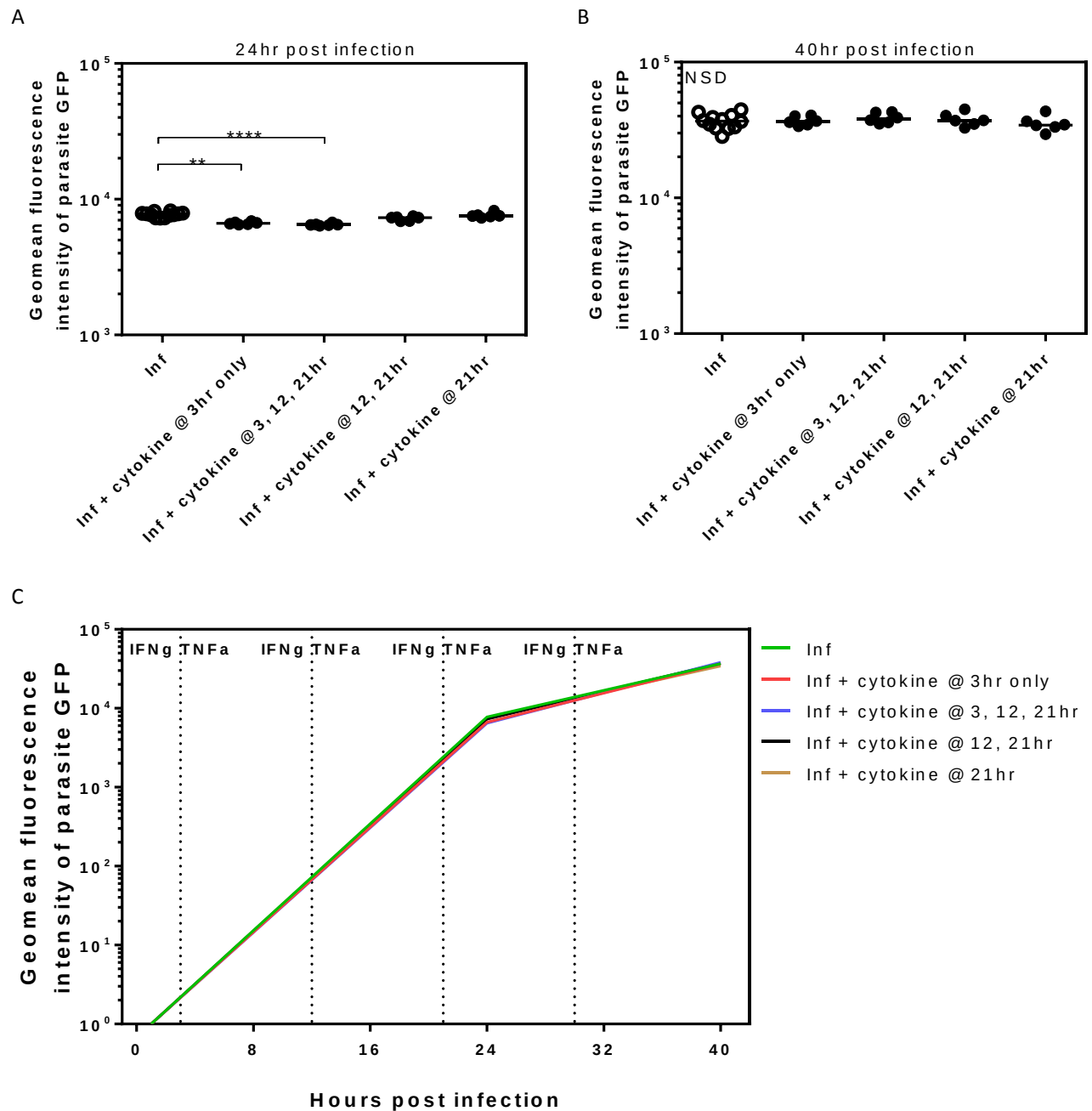


Figure 6.23 Infection time course with cytokine cocktail

20,000/well HC-04 cells were plated in half surface area 96 well plates. 24 hours later, 12,500 *PbPf*TRAP sporozoites were added per well according to the standard infection protocol. 3 hours later, sporozoites were removed and HC-04 washed in fresh media, before the addition of rh IFN- γ and TNF- α at 500U/mL at sequential time points of 3, 12 and 21 hours. Cells were acquired at A) 24 HPI and B) 40 HPI, according to the standard acquisition protocol, with geometric mean fluorescence intensity reported. Kruskal-Wallis test with Dunn's correction for multiple comparisons against infected control group, ** $p < 0.01$, **** $p < 0.0001$. Medians displayed. C) Time course summary of geometric mean fluorescence intensity of GFP. Inf = infected

6.2.6 Extension of *in vitro* assay to evaluate inhibition of *Plasmodium falciparum* infection of primary human hepatocytes

The newly developed *in vitro* assay was extended to assess TRAP-specific inhibition of *P. falciparum*-infected primary human hepatocytes (PHH). Briefly, a similar but slightly extended model of ChAd63.MVA ME-TRAP vaccinee T cell culture and subsequent FACS sorting using MHC class I pentamers was employed. E:T matched FLIFFDLFL-specific and GILGFVFTL-specific CD8⁺ T cells, as well as the CD8⁺ pentamer- fractions, were added to primary human hepatocytes on day 2 of *P. falciparum* infection. Vaccinee T cells and commercial primary hepatocytes were matched on their HLA-A2 serotype. An additional mismatched HLA-A1 hepatocyte culture was also used as an MHC negative control. Two days later, cultures were fixed and stained using anti-PfHSP70 antibody, and counted using an automated scanner. All *P. falciparum* experiments were carried out at CIMI-Paris, alongside Dr. Valérie Soulard's research team.

Percentage infection of the 2 primary human hepatocyte HLA batches was ~0.1%. No differences were measured between separate plates of the same hepatocyte batch, however there were significantly fewer infected HLA-A1 hepatocytes compared with HLA-A2 hepatocytes when comparisons were made between batches of different HLA type. Percentage inhibition for individual plates was measured against the internal control wells present on each plate (Figure 6.24).

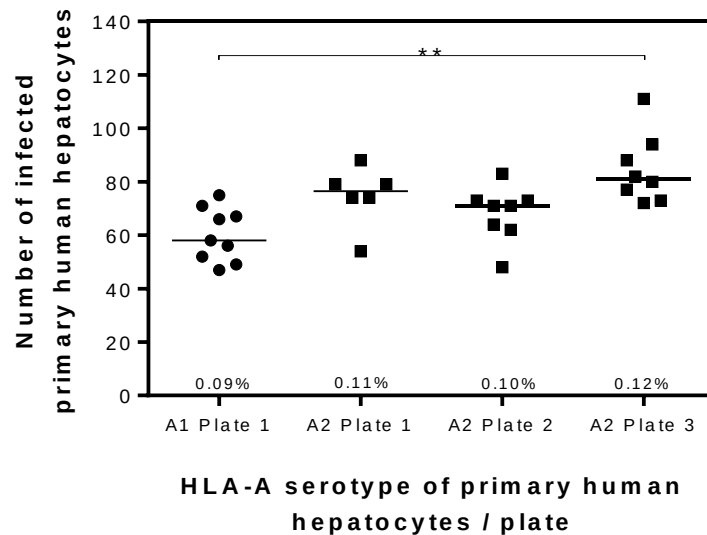


Figure 6.24 Infection summary of HLA-A1 and HLA-A2 primary human hepatocytes.

70,000/well primary human hepatocytes cells were plated in 96 well plates, and infected with 20,000 *P. falciparum* sporozoites 4 days later. Sporozoites were left to infect for 7 hours at 37°C, and then removed from the plates. Three days later, PHH cultures were DAPI stained, followed by fixation and staining using an anti-HSP70 antibody. Number of infected cells were counted by an automated plate scanner. Kruskal-Wallis test with Dunn's correction for multiple comparisons between all plates, **<0.01. Median number of infected PHH displayed. Percentages represent percentage infection per plate.

6.2.6.1 TRAP-specific inhibition

To measure TRAP-specific inhibition of *P. falciparum*, T cells from five vaccinees were tested. Three vaccinees were evaluated at multiple E:T ratios for matched CD8⁺ FLIFFDLFL⁺ (TRAP-specific) and CD8⁺ GILGFVFTL⁺ (influenza matrix protein 1- specific) cells, in addition to the pentamer⁻ fraction from each culture. The other 2 vaccinees' T cells were tested at a single E:T only. Three vaccinees has received the standard 8 week ChAd63.MVA ME-TRAP regimen, one received ChAd63 ME-TRAP only and one received the standard ChAd63.MVA ME-TRAP regimen alongside 3 shots of RTS,S vaccine. All volunteers were part of CHMI trials.

Antigen was not a significant determinant of the parasite inhibition measured with CD8⁺ pentamer⁺ T cells (Figure 6.25) and CD8⁺ pentamer⁻ T cells (Figure 6.26), indicating that inhibition measured was not TRAP-specific. For a single volunteer (047), significant parasite inhibition of 37% was measured in cultures with TRAP-specific CD8⁺ T cells compared with influenza-specific T cells, at

an E:T of 13:1. Interestingly, this volunteer had the longest delay to parasitaemia following CHMI of the 5 vaccinees.

A similar level of parasite inhibition was measured with CD8⁺ pentamer⁺ cells from 2 vaccinees. Vaccinee 019 exhibited significant TRAP-specific parasite inhibition at E:T of 54:1, and vaccinee 032 exhibited parasite inhibition with the control influenza cells (032) at a low E:T of 3:1.

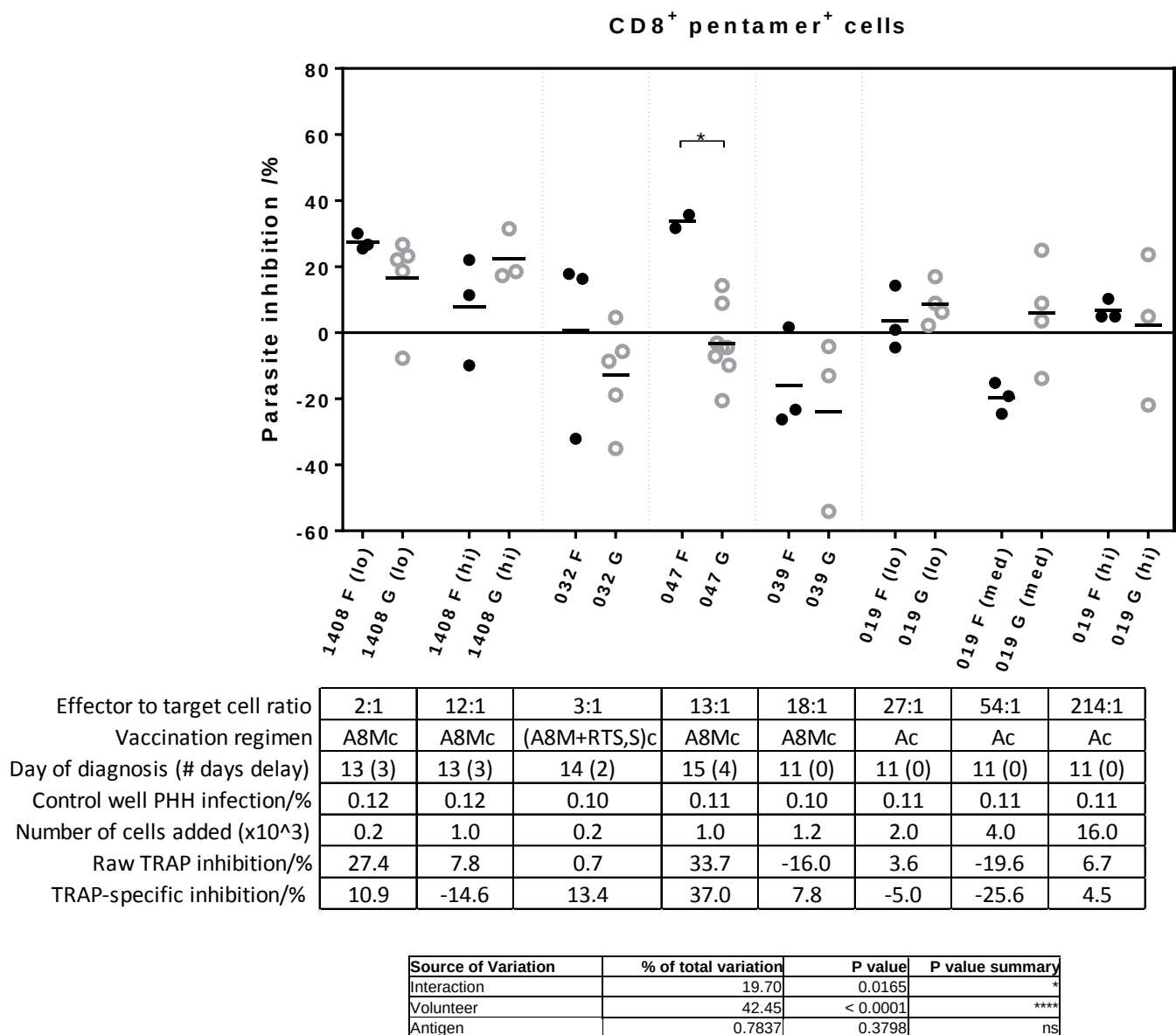
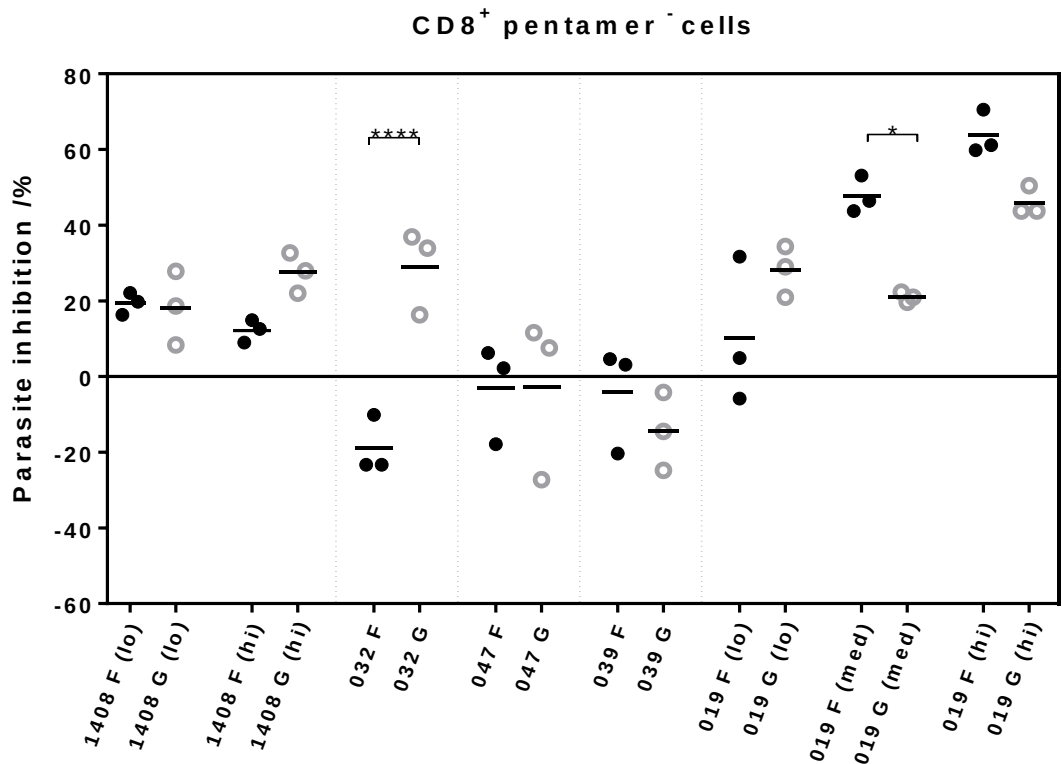


Figure 6.25 Summary of *P. falciparum* parasite inhibition assay with E:T-matched FLIFFDLFL-specific (F) and GILGFVFTL-specific (G) CD8⁺ pentamer⁺ T cells

Using the infection model outlined in Figure 6.24, E:T matched CD8⁺ pentamer⁺ T cells were added into HLA-A2 PHH cultures on the day following *P. falciparum* infection. Two days later, PHH cultures were DAPI stained, followed by fixation and staining using an anti-HSP70 antibody. Number of infected cells were counted by an automated plate scanner. Percentage inhibition calculated by reduction in infected PHH numbers in test wells compared to control wells. 2-way ANOVA with Sidak post-test for comparisons between FLIFFDLFL and GILGFVFTL cell populations, * p<0.05, **** p<0.0001. Mean inhibition displayed.



Effector to target cell ratio	2:1	12:1	3:1	13:1	18:1	27:1	54:1	214:1
Vaccination regimen	A8Mc	A8Mc	(A8M+RTS,S)c	A8Mc	A8Mc	Ac	Ac	Ac
Day of diagnosis (# days delay)	13 (3)	13 (3)	14 (2)	15 (4)	11 (0)	11 (0)	11 (0)	11 (0)
Control well PHH infection/%	0.12	0.12	0.10	0.11	0.10	0.11	0.11	0.11
Number of cells added (x10 ³)	0.2	1	0.2	1	1.2	2	4	16
Raw TRAP inhibition/%	19.4	12.2	-18.9	-3.1	-4.2	10.3	47.8	63.8
TRAP-specific inhibition/%	1.1	-15.4	-48.0	-0.4	10.3	-17.9	26.8	17.9

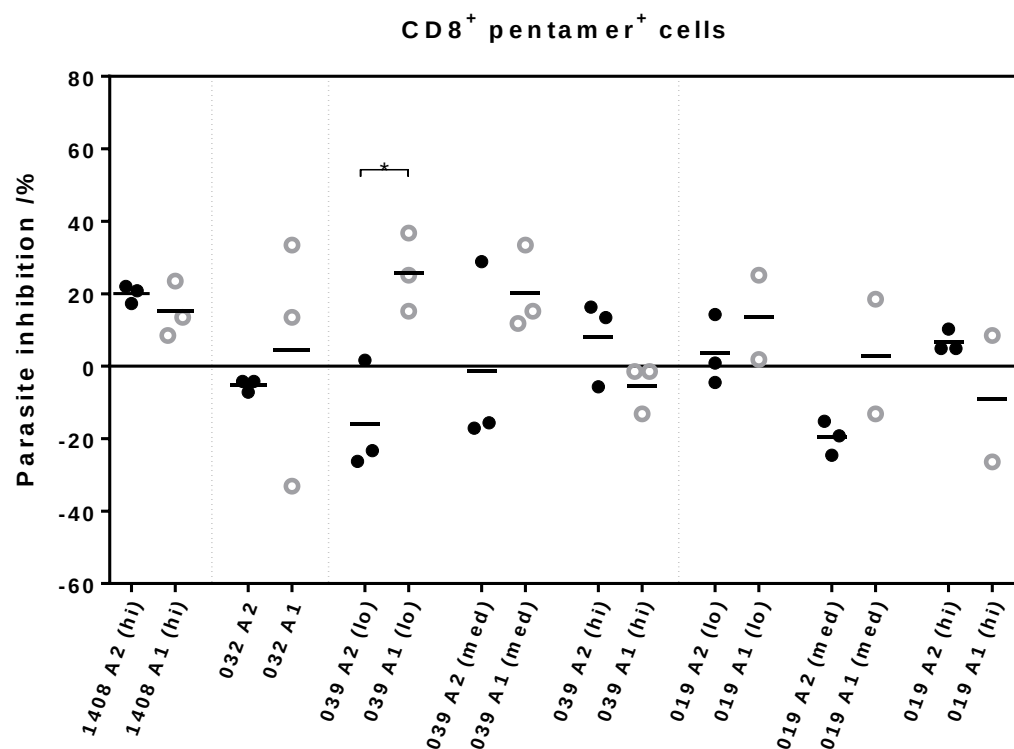
Source of Variation	% of total variation	P value	P value summary
Interaction	21.44	< 0.0001	****
Volunteer	65.41	< 0.0001	****
Antigen	0.4469	0.2966	ns

Figure 6.26 Summary of *P. falciparum* parasite inhibition assay with E:T-matched CD8⁺ pentamer⁺ T cells from FLIFFDLFLV (F) and GILGFVFTL (G) culture

Using the infection model outlined in Figure 6.24, E:T matched CD8⁺ pentamer⁺ T cells were added into HLA-A2 PHH cultures on the day following *P. falciparum* infection. Two days later, PHH cultures were DAPI stained, followed by fixation and staining using an anti-HSP70 antibody. Number of infected cells were counted by an automated plate scanner. Percentage inhibition calculated by reduction in infected PHH numbers in test wells compared to control wells. 2-way ANOVA with Sidak post-test for comparisons between FLIFFDLFLV and GILGFVFTL cell populations, * p<0.05, **** p<0.0001. Mean inhibition displayed.

6.2.6.2 MHC class I mismatch

To assess whether hepatocytes were signalling to HLA-A2 CD8⁺ T cells via MHC class I, CD8⁺ pentamer⁺ and CD8⁺ pentamer⁻ cells were plated on *P. falciparum*-infected HLA-A1 primary human hepatocytes alongside the HLA-A2 hepatocytes, when sufficient cell numbers were available. Disappointingly, there were not sufficient cells to test volunteer 047, who had exhibited positive TRAP-specific parasite inhibition in the HLA-A2 hepatocytes. HLA type was not a significant factor affecting in the CD8⁺ pentamer⁺ inhibition measured (Figure 6.27). However with the CD8⁺ pentamer⁻ cell fractions, volunteer 019 who exhibited TRAP-specific inhibition in HLA-A2 infected hepatocytes, measured significantly lower inhibition in the HLA-A1 mismatched assay (Figure 6.28).



Effector to target cell ratio	47:1	67:1	13:1	15:1	18:1	20:1	59:1	67:1	754:1	855:1	27:1	33:1	54:1	67:1	214:1	266:1
Vaccination regimen	A8Mc		(A8M+RTS,S)		A8Mc		A8Mc		A8Mc		Ac		Ac		Ac	
Day of diagnosis (# days delay)	13 (3)		14 (2)		11 (0)		11 (0)		11 (0)		11 (0)		11 (0)		11 (0)	
Control well PHH infection/%	0.12	0.09	0.10	0.09	0.10	0.09	0.10	0.09	0.10	0.09	0.11	0.09	0.11	0.09	0.11	0.09
Number of cells added (x10 ³)	4.0		0.88		1.2		4.0		51.4		2.0		4.0		16.0	
Raw HLA A2 or A1 inhibition/%	20.0	15.2	-5.2	4.6	-16.0	25.7	-1.3	20.2	8.0	-5.4	3.6	13.5	-19.6	2.7	6.7	-9.0
MHC class I-specific inhibition/%	4.9		-9.8		-41.7		-21.5		13.4		-9.9		-22.3		15.7	

Source of Variation	% of total variation	P value	P value summary
Interaction	26.85	0.0522	ns
Volunteer	16.96	0.2183	ns
HLA-A matching	6.125	0.0641	ns

Figure 6.27 Summary of *P. falciparum* parasite inhibition assay with E:T-matched FLIFFDLFL-specific (F) and GILGFVFTL-specific (G) CD8⁺ pentamer⁺ T cells and HLA mis-matched hepatocytes

Using the infection model outlined in Figure 6.24, E:T matched CD8⁺ pentamer⁺ T cells were added into HLA-A1 PHH cultures on the day following *P. falciparum* infection. Two days later, PHH cultures were DAPI stained, followed by fixation and staining using an anti-HSP70 antibody. Number of infected cells were counted by an automated plate scanner. Percentage inhibition calculated by reduction in infected PHH numbers in test wells compared to control wells. 2-way ANOVA with Sidak post-test for comparisons between FLIFFDLFL and GILGFVFTL cell populations, * p<0.05. Mean inhibition displayed.

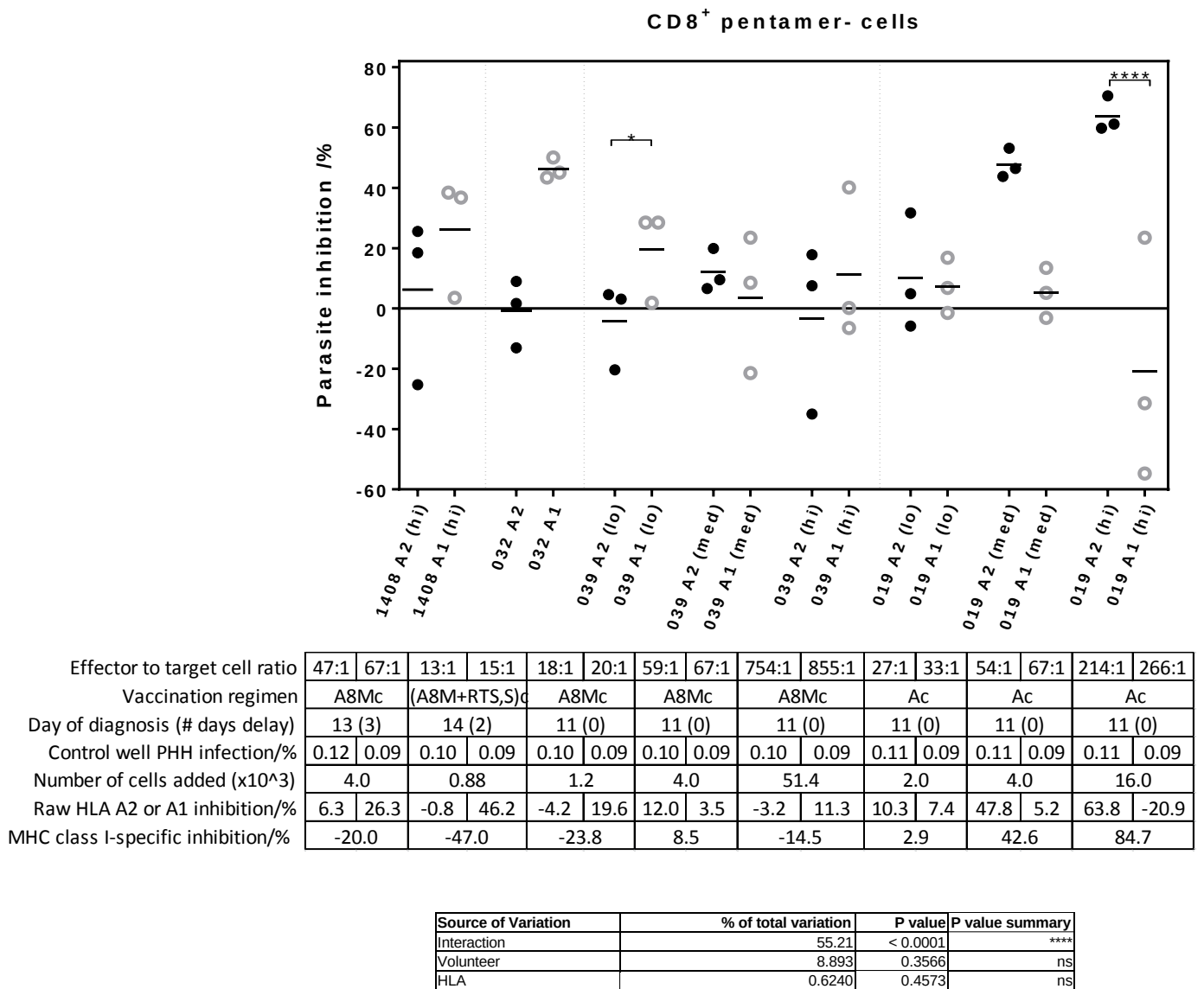


Figure 6.28 Summary of *P. falciparum* parasite inhibition assay with E:T-matched CD8⁺ pentamer⁺ T cells from FLIFFDLFLV (F) and GILGFVFTL (G) culture and HLA mis-matched hepatocytes

Using the infection model outlined in Figure 6.24, E:T matched CD8⁺ pentamer⁺ T cells were added into HLA-A1 PHH cultures on the day following *P. falciparum* infection. Two days later, PHH cultures were DAPI stained, followed by fixation and staining using an anti-HSP70 antibody. Number of infected cells were counted by an automated plate scanner. Percentage inhibition calculated by reduction in infected PHH numbers in test wells compared to control wells. 2-way ANOVA with Sidak post-test for comparisons between FLIFFDLFLV and GILGFVFTL cell populations, * p<0.05, **** p<0.0001. Mean inhibition displayed.

6.3 Discussion

The results generated from this newly developed assay provide insight into the scope of the model, as well as some key questions going forward. Some key limitations of the assay were also highlighted. The aim was to develop a high throughput assay, capable of quantifying TRAP-specific parasite inhibition from vaccine-induced T cells, and elucidate the cellular mechanisms involved. The assay structure was loosely based on a similar model using *P. berghei*-infected murine hepatoma cells to evaluate cell-mediated parasite inhibition of mouse antigen-specific CD8⁺ splenocytes. The human variation of this assay required further optimisation to assess the effects of HLA mismatch, overcome the lower frequencies of antigen-specific T cell induced by viral vector vaccines in humans compared with mice, and explore the use of suitable negative controls within the experiment.

The use of human hepatoma cell lines in the *in vitro* parasite inhibition assay provides a panel of HLA serotypes for the assay to be run against, including common serotypes HLA-A2, HLA-A3 and HLA-A11, although due to frequencies of vaccine-induced T cells being <1% at the peak time point following vaccination, enrichment of these cells using MHC class I pentamers was required, thus restricting the assay to a single epitope. This requires the MHC restriction of epitopes to be accurately predicted and limits the breadth of response that can be tested, however it does provide specificity within the assay and reduces the background parasite inhibition measured, which was a key parameter identified in the murine *in vitro* assay [363].

The use of transgenic *P. berghei* parasites in human hepatocellular carcinoma lines provides an infection model with much higher levels of infectivity than that measured with *P. falciparum* in any human cell type. In addition, the model has scope to test T cell responses to other candidate malaria antigens, as several *P. berghei* transgenic parasite lines expressing *P. falciparum* antigens are available. These include CSP, LSAP-1, LSAP-2, CelTOS, LSA1, LSA3, falstatin, UIS3 and ETRAMP5 [333, 360].

Using the newly developed *in vitro* assay, TRAP-specific parasite inhibition was measured even in volunteers who exhibited little or no delay to parasitaemia following CHMI. For conclusive results regarding the assay's true correlation with CHMI results, many more volunteers across the spectrum of CHMI protection would need to be tested, encompassing volunteers of several different HLA types

and a wider range of epitope responses. However, these data indicate that vaccine-induced CD8⁺ T cells are capable of parasite inhibition if in proximity to infected hepatocytes. *In vivo*, these CD8⁺ T cells may not home or relocate to the liver from the periphery during the pre-erythrocytic stages of parasite development. This has implications in a vaccine setting, as for effective vaccine efficacy, protective T cells need to be located or be able to home to the correct infected tissue in order to exert their effects. This highlights the need to understand homing of T cells to the liver, the associated liver homing markers and inducing such cells through vaccination.

The importance of liver resident T cells and their role in liver stage malaria vaccination have recently been demonstrated [216], reviewed in [546]. Furthermore, induction of increased numbers of such cells has been achieved through vaccination with a “prime-and-trap” vaccination strategy in mice: a T cell response is first primed in the spleen, then using an adeno-associated virus with liver tropism, cognate antigen is expressed in the liver to relocate the primed T cells, forming a liver resident T cell population. Using this approach followed by a *P. berghei* challenge, protection correlated strongly with number of liver resident memory T cells, and to a lesser extent number effector memory T cells in the liver [547]. Similar work is also being conducted at the Jenner Institute (Gola *et al*, submitted 2016) in a “prime-target” approach, whereby i.m. vaccination with ChAd63 (encoding a *P. falciparum* liver stage malaria antigen) induces antigen-specific T cells that are targeted to the liver at the peak of the immune response 2 weeks later. This is achieved through i.v. administration of homologous ChAd63 vaccine, which due to the liver tropism of adenoviruses, expresses the encoded antigen in the liver of vaccinated mice, leading to T cell relocation and the formation of a liver resident population. This model was tested with ChAd63 encoding *P. falciparum* liver stage antigens ME-TRAP, TRAP, LSA1 and LSAP2, where vaccinated mice were challenged with transgenic *P. berghei* parasites expressing the relevant *P. falciparum* antigen. 100% protection against sporozoite challenge was measured with each of the 4 vaccine antigens tested, and liver tissue-resident CD8⁺ T cells correlated with protection.

The requirement for T cells to be located in the liver also links to the hypothesis that a fast mode of action may be required to prevent liver stage infection before it becomes too advanced. This is supported by the data whereby both Atovaquone and IFN- γ yielded improved parasite

inhibition in the infection model when they were added at earlier time point following sporozoite infection. This rapid response may not be possible if CD8⁺ T cells first need to relocate to liver, although with the pre-erythrocytic period of *P. falciparum* approximately 5 days longer than *P. berghei*, these time scales could vary by several days.

In relation to the TRAP-specific CD8⁺ pentamer⁺ parasite inhibition measured, and the associated correlations and trends measured with percentage infection and E:T within the assay, it is hypothesised that the number of T cells in proportion to the number of infected cell targets and subsequent interaction between these cells, may be key in determining cell-mediated parasite inhibition. This is consistent with findings from a recent murine adoptive transfer model [327], whereby the percentage of donor cells recruited into division was indicative of the level of antigen presentation from infected hepatocytes. Furthermore, through titrating down the numbers of antigen-specific effector CD8⁺ T cells transferred and the number of sporozoites used in the *in vivo* infection, it was concluded that protection was reliant on a fine balance of number of infected cells versus the number of effector cells, with a high E:T achieved through increased numbers of effector cells, not low numbers of infected target cells [327]. Finally, the trend towards a positive correlation between E:T ratio and percentage TRAP-specific inhibition in this model was consistent with data from the murine *in vitro* parasite inhibition model, where a similar association between percentage infection of the mouse hepatoma cell line and level of antigen-specific inhibition achieved [363].

A protective correlate or mechanism related to TRAP-specific CD8⁺ pentamer⁺ parasite inhibition was not clear from the analysis of assay supernatants. Many analytes were of a similar level in assay supernatants using CD8⁺ T cells from FLIFFDLFLV and GILGFVFTL cultures. This is likely due to the background activation of the cells from peptide culture with rhIL-2, which has been previously reported for Granzyme B production from naïve mouse CD8⁺ T cells cultured with IL-2 [537]. Indeed, levels of Granzyme A were higher from FLIFFDLFL-specific CD8⁺ T cell assay supernatants, although of a similar order of magnitude to GILGFVFTL-specific CD8⁺ cells. While expression of Granzyme A could be a product of the 8 day T cell culture with rhIL-2, its upregulation and targeting towards infected cells could be better mediated by TRAP-specific CD8⁺ cells recognising their cognate antigen, therefore providing more directed parasite-specific cytotoxicity. The

relevance of Granzyme A in this model is supported by the clearly upregulated levels of Granzyme A in FLIFFDLFL-specific CD8⁺ T cells in the presence of their cognate antigen on peptide-pulsed HC-04 cells. Further experimentation would be needed to elucidate its role in parasite inhibition in this model, potentially by blocking its mode of action using serpin serine protease inhibitors such as SERPINB12, or using a perforin inhibitor such as Concanamycin A, to limit its effects [548, 549].

The role of Fas and the Fas/Fas ligand interaction could also be further assessed using this model. To identify whether Fas receptor is indeed upregulated on infected hepatocytes, a similar supernatant analysis of soluble Fas receptor could be performed on hepatoma cells previously sorted on GFP positivity. This should increase the sensitivity of the supernatant analysis by removing the 90-98% of uninfected cells still present in infected wells. Secondly, the role of nitric oxide (NO) in parasite inhibition could be explored using this assay. Due to the very short physiological half-life of this gaseous free radical, NO cannot be tested through standard Luminex analysis of supernatants and would require an additional analysis in real time, such as a colorimetric or fluorometric readout for NO and its associated products [550]. Alternatively, blocking NO production can be achieved through use of inducible nitric oxide synthase (iNOS) inhibition [551]. Equally, with the introduction of Kupffer cells into the assay, NO production may become a key pathway of parasite killing induced by infected hepatocytes or through the pro-inflammatory cytokines produced by NK cells, CD4⁺ T cells and/or CD8⁺ T cells.

The TRAP-specific effects measured from CD4⁺ T cells using the *in vitro* parasite inhibition assay are more complex. While many of the vaccinees exhibiting increased TRAP-specific killing over that of the control were matched at HLA class II loci, the assay structure was not designed to enrich CD4⁺ T cells through the MHC class I pentamer sort. Furthermore, the HC-04 cells express undetectable levels of HLA-DR. It is possible that CD4⁺ T cells with TCRs specific to the previously identified CD8⁺ T cell epitope FLIFFDLFLV were present in the PBMC cultures.

CD4⁺ T cell epitopes are typically 14-20 amino acids in length, however the peptide binding segment consists of 9 amino acids, with the remainder of the sequence flanking the open binding groove. Most MHC class II molecules have 4 binding pockets occupied by amino acids 1, 4, 6, and 9 of the minimal peptide epitope, and anchoring the peptide in position. Amino acid residues at

positions 2, 5, and 8 are available to interact with the TCR on the CD4⁺ T cell, and in some cases residue 3 and 7 also interact with the TCR. [552, 553]. While these flanking residues are necessary for optimal recognition of the peptide-MHC class II complex by the cognate CD4⁺ T cell, there are several reports of shorter peptides binding MHC class II and successfully interacting with the TCR on CD4⁺ T cells. Peptide is often required in higher concentrations for shorter sequences (~250µg/mL for a 10mer peptide compared to 1µg/ml for 15-mer), however the use of IL-2 in the culture of T cells in the assay could overcome this [554, 555].

Epitope prediction software is generally less comprehensive for MHC class II sequences compared to MHC class I, especially when predicting binding of peptides less than 15 amino acids. To overcome this, the 10mer sequence of FLIFFDLFLV was run epitope prediction software, SYFPEITHI®, with additional phenylalanine (F) amino acids flanking the sequence. The epitope mapped to several HLA-DR types, with anchor residue and auxiliary anchor residue scoring similar to that of the prediction of the 9mer FLIFFDLFL sequence to HLA-A2 (Figure 6.29, SYFPEITHI score 24-27). Similar predictions run with GILGFVFTL flanked in the same manner produced much lower epitope prediction scores to HLA-DR (range 10-19), and a 9mer GILGFVFTL HLA-A2:01 epitope prediction score of 30.

If higher frequencies of CD4⁺ T cells responding to the TRAP epitope over the influenza epitope were present in the original PBMC cultures, the culture conditions could activate TRAP-specific CD4⁺ T cells to a higher degree than their autologous influenza counterparts in the parallel GILGFVFTL culture. With the addition of IL-2 to the T cell cultures, the signal from peptide could be enhanced, and therefore result in more highly activated CD4⁺ T cells from TRAP culture, compared to influenza matrix protein 1 culture. As raw number of CD4⁺ T cells from the cell sort were matched and added into the assay, the enhanced parasite inhibition from the CD4⁺ T cells from TRAP culture could be simply due to the addition of more highly activated CD4⁺ T cells. So while the class I epitopes are well matched for their MHC class I binding, FLIFFDLFLV is more likely to interact with MHC class II molecules, and therefore could activate CD4⁺ T cells to a higher extent than in GILGFVFTL culture and explain why higher inhibition was measured with CD4⁺ T cells from TRAP culture. Measuring E:T-matched CD4⁺ T cells would give more insight on this hypothesis. Furthermore, these results on CD4⁺

T cell parasite inhibition highlight the requirement to not only focus on CD8⁺ T cell responses following vaccination.

A

1	2	3	4	5	6	7	8	9
F	L	I	F	F	D	L	F	L

HLA-A*02:01 nonamer binding score = 24

B

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
F	F	F	F	F	F	L	I	F	F	D	L	F	L	V

HLA-DRB1*0101 binding score for 10mer flanked with N-terminal phenylalanine = 27

C

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
F	F	F	F	L	I	F	F	D	L	F	L	V	F	F

HLA-DRB1*0101 binding score for 10mer flanked with N- and C-terminal phenylalanine = 21

D

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
F	L	I	F	F	D	L	F	L	V	F	F	F	F	F

HLA-DRB1*0101 binding score for 10mer flanked with C-terminal phenylalanine = 26

Figure 6.29 MHC class I and II epitope prediction using FLIFFDLFLV

A) FLIFFDLFL -9mer sequence run as MHC class I epitope on SYFPEITHI® epitope prediction software. B-D) FLIFFDLFLV -10mer sequence run as MHC class II epitope on SYFPEITHI® epitope prediction software, using additional flanking phenylalanine (F) amino acids. The SYFPEITHI® epitope prediction score is based on published motifs (pool sequencing, natural ligands) and takes into consideration the amino acids in the anchor and auxiliary anchor positions. (See appendix 4 for full SYFPEITHI readout).

The *in vitro* assay also explored NK cell-mediated parasite inhibition. The positive correlation measured between NK cell parasite inhibition and percentage infection could be linked to level of infection determining level of NK cell activation, with a higher percentage infection causing higher activation, leading to higher cytotoxicity. While a negative control for this parasite inhibition was unavailable, inhibition was based on a reduction in percentage of infected cells, thus the effects of NK cells were targeted at infected HC-04s over that of uninfected cells. Unfortunately no correlation was measured between percentage parasite inhibition by NK cells and the levels of analytes associated with NK activation (MIP 1- α , TNF- α , IFN- γ , IL-17, GrA), however the secretion of MIP1 α from NK cells could act as a positive feedback loop for NK cell recruitment to the site of infected cells, thus directing the NK response [556]. NK cells are also activated by IL-2, and with the high levels of this cytokine measured in the supernatants of both uninfected and infected wells of the assay, IL-2 could be driving this NK activation: however, no correlation was measured between IL-2 levels in assay supernatant and NK parasite inhibition.

The extension of this assay to that in *P. falciparum* infected primary human hepatocytes had limited success. This could be due to the T cell protocol used in this assay, whereby cells were rested for more days than optimisation experiments suggested. Additionally, E:T ratios achieved in this assay were in the region 2:1 to 18:1 for ChAd63.MVA ME-TRAP vaccinees, compared to 7:1 to 72:1 in the transgenic *P. berghei* model. The expression of parasite TRAP, and the subsequent presentation of TRAP epitopes on the surface of infected primary human hepatocytes, is likely to be lower in wildtype *P. falciparum* than that in the transgenic model, due to transgenic *PbPf*TRAP having both the wildtype and transgenic copy of TRAP transcribed, in addition to the transgenic *P. falciparum* TRAP copy being under a stronger promotor (UIS4) than found in wildtype *P. falciparum* parasites. Sensitivity of this *P. falciparum* assay is also likely to be considerably lower than that of the transgenic model, with percentage infectivity of *P. falciparum* in PHH over an order of magnitude lower than *PbPf*TRAP infection of HC-04 cells. Additional experiments could determine the suitability of this assay, particularly focussing on using the fully optimised T cell culture protocol and higher E:T ratios.

Chapter 7

Discussion

7 Discussion

7.1 Overview

A malaria vaccine with protective, long lasting efficacy is yet to be developed. The completion of a large phase III trial of RTS,S/AS01 is a huge advance in the field of malaria vaccine development, however this vaccine is suboptimal in many respects: the RTS,S schedule has an essential requirement for an 18 month booster; there is reduced efficacy in the youngest cohort [304, 403], which incidentally are the most at-risk group of severe malaria; and there is no CD8⁺ T cell activity induced by the regimen [243], which has been identified as a key cellular component of many pre-erythrocytic malaria vaccines, namely those with targeted at the liver stage of infection; and regimen safety is being further evaluated. RTS,S does demonstrate protective efficacy to the extent of up to 6000 cases per 1000 children and 1000 cases per 1000 infants over a 4 year follow up period, indicating its potential to reduce the paediatric malaria burden [557]. However it is reported that by this fourth year, vaccine efficacy wanes substantially, with follow up efficacy in Kilifi reported to be undetectable by year 7 [558]. Despite this, RTS,S will undoubtedly act as a yardstick for future vaccines to be compared against, alongside the vaccine aims outlined in the Malaria Vaccine Technology Roadmap [99].

The development of viral vector regimen ChAd63.MVA ME-TRAP targets some of these areas for improvement, particularly the induction of high numbers of antigen-specific CD8⁺ T cells [287]. More importantly, there are still areas within this regimen that can undergo further improvement, including the optimisation of regimen administration and wider testing in the target population.

Interestingly, neither the RTS,S or ChAd63.MVA ME-TRAP subunit approaches have replicated the vaccine efficacy achieved through the most efficacious whole sporozoite vaccination regimens. One of the main hypotheses for the higher efficacy using whole sporozoite vaccines is the vast array of antigens exposed to the immune system during vaccination [201]. This supports the suggestion of including additional antigenic components within a subunit vaccination strategy, in order to broaden the response across more than a single antigen. Importantly a multi-antigen, or even multi-stage, approach to vaccination still has more feasible deployability than whole sporozoite

approaches, because of the reduced resources required for vaccine generation, distribution and storage. The route of vaccine administration is also a key consideration in deployability, with PfSPZ requiring intravenous administration.

Key to improving a suboptimal vaccine is understanding its exact mechanism of action. As previously discussed, *in vivo* models for vaccine assessment exist but have several limitations, including variability in results measured using murine models depending on the combination of mouse strain, vaccination platform and parasite strain used in the model. Furthermore, the immune system of a mouse has many differences compared to that of humans. Non-human primates are useful and important models of human immunity to malaria [361], with a robust model of *P. falciparum* established in *Aotus* monkeys. However consistent with mice, vaccine efficacy has not always translated into humans [348]. Particularly with vaccines inducing cell-mediated immunity, differences in MHC class I genes in mice, and indeed NHPs, may account for some of these differences.

Finally, while assays exist for functional testing of human humoral responses *in vitro*, for example with the ILSDA, GIA and ISI assay, no such assay exists for the functional evaluation of human T cells against intracellular liver stage parasites. This thesis addressed many of the above issues, and explored them in more depth.

7.2 Conclusions and future directions

7.2.1 Clinical outcomes

The analysis of immunogenicity data from ChAd63.MVA ME-TRAP paediatric vaccinees indicated that the regimen induced strong cell-mediated immune responses to ME-TRAP [417]. Through deployment of a novel analysis strategy of *ex vivo* IFN- γ ELISpot data, which permits direct comparisons between adult and paediatric immune responses, it was demonstrated that vaccine immunogenicity was not reduced when the ChAd63.MVA ME-TRAP regimen was administered in age de-escalating paediatric populations. Thus in this vaccination regimen, the immune system of infants is sufficiently mature to induce T cell responses comparable to those seen in malaria-naïve adults exhibiting vaccine-induced protection against CHMI.

The adaptation of a whole blood ICS assay, permitted for the first time, robust flow cytometry analysis of T cell phenotypes induced by viral vectored malaria vaccine administration in infants. Such data suggests that ChAd63.MVA ME-TRAP administered to infants at 1 week of age alongside EPI vaccines may induce lower immune responses than those measured in infants of 2 months and older. However, the cytokine staining showed TRAP-specific CD4⁺ and CD8⁺ T cells responses producing pro-inflammatory cytokines peaking at ~0.1%. While these are the first data of its kind, limited conclusions can be drawn against other ChAd63.MVA ME-TRAP vaccinated populations. However, the validated assay can now be used across further clinical trials testing ChAd63.MVA ME-TRAP, building a larger dataset and thus enabling comparative immune responses to be measured between vaccine groups of different age, malaria exposure and EPI status. As more data is generated with this assay, a similar analysis methodology to the ELISpot response per millilitre of whole blood could be used to interpret ICS data and make more robust comparisons between datasets. With ELISpot responses increasing six fold using this approach in 10 week old Gambian infants, a similar increase in cytokine response by whole blood ICS would likely be measured.

The evaluation of 7 novel vaccine schedules involving the differential administration of vaccine candidates ChAd63 ME-TRAP and MVA ME-TRAP revealed that the standard 8 week prime-boost regimen can be further improved. ChAd63 ME-TRAP and MVA ME-TRAP were tested according

to 4- and 8- week schedules, including regimens with multiple ChAd63 ME-TRAP priming vaccinations, multiple MVA ME-TRAP boosts and regimens with alternating vectors. Vaccine-induced responses to TRAP were measured, as were the associated anti-vector immune responses. Improved CD8⁺ T cell immunogenicity over the previously reported 8 week prime-boost regimen was measured with a reduced ChAd63 ME-TRAP to MVA ME-TRAP interval of 4 weeks, and with the incorporation of an additional priming vaccination of homologous ChAd63 ME-TRAP at a 4 or 8 week interval (AAM or A_A_M). T cell immunity against the ChAd63 hexon did not affect T cell responses to the vaccine insert, however pre-vaccination ChAd63 hexon-specific T cells correlated with reduced anti-TRAP antibodies. This result indicated that any anti-vector T cell immunity induced by the more immunogenic double ChAd63 ME-TRAP prime regimens did not impact upon vaccine immunogenicity. Antibodies against ChAd63 were not measured in this study. Antibodies directed towards the hexon of AdHu5 have been reported to attenuate vaccine immunogenicity [452, 559]. However, with the double priming schedules more immunogenic in this study, it could be hypothesised that any anti-ChAd63 antibodies induced did not reverse the positive impact of a double ChAd63 ME-TRAP prime. It may be that such antibodies played a role in the lower responses measured with a triple ChAd63 ME-TRAP prime regimen. In populations with higher baseline anti-vector T cell and antibody responses, this result may differ. Vaccine-induced anti-MVA antibodies against multiple MVA ME-TRAP administrations did not affect anti-TRAP antibody induction, and correlated positively with the magnitude of ME-TRAP-specific T cells induced, and thus were not concluded to impact vaccine immunogenicity. Induction of TRAP-specific antibodies was not significantly affected by vaccine regimen.

These clinical data demonstrate the potential for improved vaccine-induced protection over the standard 8 week prime-boost ChAd63.MVA ME-TRAP. In addition, a degree of flexibility with the timing of vaccine administration has been observed, thus aiding incorporation of a prime-boost regimen into existing vaccination strategies, such as the paediatric EPI regimen or a multi-component vaccine regimen. Furthermore, the validation of a robust flow cytometry assay permits high throughput processing and batched, central flow cytometry analysis of vaccinee blood samples, using less than 1.5ml of whole blood. This assay provides an analysis platform for several types of vaccine

study including multi-site vaccine studies, such as large-scale efficacy studies; studies in very young infants with low blood volumes available for analysis, such as interference studies with childhood EPI vaccines; and clinical vaccine studies located in resource poor settings, where evaluation of malaria vaccines is most relevant.

7.2.2 Outcomes of *in vitro* parasite inhibition assay

The development of an *in vitro* assay measuring parasite inhibition of human T cells provides a solid basis for further work on human T cell responses. The optimisation performed has provided assay parameters for several of the model's components, including the infection parameters, the staining protocol for T cell and hepatoma cell differentiation by FACS readout, methodologies for measuring inhibition, maximum numbers of T cells for optimal background inhibition, inclusion of negative controls, and the timing of infection and readout. The work has also led to the robust infection of several different human hepatoma cell lines, providing a wider basis of HLA types for further testing, and resulted in far higher infectivity of *P. berghei* sporozoites in human hepatoma lines than previously published [461, 494, 495]. This work contributes to the infection model associated with many assays including the ISI assay, ILSDA and work investigating liver stage EEF development, with such detailed optimisation similar to that of Zou and colleagues, which greatly enhanced the application of the ILSDA antibody assay [184].

Upon use of vaccinee T cells in this assay, antigen-specific parasite inhibition was detected. Interestingly, vaccinees that demonstrated such inhibition were of a spectrum of protection demonstrated in CHMI studies. This could indicate that vaccine-induced T cells are capable of parasite inhibition when in contact with infected liver cells: *in vitro*, these T cells are put in direct contact with infected liver cells, however *in vivo*, T cells are required to migrate to the correct tissue, in this case the liver, in order to exert their protective effector functions. Thus it may be the case that all vaccinees generate T cells capable of liver stage parasite inhibition, but only some vaccinees have the correct T cell homing or sufficient T cell surveillance of the liver to efficiently eliminate all liver stage parasites during the pre-erythrocytic period. This hypothesis has implications in vaccine

development, with specific reference to enhanced T cell homing or recruitment of vaccine-induced T cells to the liver. Additional reasons these two readouts may not correlate include the fact that protection by CHMI is a result of the collective contribution of responses towards several epitopes, compared with just one measured currently with the *in vitro* T cell assay. Variation in sporozoite inoculum during CHMI could also be a contributing factor.

This assay has many potential uses for answering clinically relevant questions. To date, the assay has measured antigen-specific T cell parasite inhibition, however testing on a larger group of vaccinees would further validate these results. A much larger pool of volunteers could be evaluated if the assay was extended to use other hepatoma cell line HLA types beyond the HLA-A2 hepatoma line predominantly used in this thesis. This could encompass epitopes of different MHC restriction, with particular emphasis on the use of HLA-A3 pentamers in the HLA-A3 hepatoma cell line PLC/PRF/5. Exploration of additional epitopes within TRAP could be explored through the use of a panel of MHC class I pentamers to sort CD8⁺ T cells specific to other identified epitopes within TRAP. MHC class II multimers could also be introduced, to explore the CD4⁺ T cell compartment and the antigen-specific role these cells play in parasite inhibition. This would require some ground work regarding the identification of CD4⁺ T cell epitopes within TRAP, however the assay has already identified CD4⁺ T cells as a key area to investigate further and is supported by strong CD4⁺ T cell responses measured by ICS following vaccination with ChAd63.MVA ME-TRAP [284, 285, 287, 417]. Finally, the option to test the specificity of the signalling through MHC is possible, with direct comparison of inhibition in HLA-matched versus HLA-mismatched hepatoma cells, and assessments of the importance of hepatocyte to T cell contact through a transwell plate system.

The effector mechanisms underpinning parasite inhibition in this assay have only been briefly explored. Blocking experiments of key effector mechanisms identified in this thesis, particularly Granzyme A and the Fas/Fas ligand interaction, and identified in the literature, particularly the role of nitric oxide, could also be carried out using this assay platform, in order to further elucidate the pathways and effector molecules involved in vaccine-induced T cell parasite inhibition. Furthermore, different combinations of cell types could be evaluated, particularly that of TRAP-specific CD4⁺ and CD8⁺ T cells, to specifically explore the direct versus helper role of CD4⁺ T cells. Finally, the role of NK

cells in amplifying the adaptive IFN- γ response could be evaluated through addition of pre-vaccination NK cells alongside vaccine-induced T cells.

As the assay model develops, there is scope for further assay extension into the *P. falciparum* infection of primary human hepatocyte infection. This model permits less assay optimisation and exploration, due to the financial implications of raising *P. falciparum*-infected mosquitoes and purchasing commercially available primary human hepatocytes, in addition to the lower throughput of the assay related to length of liver stage cycle and the heightened resources associated with primary cell culture. However, a *P. falciparum* model will likely provide a more clinically relevant model of vaccine-induced responses, and could be used to answer key questions raised in the transgenic *P. berghei* parasite assay model.

7.3 Final remarks

As subunit vaccination strategies develop and new candidate antigens are explored, it is important to fully optimise the protective efficacy that can be achieved with a regimen, and accurately assess its immunogenicity and efficacy within the target population through the deployment of robust immunological and functional assays. The availability of a functional *in vitro* assay could aid the down-selection or support of novel candidate antigens in early Phase clinical testing, in addition to identifying key mechanisms of protection associated with vaccination, permitting informed correlates of protection to be measured from human vaccinees. These key areas can aid assessment of vaccine immunogenicity and effector function going forward, and subsequently feed back into improved vaccine design and evaluation.

8 Appendices

8.1 Appendix 1 – Peptide sequences

Table S1. TRAP T9/96 peptide sequences

TRAP 20mer peptides overlapping by 10 amino acids. Separate ELISpot pools shaded.

Peptide Name	T9/96 Peptide Sequence	T9/96 Peptide Pool
1T	MNHLGNVKYLIVFLIFFDL	TT 1-10
2T	VIVFLIFFDLFLVNGRDVQN	TT 1-10
3T	FLVNGRDVQNNIVDEIKYSE	TT 1-10
4T	NIVDEIKYSEEVCDNDQVDLY	TT 1-10
5T	EVCNDQVDLYLLMDCSGSIR	TT 1-10
6T	LLMDCSGSIRRHNVVNHAVP	TT 1-10
7T	RHNWVNHAVPLAMKLIQQLN	TT 1-10
8T	LAMKLIQQLNLNDNAIHLYV	TT 1-10
9T	LNDNAIHLYVNVFSNNAKEI	TT 1-10
10T	LNDNAIHLYVNVFSNNAKEI	TT 1-10
11T	IRLHSDASKNKEKALIIRS	TT 11-20
12T	KEKALIIRSLLSTNLPYGR	TT 11-20
13T	LLSTNLPYGRNLTDALQV	TT 11-20
14T	TNLTDALQVRKHLNDRINR	TT 11-20
15T	RKHLNDRINRENANQLVVIL	TT 11-20
16T	ENANQLVVILTDGIPDSIQD	TT 11-20
17T	TDGIPDSIQDSLKESRKLS	TT 11-20
18T	SLKESRKLSDRGVKIAVFGI	TT 11-20
19T	RGVKIAVFGIGQGGINVAFNR	TT 11-20
20T	GQGGINVAFNRFLVGCHPSDG	TT 11-20
21T	FLVGCHPSDGKCNLYADSAW	TT 21-30
22T	KCNLYADSAWENVKNVIGPF	TT 21-30
23T	ENVKNVIGPFMKAVCVEVEK	TT 21-30
24T	MKAVCVEVEKTASCGVWDEW	TT 21-30
25T	TASCGVWDEWSPCSVTCGKG	TT 21-30
26T	SPCSVTCGKGTRSRKREILH	TT 21-30
27T	TRSRKREILHEGCTSEIQEQ	TT 21-30
28T	EGCTSEIQEQCEEERCPKWK	TT 21-30
29T	CEEERCPKWEPDVPDEPE	TT 21-30
30T	EPLDVPDEPEDDQPRPRGDN	TT 21-30
31T	DDQPRPRGDNSSVQKPEENI	TT 31-40
32T	SSVQKPEENIIDNNPQEPSP	TT 31-40
33T	IDNNPQEPSPNPEEGKDENP	TT 31-40
34T	NPEEGKDENPNNGFDLDENPE	TT 31-40
35T	NGFDLDENPENPPNDIPEQ	TT 31-40
36T	NPPNDIPEQKPNIPEDSEK	TT 31-40
37T	Not present in TRAP T9/96	N/A
38T	DIPEQKPNIPEDSEKEVPSD	TT 31-40
39T	EDSEKEVPSDVPKNPEDDRE	TT 31-40
40T	VPKNPEDDREENFDIPKKPE	TT 31-40

41T	ENFDIPKKPENKHDNQNNLP	TT 41-50
42T	NKHDNQNNLPNDKSDRNIPY	TT 41-50
43T	NDKSDRNIPYSPLPPKVLDN	TT 41-50
44T	SPLPPKVLDNERKQSDPQSQ	TT 41-50
45T	ERKQSDPQSQDNNNGNRHVPN	TT 41-50
46T	DNNGNRHVPNSEDRETRPHG	TT 41-50
47T	SEDRETRPHGRNNENRSYNR	TT 41-50
48T	RNNENRSYNRKYNDTPKHPE	TT 41-50
49T	KYNDTPKHPEREEHEKPDNN	TT 41-50
50T	REEHEKPDNNKKKGESDNKY	TT 41-50
51T	KKKGESDNKYKIAGGIAGGL	TT 51-57
52T	KIAGGIAGGLALLACAGLAY	TT 51-57
53T	ALLACAGLAYKFVPGAATP	TT 51-57
54T	KFVPGAATPYAGEPAPFDE	TT 51-57
55T	YAGEPAPFDETLGEEDKDLD	TT 51-57
56T	TLGEEDKDLDEPEQFRLPEE	TT 51-57
57T	EPEQFRLPEENEWN	TT 51-57

Table S2. Peptide sequences for non-overlapping Multi-Epitope string.

Peptide Name	Epitope Peptide Sequence	ME Peptide Pool
st8	MINAYLDKL	ME pool
ls50	ISKYEDEI	
pb9	SYIPSAEKI	
ls8	KPNDKSLY	
cp26	KPKDELDY	
ls6	KPIVQYDNF	
tr42/43	ASKNKEKALII	
tr39	GIAGGLALL	
cp6	MNPNDPNRNV	
tr26	HLGNVKYLV	
ls53	KSLEYDEHI	
tr29	LLMDCSGSI	
csp	DPNANPNVDPNANPNV	
38H BCG	QVHFQPLPPAVVKL	
FTTp	QFIKANSKFIGITE	
cp39	YLNKIQNSL	
la72	MEKLKELEK	
ex23	ATSVLAGL	
nanp	NANPNANPNANPNANP	
trapAM	DEWSPCSVTGKGTRSRKRE	

Table S3. Peptide sequences for hexon peptides.

Hexon 20mer peptides overlapping by 10 amino acids. "Common" denotes peptide sequences common to both ChAd63 and AdHu4 adenoviruses. "ChAd63-specific" denotes dissimilar peptide sequences between ChAd63 and AdHu4 adenoviruses.

Peptide Name	ChAd63 Peptide Sequence	Hexon peptide pool
AdC63-1	MATPSMLPQWAYMHIAGQDA	Common
AdC63-2	AYMHIAGQDASEYLSPGLVQ	ChAd63-specific
AdC63-3	SEYLSPLVQFARATDTYFS	Common
AdC63-4	FARATDTYFSLGNKFRNPTV	Common
AdC63-5	LGNKFRNPTVAPTHDVTDDR	ChAd63-specific
AdC63-6	APTHDVTDRSQRLLRFVP	Common
AdC63-7	SQRLLRFVPVDREDNTYSY	Common
AdC63-8	VDREDNTYSYKVRTLAVGD	Common
AdC63-9	KVRTLAVGDNRVLDMASTY	ChAd63-specific
AdC63-10	NRVLDMASTYFDIRGVLDRG	Common
AdC63-11	FDIRGVLDRGPSFKPYSGTA	Common
AdC63-12	PSFKPYSGTAYNSLAPKGAP	Common
AdC63-13	YNSLAPKGAPNTSQWKSDS	ChAd63-specific
AdC63-14	NTSQWKSDSKMHTFGVAAM	ChAd63-specific
AdC63-15	KMHTFGVAAMPGVVGKKIEA	ChAd63-specific
AdC63-16	PGVVGKKIEADGLPIGIDSS	ChAd63-specific
AdC63-17	DGLPIGIDSSSGTDITIIAD	ChAd63-specific
AdC63-18	SGTDITIIADKTFQPEPQVG	ChAd63-specific
AdC63-19	KTFQPEPQVGSDSWVDTNGA	ChAd63-specific
AdC63-20	SDSWVDTNGAEEKYGGRAK	ChAd63-specific
AdC63-21	EEKYGGRAKDTTNMKPCYG	ChAd63-specific
AdC63-22	DTTNMKPCYGSFARPTNKEG	Not present
AdC63-23	SFARPTNKEGGQANIKDSET	ChAd63-specific
AdC63-24	GQANIKDSETASTTPNYDID	ChAd63-specific
AdC63-25	ASTTPNYDIDLAFDKNIA	ChAd63-specific
AdC63-26	LAFDKNIAANYDPDIVMY	ChAd63-specific
AdC63-27	ANYDPDIVMYTENVELQTPD	ChAd63-specific
AdC63-28	TENVELQTPDTHIVFKPGTS	ChAd63-specific
AdC63-29	THIVFKPGTSDASSEANLGQ	ChAd63-specific
AdC63-30	DESSEANLGQQAMPNRPNYI	ChAd63-specific
AdC63-31	QAMPNRPNYIGFRDNFIGLM	ChAd63-specific
AdC63-32	GFRDNFIGLMYYNSTGNMGV	ChAd63-specific
AdC63-33	YYNSTGNMGVLAGQASQLNA	ChAd63-specific
AdC63-34	LAGQASQLNAVVDLQDRNTE	ChAd63-specific
AdC63-35	VVDLQDRNTELSYQLLLDSL	ChAd63-specific
AdC63-36	LSYQLLLDSLGRTRYFSMW	ChAd63-specific
AdC63-37	GDRTRYFSMWNAVDSYDPD	ChAd63-specific
AdC63-38	NQAVDSYDPDVRIENHGVE	ChAd63-specific
AdC63-39	VRIENHGVEDELPNYCFPL	ChAd63-specific

AdC63-40	DELPNYCFPLNGVGFTDTYQ	ChAd63-specific
AdC63-41	NGVGFTDTYQGVKVKTDATA	ChAd63-specific
AdC63-42	GVKVKTDATGTNGTQWDK	ChAd63-specific
AdC63-43	TGTNGTQWDKDDTTVSTANE	ChAd63-specific
AdC63-44	DDTTVSTANEIHSGNPFAME	ChAd63-specific
AdC63-45	IHSGNPFAMEINIQANLWRN	ChAd63-specific
AdC63-46	INIQANLWRNFLYANVALYL	ChAd63-specific
AdC63-47	FLYANVALYLPDSYKYTPAN	ChAd63-specific
AdC63-48	PDSYKYTPANITLPTNTNTY	ChAd63-specific
AdC63-49	ITLPTNTNTYDYMNGRVVAP	ChAd63-specific
AdC63-50	DYMNGRVVAPSLVDAYINIG	ChAd63-specific
AdC63-51	SLVDAYINIGARWSLDPMDN	Not present
AdC63-52	ARWSLDPMDNVNPFNHHRNA	ChAd63-specific
AdC63-53	VNPFNHHRNAGLRYRSMMLG	ChAd63-specific
AdC63-54	GLRYRSMMLGNGRYVPFHIQ	ChAd63-specific
AdC63-55	NGRYVPFHIQVPQKFFAICS	ChAd63-specific
AdC63-56	VPQKFFAICSLLLPGSYTY	ChAd63-specific
AdC63-57	LLLLPGSYTYEWNFRKDVNM	ChAd63-specific
AdC63-58	EWNFRKDVNMILQSSLGNDL	ChAd63-specific
AdC63-59	ILQSSLGNDLRTDGASIAFT	ChAd63-specific
AdC63-60	RTDGASIAFTSINLYATFFP	ChAd63-specific
AdC63-61	SINLYATFFPMAHNTASTLE	ChAd63-specific
AdC63-62	MAHNTASTLEAMLRNDTNDQ	ChAd63-specific
AdC63-63	AMLRNDTNDQSFNDYLSAAN	ChAd63-specific
AdC63-64	SFNDYLSAANMLYPIANAT	ChAd63-specific
AdC63-65	MLYPIANATNVPISPSRN	ChAd63-specific
AdC63-66	NVPISPSRNWAAFRGWSFT	ChAd63-specific
AdC63-67	WAAFRGWSFTRLKTRTPSL	ChAd63-specific
AdC63-68	RLKTRTPSLGSGFDPPYFVY	ChAd63-specific
AdC63-69	GSGFDPPYFVYSGSIPYLDGT	ChAd63-specific
AdC63-70	SGSIPYLDGTFYLNHTFKKV	ChAd63-specific
AdC63-71	FYLNHTFKKVSITFDSSVSW	Not present
AdC63-72	SITFDSSVSWPGNDRLLTPN	ChAd63-specific
AdC63-73	PGNDRLLTPNEFEIKRTVDG	ChAd63-specific
AdC63-74	EFEIKRTVDGEGYNVAQCNM	ChAd63-specific
AdC63-75	EGYNVAQCNMTKDWFLVQML	ChAd63-specific
AdC63-76	TKDWFLVQMLAHYNIGYQGF	ChAd63-specific
AdC63-77	AHYNIGYQGFYVPEGYKDRM	ChAd63-specific
AdC63-78	YVPEGYKDRMYSFFRNQPM	ChAd63-specific
AdC63-79	YSFFRNQPMQRQVVDEVNY	ChAd63-specific
AdC63-80	SRQVVDEVNYKDYQAVTLAY	ChAd63-specific
AdC63-81	KDYQAVTLAYQHNNSGFVGY	ChAd63-specific
AdC63-82	QHNNSGFVGYLAPTMRQGQP	ChAd63-specific
AdC63-83	LAPTMRQGQPYPANYPYPLI	ChAd63-specific
AdC63-84	YPANYPYPLIGKSAVASVTQ	ChAd63-specific
AdC63-85	GKSAVASVTQKKFLCDRVMW	ChAd63-specific

AdC63-86	KKFLCDRVMWRIPFSSNFMS	ChAd63-specific
AdC63-87	RIPFSSNFMSMGALTDLGQN	ChAd63-specific
AdC63-88	MGALTDLGQNMLYANSAHAL	ChAd63-specific
AdC63-89	MLYANSAHALDMNFEVDPM	ChAd63-specific
AdC63-90	DMNFEVDPMDESTLLYVFE	ChAd63-specific
AdC63-91	ESTLLYVFEVDVVRVHQP	ChAd63-specific
AdC63-92	VFDVVRVHQPVRGVEAVYL	ChAd63-specific
AdC63-93	HRGVIEAVYLRTPFSAAGAT	ChAd63-specific

Table S4. Peptide sequences for non-overlapping CTL peptides.

Name	Amino acid Sequence	Source organism	Protein (region)	Number of amino acids
CTL1	VSDGGPNLY	Influenza A	PB1 (591-599)	9
CTL2	CTELKLSDY	Influenza A	NP (44-52)	9
CTL3	GLCTLVAML	EBV	BMLF1 (259-267)	9
CTL4	GILGFVFTL	Influenza A	Matrix 1 (58-66)	9
CTL5	NLVPMVATV	HCMV	pp65 (495-503)	9
CTL6	ILRGSAVHK	Influenza A	NP (265-273)	9
CTL7	RVRATYSK	EBV	BRLF1 (148-156)	9
CTL8	RLRAEAQVK	EBV	EBNA3A (603-611)	9
CTL9	IVTDFSVIK	EBV	EBNA3B (416-424)	9
CTL10	ATIGTAMYK	EBV	BRLF1 (134-143)	9
CTL11	DYCNVLNKEF	EBV	BRLF1 (28-37)	10
CTL12	KTGGPIYKR	Influenza A	NP (91-99)	9
CTL13	RPPIFIRRL	EBV	EBNA3A (379-387)	9
CTL14	TPRVTGGGAM	HCMV	pp65	10
CTL15	QAKWRLQTL	EBV	EBNA3A (158-166)	9
CTL16	FLRGRAYGL	EBV	EBNA3A (325-333)	9
CTL17	RAKFKQLL	EBV	BZLF1 (190-197)	8
CTL18	ELRSRYWAI	Influenza A	NP (380-388)	9
CTL19	SRYWAIRTR	Influenza A	NP (383-391)	9
CTL20	RRIYDLIEL	EBV	EBNA3C (258-266)	9
CTL21	YPLHEQHGM	EBV	EBNA3A (458-466)	9
CTL22	EENLLDFVRF	EBV	EBNA3C (281-290)	10
CTL23	EFFWDANDIY	HCMV	pp65 (511-525)	10

8.2 Appendix 2 – T cell gating strategy

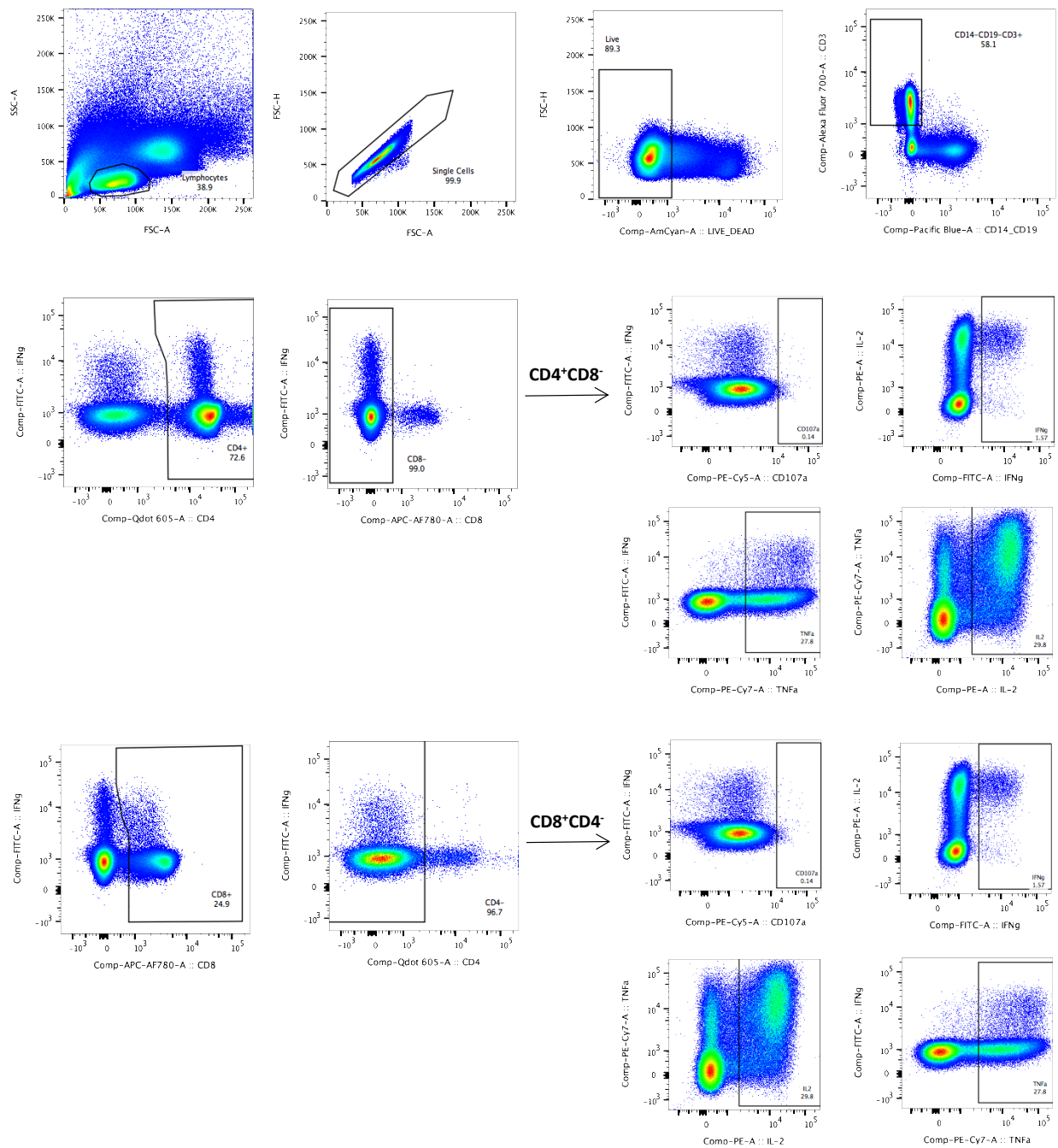


Figure S1. Sample gating strategy for analysis of flow cytometry with ICS.

8.3 Appendix 3 – Luminex data

Luminex plate 1 (mean of replicates)		Analyte pg/mL																		
TNF- alpha	IL-6	IL-3	CXCL8/IL-8	IL-10	IL-1beta	IFN-g	MIP-1 alpha	IL-1 alpha	FAS Ligand	IL-17A	IL-2	GM-CSF	Granzyme A	CXCL9/MIG	IL-12 p70	Granzyme B	IL-21	FAS	IL-18	
1429 U	1.3	31.7	1143.1	4.7	4.6	17.2	68.8	1.3	4.1	2.9	143.4	4.2	7.7	126.1	38.6	5.9	8.4	77.5	15.8	
1429 I	1.3	30.1	1097.3	3.2	4.6	18.1	65.5	1.7	4.6	3.3	160.6	5.2	10.3	117.9	37.9	6.3	8.8	75.3	15.9	
1429 CD8 flff p+	1.3	30.9	916.8	4.0	4.6	19.0	60.0	1.7	4.5	3.0	179.5	4.4	9.2	117.9	36.0	6.1	8.4	71.6	15.6	
1429 CD4 flff	1.3	31.7	1043.1	3.2	4.3	19.9	72.3	2.0	6.0	3.1	176.8	3.9	27.2	126.1	37.3	18.9	8.4	74.5	15.9	
1429 CD8 gllg p+	1.2	30.9	1078.8	3.6	4.3	18.1	57.9	2.0	4.3	2.9	137.7	4.2	11.9	117.9	37.3	6.1	8.4	78.2	14.7	
1429 CD4 gllg	1.3	30.9	1220.8	2.9	4.3	19.0	65.5	1.7	5.5	3.0	179.7	4.4	28.6	117.9	36.0	9.9	8.4	81.2	15.8	
1429 NK	1.5	32.1	1357.0	3.6	4.3	18.1	74.0	1.7	4.6	2.8	176.2	4.4	9.5	117.9	36.0	5.8	8.4	78.2	15.9	
1166 80/20 peptide only	1.3	30.9	331.8	4.0	4.3	20.0	51.1	1.7	3.8	2.9	191.4	4.4	7.7	117.9	38.6	5.9	8.0	60.0	14.7	
1407 U	1.3	30.5	409.8	4.0	4.6	16.2	53.9	1.3	4.1	2.9	164.0	3.9	7.7	117.9	39.9	5.7	7.5	85.0	16.1	
1407 I	1.3	29.3	1002.5	3.6	4.3	18.1	62.0	1.3	4.1	2.9	140.8	4.3	7.7	109.2	36.6	5.9	7.5	62.9	15.2	
1407 CD8 flff p+	1.4	26.1	556.1	2.1	4.1	19.9	56.0	1.7	5.0	2.8	164.8	4.2	61.1	117.9	36.0	12.4	8.4	60.0	15.4	
1407 CD4 flff	1.2	30.9	1310.7	2.9	4.3	17.2	67.4	1.3	4.3	3.0	169.5	4.9	11.4	117.9	37.3	6.2	8.0	67.6	15.1	
1407 CD8 gllg p+	1.3	29.3	995.3	3.2	4.3	15.4	72.3	1.7	5.9	2.9	142.0	4.9	74.2	113.3	39.9	14.7	8.0	64.0	15.4	
1407 CD4 gllg	1.2	28.1	1165.4	3.6	4.6	17.2	62.0	1.7	4.3	2.9	162.5	4.3	8.2	117.9	37.3	6.2	8.0	64.3	15.9	
1407 NK	9.3	29.3	> 1140	3.6	8.8	21.8	270.3	2.6	12.4	3.1	140.5	9.9	28.0	109.2	37.3	6.9	8.4	61.5	15.8	
1166 80/20 flff cd8 p+	1.3	30.9	391.0	4.0	4.6	17.2	53.9	0.7	4.1	2.9	171.6	4.2	7.2	117.9	41.2	5.9	7.5	84.3	16.1	
1166 80/20 NK	1.3	30.1	428.5	4.0	4.6	15.3	53.9	2.0	4.1	2.9	156.4	3.7	8.2	117.9	38.6	5.5	7.5	85.8	16.1	
1454 U	1.0	26.9	360.1	2.9	3.9	18.1	53.1	1.0	4.1	2.5	144.1	4.2	7.2	113.6	33.4	5.4	7.5	77.1	15.8	
1454 I	1.3	27.7	992.8	2.9	4.3	17.6	53.9	1.7	4.1	2.6	144.9	3.4	7.2	117.9	33.4	5.7	8.0	63.6	15.8	
1454 CD8 flff p+ (lo)	1.3	28.5	855.9	3.1	3.8	19.0	72.3	1.0	6.4	2.7	154.5	4.2	46.7	117.9	34.7	9.5	8.0	62.9	15.4	
1454 CD4 flff (lo +hi)	1.3	27.7	1056.4	3.1	3.9	16.2	62.0	1.7	4.3	2.8	175.7	5.2	9.2	117.9	36.0	6.1	7.5	67.2	15.4	
1454 CD8 gllg p+ (lo)	1.3	29.3	1006.0	2.9	4.6	18.1	62.0	1.7	5.7	2.7	144.7	4.2	31.5	100.5	38.6	9.1	7.5	67.2	15.4	
1454 CD4 gllg (lo)	1.3	27.7	990.0	2.5	4.3	17.2	53.9	1.0	4.1	2.9	154.3	3.9	7.7	109.2	36.0	5.9	8.0	65.8	15.1	
1454 NK (lo)	3.5	29.3	> 1140	3.6	5.0	21.8	238.4	1.7	18.2	2.8	150.7	16.8	62.0	113.6	37.3	9.2	8.0	86.9	15.8	
1166 80/20 gllg CD8 p+	1.2	28.1	271.4	3.2	4.0	18.1	53.9	0.7	4.3	2.5	170.9	4.4	17.9	109.2	37.3	8.2	7.5	58.6	15.4	
1113 80/20 flff CD8 p+ hi	1.3	35.8	674.6	3.2	4.3	244.7	2016.8	2.0	31.3	2.9	223.2	58.6	504.4	117.9	38.6	83.5	8.0	64.3	15.8	
1113 80/20 gllg CD8 p+	1.2	27.7	305.5	3.2	3.9	17.2	53.9	1.7	4.8	2.9	201.7	4.2	39.9	117.9	38.6	14.7	7.5	60.8	15.4	
1454 CD8 flff p+ (hi)	1.2	28.1	991.9	2.7	4.3	20.9	110.1	1.3	9.5	2.8	185.7	5.5	126.7	109.2	36.0	20.4	8.0	69.4	15.4	
1454 CD4 flff (hi)	1.1	26.9	753.3	1.7	4.6	17.2	62.0	0.7	4.6	2.6	144.9	5.2	10.3	117.9	33.4	5.9	7.5	65.0	14.7	
? 1113 80/20 NK	1.3	26.9	318.0	2.5	3.3	19.0	53.9	0.7	5.5	2.9	150.7	4.2	28.0	117.9	34.7	5.9	7.5	57.9	15.4	
1454 CD8 gllg p+ (hi)	1.2	29.3	1011.6	2.9	3.9	19.9	75.5	1.7	7.4	2.9	168.8	4.3	48.5	117.9	34.7	11.6	7.5	68.7	15.8	
1454 CD4 gllg (hi)	1.2	27.7	886.2	2.5	4.6	18.1	56.0	1.0	4.0	2.8	167.5	3.7	8.2	117.9	37.3	5.7	8.0	63.6	15.4	
1454 NK (hi)	5.8	28.5	> 1140	4.0	9.2	29.7	335.3	2.0	22.4	2.9	149.4	32.5	42.0	117.9	34.7	10.5	8.4	63.6	15.4	
1113 80/20 peptide only	1.2	27.7	342.7	2.5	3.9	16.2	51.6	1.7	4.1	2.5	152.7	3.9	7.4	109.2	34.1	5.5	7.5	55.8	15.1	
1431 U	1.2	28.5	400.0	2.3	4.3	18.1	49.3	1.7	4.0	2.8	139.7	3.7	6.1	109.2	36.0	5.3	7.1	70.8	14.7	
1431 I	1.2	26.1	762.8	3.2	4.3	15.3	57.9	1.7	3.8	2.5	149.2	3.7	6.6	109.2	36.0	5.5	7.1	59.7	14.9	
1431 CD8 flff p+	1.2	27.7	712.4	2.5	3.9	17.2	65.5	1.7	7.2	2.6	132.9	3.6	64.1	100.5	34.7	8.9	7.3	60.0	14.4	
1431 CD4 flff	1.2	26.1	820.4	1.7	3.9	16.2	49.0	2.0	4.1	2.6	159.2	4.2	9.8	117.9	35.4	5.7	7.5	62.2	15.4	
1431 CD8 gllg p+	1.3	26.1	705.2	2.1	3.9	17.2	118.1	1.7	12.0	2.4	156.0	5.2	44.3	109.2	33.4	7.5	7.5	62.2	15.4	
1431 CD4 gllg	1.1	26.9	720.0	2.9	4.1	18.1	49.0	1.3	4.3	2.7	155.0	3.7	8.2	109.2	34.7	5.8	7.5	58.6	15.8	
1431 NK	5.3	25.4	> 1140	3.6	7.8	18.1	199.8	2.0	10.1	2.6	134.4	5.8	13.0	117.9	36.0	6.5	7.5	57.9	15.1	
1113 80/20 flff CD8 p+	1.2	28.1	470.5	2.3	3.9	36.9	293.4	1.3	7.8	2.6	147.9	12.1	51.5	117.9	34.7	10.1	7.5	62.2	14.7	

Luminex plate 2 (mean of replicates)		Analyte pg/mL																		
Well	TNF- alpha	IL-6	IL-3	CXCL8/IL-8	IL-10	IL-1 beta	IFN-γ	MIP-1 alpha	IL-1 alpha	FAS Ligand	IL-17A	IL-2	GM-CSF	Granzyme A	CXCL9/MIG	IL-12 p70	Granzyme B	IL-21	FAS	IL-18
1451 U	4.6	1.5	33.1	548.7	2.3	5.1	22.3	61.3	2.8	4.3	3.2	315.6	5.5	11.2	138.8	41.6	6.1	8.7	79.1	16.8
1451 I	4.4	1.6	33.8	813.7	1.9	5.3	20.5	58.5	2.3	4.3	3.1	267.3	5.1	11.2	119.3	40.5	6.1	7.9	63.5	17.2
1451 CD8 flttf p+	5.4	1.5	33.8	1020.1	2.1	4.8	19.7	83.5	2.4	8.1	3.1	256.5	5.6	234.6	129.3	39.5	10.0	8.7	67.8	16.6
1451 CD4 flttf	5.2	1.5	34.5	957.1	2.1	5.2	20.5	66.8	2.3	4.5	3.3	222.4	5.5	13.1	129.3	40.5	6.5	8.3	66.8	16.9
1451 CD8 gltg p+	6.2	1.5	32.4	617.6	2.2	5.1	21.8	153.1	2.6	13.9	3.2	251.7	6.5	74.0	129.3	39.5	7.5	8.3	63.8	17.1
1451 CD4 gltg	4.8	1.3	33.8	917.1	1.9	5.1	19.7	61.3	2.8	4.6	3.2	277.2	5.3	13.1	138.8	41.6	6.1	7.9	64.1	16.5
1451 NK	322.0	392.2	34.5	>1140	159.2	166.8	106.8	3832.0	6.6	39.0	3.1	288.6	42.2	124.1	166.2	45.9	13.1	10.0	65.3	17.5
1407 80/20 peptide only	3.6	1.3	29.8	508.9	1.8	4.8	15.7	55.4	2.3	4.0	2.6	178.3	4.6	8.4	119.3	37.4	5.4	7.9	76.8	15.6
1134 U	4.2	1.4	33.1	1175.5	2.1	5.1	20.5	62.6	2.8	4.5	3.1	217.0	4.8	10.2	129.3	38.4	6.0	8.3	138.6	16.2
1134 I	4.6	1.5	32.5	>1140	2.4	5.1	20.5	74.4	3.0	4.3	3.1	244.5	5.3	11.2	129.3	40.5	6.4	9.1	113.1	16.5
1134 CD8 flttf p+	6.6	1.6	32.4	>1140	2.3	5.6	21.8	86.7	3.0	10.4	3.3	243.3	5.4	107.2	138.8	37.4	9.4	8.7	108.9	16.5
1134 CD4 flttf	4.8	1.6	31.8	>1140	2.0	4.6	21.0	74.2	2.7	4.5	3.2	214.9	5.1	11.2	129.3	37.4	6.1	8.7	111.3	15.6
1134 CD8 gltg p+	6.0	1.5	31.8	>1140	2.2	4.5	19.7	83.5	2.8	8.8	3.2	227.5	5.9	119.9	138.8	41.1	6.2	8.7	113.7	16.5
1134 CD4 gltg	4.6	1.5	33.1	>1140	2.1	5.3	20.5	71.9	2.6	4.5	3.3	234.2	5.8	11.2	138.8	41.1	6.2	8.7	113.7	16.5
1134 NK	58.5	4.8	33.8	>1140	2.7	6.5	23.5	1010.9	3.0	34.7	3.1	271.6	16.9	137.8	157.2	40.5	8.6	9.6	109.6	16.8
1407 80/20 CD8 flttf p+	10.7	1.3	32.4	498.4	1.7	5.3	21.4	170.0	2.3	5.6	3.0	177.2	30.0	44.5	129.3	37.4	7.1	8.3	82.9	15.6
1407 80/20 NK	5.8	2.0	29.8	809.1	1.9	4.8	18.1	69.4	2.3	7.0	2.6	177.2	4.6	24.6	129.3	35.3	5.6	7.5	74.0	16.2
1168 U	3.8	1.5	29.8	1322.1	1.9	4.5	16.5	64.1	2.3	4.3	3.1	217.1	5.1	10.2	129.3	37.4	5.5	8.3	140.8	16.2
1168 I	4.6	1.5	33.1	>1140	2.2	5.3	20.5	69.4	2.6	4.5	3.0	271.3	5.3	12.1	138.8	38.4	6.1	8.7	112.0	16.8
1168 CD8 flttf p+	4.8	1.6	32.4	>1140	2.1	5.3	18.9	76.7	2.8	6.3	3.2	279.3	5.8	116.7	129.3	40.5	12.3	9.1	108.9	16.5
1168 CD4 flttf	4.8	1.5	33.1	>1140	2.3	5.1	23.1	71.9	3.0	4.5	3.1	321.0	6.2	12.1	148.2	41.1	6.5	8.7	115.9	17.1
1168 CD8 gltg p+	5.0	1.4	31.8	>1140	2.5	4.8	21.4	76.7	2.8	7.7	3.0	257.7	6.2	108.3	119.3	39.5	13.3	9.1	116.9	16.8
1168 CD4 gltg	4.6	1.4	31.8	>1140	2.0	4.8	20.1	73.1	2.4	4.5	3.0	234.6	5.3	11.2	119.3	37.9	6.1	9.1	111.7	15.9
1168 NK	30.0	7.9	32.4	>1140	2.8	6.2	19.7	504.3	2.7	17.6	2.9	183.6	12.8	35.5	138.8	39.5	6.6	8.9	99.4	15.9
1407 80/20 CD8 gltg p+	4.4	1.3	29.1	574.7	1.6	4.2	16.9	52.0	2.1	4.6	2.7	170.8	4.6	36.0	119.3	35.3	7.1	7.5	78.4	15.7
1127 U	4.2	1.5	32.5	1125.0	1.9	4.6	18.9	64.1	2.3	4.3	2.9	250.0	5.3	9.3	129.3	39.5	6.0	8.3	128.2	16.5
1127 I	4.5	1.4	33.1	2844.7	2.2	5.1	20.1	68.0	2.8	4.3	3.1	310.1	5.3	11.2	129.3	40.5	6.2	8.7	100.7	17.4
1127 CD8 flttf p+	6.0	1.6	32.5	>1140	2.1	5.1	21.4	74.2	2.8	7.7	3.6	309.8	6.2	191.3	138.8	39.5	14.4	8.7	111.7	17.1
1127 CD4 flttf	5.0	1.5	32.4	>1140	2.4	5.3	21.4	69.4	2.8	4.8	3.3	331.1	6.5	13.1	129.3	42.7	6.6	9.1	107.9	17.1
1127 CD8 gltg p+	6.0	1.6	32.5	>1140	2.2	5.3	23.1	72.9	2.7	7.4	3.3	311.6	6.0	173.0	128.8	43.7	13.3	9.1	110.3	17.7
1127 CD4 gltg	4.8	1.6	32.4	>1140	2.3	5.3	22.2	69.4	2.8	4.7	3.4	291.4	5.8	12.1	129.3	40.5	6.1	8.7	106.2	17.1
1127 NK	61.5	94.7	31.8	>1140	15.2	51.3	31.1	1024.5	5.5	21.4	3.0	181.3	31.0	80.7	129.3	40.5	12.1	8.7	89.7	15.6
1454 80/20 CD8 flttf p+	47.6	1.3	39.4	843.7	1.8	4.8	37.7	537.0	2.3	9.6	2.6	183.6	124.8	48.5	129.3	35.3	10.0	8.3	82.9	16.8
1454 80/20 NK	7.0	1.6	28.5	785.2	1.7	4.8	14.9	64.1	1.9	6.7	2.8	161.0	4.6	15.0	119.5	37.4	5.9	7.5	75.2	15.6
1170 U	4.2	1.3	31.1	967.8	2.0	4.6	17.3	58.2	2.1	4.1	2.9	189.6	4.4	10.2	129.3	37.9	5.5	7.9	113.8	16.2
1170 I	3.9	1.4	31.8	2235.7	2.1	4.5	18.1	69.4	2.8	4.3	2.8	226.9	4.6	10.2	129.3	36.3	5.9	8.1	96.7	15.6
1170 CD8 flttf p+	4.8	1.5	32.4	2147.6	2.1	5.1	18.9	71.9	2.6	4.8	3.0	268.9	5.5	39.0	138.8	37.4	6.5	7.9	102.1	17.1
1170 CD4 flttf	4.8	1.5	32.4	3631.9	2.1	5.1	20.5	74.2	2.8	4.5	3.0	305.7	6.5	12.1	129.3	41.6	6.1	8.3	106.2	16.5
1170 CD8 gltg p+	5.4	1.6	32.8	2839.8	2.2	5.3	21.4	71.9	2.8	5.3	3.2	322.7	6.5	46.5	138.8	40.6	7.6	8.3	104.8	16.8
1170 CD4 gltg	4.6	1.5	32.4	2853.6	2.3	5.1	22.2	70.4	3.3	4.5	3.2	300.8	6.0	10.2	138.8	41.6	6.4	9.1	108.9	16.8
1170 NK	22.8	14.6	33.8	>1140	9.1	9.6	22.2	329.1	3.5	10.7	3.2	231.2	8.2	43.5	148.2	39.5	6.6	9.1	94.7	16.5
1454 80/20 CD8 gltg p+	4.0	1.2	28.8	502.6	1.7	4.5	15.7	57.0	2.1	4.6	2.6	179.2	4.4	23.7	109.3	35.3	6.9	7.5	75.9	15.6

8.4 Appendix 4 – SYFPYTHEI epitope prediction

SYFPYTHEI HLA-DR prediction FFFFFLIFFDLFLV

HLA-DRB1*0101 15 - mers

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
1	FFFFFLLIFFDLFLV	27

HLA-DRB1*0301 (DR17) 15 - mers

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
1	FFFFFLLIFFDLFLV	16

HLA-DRB1*0401 (DR4Dw4) 15 - mers

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
1	FFFFFLLIFFDLFLV	22

HLA-DRB1*0701 15 - mers

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
1	FFFFFLLIFFDLFLV	18

HLA-DRB1*1101 15 - mers

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
1	FFFFFLLIFFDLFLV	17

HLA-DRB1*1501 (DR2b) 15 - mers

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
1	FFFFFLLIFFDLFLV	18

HLA-DRB1*0101 15 - mers

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
1	FFFFLIFFDLFLVFF	21

HLA-DRB1*0301 (DR17) 15 - mers

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
1	FFFFLIFFDLFLVFF	19

HLA-DRB1*0401 (DR4Dw4) 15 - mers

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
1	FFFFLIFFDLFLVFF	22

HLA-DRB1*0701 15 - mers

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
1	FFFFLIFFDLFLVFF	24

HLA-DRB1*1101 15 - mers

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
1	FFFFLIFFDLFLVFF	10

HLA-DRB1*1501 (DR2b) 15 - mers

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
1	FFFFLIFFDLFLVFF	28

SYFPYTHEI HLA-DR prediction FLIFFDLFLVFFFFF

HLA-DRB1*0101 15 - mers

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
1	F L I F F D L F L V F F F F F	26

HLA-DRB1*0301 (DR17) 15 - mers

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
1	F L I F F D L F L V F F F F F	17

HLA-DRB1*0401 (DR4Dw4) 15 - mers

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
1	F L I F F D L F L V F F F F F	16

HLA-DRB1*0701 15 - mers

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
1	F L I F F D L F L V F F F F F	16

HLA-DRB1*1101 15 - mers

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
1	F L I F F D L F L V F F F F F	17

HLA-DRB1*1501 (DR2b) 15 - mers

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
1	F L I F F D L F L V F F F F F	14

SYFPYTHEI HLA-A2 prediction FLIFFDLFL

HLA-A*02:01 nonamers

Pos	1 2 3 4 5 6 7 8 9	score
1	F L I F F D L F L	24

SYFPYTHEI HLA-DR prediction FFFFFGILGFVFTL

HLA-DRB1*0101 15 - mers

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
1	F F F F F G I L G F V F T L	19

HLA-DRB1*0301 (DR17) 15 - mers

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
1	F F F F F G I L G F V F T L	8

HLA-DRB1*0401 (DR4Dw4) 15 - mers

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
1	F F F F F G I L G F V F T L	16

HLA-DRB1*0701 15 - mers

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
1	F F F F F G I L G F V F T L	18

HLA-DRB1*1101 15 - mers

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
1	F F F F F G I L G F V F T L	11

HLA-DRB1*1501 (DR2b) 15 - mers

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
1	F F F F F G I L G F V F T L	12

SYFPYTHEI HLA-DR prediction FFF GILGFVFTLFFF

HLA-DRB1*0101 15 - mers

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
1	F F F G I L G F V F T L F F F	9

HLA-DRB1*0301 (DR17) 15 - mers

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
1	F F F G I L G F V F T L F F F	10

HLA-DRB1*0401 (DR4Dw4) 15 - mers

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
1	F F F G I L G F V F T L F F F	6

HLA-DRB1*0701 15 - mers

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
1	F F F G I L G F V F T L F F F	8

HLA-DRB1*1101 15 - mers

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
1	F F F G I L G F V F T L F F F	0

HLA-DRB1*1501 (DR2b) 15 - mers

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
1	F F F G I L G F V F T L F F F	10

SYFPYTHEI HLA-DR prediction GILGFVFTLFFFFFFF

HLA-DRB1*0101 15 - mers

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
1	G I L G F V F T L F F F F F F	8

HLA-DRB1*0301 (DR17) 15 - mers

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
1	G I L G F V F T L F F F F F F	8

HLA-DRB1*0401 (DR4Dw4) 15 - mers

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
1	G I L G F V F T L F F F F F F	6

HLA-DRB1*0701 15 - mers

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
1	G I L G F V F T L F F F F F F	6

HLA-DRB1*1101 15 - mers

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
1	G I L G F V F T L F F F F F F	2

HLA-DRB1*1501 (DR2b) 15 - mers

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
1	G I L G F V F T L F F F F F F	16

SYFPYTHEI HLA-A2 prediction GILGFVFTL

HLA-A*02:01 nonamers

Pos	1 2 3 4 5 6 7 8 9	score
1	G I L G F V <u>F</u> T L	30

9 References

1. Lee AH, Symington LS, Fidock DA: **DNA repair mechanisms and their biological roles in the malaria parasite *Plasmodium falciparum***. *Microbiology and Molecular Biology Reviews* 2014, **78**(3):469-486.
2. Marsh K, Kinyanjui S: **Immune effector mechanisms in malaria**. *Parasite immunology* 2006, **28**(1-2):51-60.
3. Langhorne J, Ndungu FM, Sponaas A-M, Marsh K: **Immunity to malaria: more questions than answers**. *Nature immunology* 2008, **9**(7):725-732.
4. FRS P: **The life-cycle of primate malaria parasites**. *British Medical Bulletin* 1982, **38**(2):117-122.
5. Riley EM, Stewart VA: **Immune mechanisms in malaria: new insights in vaccine development**. *Nature medicine* 2013, **19**(2):168-178.
6. Killick-Kendrick R: **Rodent malaria**: Elsevier; 2012.
7. Carter R, Diggs CL: ***Plasmodia* of rodents**. *Parasitic protozoa* 1977, **3**:359-451.
8. WHO: **World Malaria Report 2016**. 2016.
9. Jones CO, Williams HA: **The social burden of malaria: what are we measuring?** *The American Journal of Tropical Medicine and Hygiene* 2004, **71**(2 suppl):156-161.
10. Tusting LS, Willey B, Lucas H, Thompson J, Kafy HT, Smith R, Lindsay SW: **Socioeconomic development as an intervention against malaria: a systematic review and meta-analysis**. *The Lancet* 2013, **382**(9896):963-972.
11. Sicuri E, Vieta A, Lindner L, Constenla D, Sauboin C: **The economic costs of malaria in children in three sub-Saharan countries: Ghana, Tanzania and Kenya**. *Malaria journal* 2013, **12**(1):307.
12. Teklehaimanot A, McCord GC, Sachs JD: **Scaling up malaria control in Africa: an economic and epidemiological assessment**. *The American journal of tropical medicine and hygiene* 2007, **77**(6 Suppl):138-144.
13. Sachs J, Malaney P: **The economic and social burden of malaria**. *Nature* 2002, **415**(6872):680-685.
14. Gelband H, Panosian C, Arrow KJ: **Saving lives, buying time: economics of malaria drugs in an age of resistance**: National Academies Press; 2004.
15. Malaria RB: **Economic costs of malaria**. *World Health Organization Available from: URL: http://www.rbm.who.int/cmc_upload/0/000/015/363/RBMInfosheet_10.htm* 2011.
16. Battle KE, Gething PW, Elyazar IR, Moyes CL, Sinka ME, Howes RE, Guerra CA, Price RN, Baird JK, Hay SI: **The global public health significance of *Plasmodium vivax***. 2012.
17. Rodriguez-Morales AJ, Bolívar-Mejía A, Alarcón-Olave C, Calvo-Betancourt LS: ***Plasmodium vivax* malaria in Latin America**. In: *Neglected Tropical Diseases-Latin America and the Caribbean*. Springer; 2015: 89-111.
18. Miller LH, Mason SJ, Dvorak JA, McGinniss MH, Rothman IK: **Erythrocyte receptors for (*Plasmodium knowlesi*) malaria: Duffy blood group determinants**. *Science* 1975, **189**(4202):561-563.
19. Miller LH, Aikawa M, Johnson JG, Shiroishi T: **Interaction between cytochalasin B-treated malarial parasites and erythrocytes. Attachment and junction formation**. *Journal of Experimental Medicine* 1979, **149**(1):172-184.
20. Barnwell JW, Nichols ME, Rubinstein P: **In vitro evaluation of the role of the Duffy blood group in erythrocyte invasion by *Plasmodium vivax***. *J Exp Med* 1989, **169**(5):1795-1802.
21. Harbach R: **Genus *Anopheles* Meigen, 1818**. *Mosquito Taxonomic Inventory Book Genus ANOPHELES Meigen, 1818 Mosquito Taxonomic Inventory City* 2011.
22. Hay SI, Sinka ME, Okara RM, Kabaria CW, Mbithi PM, Tago CC, Benz D, Gething PW, Howes RE, Patil AP: **Developing global maps of the dominant *Anopheles* vectors of human malaria**. *PLoS Med* 2010, **7**(2):e1000209.
23. Stark KR, James AA: **Salivary gland anticoagulants in culicine and anopheline mosquitoes (Diptera: Culicidae)**. *Journal of medical entomology* 1996, **33**(4):645-650.
24. Kerkut G: **Regulation: Digestion, Nutrition, Excretion**, vol. 4: Elsevier; 2013.
25. Rosenberg R, Wirtz RA, Schneider I, Burge R: **An estimation of the number of malaria sporozoites ejected by a feeding mosquito**. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 1990, **84**(2):209-212.
26. BEIER JC, ONYANGO FK, KOROS JK, RAMADHAN M, OGWANG R, WIRTZ RA, KOECH DK, ROBERTS CR: **Quantitation of malaria sporozoites transmitted in vitro during salivation by wild Afrotropical *Anopheles***. *Medical and veterinary entomology* 1991, **5**(1):71-79.
27. Yamauchi LM, Coppi A, Snounou G, Sinnis P: ***Plasmodium* sporozoites trickle out of the injection site**. *Cellular microbiology* 2007, **9**(5):1215-1222.

28. Amino R, Thiberge S, Martin B, Celli S, Shorte S, Frischknecht F, Ménard R: **Quantitative imaging of *Plasmodium* transmission from mosquito to mammal.** *Nature medicine* 2006, **12**(2):220-224.
29. Chakravarty S, Baldeviano GC, Overstreet MG, Zavala F: **Effector CD8+ T lymphocytes against liver stages of *Plasmodium yoelii* do not require gamma interferon for antiparasite activity.** *Infection and immunity* 2008, **76**(8):3628-3631.
30. Duffy PE, Sahu T, Akue A, Milman N, Anderson C: **Pre-erythrocytic malaria vaccines: identifying the targets.** *Expert review of vaccines* 2012, **11**(10):1261-1280.
31. Frevort U: **Malaria sporozoite-hepatocyte interactions.** *Experimental parasitology* 1994, **79**(2):206-210.
32. Coppi A, Tewari R, Bishop JR, Bennett BL, Lawrence R, Esko JD, Billker O, Sinnis P: **Heparan sulfate proteoglycans provide a signal to *Plasmodium* sporozoites to stop migrating and productively invade host cells.** *Cell host & microbe* 2007, **2**(5):316-327.
33. Sihden R, Smith J: **The role of the Kupffer cell in the infection of rodents by sporozoites of *Plasmodium*: uptake of sporozoites by perfused liver and the establishment of infection in vivo.** *Acta tropica* 1982, **39**(1):11-27.
34. Baer K, Roosevelt M, Clarkson AB, Van Rooijen N, Schnieder T, Frevort U: **Kupffer cells are obligatory for *Plasmodium yoelii* sporozoite infection of the liver.** *Cellular microbiology* 2007, **9**(2):397-412.
35. Frevort U, Usynin I, Baer K, Klotz C: **Nomadic or sessile: can Kupffer cells function as portals for malaria sporozoites to the liver?** *Cellular microbiology* 2006, **8**(10):1537-1546.
36. Mota MM, Pradel G, Vanderberg JP, Hafalla JC, Frevort U, Nussenzweig RS, Nussenzweig V, Rodríguez A: **Migration of *Plasmodium* sporozoites through cells before infection.** *Science* 2001, **291**(5501):141-144.
37. Ishino T, Yano K, Chinzei Y, Yuda M: **Cell-passage activity is required for the malarial parasite to cross the liver sinusoidal cell layer.** *PLoS Biol* 2004, **2**(1):e4.
38. Mota MM, Rodriguez A: **Invasion of mammalian host cells by *Plasmodium* sporozoites.** *Bioessays* 2002, **24**(2):149-156.
39. Lindner SE, Miller JL, Kappe SH: **Malaria parasite pre-erythrocytic infection: preparation meets opportunity.** *Cellular microbiology* 2012, **14**(3):316-324.
40. Müller H, Reckmann I, Hollingdale MR, Bujard H, Robson K, Crisanti A: **Thrombospondin related anonymous protein (TRAP) of *Plasmodium falciparum* binds specifically to sulfated glycoconjugates and to HepG2 hepatoma cells suggesting a role for this molecule in sporozoite invasion of hepatocytes.** *The EMBO journal* 1993, **12**(7):2881.
41. Sultan AA, Thathy V, Frevort U, Robson KJ, Crisanti A, Nussenzweig V, Nussenzweig RS, Ménard R: **TRAP is necessary for gliding motility and infectivity of *Plasmodium* sporozoites.** *Cell* 1997, **90**(3):511-522.
42. Morahan BJ, Wang L, Coppel RL: **No TRAP, no invasion.** *Trends in parasitology* 2009, **25**(2):77-84.
43. Rogers WO, Malik A, Mellouk S, Nakamura K, Rogers MD, Szarfman A, Gordon DM, Nussler AK, Aikawa M, Hoffman SL: **Characterization of *Plasmodium falciparum* sporozoite surface protein 2.** *Proceedings of the National Academy of Sciences* 1992, **89**(19):9176-9180.
44. Cowman AF, Crabb BS: **Invasion of red blood cells by malaria parasites.** *Cell* 2006, **124**(4):755-766.
45. Reilly HB, Wang H, Steuter JA, Marx AM, Ferdig MT: **Quantitative dissection of clone-specific growth rates in cultured malaria parasites.** *International journal for parasitology* 2007, **37**(14):1599-1607.
46. Day K, Hayward R, Dyer M: **The biology of *Plasmodium falciparum* transmission stages.** *Parasitology* 1998, **116**(S1):S95-S109.
47. Hafalla JC, Silvie O, Matuschewski K: **Cell biology and immunology of malaria.** *Immunological reviews* 2011, **240**(1):297-316.
48. Naing C, Whittaker MA, Wai VN, Mak JW: **Is *Plasmodium vivax* malaria a severe malaria?: a systematic review and meta-analysis.** *PLoS Negl Trop Dis* 2014, **8**(8):e3071.
49. Looareesuwan S, Ho M, Wattanagoon Y, White NJ, Warrell DA, Bunnag D, Harinasuta T, Wyler DJ: **Dynamic alteration in splenic function during acute falciparum malaria.** *New England Journal of Medicine* 1987, **317**(11):675-679.
50. Haldar K, Mohandas N: **Malaria, erythrocytic infection, and anemia.** *ASH Education Program Book* 2009, **2009**(1):87-93.
51. Clark IA, Cowden WB: **The pathophysiology of falciparum malaria.** *Pharmacology & therapeutics* 2003, **99**(2):221-260.
52. Ayimba E, Hegewald J, Segbena A, Gantin R, Lechner C, Agossou A, Banla M, Soboslay P: **Proinflammatory and regulatory cytokines and chemokines in infants with uncomplicated and severe *Plasmodium falciparum* malaria.** *Clinical & Experimental Immunology* 2011, **166**(2):218-226.

53. Awandare GA, Goka B, Boeuf P, Tetteh JK, Kurtzhals JA, Behr C, Akanmori BD: **Increased levels of inflammatory mediators in children with severe *Plasmodium falciparum* malaria with respiratory distress.** *Journal of Infectious Diseases* 2006, **194**(10):1438-1446.
54. Angulo I, Fresno M: **Cytokines in the pathogenesis of and protection against malaria.** *Clinical and diagnostic laboratory immunology* 2002, **9**(6):1145-1152.
55. Martiney JA, Sherry B, Metz CN, Espinoza M, Ferrer AS, Calandra T, Broxmeyer HE, Bucala R: **Macrophage migration inhibitory factor release by macrophages after ingestion of *Plasmodium chabaudi*-infected erythrocytes: possible role in the pathogenesis of malarial anemia.** *Infection and Immunity* 2000, **68**(4):2259-2267.
56. Ochola LB, Siddondo BR, Ocholla H, Nkya S, Kimani EN, Williams TN, Makale JO, Liljander A, Urban BC, Bull PC: **Specific receptor usage in *Plasmodium falciparum* cytoadherence is associated with disease outcome.** *PLoS one* 2011, **6**(3):e14741.
57. Mackintosh CL, Beeson JG, Marsh K: **Clinical features and pathogenesis of severe malaria.** *Trends in parasitology* 2004, **20**(12):597-603.
58. White NJ, Pukrittayakamee S, Hien TT, Faiz MA, Mokuolu OA, Dondorp AM: **Malaria.** *The Lancet* 2014, **383**(9918):723-735.
59. Newbold C, Craig A, Kyes S, Berendt A, Snow R, Peshu N, Marsh K: **PfEMP1, polymorphism and pathogenesis.** *Annals of Tropical Medicine & Parasitology* 1997, **91**(5):551-557.
60. Pereira MA, Clausen TM, Pehrson C, Mao Y, Resende M, Daugaard M, Kristensen AR, Spliid C, Mathiesen L, Knudsen LE: **Placental Sequestration of *Plasmodium falciparum* Malaria Parasites Is Mediated by the Interaction Between VAR2CSA and Chondroitin Sulfate A on Syndecan-1.** *PLoS Pathog* 2016, **12**(8):e1005831.
61. Pain A, Ferguson DJ, Kai O, Urban BC, Lowe B, Marsh K, Roberts DJ: **Platelet-mediated clumping of *Plasmodium falciparum*-infected erythrocytes is a common adhesive phenotype and is associated with severe malaria.** *Proceedings of the National Academy of Sciences* 2001, **98**(4):1805-1810.
62. Doumbo OK, Thera MA, Koné AK, Raza A, Tempest LJ, Lyke KE, Plowe CV, Rowe JA: **High levels of *Plasmodium falciparum* rosetting in all clinical forms of severe malaria in African children.** *The American journal of tropical medicine and hygiene* 2009, **81**(6):987-993.
63. Grau GER, Craig AG: **Cerebral malaria pathogenesis: revisiting parasite and host contributions.** *Future microbiology* 2012, **7**(2):291-302.
64. Moxon CA, Grau GE, Craig AG: **Malaria: modification of the red blood cell and consequences in the human host.** *British journal of haematology* 2011, **154**(6):670-679.
65. Roberts L, Enserink M: **Did they really say... eradication?** *Science* 2007, **318**(5856):1544-1545.
66. Greenwood B: **Anti-malarial drugs and the prevention of malaria in the population of malaria endemic areas.** *Malaria Journal* 2010, **9**(3):S2.
67. O'Meara WP, Mangeni JN, Steketee R, Greenwood B: **Changes in the burden of malaria in sub-Saharan Africa.** *The Lancet infectious diseases* 2010, **10**(8):545-555.
68. Ceesay SJ, Casals-Pascual C, Erskine J, Anya SE, Duah NO, Fulford AJ, Sesay SS, Abubakar I, Dunyo S, Sey O: **Changes in malaria indices between 1999 and 2007 in The Gambia: a retrospective analysis.** *The Lancet* 2008, **372**(9649):1545-1554.
69. O'Meara WP, Bejon P, Mwangi TW, Okiro EA, Peshu N, Snow RW, Newton CR, Marsh K: **Effect of a fall in malaria transmission on morbidity and mortality in Kilifi, Kenya.** *The lancet* 2008, **372**(9649):1555-1562.
70. Cairns M, Carneiro I, Milligan P, Owusu-Agyei S, Awine T, Gosling R, Greenwood B, Chandramohan D: **Duration of protection against malaria and anaemia provided by intermittent preventive treatment in infants in Navrongo, Ghana.** *PLoS One* 2008, **3**(5):e2227.
71. Mwesigwa J, Okebe J, Affara M, Di Tanna GL, Nwakanma D, Janha O, Opondo K, Grietens KP, Achan J, D'Alessandro U: **On-going malaria transmission in The Gambia despite high coverage of control interventions: a nationwide cross-sectional survey.** *Malaria journal* 2015, **14**(1):314.
72. Cairns ME, Walker PG, Okell LC, Griffin JT, Garske T, Asante KP, Owusu-Agyei S, Diallo D, Dicko A, Cisse B: **Seasonality in malaria transmission: implications for case-management with long-acting artemisinin combination therapy in sub-Saharan Africa.** *Malaria journal* 2015, **14**(1):321.
73. Bhattarai A, Ali AS, Kachur SP, Mårtensson A, Abbas AK, Khatib R, Al-Mafazy A-w, Ramsan M, Rotllant G, Gerstenmaier JF: **Impact of artemisinin-based combination therapy and insecticide-treated nets on malaria burden in Zanzibar.** *PLoS Med* 2007, **4**(11):e309.
74. Steketee RW, Sipilanyambe N, Chimumbwa J, Banda JJ, Mohamed A, Miller J, Basu S, Miti SK, Campbell CC: **National malaria control and scaling up for impact: the Zambia experience through 2006.** *The American journal of tropical medicine and hygiene* 2008, **79**(1):45-52.

75. Chizema-Kawesha E, Miller JM, Steketee RW, Mukonka VM, Mukuka C, Mohamed AD, Miti SK, Campbell CC: **Scaling up malaria control in Zambia: progress and impact 2005–2008.** *The American journal of tropical medicine and hygiene* 2010, **83**(3):480-488.
76. Otten M, Aregawi M, Were W, Karema C, Medin A, Bekele W, Jima D, Gausi K, Komatsu R, Korenromp E: **Initial evidence of reduction of malaria cases and deaths in Rwanda and Ethiopia due to rapid scale-up of malaria prevention and treatment.** *Malaria journal* 2009, **8**(1):14.
77. van Schalkwyk DA: **History of Antimalarial Agents.** *eLS*.
78. Baird JK, Hoffman SL: **Primaquine therapy for malaria.** *Clinical infectious diseases* 2004, **39**(9):1336-1345.
79. Llanos-Cuentas A, Lacerda MV, Rueangweerayut R, Krudsood S, Gupta SK, Kochar SK, Arthur P, Chuenchom N, Möhrle JJ, Duparc S: **Tafenoquine plus chloroquine for the treatment and relapse prevention of *Plasmodium vivax* malaria (DETECTIVE): a multicentre, double-blind, randomised, phase 2b dose-selection study.** *The Lancet* 2014, **383**(9922):1049-1058.
80. Payne D: **Spread of chloroquine resistance in *Plasmodium falciparum*.** *Parasitology today* 1987, **3**(8):241-246.
81. Peters W: **Resistance in human malaria IV: 4-aminoquinolines and multiple resistance.** *Chemotherapy and drug resistance in malaria* 1987, **2**:659-786.
82. Roper C, Pearce R, Nair S, Sharp B, Nosten F, Anderson T: **Intercontinental spread of pyrimethamine-resistant malaria.** *Science* 2004, **305**(5687):1124-1124.
83. Price RN, Uhlemann A-C, Brockman A, McGready R, Ashley E, Phaipun L, Patel R, Laing K, Looareesuwan S, White NJ: **Mefloquine resistance in *Plasmodium falciparum* and increased pfmdr1 gene copy number.** *The Lancet* 2004, **364**(9432):438-447.
84. Organization WH: **Guidelines for the treatment of malaria:** World Health Organization; 2006.
85. Alker AP, Lim P, Sem R, Shah NK, Yi P, Bouth DM, Tsuyuoka R, Maguire JD, Fandeur T, Arieu F: **Pfmdr1 and in vivo resistance to artesunate-mefloquine in falciparum malaria on the Cambodian–Thai border.** *The American journal of tropical medicine and hygiene* 2007, **76**(4):641-647.
86. Denis MB, Tsuyuoka R, Poravuth Y, Narann TS, Seila S, Incardona S, Lim P, Sem R, Socheat D, Christophel EM: **Surveillance of the efficacy of artesunate and mefloquine combination for the treatment of uncomplicated falciparum malaria in Cambodia.** *Tropical Medicine & International Health* 2006, **11**(9):1360-1366.
87. Noedl H, Se Y, Schaecher K, Smith BL, Socheat D, Fukuda MM: **Evidence of artemisinin-resistant malaria in western Cambodia.** *New England Journal of Medicine* 2008, **359**(24):2619-2620.
88. Brown A, Pal R: **Insecticide Resistance in Arthropods World Health Organization.** In.: Geneva; 1958.
89. Duchet C, Allan R, Carnevale P: ***Anopheles* mosquitoes: new insights into malaria vectors.** 2013.
90. Hemingway J, Ranson H: **Insecticide resistance in insect vectors of human disease.** *Annual review of entomology* 2000, **45**(1):371-391.
91. Ryciak R, Vujoen J: **DDT, dieldrin and pyrethroid Insecticide resistance In African malaria vector mosquitoes: an historical review and implications for future malaria control in southern Africa.** *South African Journal of Science* 1999, **95**:215.
92. Etang J, Manga L, Chandre F, Guillet P, Fondjo E, Mimpfoundi R, Toto J-c, Fontenille D: **Insecticide susceptibility status of *Anopheles gambiae* sl (Diptera: Culicidae) in the Republic of Cameroon.** *Journal of Medical Entomology* 2003, **40**(4):491-497.
93. Muregi FW, Ishih A: **Next-generation antimalarial drugs: hybrid molecules as a new strategy in drug design.** *Drug development research* 2010, **71**(1):20-32.
94. Wani WA, Jameel E, Baig U, Mumtazuddin S, Hun LT: **Ferroquine and its derivatives: new generation of antimalarial agents.** *European journal of medicinal chemistry* 2015, **101**:534-551.
95. Flannery EL, Chatterjee AK, Winzeler EA: **Antimalarial drug discovery [mdash] approaches and progress towards new medicines.** *Nature Reviews Microbiology* 2013, **11**(12):849-862.
96. Plowe CV, Alonso P, Hoffman SL: **The potential role of vaccines in the elimination of falciparum malaria and the eventual eradication of malaria.** *Journal of Infectious Diseases* 2009, **200**(11):1646-1649.
97. Henderson DA: **Principles and lessons from the smallpox eradication programme.** *Bulletin of the World Health Organization* 1987, **65**(4):535.
98. John J: **Role of injectable and oral polio vaccines in polio eradication.** 2009.
99. WHO: **Malaria vaccine technology roadmap** 2013.
100. Liehl P, Mota MM: **Innate recognition of malarial parasites by mammalian hosts.** *International journal for parasitology* 2012, **42**(6):557-566.

101. Liehl P, Zuzarte-Luís V, Chan J, Zillinger T, Baptista F, Carapau D, Konert M, Hanson KK, Carret C, Lassnig C: **Host-cell sensors for *Plasmodium* activate innate immunity against liver-stage infection.** *Nature medicine* 2014, **20**(1):47-53.
102. Miller JL, Sack BK, Baldwin M, Vaughan AM, Kappe SH: **Interferon-mediated innate immune responses against malaria parasite liver stages.** *Cell reports* 2014, **7**(2):436-447.
103. Nussler AK, Rénia L, Pasquetto V, Miltgen F, Matile H, Mazier D: **In vivo induction of the nitric oxide pathway in hepatocytes after injection with irradiated malaria sporozoites, malaria blood parasites or adjuvants.** *European journal of immunology* 1993, **23**(4):882-887.
104. Seguin MC, Klotz FW, Schneider I, Weir JP, Goodbary M, Slayter M, Raney JJ, Aniagolu JU, Green SJ: **Induction of nitric oxide synthase protects against malaria in mice exposed to irradiated *Plasmodium berghei* infected mosquitoes: involvement of interferon gamma and CD8+ T cells.** *Journal of Experimental Medicine* 1994, **180**(1):353-358.
105. Torgler R, Bongfen SE, Romero JC, Tardivel A, Thome M, Corradin G: **Sporozoite-mediated hepatocyte wounding limits *Plasmodium* parasite development via MyD88-mediated NF-κB activation and inducible NO synthase expression.** *The Journal of Immunology* 2008, **180**(6):3990-3999.
106. Singh AP, Buscaglia CA, Wang Q, Levay A, Nussenzweig DR, Walker JR, Winzeler EA, Fujii H, Fontoura BM, Nussenzweig V: ***Plasmodium* circumsporozoite protein promotes the development of the liver stages of the parasite.** *Cell* 2007, **131**(3):492-504.
107. Usynin I, Klotz C, Frevert U: **Malaria circumsporozoite protein inhibits the respiratory burst in Kupffer cells.** *Cellular microbiology* 2007, **9**(11):2610-2628.
108. Miller LH, Baruch DI, Marsh K, Doumbo OK: **The pathogenic basis of malaria.** *Nature* 2002, **415**(6872):673-679.
109. Walther M, Woodruff J, Edele F, Jeffries D, Tongren JE, King E, Andrews L, Bejon P, Gilbert SC, De Souza JB: **Innate immune responses to human malaria: heterogeneous cytokine responses to blood-stage *Plasmodium falciparum* correlate with parasitological and clinical outcomes.** *The Journal of Immunology* 2006, **177**(8):5736-5745.
110. Phanuphak P, Hanvanich M, Sakulramrung R, Moollaor P, Sitprija V, Phanthumkosol D: **Complement changes in falciparum malaria infection.** *Clinical and experimental immunology* 1985, **59**(3):571.
111. Ade-Serrano M, Ejezie G, Kassim O: **Correlation of *Plasmodium falciparum* gametocytemia with complement component titers in rural Nigerian school children.** *Journal of clinical microbiology* 1981, **13**(1):195-198.
112. Dulaney A: **Complement activity of human serum with especial reference to malaria.** In: *Federation proceedings: 1947; 1947:* 425-425.
113. Parroche P, Lauw FN, Goutagny N, Latz E, Monks BG, Visintin A, Halmen KA, Lamphier M, Olivier M, Bartholomeu DC: **Malaria hemozoin is immunologically inert but radically enhances innate responses by presenting malaria DNA to Toll-like receptor 9.** *Proceedings of the National Academy of Sciences* 2007, **104**(6):1919-1924.
114. Gazzinelli RT, Denkers EY: **Protozoan encounters with Toll-like receptor signalling pathways: implications for host parasitism.** *Nature Reviews Immunology* 2006, **6**(12):895-906.
115. Coban C, Ishii KJ, Kawai T, Hemmi H, Sato S, Uematsu S, Yamamoto M, Takeuchi O, Itagaki S, Kumar N: **Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin.** *Journal of Experimental Medicine* 2005, **201**(1):19-25.
116. Krishnegowda G, Hajjar AM, Zhu J, Douglass EJ, Uematsu S, Akira S, Woods AS, Gowda DC: **Induction of proinflammatory responses in macrophages by the glycosylphosphatidylinositols of *Plasmodium falciparum* cell signaling receptors, glycosylphosphatidylinositol (GPI) structural requirement, and regulation of GPI activity.** *Journal of Biological Chemistry* 2005, **280**(9):8606-8616.
117. Gowda DC: **TLR-mediated cell signaling by malaria GPIs.** *Trends in parasitology* 2007, **23**(12):596-604.
118. Dostert C, Guarda G, Romero JF, Menu P, Gross O, Tardivel A, Suva M-L, Stehle J-C, Kopf M, Stamenkovic I: **Malarial hemozoin is a Nalp3 inflammasome activating danger signal.** *PloS one* 2009, **4**(8):e6510.
119. Nagel R: **Innate resistance to malaria: the intraerythrocytic cycle.** *Blood cells* 1989, **16**(2-3):321-339; discussion 340-329.
120. Hedrick PW: **Resistance to malaria in humans: the impact of strong, recent selection.** *Malaria journal* 2012, **11**(1):349.
121. Kwiatkowski DP: **How malaria has affected the human genome and what human genetics can teach us about malaria.** *The American Journal of Human Genetics* 2005, **77**(2):171-192.
122. Travers P, Walport MJ, Janeway C, Murphy KP: **Janeway's immunobiology:** Garland Science; 2008.

123. Kambayashi T, Laufer TM: **Atypical MHC class II-expressing antigen-presenting cells: can anything replace a dendritic cell?** *Nature Reviews Immunology* 2014, **14**(11):719-730.
124. Zhu J, Paul WE: **Peripheral CD4+ T-cell differentiation regulated by networks of cytokines and transcription factors.** *Immunological reviews* 2010, **238**(1):247-262.
125. Abbas AK, Murphy KM, Sher A: **Functional diversity of helper T lymphocytes.** *Nature* 1996, **383**(6603):787.
126. Murphy JR, Baqar S, Davis JR, Herrington DA, Clyde DF: **Evidence for a 6.5-day minimum exoerythrocytic cycle for *Plasmodium falciparum* in humans and confirmation that immunization with a synthetic peptide representative of a region of the circumsporozoite protein retards infection.** *Journal of Clinical Microbiology* 1989, **27**(7):1434-1437.
127. Neefjes J, Jongsma ML, Paul P, Bakke O: **Towards a systems understanding of MHC class I and MHC class II antigen presentation.** *Nature Reviews Immunology* 2011, **11**(12):823-836.
128. Belz GT, Carbone FR, Heath WR: **Cross-presentation of antigens by dendritic cells.** *Critical Reviews™ in Immunology* 2002, **22**(5-6).
129. Winau F, Hegasy G, Weiskirchen R, Weber S, Cassan C, Sieling PA, Modlin RL, Liblau RS, Gressner AM, Kaufmann SH: **Ito cells are liver-resident antigen-presenting cells for activating T cell responses.** *Immunity* 2007, **26**(1):117-129.
130. Knolle PA, Gerken G: **Local control of the immune response in the liver.** *Immunological reviews* 2000, **174**(1):21-34.
131. Jenne CN, Kubes P: **Immune surveillance by the liver.** *Nature immunology* 2013, **14**(10):996-1006.
132. Herkel J, Jagemann B, Wiegand C, Lazaro JFG, Lueth S, Kanzler S, Blessing M, Schmitt E, Lohse AW: **MHC class II-expressing hepatocytes function as antigen-presenting cells and activate specific CD4 T lymphocytes.** *Hepatology* 2003, **37**(5):1079-1085.
133. Renia L, Marussig MS, Grillot D, Pied S, Corradin G, Miltgen F, Del Giudice G, Mazier D: **In vitro activity of CD4+ and CD8+ T lymphocytes from mice immunized with a synthetic malaria peptide.** *Proceedings of the National Academy of Sciences* 1991, **88**(18):7963-7967.
134. Renia L, Grillot D, Marussig M, Corradin G, Miltgen F, Lambert P, Mazier D, Del Giudice G: **Effector functions of circumsporozoite peptide-primed CD4+ T cell clones against *Plasmodium yoelii* liver stages.** *The Journal of Immunology* 1993, **150**(4):1471-1478.
135. Kurosaki T, Kometani K, Ise W: **Memory B cells.** *Nature Reviews Immunology* 2015, **15**(3):149-159.
136. Lanzavecchia A: **Antigen-specific interaction between T and B cells.** 1985.
137. Struik SS, Riley EM: **Does malaria suffer from lack of memory?** *Immunological reviews* 2004, **201**(1):268-290.
138. Carter R, Mendis KN: **Evolutionary and historical aspects of the burden of malaria.** *Clinical microbiology reviews* 2002, **15**(4):564-594.
139. Doolan DL, Dobaño C, Baird JK: **Acquired immunity to malaria.** *Clinical microbiology reviews* 2009, **22**(1):13-36.
140. Beck H, Felger I, Genton B, Alexander N, Al-Yaman F, Anders R, Alpers M: **Humoral and cell-mediated immunity to the *Plasmodium falciparum* ring-infected erythrocyte surface antigen in an adult population exposed to highly endemic malaria.** *Infection and immunity* 1995, **63**(2):596-600.
141. DOOLAN DL, BECK HP, GOOD MF: **Evidence for limited activation of distinct CD4+ T cell subsets in response to the *Plasmodium falciparum* circumsporozoite protein in Papua New Guinea.** *Parasite immunology* 1994, **16**(3):129-136.
142. Zevering Y, Houghten RA, Frazer IH, Good MF: **Major population differences in T cell response to a malaria sporozoite vaccine candidate.** *International immunology* 1990, **2**(10):945-955.
143. Owusu-Agyei S, Koram K, Baird JK, Utz G, Binka FN, Nkrumah F, Fryauff D, Hoffman S: **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* 2001, **65**(3):197-203.
144. Hoffman SL, Oster CN, Plowe CV, Woollett GR, Beier JC, Chulay JD, Wirtz RA, Hollingdale MR, Mugambi M: **Naturally acquired antibodies to sporozoites do not prevent malaria: vaccine development implications.** *Science* 1987, **237**(4815):639-642.
145. Cohen S, McGregor I, Carrington S: **Gamma-globulin and acquired immunity to human malaria.** *Nature* 1961, **192**:733-737.
146. Sabchareon A, Burnouf T, Ouattara D, Attanath P, Bouharoun-Tayoun H, Chantavanich P, Foucault C, Chongsuphajaisiddhi T, Druilhe P: **Parasitologic and clinical human response to immunoglobulin administration in *falciparum* malaria.** *The American journal of tropical medicine and hygiene* 1991, **45**(3):297-308.

147. Wipasa J, Elliott S, Xu H, Good MF: **Immunity to asexual blood stage malaria and vaccine approaches.** *Immunology and cell biology* 2002, **80**(5):401-414.
148. Cohen S, Butcher G, Crandall R: **Action of malarial antibody in vitro.** *Nature* 1969, **223**:368-371.
149. 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.** *Journal of Experimental Medicine* 1990, **172**(6):1633-1641.
150. Osier FH, Fegan G, Polley SD, Murungi L, Verra F, Tetteh KK, Lowe B, Mwangi T, Bull PC, Thomas AW: **Breadth and magnitude of antibody responses to multiple *Plasmodium falciparum* merozoite antigens are associated with protection from clinical malaria.** *Infection and immunity* 2008, **76**(5):2240-2248.
151. Bull PC, Lowe BS, Kortok M, Molyneux CS, Newbold CI, Marsh K: **Parasite antigens on the infected red cell surface are targets for naturally acquired immunity to malaria.** *Nature medicine* 1998, **4**(3):358.
152. Giha HA, Staalsoe T, Dodo D, Elhassan IM, Roper C, Satti GM, Arnot DE, Theander TG, Hviid L: **Nine-year longitudinal study of antibodies to variant antigens on the surface of *Plasmodium falciparum*-infected erythrocytes.** *Infection and immunity* 1999, **67**(8):4092-4098.
153. Ofori MF, Dodo D, Staalsoe T, Kurtzhals JA, Koram K, Theander TG, Akanmori BD, Hviid L: **Malaria-induced acquisition of antibodies to *Plasmodium falciparum* variant surface antigens.** *Infection and immunity* 2002, **70**(6):2982-2988.
154. Xu H, Wipasa J, Yan H, Zeng M, Makobongo MO, Finkelman FD, Kelso A, Good MF: **The mechanism and significance of deletion of parasite-specific CD4+ T cells in malaria infection.** *Journal of Experimental Medicine* 2002, **195**(7):881-892.
155. Portugal S, Carret C, Recker M, Armitage AE, Gonçalves LA, Epiphany S, Sullivan D, Roy C, Newbold CI, Drakesmith H: **Host-mediated regulation of superinfection in malaria.** *Nature medicine* 2011, **17**(6):732-737.
156. John C, Sumba P, Ouma J, Nahlen B, King C, Kazura J: **Cytokine responses to *Plasmodium falciparum* liver-stage antigen 1 vary in rainy and dry seasons in highland Kenya.** *Infection and immunity* 2000, **68**(9):5198-5204.
157. Migot-Nabias F, Deloron P, Ringwald P, Dubois B, Mayombo J, Minh T, Fievet N, Millet P, Luty A: **Immune response to *Plasmodium falciparum* liver stage antigen-1: geographical variations within Central Africa and their relationship with protection from clinical malaria.** *Transactions of the Royal Society of Tropical Medicine and Hygiene* 2000, **94**(5):557-562.
158. Chelimo K, Sumba PO, Kazura JW, Ofula AV, John CC: **Interferon-gamma responses to *Plasmodium falciparum* liver-stage antigen-1 and merozoite-surface protein-1 increase with age in children in a malaria holoendemic area of western Kenya.** *Malaria journal* 2003, **2**(1):37.
159. Todryk SM, Bejon P, Mwangi T, Plebanski M, Urban B, Marsh K, Hill AV, Flanagan KL: **Correlation of memory T cell responses against TRAP with protection from clinical malaria, and CD4+ CD25 high T Cells with susceptibility in Kenyans.** *PLoS One* 2008, **3**(4):e2027.
160. Flanagan KL, Mwangi T, Plebanski M, Odhiambo K, Ross A, Sheu E, Kortok M, Lowe B, Marsh K, Hill AV: **Ex vivo interferon-gamma immune response to thrombospondin-related adhesive protein in coastal Kenyans: longevity and risk of *Plasmodium falciparum* infection.** *The American journal of tropical medicine and hygiene* 2003, **68**(4):421-430.
161. Reece WH, Pinder M, Gothard PK, Milligan P, Bojang K, Doherty T, Plebanski M, Akinwunmi P, Everaere S, Watkins KR: **A CD4+ T-cell immune response to a conserved epitope in the circumsporozoite protein correlates with protection from natural *Plasmodium falciparum* infection and disease.** *Nature medicine* 2004, **10**(4):406-410.
162. Pombo DJ, Lawrence G, Hirunpetcharat C, Rzepczyk C, Bryden M, Cloonan N, Anderson K, Mahakunkijcharoen Y, Martin LB, Wilson D: **Immunity to malaria after administration of ultra-low doses of red cells infected with *Plasmodium falciparum*.** *The Lancet* 2002, **360**(9333):610-617.
163. Riley E, Allen S, Wheeler J, Blackman M, Bennett S, Takacs B, SCHONFELD HJ, Holder A, Greenwood B: **Naturally acquired cellular and humoral immune responses to the major merozoite surface antigen (Pf MSP1) of *Plasmodium falciparum* are associated with reduced malaria morbidity.** *Parasite immunology* 1992, **14**(3):321-337.
164. Troye-Blomberg M: **Human T-cell responses to blood stage antigens in *Plasmodium falciparum* malaria.** *Immunology letters* 1994, **41**(2):103-107.
165. Walker KM, Okitsu S, Porter DW, Duncan C, Amacker M, Pluschke G, Cavanagh DR, Hill AV, Todryk SM: **Antibody and T-cell responses associated with experimental human malaria infection or vaccination show limited relationships.** *Immunology* 2015, **145**(1):71-81.

166. Good MF, Pombo D, Quakyi IA, Riley EM, Houghten RA, Menon A, Alling DW, Berzofsky JA, Miller LH: **Human T-cell recognition of the circumsporozoite protein of *Plasmodium falciparum*: immunodominant T-cell domains map to the polymorphic regions of the molecule.** *Proceedings of the National Academy of Sciences* 1988, **85**(4):1199-1203.
167. Day KP, Marsh K: **Naturally acquired immunity to *Plasmodium falciparum*.** *Immunology today* 1991, **12**(3):A68-A71.
168. Lockyer MJ, Marsh K, Newbold CI: **Wild isolates of *Plasmodium falciparum* show extensive polymorphism in T cell epitopes of the circumsporozoite protein.** *Molecular and biochemical parasitology* 1989, **37**(2):275-280.
169. Carvalho LH, Fontes C, Krettli AU: **Cellular responses to *Plasmodium falciparum* major surface antigens and their relationship to human activities associated with malaria transmission.** *The American journal of tropical medicine and hygiene* 1999, **60**(4):674-679.
170. Rémy V, Zöllner Y, Heckmann U: **Vaccination: The cornerstone of an efficient healthcare system.** *Journal of market access & health policy* 2015, **3**.
171. Cruz A, Torrado E, Carmona J, Fraga AG, Costa P, Rodrigues F, Appelberg R, Correia-Neves M, Cooper AM, Saraiva M: **BCG vaccination-induced long-lasting control of *Mycobacterium tuberculosis* correlates with the accumulation of a novel population of CD4+ IL-17+ TNF+ IL-2+ T cells.** *Vaccine* 2015, **33**(1):85-91.
172. Plotkin SA: **Correlates of protection induced by vaccination.** *Clinical and Vaccine Immunology* 2010, **17**(7):1055-1065.
173. John J: **Role of injectable and oral polio vaccines in polio eradication.** *Expert review of vaccines* 2009, **8**(1):5-8.
174. Vogelzang A, Perdomo C, Zedler U, Kuhlmann S, Hurwitz R, Gengenbacher M, Kaufmann SH: **Central memory CD4+ T cells are responsible for the recombinant bacillus Calmette-Guerin ΔureC:: hly vaccine's superior protection against tuberculosis.** *Journal of Infectious Diseases* 2014, **210**(12):1928-1937.
175. Koff WC, Burton DR, Johnson PR, Walker BD, King CR, Nabel GJ, Ahmed R, Bhan MK, Plotkin SA: **Accelerating next-generation vaccine development for global disease prevention.** *Science* 2013, **340**(6136):1232910.
176. Zinkernagel RM, Hengartner H: **Protective 'immunity' by pre-existent neutralizing antibody titers and preactivated T cells but not by so-called 'immunological memory'.** *Immunological reviews* 2006, **211**(1):310-319.
177. Russell PF, Mohan B: **The immunization of fowls against mosquito-borne *Plasmodium gallinaceum* by injections of serum and of inactivated homologous sporozoites.** *The Journal of experimental medicine* 1942, **76**(5):477-495.
178. Schofield L, Villalquiran J, Ferreira A, Schellekens H, Nussenzweig R, Nussenzweig V: **γ Interferon, CD8+ T cells and antibodies required for immunity to malaria sporozoites.** *Nature* 1987, **330**(6149):664-666.
179. Ewer KJ, Sierra-Davidson K, Salman AM, Illingworth JJ, Draper SJ, Biswas S, Hill AVS: **Progress with viral vectored malaria vaccines: A multi-stage approach involving "unnatural immunity".** *Vaccine* 2015, **33**(52):7444-7451.
180. Cornelissen A, Schetters TP: **Vaccines against protozoal diseases of veterinary importance.** *FEMS Immunology & Medical Microbiology* 1996, **15**(2-3):61-72.
181. Hollingdale MR, Nardin EH, Tharavani S, Schwartz A, Nussenzweig R: **Inhibition of entry of *Plasmodium falciparum* and *P. vivax* sporozoites into cultured cells; an in vitro assay of protective antibodies.** *The Journal of Immunology* 1984, **132**(2):909-913.
182. Chattopadhyay R, de la Vega P, Paik SH, Murata Y, Ferguson EW, Richie TL, Ooi GT: **Early transcriptional responses of HepG2-A16 liver cells to infection by *Plasmodium falciparum* sporozoites.** *Journal of Biological Chemistry* 2011, **286**(30):26396-26405.
183. Dups JN, Pepper M, Cockburn IA: **Antibody and B cell responses to *Plasmodium sporozoites*.** 2014.
184. Zou X, House BL, Zyzak MD, Richie TL, Gerbasi VR: **Towards an optimized inhibition of liver stage development assay (ILSDA) for *Plasmodium falciparum*.** *Malaria journal* 2013, **12**(1):1.
185. Lindner SE, Swearingen KE, Harupa A, Vaughan AM, Sinnis P, Moritz RL, Kappe SH: **Total and putative surface proteomics of malaria parasite salivary gland sporozoites.** *Molecular & Cellular Proteomics* 2013, **12**(5):1127-1143.
186. Cheng Q, Lawrence G, Reed C, Stowers A, RANFORD—CARTWRIGHT L, Creasey A, Carter R, Saul A: **MEASUREMENT OF PLASMODIUM FALCIPARUM GROWTH RATES IN VIVO: A TEST OF MALARIA VACCINES.** *Am J Trop Med va* 1997, **57**(4):495-500.

187. Rickman LS, Jones TR, Long GW, Paparello S, Schneider I, Paul CF, Beaudoin RL, Hoffman SL: ***Plasmodium falciparum*-infected *Anopheles stephensi* inconsistently transmit malaria to humans.** *American Journal of Tropical Medicine and Hygiene* 1990, **43**(5):441-445.
188. Cockburn IA, Tse S-W, Radtke AJ, Srinivasan P, Chen Y-C, Sinnis P, Zavala F: **Dendritic cells and hepatocytes use distinct pathways to process protective antigen from *Plasmodium* in vivo.** *PLoS Pathog* 2011, **7**(3):e1001318.
189. Bongfen SE, Torgler R, Romero JF, Renia L, Corradin G: ***Plasmodium berghei*-infected primary hepatocytes process and present the circumsporozoite protein to specific CD8+ T cells in vitro.** *The Journal of Immunology* 2007, **178**(11):7054-7063.
190. Silvie O, Franetich J-F, Charrin S, Mueller MS, Siau A, Bodescot M, Rubinstein E, Hannoun L, Charoenvit Y, Kocken CH: **A role for apical membrane antigen 1 during invasion of hepatocytes by *Plasmodium falciparum* sporozoites.** *Journal of Biological Chemistry* 2004, **279**(10):9490-9496.
191. Fidock DA, Gras-Masse H, Lepers J-P, Brahimi K, Benmohamed L, Mellouk S, Guerin-Marchand C, Londono A, Raharimalala L, Meis J: ***Plasmodium falciparum* liver stage antigen-1 is well conserved and contains potent B and T cell determinants.** *The Journal of Immunology* 1994, **153**(1):190-204.
192. García JE, Curtidor H, López R, Rodríguez L, Vera R, Valbuena J, Rosas J, Ocampo M, Puentes A, Forero M: **Liver stage antigen 3 *Plasmodium falciparum* peptides specifically interacting with HepG2 cells.** *Journal of molecular medicine* 2004, **82**(9):600-611.
193. Sá e Cunha C, Nyboer B, Heiss K, Sanches-Vaz M, Fontinha D, Wiedtke E, Grimm D, Przyborski JM, Mota MM, Prudêncio M *et al*: ***Plasmodium berghei* EXP-1 interacts with host Apolipoprotein H during *Plasmodium* liver-stage development.** *Proceedings of the National Academy of Sciences* 2017.
194. RUSSELL PF, Mulligan H: **Active Immunization of Fowls against Sporozoites but not Trophozoites of *Plasmodium gallinaceum* by Injections of Homologous Sporozoites.** *Journal of the Malaria Institute of India* 1942, **4**(3).
195. Nussenzweig R, Vanderberg J, Most H, Orton C: **Specificity of protective immunity produced by x-irradiated *Plasmodium berghei* sporozoites.** 1969.
196. Nussenzweig R, Vanderberg J, Most H, Orton C: **Protective immunity produced by the injection of x-irradiated sporozoites of *Plasmodium berghei*.** 1967.
197. Hoffman SL, Goh LM, Luke TC, Schneider I, Le TP, Doolan DL, Sacchi J, de la Vega P, Dowler M, Paul C: **Protection of humans against malaria by immunization with radiation-attenuated *Plasmodium falciparum* sporozoites.** *Journal of Infectious Diseases* 2002, **185**(8):1155-1164.
198. Clyde DF: **Immunization of man against *falciparum* and *vivax* malaria by use of attenuated sporozoites.** *The American journal of tropical medicine and hygiene* 1975, **24**(3):397-401.
199. Billingsley P, Sim BK, James E, Richie T, Shekalaghe S, Healy S, Sissoko M, Mordmueller B, Ledgerwood J, Graham B: **Progress with PfSPZ Vaccine, a radiation attenuated *Plasmodium falciparum* sporozoite vaccine.** *Malaria Journal* 2014, **13**(Suppl 1):O34.
200. Edelman R, Hoffman SL, Davis JR, Beier M, Szein MB, Losonsky G, Herrington DA, Eddy HA, Hollingdale MR, Gordon DM: **Long-term persistence of sterile immunity in a volunteer immunized with X-irradiated *Plasmodium falciparum* sporozoites.** *Journal of Infectious Diseases* 1993, **168**(4):1066-1070.
201. Mellouk S, Lunel F, Sedegah M, Beaudoin R-L, Druilhe P: **Protection against malaria induced by irradiated sporozoites.** *The Lancet* 1990, **335**(8691):721.
202. Potocnjak P, Yoshida N, Nussenzweig RS, Nussenzweig V: **Monovalent fragments (Fab) of monoclonal antibodies to a sporozoite surface antigen (Pb44) protect mice against malarial infection.** *Journal of Experimental Medicine* 1980, **151**(6):1504-1513.
203. Yoshida N, Nussenzweig RS, Potocnjak P, Nussenzweig V, Aikawa M: **Hybridoma produces protective antibodies directed against the sporozoite stage of malaria parasite.** *Science* 1980, **207**(4426):71-73.
204. Hoffman SL, Isenbarger D, Long GW, Sedegah M, Szarfman A, Waters L, Hollingdale MR, Van der Meide PH, Finbloom DS, Ballou WR: **Sporozoite vaccine induces genetically restricted T cell elimination of malaria from hepatocytes.** *Science* 1989, **244**(4908):1078-1082.
205. Weiss WR, Jiang CG: **Protective CD8+ T lymphocytes in primates immunized with malaria sporozoites.** *PLoS one* 2012, **7**(2):e31247.
206. Rodrigues MM, Cordey A-S, Arreaza G, Corradin G, Romero P, Maryanski JL, Nussenzweig RS, Zavala F: **CD8+ cytolytic T cell clones derived against the *Plasmodium yoelii* circumsporozoite protein protect against malaria.** *International immunology* 1991, **3**(6):579-585.
207. Romero P, Maryanskii JL, Corradini G, Nussenzweig RS, Nussenzweig V, Zavala F: **Cloned cytotoxic T cells recognize an epitope in the circumsporozoite protein and protect against malaria.** *Nature* 1989, **341**:28.

208. Weiss WR, Sedegah M, Beaudoin RL, Miller LH, Good MF: **CD8+ T cells (cytotoxic/suppressors) are required for protection in mice immunized with malaria sporozoites.** *Proceedings of the National Academy of Sciences* 1988, **85**(2):573-576.
209. White KL, Snyder H, Krzych U: **MHC class I-dependent presentation of exoerythrocytic antigens to CD8+ T lymphocytes is required for protective immunity against *Plasmodium berghei*.** *The Journal of Immunology* 1996, **156**(9):3374-3381.
210. Krzych U, Schwenk R: **The dissection of CD8 T cells during liver-stage infection.** In: *Immunology and Immunopathogenesis of Malaria*. Springer; 2005: 1-24.
211. Doolan DL, Hoffman SL: **The complexity of protective immunity against liver-stage malaria.** *The Journal of Immunology* 2000, **165**(3):1453-1462.
212. Hoffman SL, Billingsley PF, James E, Richman A, Loyevsky M, Li T, Chakravarty S, Gunasekera A, Chattopadhyay R, Li M: **Development of a metabolically active, non-replicating sporozoite vaccine to prevent *Plasmodium falciparum* malaria.** *Human Vaccines* 2010, **6**(1):97-106.
213. Akanmori B, Genton B, Brown G: **Progress and Challenges in Development of Whole Organism Malaria Vaccines for Endemic Countries.** 2009.
214. Seder RA, Chang L-J, Enama ME, Zephir KL, Sarwar UN, Gordon IJ, Holman LA, James ER, Billingsley PF, Gunasekera A: **Protection against malaria by intravenous immunization with a nonreplicating sporozoite vaccine.** *Science* 2013, **341**(6152):1359-1365.
215. Epstein JE, Tewari K, Lyke K, Sim B, Billingsley P, Laurens M, Gunasekera A, Chakravarty S, James E, Sedegah M: **Live attenuated malaria vaccine designed to protect through hepatic CD8+ T cell immunity.** *Science* 2011, **334**(6055):475-480.
216. Ishizuka AS, Lyke KE, DeZure A, Berry AA, Richie TL, Mendoza FH, Enama ME, Gordon IJ, Chang L-J, Sarwar UN: **Protection against malaria at 1 year and immune correlates following PfSPZ vaccination.** *Nature medicine* 2016.
217. Roestenberg M, McCall M, Hopman J, Wiersma J, Luty AJ, van Gemert GJ, van de Vegte-Bolmer M, van Schaijk B, Teelen K, Arens T: **Protection against a malaria challenge by sporozoite inoculation.** *New England Journal of Medicine* 2009, **361**(5):468-477.
218. Roestenberg M, Teirlinck AC, McCall MB, Teelen K, Makamdop KN, Wiersma J, Arens T, Beckers P, Van Gemert G, van de Vegte-Bolmer M: **Long-term protection against malaria after experimental sporozoite inoculation: an open-label follow-up study.** *The Lancet* 2011, **377**(9779):1770-1776.
219. Bijker EM, Bastiaens GJ, Teirlinck AC, van Gemert G-J, Graumans W, van de Vegte-Bolmer M, Siebelink-Stoter R, Arens T, Teelen K, Nahrendorf W: **Protection against malaria after immunization by chloroquine prophylaxis and sporozoites is mediated by preerythrocytic immunity.** *Proceedings of the National Academy of Sciences* 2013, **110**(19):7862-7867.
220. Belnoue E, Costa FT, Frankenberg T, Vigário AM, Voza T, Leroy N, Rodrigues MM, Landau I, Snounou G, Rénia L: **Protective T cell immunity against malaria liver stage after vaccination with live sporozoites under chloroquine treatment.** *The Journal of Immunology* 2004, **172**(4):2487-2495.
221. Mueller A-K, Labaied M, Kappe SH, Matuschewski K: **Genetically modified *Plasmodium* parasites as a protective experimental malaria vaccine.** *Nature* 2005, **433**(7022):164-167.
222. Douradinha B, van Dijk M, van Gemert G-J, Khan SM, Janse CJ, Waters AP, Sauerwein RW, Luty AJ, Silva-Santos B, Mota MM: **Immunization with genetically attenuated P52-deficient *Plasmodium berghei* sporozoites induces a long-lasting effector memory CD8+ T cell response in the liver.** *Journal of immune based therapies and vaccines* 2011, **9**(1):6.
223. Mueller A-K, Camargo N, Kaiser K, Andorfer C, Frevert U, Matuschewski K, Kappe SH: ***Plasmodium* liver stage developmental arrest by depletion of a protein at the parasite-host interface.** *Proceedings of the National Academy of Sciences of the United States of America* 2005, **102**(8):3022-3027.
224. Van Dijk MR, Douradinha B, Franke-Fayard B, Heussler V, van Dooren MW, van Schaijk B, van Gemert G-J, Sauerwein RW, Mota MM, Waters AP: **Genetically attenuated, P36p-deficient malarial sporozoites induce protective immunity and apoptosis of infected liver cells.** *Proceedings of the National Academy of Sciences of the United States of America* 2005, **102**(34):12194-12199.
225. Jobe O, Lumsden J, Mueller A-K, Williams J, Silva-Rivera H, Kappe SH, Schwenk RJ, Matuschewski K, Krzych U: **Genetically attenuated *Plasmodium berghei* liver stages induce sterile protracted protection that is mediated by major histocompatibility complex class I-dependent interferon-γ-producing CD8+ T cells.** *Journal of Infectious Diseases* 2007, **196**(4):599-607.
226. Butler NS, Schmidt NW, Vaughan AM, Aly AS, Kappe SH, Harty JT: **Superior antimalarial immunity after vaccination with late liver stage-arresting genetically attenuated parasites.** *Cell host & microbe* 2011, **9**(6):451-462.

227. Spring M, Murphy J, Nielsen R, Dowler M, Bennett JW, Zarling S, Williams J, de la Vega P, Ware L, Komisar J: **First-in-human evaluation of genetically attenuated *Plasmodium falciparum* sporozoites administered by bite of *Anopheles* mosquitoes to adult volunteers.** *Vaccine* 2013, **31**(43):4975-4983.
228. van Schaijk BC, Ploemen IH, Annoura T, Vos MW, Foquet L, van Gemert G-J, Chevalley-Maurel S, van de Vegte-Bolmer M, Sajid M, Franetich J-F: **A genetically attenuated malaria vaccine candidate based on *P. falciparum* b9/slarp gene-deficient sporozoites.** *Elife* 2014, **3**:e03582.
229. Mikolajczak SA, Lakshmanan V, Fishbaugher M, Camargo N, Harupa A, Kaushansky A, Douglass AN, Baldwin M, Healer J, O'Neill M: **A next-generation genetically attenuated *Plasmodium falciparum* parasite created by triple gene deletion.** *Molecular therapy* 2014, **22**(9):1707-1715.
230. Kublin JG, Mikolajczak SA, Sack BK, Fishbaugher ME, Seillie A, Shelton L, VonGoedert T, Firat M, Magee S, Fritzen E: **Complete attenuation of genetically engineered *Plasmodium falciparum* sporozoites in human subjects.** *Science Translational Medicine* 2017, **9**(371):eaad9099.
231. Annoura T, Ploemen IH, van Schaijk BC, Sajid M, Vos MW, van Gemert G-J, Chevalley-Maurel S, Franke-Fayard BM, Hermesen CC, Gego A: **Assessing the adequacy of attenuation of genetically modified malaria parasite vaccine candidates.** *Vaccine* 2012, **30**(16):2662-2670.
232. Purcell LA, Yanow SK, Lee M, Spithill TW, Rodriguez A: **Chemical attenuation of *Plasmodium berghei* sporozoites induces sterile immunity in mice.** *Infection and immunity* 2008, **76**(3):1193-1199.
233. Purcell LA, Wong KA, Yanow SK, Lee M, Spithill TW, Rodriguez A: **Chemically attenuated *Plasmodium* sporozoites induce specific immune responses, sterile immunity and cross-protection against heterologous challenge.** *Vaccine* 2008, **26**(38):4880-4884.
234. Raja AI, Cai Y, Reiman JM, Groves P, Chakravarty S, McPhun V, Doolan DL, Cockburn I, Hoffman SL, Stanisic DI: **Chemically attenuated blood-stage *Plasmodium yoelii* parasites induce long-lived and strain-transcending protection.** *Infection and immunity* 2016, **84**(8):2274-2288.
235. Barbacid M, Breitman M, Lauver A, Long L, Vogt P, Beemon K, Wang L: **Synthesis and assembly of hepatitis B virus surface antigen particles in yeast.** *Nature* 1982, **298**:347.
236. Liljeqvist S, Ståhl S: **Production of recombinant subunit vaccines: protein immunogens, live delivery systems and nucleic acid vaccines.** *Journal of biotechnology* 1999, **73**(1):1-33.
237. Nussenzweig R, Nussenzweig V: **Antisporozoite vaccine for malaria: experimental basis and current status.** *Review of Infectious Diseases* 1989, **11**(Supplement 3):S579-S585.
238. Gwadz R, Cochrane A, Nussenzweig V, Nussenzweig R: **Preliminary studies on vaccination of rhesus monkeys with irradiated sporozoites of *Plasmodium knowlesi* and characterization of surface antigens of these parasites.** *Bulletin of the world Health Organization* 1978, **57**:165-173.
239. Nardin EH, Nussenzweig V, Nussenzweig RS, Collins WE, Harinasuta KT, Tapchaisri P, Chomcharn Y: **Circumsporozoite proteins of human malaria parasites *Plasmodium falciparum* and *Plasmodium vivax*.** *Journal of Experimental Medicine* 1982, **156**(1):20-30.
240. Schwartz L, Brown GV, Genton B, Moorthy VS: **A review of malaria vaccine clinical projects based on the WHO rainbow table.** *Malaria journal* 2012, **11**(1):11.
241. Macete EV, Sacarlal J, Aponte JJ, Leach A, Navia MM, Milman J, Guinovart C, Mandomando I, López-Púa Y, Lievens M: **Evaluation of two formulations of adjuvanted RTS, S malaria vaccine in children aged 3 to 5 years living in a malaria-endemic region of Mozambique: a Phase I/IIb randomized double-blind bridging trial.** *Trials* 2007, **8**(1):11.
242. White MT, Bejon P, Olotu A, Griffin JT, Riley EM, Kester KE, Ockenhouse CF, Ghani AC: **The relationship between RTS, S vaccine-induced antibodies, CD4+ T cell responses and protection against *Plasmodium falciparum* infection.** *PLOS one* 2013, **8**(4):e61395.
243. Kester KE, Cummings JF, Ofori-Anyinam O, Ockenhouse CF, Krzych U, Moris P, Schwenk R, Nielsen RA, Debebe Z, Pinelis E: **Randomized, double-blind, phase 2a trial of falciparum malaria vaccines RTS, S/AS01B and RTS, S/AS02A in malaria-naïve adults: safety, efficacy, and immunologic associates of protection.** *Journal of Infectious Diseases* 2009, **200**(3):337-346.
244. Cummings JF, Spring MD, Schwenk RJ, Ockenhouse CF, Kester KE, Polhemus ME, Walsh DS, Yoon I-K, Prosperi C, Juompan LY: **Recombinant Liver Stage Antigen-1 (LSA-1) formulated with AS01 or AS02 is safe, elicits high titer antibody and induces IFN-γ/IL-2 CD4+ T cells but does not protect against experimental *Plasmodium falciparum* infection.** *Vaccine* 2010, **28**(31):5135-5144.
245. Reed SG, Orr MT, Fox CB: **Key roles of adjuvants in modern vaccines.** *Nature medicine* 2013, **19**(12):1597-1608.
246. Hutchings CL, Gilbert SC, Hill AV, Moore AC: **Novel protein and poxvirus-based vaccine combinations for simultaneous induction of humoral and cell-mediated immunity.** *The Journal of Immunology* 2005, **175**(1):599-606.

247. Sedegah M, Hedstrom R, Hobart P, Hoffman SL: **Protection against malaria by immunization with plasmid DNA encoding circumsporozoite protein.** *Proceedings of the National Academy of Sciences* 1994, **91**(21):9866-9870.
248. Wang R, Doolan DL, Le TP, Hedstrom RC, Coonan KM, Charoenvit Y, Jones TR, Hobart P, Margalith M, Ng J: **Induction of antigen-specific cytotoxic T lymphocytes in humans by a malaria DNA vaccine.** *Science* 1998, **282**(5388):476-480.
249. Wang R, Epstein J, Baraceros FM, Gorak EJ, Charoenvit Y, Carucci DJ, Hedstrom RC, Rahardjo N, Gay T, Hobart P: **Induction of CD4+ T cell-dependent CD8+ type 1 responses in humans by a malaria DNA vaccine.** *Proceedings of the National Academy of Sciences* 2001, **98**(19):10817-10822.
250. Doolan DL, Hoffman SL: **DNA-based vaccines against malaria: status and promise of the Multi-Stage Malaria DNA Vaccine Operation.** *International journal for parasitology* 2001, **31**(8):753-762.
251. Schneider J, Gilbert SC, Blanchard TJ, Hanke T, Robson KJ, Hannan CM, Becker M, Sinden R, Smith GL, Hill AV: **Enhanced immunogenicity for CD8+ T cell induction and complete protective efficacy of malaria DNA vaccination by boosting with modified vaccinia virus Ankara.** *Nature medicine* 1998, **4**(4):397-402.
252. Dunachie S, Walther M, Epstein J, Keating S, Berthoud T, Andrews L, Andersen R, Bejon P, Goonetilleke N, Poulton I: **A DNA prime-modified vaccinia virus ankara boost vaccine encoding thrombospondin-related adhesion protein but not circumsporozoite protein partially protects healthy malaria-naive adults against *Plasmodium falciparum* sporozoite challenge.** *Infection and immunity* 2006, **74**(10):5933-5942.
253. McConkey SJ, Reece WH, Moorthy VS, Webster D, Dunachie S, Butcher G, Vuola JM, Blanchard TJ, Gothard P, Watkins K: **Enhanced T-cell immunogenicity of plasmid DNA vaccines boosted by recombinant modified vaccinia virus Ankara in humans.** *Nature medicine* 2003, **9**(6):729-735.
254. Tatsis N, Ertl HC: **Adenoviruses as vaccine vectors.** *Molecular Therapy* 2004, **10**(4):616-629.
255. Swadling L, Capone S, Antrobus RD, Brown A, Richardson R, Newell EW, Halliday J, Kelly C, Bowen D, Fergusson J: **A human vaccine strategy based on chimpanzee adenoviral and MVA vectors that primes, boosts, and sustains functional HCV-specific T cell memory.** *Science translational medicine* 2014, **6**(261):261ra153-261ra153.
256. Barnes E, Folgori A, Capone S, Swadling L, Aston S, Kurioka A, Meyer J, Huddart R, Smith K, Townsend R *et al*: **Novel adenovirus-based vaccines induce broad and sustained T cell responses to HCV in man.** *Science translational medicine* 2012, **4**(115):115ra111-115ra111.
257. Borthwick N, Ahmed T, Ondondo B, Hayes P, Rose A, Ebrahimsa U, Hayton E-J, Black A, Bridgeman A, Rosario M: **Vaccine-elicited human T cells recognizing conserved protein regions inhibit HIV-1.** *Molecular Therapy* 2014, **22**(2):464-475.
258. Afolabi MO, Ndure J, Drammeh A, Darboe F, Mehedi S-R, Rowland-Jones SL, Borthwick N, Black A, Ambler G, John-Stewart GC: **A phase I randomized clinical trial of candidate human immunodeficiency virus type 1 vaccine MVA. HIVA administered to Gambian infants.** *PloS one* 2013, **8**(10):e78289.
259. Buchbinder SP, Mehrotra DV, Duerr A, Fitzgerald DW, Mogg R, Li D, Gilbert PB, Lama JR, Marmor M, del Rio C: **Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial.** *The Lancet* 2008, **372**(9653):1881-1893.
260. Ewer K, Rampling T, Venkatraman N, Bowyer G, Wright D, Lambe T, Imoukhuede EB, Payne R, Fehling SK, Strecker T *et al*: **A Monovalent Chimpanzee Adenovirus Ebola Vaccine Boosted with MVA.** *New England Journal of Medicine* 2016, **374**(17):1635-1646.
261. De Santis O, Audran R, Pothin E, Warpelin-Decrausaz L, Vallotton L, Wuerzner G, Cochet C, Estoppey D, Steiner-Monard V, Lonchampt S: **Safety and immunogenicity of a chimpanzee adenovirus-vectored Ebola vaccine in healthy adults: a randomised, double-blind, placebo-controlled, dose-finding, phase 1/2a study.** *The Lancet Infectious Diseases* 2016, **16**(3):311-320.
262. Keating SM, Bejon P, Berthoud T, Vuola JM, Todryk S, Webster DP, Dunachie SJ, Moorthy VS, McConkey SJ, Gilbert SC: **Durable human memory T cells quantifiable by cultured enzyme-linked immunospot assays are induced by heterologous prime boost immunization and correlate with protection against malaria.** *The Journal of Immunology* 2005, **175**(9):5675-5680.
263. McShane H, Pathan AA, Sander CR, Goonetilleke NP, Fletcher HA, Hill AV: **Boosting BCG with MVA85A: the first candidate subunit vaccine for tuberculosis in clinical trials.** *Tuberculosis* 2005, **85**(1):47-52.
264. Sutter G, Wyatt LS, Foley PL, Bennink JR, Moss B: **A recombinant vector derived from the host range-restricted and highly attenuated MVA strain of vaccinia virus stimulates protective immunity in mice to influenza virus.** *Vaccine* 1994, **12**(11):1032-1040.

265. Lambe T, Carey JB, Li Y, Spencer AJ, van Laarhoven A, Mullarkey CE, Vrdoljak A, Moore AC, Gilbert SC: **Immunity against heterosubtypic influenza virus induced by adenovirus and MVA expressing nucleoprotein and matrix protein-1.** *Scientific reports* 2013, **3**:1443.
266. O'Hara GA, Duncan CJ, Ewer KJ, Collins KA, Elias SC, Halstead FD, Goodman AL, Edwards NJ, Reyes-Sandoval A, Bird P: **Clinical assessment of a recombinant simian adenovirus ChAd63: a potent new vaccine vector.** *Journal of Infectious Diseases* 2012, **205**(5):772-781.
267. Sheehy SH, Duncan CJ, Elias SC, Choudhary P, Biswas S, Halstead FD, Collins KA, Edwards NJ, Douglas AD, Anagnostou NA: **ChAd63-MVA-vectored blood-stage malaria vaccines targeting MSP1 and AMA1: assessment of efficacy against mosquito bite challenge in humans.** *Molecular Therapy* 2012, **20**(12):2355-2368.
268. Sheehy SH, Duncan CJ, Elias SC, Biswas S, Collins KA, O'Hara GA, Halstead FD, Ewer KJ, Mahungu T, Spencer AJ: **Phase Ia clinical evaluation of the safety and immunogenicity of the *Plasmodium falciparum* blood-stage antigen AMA1 in ChAd63 and MVA vaccine vectors.** *PLoS One* 2012, **7**(2):e31208.
269. Ogwang C, Afolabi M, Kimani D, Jagne YJ, Sheehy SH, Bliss CM, Duncan CJ, Collins KA, Knight MAG, Kimani E: **Safety and immunogenicity of heterologous prime-boost immunisation with *Plasmodium falciparum* malaria candidate vaccines, ChAd63 ME-TRAP and MVA ME-TRAP, in healthy Gambian and Kenyan adults.** *PLoS One* 2013, **8**(3):e57726.
270. Webster D, Dunachie S, McConkey S, Poulton I, Moore A, Walther M, Laidlaw S, Peto T, Skinner M, Gilbert S: **Safety of recombinant fowlpox strain FP9 and modified vaccinia virus Ankara vaccines against liver-stage *P. falciparum* malaria in non-immune volunteers.** *Vaccine* 2006, **24**(15):3026-3034.
271. Colloca S, Barnes E, Folgori A, Ammendola V, Capone S, Cirillo A, Siani L, Naddeo M, Grazioli F, Esposito ML: **Vaccine vectors derived from a large collection of simian adenoviruses induce potent cellular immunity across multiple species.** *Science translational medicine* 2012, **4**(115):115ra112-115ra112.
272. Hill AV, Reyes-Sandoval A, O'Hara G, Ewer K, Lawrie A, Goodman A, Nicosia A, Folgori A, Colloca S, Cortese R: **Prime-boost vectored malaria vaccines: progress and prospects.** *Human vaccines* 2010, **6**(1):78-83.
273. Norbury CC, Malide D, Gibbs JS, Bennink JR, Yewdell JW: **Visualizing priming of virus-specific CD8+ T cells by infected dendritic cells in vivo.** *Nature immunology* 2002, **3**(3):265-271.
274. Reyes-Sandoval A, Wyllie DH, Bauza K, Milicic A, Forbes EK, Rollier CS, Hill AV: **CD8+ T effector memory cells protect against liver-stage malaria.** *The Journal of Immunology* 2011, **187**(3):1347-1357.
275. Draper SJ, Moore AC, Goodman AL, Long CA, Holder AA, Gilbert SC, Hill F, Hill AV: **Effective induction of high-titer antibodies by viral vector vaccines.** *Nature medicine* 2008, **14**(8):819-821.
276. Reyes-Sandoval A, Wyllie DH, Bauza K, Milicic A, Rollier CS, Forbes EK, Hill AVS: **CD8(+) T Effector Memory Cells Protect against Liver-Stage Malaria.** *Journal of immunology (Baltimore, Md : 1950)* 2011, **187**(3):1347-1357.
277. Mayr A, Stickl H, Müller H, Danner K, Singer H: **The smallpox vaccination strain MVA: marker, genetic structure, experience gained with the parenteral vaccination and behavior in organisms with a debilitated defence mechanism (author's transl).** *Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene Erste Abteilung Originale Reihe B: Hygiene, Betriebshygiene, präventive Medizin* 1978, **167**(5-6):375-390.
278. Reyes-Sandoval A, Harty JT, Todryk SM: **Viral vector vaccines make memory T cells against malaria.** *Immunology* 2007, **121**(2):158-165.
279. Sheehy SH, Duncan CJ, Elias SC, Collins KA, Ewer KJ, Spencer AJ, Williams AR, Halstead FD, Moretz SE, Miura K: **Phase Ia clinical evaluation of the *Plasmodium falciparum* blood-stage antigen MSP1 in ChAd63 and MVA vaccine vectors.** *Molecular Therapy* 2011, **19**(12):2269-2276.
280. Moorthy VS, Pinder M, Reece WH, Watkins K, Atabani S, Hannan C, Bojang K, McAdam KP, Schneider J, Gilbert S: **Safety and immunogenicity of DNA/modified vaccinia virus ankara malaria vaccination in African adults.** *Journal of Infectious Diseases* 2003, **188**(8):1239-1244.
281. Moorthy V, McConkey S, Roberts M, Gothard P, Arulanantham N, Degano P, Schneider J, Hannan C, Roy M, Gilbert S: **Safety of DNA and modified vaccinia virus Ankara vaccines against liver-stage *P. falciparum* malaria in non-immune volunteers.** *Vaccine* 2003, **21**(17):1995-2002.
282. Webster DP, Dunachie S, Vuola JM, Berthoud T, Keating S, Laidlaw SM, McConkey SJ, Poulton I, Andrews L, Andersen RF: **Enhanced T cell-mediated protection against malaria in human challenges by using the recombinant poxviruses FP9 and modified vaccinia virus Ankara.** *Proceedings of the National Academy of Sciences of the United States of America* 2005, **102**(13):4836-4841.

283. Afolabi MO, Tiono AB, Adetifa UJ, Yaro JB, Drammeh A, Nébié I, Bliss C, Hodgson SH, Anagnostou NA, Sanou GS: **Safety and Immunogenicity of ChAd63 and MVA ME-TRAP in West African children and infants.** *Molecular Therapy* 2016.
284. Rampling T, Ewer KJ, Bowyer G, Bliss CM, Edwards NJ, Wright D, Payne R, Venkatraman N, de Barra E, Snudden CM: **Safety and high level efficiency of the combination malaria vaccine regimen of RTS, S/AS01B with ChAd-MVA vectored vaccines expressing ME-TRAP.** *The Journal of Infectious Diseases* 2016:1-26.
285. Hodgson SH, Ewer KJ, Bliss CM, Edwards NJ, Rampling T, Anagnostou NA, De Barra E, Havelock T, Bowyer G, Poulton ID: **Evaluation of the efficacy of ChAd63-MVA vectored vaccines expressing circumsporozoite protein and ME-TRAP against controlled human malaria infection in malaria-naïve individuals.** *Journal of Infectious Diseases* 2015, **211**(7):1076-1086.
286. De Barra E, Hodgson SH, Ewer KJ, Bliss CM, Hennigan K, Collins A, Berrie E, Lawrie AM, Gilbert SC, Nicosia A: **A phase Ia study to assess the safety and immunogenicity of new malaria vaccine candidates ChAd63 CS administered alone and with MVA CS.** *PLoS One* 2014, **9**(12):e115161.
287. Ewer KJ, O'Hara GA, Duncan CJ, Collins KA, Sheehy SH, Reyes-Sandoval A, Goodman AL, Edwards NJ, Elias SC, Halstead FD: **Protective CD8+ T-cell immunity to human malaria induced by chimpanzee adenovirus-MVA immunisation.** *Nature communications* 2013, **4**:2836.
288. Ouédraogo A, Tiono AB, Kargougou D, Yaro JB, Ouédraogo E, Kaboré Y, Kangoye D, Bougouma EC, Gansane A, Henri N: **A phase 1b randomized, controlled, double-blinded dosage-escalation trial to evaluate the safety, reactogenicity and immunogenicity of an adenovirus type 35 based circumsporozoite malaria vaccine in Burkinabe healthy adults 18 to 45 years of age.** *PLoS one* 2013, **8**(11):e78679.
289. Thorner AR, Vogels R, Kaspers J, Weverling GJ, Holterman L, Lemckert AA, Dilraj A, McNally LM, Jeena PM, Jepsen S: **Age dependence of adenovirus-specific neutralizing antibody titers in individuals from sub-Saharan Africa.** *Journal of clinical microbiology* 2006, **44**(10):3781-3783.
290. Mast TC, Kierstead L, Gupta SB, Nikas AA, Kallas EG, Novitsky V, Mbewe B, Pitisuttithum P, Schechter M, Vardas E: **International epidemiology of human pre-existing adenovirus (Ad) type-5, type-6, type-26 and type-36 neutralizing antibodies: correlates of high Ad5 titers and implications for potential HIV vaccine trials.** *Vaccine* 2010, **28**(4):950-957.
291. Dudareva M, Andrews L, Gilbert S, Bejon P, Marsh K, Mwacharo J, Kai O, Nicosia A, Hill A: **Prevalence of serum neutralizing antibodies against chimpanzee adenovirus 63 and human adenovirus 5 in Kenyan children, in the context of vaccine vector efficacy.** *Vaccine* 2009, **27**(27):3501-3504.
292. Chuang I, Sedegah M, Cicatelli S, Spring M, Polhemus M, Tamminga C, Patterson N, Guerrero M, Bennett JW, McGrath S: **DNA prime/Adenovirus boost malaria vaccine encoding P. falciparum CSP and AMA1 induces sterile protection associated with cell-mediated immunity.** *PLoS one* 2013, **8**(2):e55571.
293. Gilbert SC, Plebanski M, Harris SJ, Allsopp CE, Thomas R, Layton GT, Hill AV: **A protein particle vaccine containing multiple malaria epitopes.** *Nature biotechnology* 1997, **15**(12):1280-1284.
294. Kimani D, Jagne YJ, Cox M, Kimani E, Bliss CM, Gitau E, Ogwang C, Afolabi MO, Bowyer G, Collins KA: **Translating the immunogenicity of prime-boost immunization with ChAd63 and MVA ME-TRAP from malaria naïve to malaria-endemic populations.** *Molecular Therapy* 2014, **22**(11):1992-2003.
295. Ogwang C, Kimani D, Edwards NJ, Roberts R, Mwacharo J, Bowyer G, Bliss C, Hodgson SH, Njuguna P, Viebig NK: **Prime-boost vaccination with chimpanzee adenovirus and modified vaccinia Ankara encoding TRAP provides partial protection against Plasmodium falciparum infection in Kenyan adults.** *Science translational medicine* 2015, **7**(286):286re285-286re285.
296. Mettens P, Dubois PM, Demoitié M-A, Bayat B, Donner M-N, Bourguignon P, Stewart VA, Heppner DG, Garçon N, Cohen J: **Improved T cell responses to Plasmodium falciparum circumsporozoite protein in mice and monkeys induced by a novel formulation of RTS, S vaccine antigen.** *Vaccine* 2008, **26**(8):1072-1082.
297. Ballou WR, Rothbard J, Wirtz RA, Gordon DM, Williams JS, Gore RW, Schneider I, Hollingdale MR, Beaudoin RL, Maloy WL: **Immunogenicity of synthetic peptides form circumsporozoite protein of Plasmodium falciparum.** *Science* 1985, **228**:996-1000.
298. Malik A, Egan JE, Houghten RA, Sadoff JC, Hoffman SL: **Human cytotoxic T lymphocytes against the Plasmodium falciparum circumsporozoite protein.** *Proceedings of the National Academy of Sciences* 1991, **88**(8):3300-3304.
299. Kumar S, Miller LH, Quakyi IA, Keister DB, Houghten RA, Maloy WL, Moss B, Berzofsky JA, Good MF: **Cytotoxic T cells specific for the circumsporozoite protein of Plasmodium falciparum.** 1988.

300. Garçon N, Heppner DG, Cohen J: **Development of RTS, S/AS02: a purified subunit-based malaria vaccine candidate formulated with a novel adjuvant.** *Expert review of vaccines* 2003, **2**(2):231-238.
301. Bojang KA, Milligan PJ, Pinder M, Vigneron L, Allouche A, Kester KE, Ballou WR, Conway DJ, Reece WH, Gothard P: **Efficacy of RTS, S/AS02 malaria vaccine against *Plasmodium falciparum* infection in semi-immune adult men in The Gambia: a randomised trial.** *The Lancet* 2001, **358**(9297):1927-1934.
302. Kester KE, McKinney DA, Tornieporth N, Ockenhouse CF, Heppner DG, Hall T, Krzych U, Delchambre M, Voss G, Dowler MG: **Efficacy of recombinant circumsporozoite protein vaccine regimens against experimental *Plasmodium falciparum* malaria.** *Journal of Infectious Diseases* 2001, **183**(4):640-647.
303. Rts S: **Efficacy and safety of RTS, S/AS01 malaria vaccine with or without a booster dose in infants and children in Africa: final results of a phase 3, individually randomised, controlled trial.** *The Lancet* 2015, **386**(9988):31-45.
304. Agnandji ST, Lell B, Soulanoudjingar SS, Fernandes JF, Abossolo BP, Conzelmann C, Methogo B, Doucka Y, Flamen A, Mordmüller B: **First results of phase 3 trial of RTS, S/AS01 malaria vaccine in African children.** *The New England journal of medicine* 2011, **365**(20):1863.
305. Moorthy VS, Newman RD, Okwo-Bele JM: **Malaria vaccine technology roadmap.** *Lancet* 2013, **382**(9906):1700-1701.
306. Ndungu FM, Mwacharo J, Kimani D, Kai O, Moris P, Jongert E, Vekemans J, Olotu A, Bejon P: **A statistical interaction between circumsporozoite protein-specific T cell and antibody responses and risk of clinical malaria episodes following vaccination with RTS, S/AS01E.** *PLoS One* 2012, **7**(12):e52870.
307. Goodman A, Draper S: **Blood-stage malaria vaccines—recent progress and future challenges.** *Annals of Tropical Medicine & Parasitology* 2010, **104**(3):189-211.
308. Takala SL, Coulibaly D, Thera MA, Batchelor AH, Cummings MP, Escalante AA, Ouattara A, Traoré K, Niangaly A, Djimdé AA: **Extreme polymorphism in a vaccine antigen and risk of clinical malaria: implications for vaccine development.** *Science translational medicine* 2009, **1**(2):2ra5-2ra5.
309. Spring MD, Cummings JF, Ockenhouse CF, Dutta S, Reidler R, Angov E, Bergmann-Leitner E, Stewart VA, Bittner S, Juompan L: **Phase 1/2a study of the malaria vaccine candidate apical membrane antigen-1 (AMA-1) administered in adjuvant system AS01B or AS02A.** *PloS one* 2009, **4**(4):e5254.
310. Thera MA, Doumbo OK, Coulibaly D, Laurens MB, Ouattara A, Kone AK, Guindo AB, Traore K, Traore I, Kouriba B: **A field trial to assess a blood-stage malaria vaccine.** *New England Journal of Medicine* 2011, **365**(11):1004-1013.
311. Genton B, Betuela I, Felger I, Al-Yaman F, Anders RF, Saul A, Rare L, Baisor M, Lorry K, Brown GV: **A recombinant blood-stage malaria vaccine reduces *Plasmodium falciparum* density and exerts selective pressure on parasite populations in a phase 1-2b trial in Papua New Guinea.** *Journal of Infectious Diseases* 2002, **185**(6):820-827.
312. Thompson FM, Porter DW, Okitsu SL, Westerfeld N, Vogel D, Todryk S, Poulton I, Correa S, Hutchings C, Berthoud T: **Evidence of blood stage efficacy with a virosomal malaria vaccine in a phase IIa clinical trial.** *PLoS One* 2008, **3**(1):e1493.
313. Crosnier C, Bustamante LY, Bartholdson SJ, Bei AK, Theron M, Uchikawa M, Mboup S, Ndir O, Kwiatkowski DP, Duraisingh MT: **Basigin is a receptor essential for erythrocyte invasion by *Plasmodium falciparum*.** *Nature* 2011, **480**(7378):534-537.
314. Douglas AD, Baldeviano GC, Lucas CM, Lugo-Roman LA, Crosnier C, Bartholdson SJ, Diouf A, Miura K, Lambert LE, Ventocilla JA: **A PfrH5-based vaccine is efficacious against heterologous strain blood-stage *Plasmodium falciparum* infection in *Aotus* monkeys.** *Cell host & microbe* 2015, **17**(1):130-139.
315. McCarthy JS, Good MF: **Whole parasite blood stage malaria vaccines: a convergence of evidence.** *Human vaccines* 2010, **6**(1):114-123.
316. Kang J-M, Ju H-L, Moon S-U, Cho P-Y, Bahk Y-Y, Sohn W-M, Park Y-K, Cha SH, Kim T-S, Na B-K: **Limited sequence polymorphisms of four transmission-blocking vaccine candidate antigens in *Plasmodium vivax* Korean isolates.** *Malaria journal* 2013, **12**(1):144.
317. Tsuboi T, Kaslow DC, Gozar M, Tachibana M, Cao Y-M, Torii M: **Sequence polymorphism in two novel *Plasmodium vivax* ookinete surface proteins, Pvs25 and Pvs28, that are malaria transmission-blocking vaccine candidates.** *Molecular medicine* 1998, **4**(12):772.
318. Zakeri S, Razavi S, Djadid ND: **Genetic diversity of transmission blocking vaccine candidate (Pvs25 and Pvs28) antigen in *Plasmodium vivax* clinical isolates from Iran.** *Acta tropica* 2009, **109**(3):176-180.
319. Li Y, Leneghan DB, Miura K, Nikolaeva D, Brian II, Dicks MD, Fyfe AJ, Zakutansky SE, de Cassan S, Long CA: **Enhancing immunogenicity and transmission-blocking activity of malaria vaccines by fusing Pfs25 to IMX313 multimerization technology.** *Scientific reports* 2016, **6**.

320. Theisen M, Jore MM, Sauerwein R: **Towards clinical development of a Pf s48/45-based transmission blocking malaria vaccine.** *Expert Review of Vaccines* 2017(just-accepted).
321. Sauerwein RW, Roestenberg M, Moorthy VS: **Experimental human challenge infections can accelerate clinical malaria vaccine development.** *Nature reviews Immunology* 2011, **11**(1):57-64.
322. Roestenberg M, Bijker EM, Sim BKL, Billingsley PF, James ER, Bastiaens GJ, Teirlinck AC, Scholzen A, Teelen K, Arens T: **Controlled human malaria infections by intradermal injection of cryopreserved *Plasmodium falciparum* sporozoites.** *The American journal of tropical medicine and hygiene* 2013, **88**(1):5-13.
323. Sheehy SH, Spencer AJ, Douglas AD, Sim BKL, Longley RJ, Edwards NJ, Poulton ID, Kimani D, Williams AR, Anagnostou NA: **Optimising controlled human malaria infection studies using cryopreserved *P. falciparum* parasites administered by needle and syringe.** *PloS one* 2013, **8**(6):e65960.
324. Hermesen CC, Telgt DS, Linders EH, van de Locht LA, Eling WM, Mensink EJ, Sauerwein RW: **Detection of *Plasmodium falciparum* malaria parasites in vivo by real-time quantitative PCR.** *Molecular and biochemical parasitology* 2001, **118**(2):247-251.
325. Andrews L, Andersen RF, Webster D, Dunachie S, Walther RM, Bejon P, Hunt-Cooke A, Bergson G, Sanderson F, Hill AV: **Quantitative real-time polymerase chain reaction for malaria diagnosis and its use in malaria vaccine clinical trials.** *The American journal of tropical medicine and hygiene* 2005, **73**(1):191-198.
326. Guebre-Xabier M, Schwenk R, Krzych U: **Memory phenotype CD 8+ T cells persist in livers of mice protected against malaria by immunization with attenuated *Plasmodium berghei* sporozoites.** *European journal of immunology* 1999, **29**(12):3978-3986.
327. Spencer AJ, Longley RJ, Gola A, Ulaszewska M, Lambe T, Hill AV: **The Threshold of Protection from Liver-Stage Malaria Relies on a Fine Balance between the Number of Infected Hepatocytes and Effector CD8+ T Cells Present in the Liver.** *The Journal of Immunology* 2017:1601209.
328. Vincke I: **Experimental transmission of *Plasmodium berghei*.** *Indian journal of malariology* 1954, **8**(4):257.
329. Hutchings CL, Birkett AJ, Moore AC, Hill AV: **Combination of protein and viral vaccines induces potent cellular and humoral immune responses and enhanced protection from murine malaria challenge.** *Infection and immunity* 2007, **75**(12):5819-5826.
330. Hoffman S, Isenbarger D, Long G, Sedegah M, Szafrman A, Mellouk S, Ballou W: **T lymphocytes from mice immunized with irradiated sporozoites eliminate malaria from hepatocytes.** *Bulletin of the World Health Organization* 1990, **68**(Suppl):132.
331. Van Braeckel-Budimir N, Harty JT: **CD8 T-cell-mediated protection against liver-stage malaria: lessons from a mouse model.** *Frontiers in Microbiology* 2014, **5**.
332. Bauza K, Atcheson E, Malinauskas T, Blagborough AM, Reyes-Sandoval A: **Tailoring a Combination Preerythrocytic Malaria Vaccine.** *Infection and immunity* 2016, **84**(3):622-634.
333. Longley RJ, Salman AM, Cottingham MG, Ewer K, Janse CJ, Khan SM, Spencer AJ, Hill AV: **Comparative assessment of vaccine vectors encoding ten malaria antigens identifies two protective liver-stage candidates.** *Scientific reports* 2015, **5**:11820.
334. Khan ZM, Vanderberg JP: **Role of host cellular response in differential susceptibility of nonimmunized BALB/c mice to *Plasmodium berghei* and *Plasmodium yoelii* sporozoites.** *Infection and immunity* 1991, **59**(8):2529-2534.
335. Kaushansky A, Austin LS, Mikolajczak SA, Lo FY, Miller JL, Douglass AN, Arang N, Vaughan AM, Gardner MJ, Kappe SH: **Susceptibility to *Plasmodium yoelii* preerythrocytic infection in BALB/c substrains is determined at the point of hepatocyte invasion.** *Infection and immunity* 2015, **83**(1):39-47.
336. Vanderberg JP, Khan ZM, Stewart MJ: **Induction of hepatic inflammatory response by *Plasmodium berghei* sporozoites protects BALB/c mice against challenge with *Plasmodium yoelii* sporozoites.** *The Journal of parasitology* 1993, **79**(5):763-767.
337. Uhl EW, Warner NJ: **Mouse models as predictors of human responses: evolutionary medicine.** *Current pathobiology reports* 2015, **3**(3):219-223.
338. Wang Z-E, Reiner SL, Zheng S, Dalton DK, Locksley RM: **CD4+ effector cells default to the Th2 pathway in interferon gamma-deficient mice infected with *Leishmania major*.** *The Journal of experimental medicine* 1994, **179**(4):1367-1371.
339. Egan AF, Blackman MJ, Kaslow DC: **Vaccine Efficacy of Recombinant *Plasmodium falciparum* Merozoite Surface Protein 1 in Malaria-Naive,-Exposed, and/or-Rechallenged *Aotus vociferans* Monkeys.** *Infection and immunity* 2000, **68**(3):1418-1427.

340. Jones TR, Obaldia N, Gramzinski RA, Charoenvit Y, Kolodny N, Kitov S, Davis HL, Krieg AM, Hoffman SL: **Synthetic oligodeoxynucleotides containing CpG motifs enhance immunogenicity of a peptide malaria vaccine in *Aotus* monkeys.** *Vaccine* 1999, **17**(23):3065-3071.
341. Kumar S, Collins W, Egan A, Yadava A, Garraud O, Blackman MJ, Patino JAG, Diggs C, Kaslow DC: **Immunogenicity and efficacy in *aotus* monkeys of four recombinant *Plasmodium falciparum* vaccines in multiple adjuvant formulations based on the 19-kilodalton C terminus of merozoite surface protein 1.** *Infection and Immunity* 2000, **68**(4):2215-2223.
342. Moreno CA, Rodriguez R, Oliveira GA, Ferreira V, Nussenzweig RS, Castro ZRM, Calvo-Calle JM, Nardin E: **Preclinical evaluation of a synthetic *Plasmodium falciparum* MAP malaria vaccine in *Aotus* monkeys and mice.** *Vaccine* 1999, **18**(1):89-99.
343. Shortt H, Garnham PCC: **Demonstration of a persisting exo-erythrocytic cycle in *Plasmodium cynomolgi* and its bearing on the production of relapses.** *Bulletin of the World Health Organization* 2000, **78**(12):1447-1449.
344. Demebele L, Gego A, Zeeman A-M, Franetich J-F, Silvie O, Rametti A, Le Grand R, Dereuddre-Bosquet N, Sauerwein R, van Gemert G-J: **Towards an in vitro model of *Plasmodium hypnozoites* suitable for drug discovery.** *PLoS one* 2011, **6**(3):e18162.
345. Su X, Hayton K, Wellems TE: **Genetic linkage and association analyses for trait mapping in *Plasmodium falciparum*.** *Nature Reviews Genetics* 2007, **8**(7):497-506.
346. Benmohamed L, Thomas A, Bossus M, Brahimi K, Wubben J, Gras-Masse H, Druilhe P: **High immunogenicity in chimpanzees of peptides and lipopeptides derived from four new *Plasmodium falciparum* pre-erythrocytic molecules.** *Vaccine* 2000, **18**(25):2843-2855.
347. WHO: **Malaria Fact Sheet No. 94.** 1998.
348. Stowers AW, Cioce V, Shimp RL, Lawson M, Hui G, Muratova O, Kaslow DC, Robinson R, Long CA, Miller LH: **Efficacy of Two Alternate Vaccines Based on *Plasmodium falciparum* Merozoite Surface Protein 1 in an *Aotus* Challenge Trial.** *Infection and immunity* 2001, **69**(3):1536-1546.
349. Adams EJ, Parham P: **Species-specific evolution of MHC class I genes in the higher primates.** *Immunological reviews* 2001, **183**(1):41-64.
350. Otting N, de Vos-Rouweler AJ, Heijmans CM, de Groot NG, Doxiadis GG, Bontrop RE: **MHC class IA region diversity and polymorphism in macaque species.** *Immunogenetics* 2007, **59**(5):367-375.
351. Doxiadis G, Heijmans C, Otting N, Bontrop R: **MIC gene polymorphism and haplotype diversity in rhesus macaques.** *Tissue antigens* 2007, **69**(3):212-219.
352. Adams EJ, Cooper S, Thomson G, Parham P, Adams E: **Common chimpanzees have greater diversity than humans at two of the three highly polymorphic MHC class I genes.** *Immunogenetics* 2000, **51**(6):410-424.
353. Teixeira C, Gomes R: **Experimental models in vaccine research: malaria and leishmaniasis.** *Brazilian Journal of Medical and Biological Research* 2013, **46**(2):109-116.
354. Wykes MN, Good MF: **What have we learnt from mouse models for the study of malaria?** *European journal of immunology* 2009, **39**(8):2004-2007.
355. Vaughan AM, Kappe SH, Ploss A, Mikolajczyk SA: **Development of humanized mouse models to study human malaria parasite infection.** *Future microbiology* 2012, **7**(5):657-665.
356. Soulard V, Bosson-Vanga H, Lorthiois A, Roucher C, Franetich J-F, Zanghi G, Bordessoulles M, Tefit M, Thellier M, Morosan S: ***Plasmodium falciparum* full life cycle and *Plasmodium ovale* liver stages in humanized mice.** *Nature communications* 2015, **6**.
357. Angulo-Barturen I, Jiménez-Díaz MB, Mulet T, Rullas J, Herreros E, Ferrer S, Jiménez E, Mendoza A, Regadera J, Rosenthal PJ: **A murine model of falciparum-malaria by in vivo selection of competent strains in non-myelodepleted mice engrafted with human erythrocytes.** *PLoS One* 2008, **3**(5):e2252.
358. Li X, Huang J, Zhang M, Funakoshi R, Sheetij D, Spaccapelo R, Crisanti A, Nussenzweig V, Nussenzweig RS, Tsuji M: **Human CD8+ T cells mediate protective immunity induced by a human malaria vaccine in human immune system mice.** *Vaccine* 2016, **34**(38):4501-4506.
359. Mlambo G, Kumar N: **Transgenic rodent *Plasmodium berghei* parasites as tools for assessment of functional immunogenicity and optimization of human malaria vaccines.** *Eukaryotic cell* 2008, **7**(11):1875-1879.
360. Salman AM, Mogollon CM, Lin J-w, van Pul FJ, Janse CJ, Khan SM: **Generation of Transgenic Rodent Malaria Parasites Expressing Human Malaria Parasite Proteins.** *Malaria Vaccines: Methods and Protocols* 2015:257-286.
361. Langhorne J, Buffet P, Galinski M, Good M, Harty J, Leroy D, Mota MM, Pasini E, Renia L, Riley E: **The relevance of non-human primate and rodent malaria models for humans.** *Malaria journal* 2011, **10**(1):23.

362. House BL, Hollingdale MR, Sacchi JB, Richie TL: **Functional immunoassays using an in-vitro malaria liver-stage infection model: where do we go from here?** *Trends in parasitology* 2009, **25**(11):525-533.
363. Longley RJ, Bauza K, Ewer KJ, Hill AV, Spencer AJ: **Development of an in vitro assay and demonstration of *Plasmodium berghei* liver-stage inhibition by TRAP-specific CD8+ T cells.** *PloS one* 2015, **10**(3):e0119880.
364. de Koning-Ward TF, Sperança MA, Waters AP, Janse CJ: **Analysis of stage specificity of promoters in *Plasmodium berghei* using luciferase as a reporter.** *Molecular and biochemical parasitology* 1999, **100**(1):141-146.
365. de Koning-Ward TF, Thomas AW, Waters AP, Janse CJ: **Stable expression of green fluorescent protein in blood and mosquito stages of *Plasmodium berghei*.** *Molecular and biochemical parasitology* 1998, **97**(1):247-252.
366. Cao Y, Zhang D, Pan W: **Construction of transgenic *Plasmodium berghei* as a model for evaluation of blood-stage vaccine candidate of *Plasmodium falciparum* chimeric protein 2.9.** *PLoS One* 2009, **4**(9):e6894.
367. Persson C, Oliveira GA, Sultan AA, Bhanot P, Nussenzweig V, Nardin E: **Cutting edge: a new tool to evaluate human pre-erythrocytic malaria vaccines: rodent parasites bearing a hybrid *Plasmodium falciparum* circumsporozoite protein.** *The Journal of Immunology* 2002, **169**(12):6681-6685.
368. Porter MD, Nicki J, Pool CD, DeBot M, Illam RM, Brando C, Bozick B, De La Vega P, Angra D, Spaccapelo R: **Transgenic parasites stably expressing full-length *Plasmodium falciparum* circumsporozoite protein as a model for vaccine down-selection in mice using sterile protection as an endpoint.** *Clinical and Vaccine Immunology* 2013, **20**(6):803-810.
369. Rammensee H-G, Bachmann J, Emmerich NPN, Bachor OA, Stevanović S: **SYFPEITHI: database for MHC ligands and peptide motifs.** *Immunogenetics* 1999, **50**(3-4):213-219.
370. Robinson HL, Amara RR: **T cell vaccines for microbial infections.** *Nature medicine* 2005, **11**:S25-S32.
371. Lambert P-H, Liu M, Siegrist C-A: **Can successful vaccines teach us how to induce efficient protective immune responses?** *Nature medicine* 2005, **11**:S54-S62.
372. Siegrist C-A: **Neonatal and early life vaccinology.** *Vaccine* 2001, **19**(25):3331-3346.
373. Riley JK, Yokoyama WM: **REVIEW ARTICLE: NK Cell Tolerance and the Maternal-Fetal Interface.** *American Journal of Reproductive Immunology* 2008, **59**(5):371-387.
374. Levy O: **Innate immunity of the newborn: basic mechanisms and clinical correlates.** *Nature Reviews Immunology* 2007, **7**(5):379-390.
375. Mohr E, Siegrist C-A: **Vaccination in early life: standing up to the challenges.** *Current opinion in immunology* 2016, **41**:1-8.
376. Ivarsson MA, Loh L, Marquardt N, Kekäläinen E, Berglin L, Björkström NK, Westgren M, Nixon DF, Michaëlsson J: **Differentiation and functional regulation of human fetal NK cells.** *The Journal of clinical investigation* 2013, **123**(9):3889-3901.
377. Schüller S, Sadeghi K, Wisgrill L, Dangel A, Diesner S, Prusa A, Klebermasz-Schrehof K, Greber-Platzer S, Neumüller J, Helmer H: **Preterm neonates display altered plasmacytoid dendritic cell function and morphology.** *Journal of leukocyte biology* 2013, **93**(5):781-788.
378. De Kleer I, Willems F, Lambrecht B, Goriely S: **Ontogeny of myeloid cells.** *Frontiers in immunology* 2014, **5**:423.
379. Angelone DF, Wessels MR, Coughlin M, Suter EE, Valentini P, Kalish LA, Levy O: **Innate immunity of the human newborn is polarized toward a high ratio of IL-6/TNF- α production in vitro and in vivo.** *Pediatric research* 2006, **60**(2):205-209.
380. Takahata Y, Nomura A, Takada H, Ohga S, Furuno K, Hikino S, Nakayama H, Sakaguchi S, Hara T: **CD25+ CD4+ T cells in human cord blood: an immunoregulatory subset with naive phenotype and specific expression of forkhead box p3 (Foxp3) gene.** *Experimental hematology* 2004, **32**(7):622-629.
381. Holt PG: **The role of genetic and environmental factors in the development of T-cell mediated allergic disease in early life.** *Paediatric respiratory reviews* 2004, **5**:S27-S30.
382. Sun C-M, Deriaud E, Leclerc C, Lo-Man R: **Upon TLR9 signaling, CD5+ B cells control the IL-12-dependent Th1-priming capacity of neonatal DCs.** *Immunity* 2005, **22**(4):467-477.
383. Simon AK, Hollander GA, McMichael A: **Evolution of the immune system in humans from infancy to old age.** In: *Proc R Soc B: 2015: The Royal Society*; 2015: 20143085.
384. Gans HA, Ren J, Yasukawa LL, Alderson A, Rinki M, DeHovitz R, Beeler J, Audet S, Maldonado Y, Arvin AM: **Humoral and cell-mediated immune responses to an early 2-dose measles vaccination regimen in the United States.** *Journal of Infectious Diseases* 2004, **190**(1):83-90.
385. Welsh RM: **Assessing CD8 T cell number and dysfunction in the presence of antigen.** *Journal of Experimental Medicine* 2001, **193**(5):F19-F22.

386. Martinez X, Regner M, Kovarik J, Zarei S, Hauser C, Lambert P-H, Leclerc C, Siegrist C-A: **CD4-independent protective cytotoxic T cells induced in early life by a non-replicative delivery system based on virus-like particles.** *Virology* 2003, **305**(2):428-435.
387. Adkins B, Leclerc C, Marshall-Clarke S: **Neonatal adaptive immunity comes of age.** *Nature Reviews Immunology* 2004, **4**(7):553-564.
388. PrabhuDas M, Adkins B, Gans H, King C, Levy O, Ramilo O, Siegrist C-A: **Challenges in infant immunity: implications for responses to infection and vaccines.** *Nature immunology* 2011, **12**(3):189.
389. Powell T, Streilein J: **In vitro suppression of cytotoxic T cell generation by lymphocytes from mice rendered neonatally tolerant of class II MHC alloantigens.** *Transplantation* 1991, **52**(2):383-386.
390. Fadel SA, Cowell LG, Cao S, Ozaki DA, Kepler TB, Steeber DA, Sarzotti M: **Neonate-primed CD8+ memory cells rival adult-primed memory cells in antigen-driven expansion and anti-viral protection.** *International immunology* 2006, **18**(2):249-257.
391. Rowe J, Macaubas C, Monger T, Holt B, Harvey J, Poolman J, Sly P, Holt P: **Antigen-specific responses to diphtheria-tetanus-acellular pertussis vaccine in human infants are initially Th2 polarized.** *Infection and immunity* 2000, **68**(7):3873-3877.
392. Ota MO, Vekemans J, Schlegel-Haueter SE, Fielding K, Sanneh M, Kidd M, Newport MJ, Aaby P, Whittle H, Lambert P-H: **Influence of *Mycobacterium bovis* bacillus Calmette-Guerin on antibody and cytokine responses to human neonatal vaccination.** *The Journal of Immunology* 2002, **168**(2):919-925.
393. Gans H, Yasukawa L, Rinki M, DeHovitz R, Forghani B, Beeler J, Audet S, Maldonado Y, Arvin AM: **Immune responses to measles and mumps vaccination of infants at 6, 9, and 12 months.** *Journal of Infectious Diseases* 2001, **184**(7):817-826.
394. Hussey GD, Watkins ML, Goddard EA, Gottschalk S, Hughes EJ, Iloni K, Kibel MA, Ress SR: **Neonatal mycobacterial specific cytotoxic T-lymphocyte and cytokine profiles in response to distinct BCG vaccination strategies.** *Immunology* 2002, **105**(3):314-324.
395. Marchant A, Goetghebuer T, Ota MO, Wolfe I, Ceesay SJ, De Groote D, Corrah T, Bennett S, Wheeler J, Huygen K: **Newborns develop a Th1-type immune response to *Mycobacterium bovis* bacillus Calmette-Guerin vaccination.** *The Journal of Immunology* 1999, **163**(4):2249-2255.
396. Vekemans J, Amedei A, Ota MO, D'Elis MM, Goetghebuer T, Ismaili J, Newport MJ, Del Prete G, Goldman M, McAdam KP: **Neonatal bacillus Calmette-Guérin vaccination induces adult-like IFN- γ production by CD4+ T lymphocytes.** *European journal of immunology* 2001, **31**(5):1531-1535.
397. Mascart F, Verscheure V, Malfroot A, Hainaut M, Piérard D, Temerman S, Peltier A, Debré A-S, Levy J, Del Giudice G: ***Bordetella pertussis* infection in 2-month-old infants promotes type 1 T cell responses.** *The Journal of Immunology* 2003, **170**(3):1504-1509.
398. Marchant A, Appay V, van der Sande M, Dulphy N, Liesnard C, Kidd M, Kaye S, Ojuola O, Gillespie GM, Cuero ALV: **Mature CD8+ T lymphocyte response to viral infection during fetal life.** *The Journal of clinical investigation* 2003, **111**(11):1747-1755.
399. Martinez X, Brandt C, Saddallah F, Tougne C, Barrios C, Wild F, Dougan G, Lambert P-H, Siegrist C-A: **DNA immunization circumvents deficient induction of T helper type 1 and cytotoxic T lymphocyte responses in neonates and during early life.** *Proceedings of the National Academy of Sciences* 1997, **94**(16):8726-8731.
400. Henderson RA, Watkins SC, Flynn J: **Activation of human dendritic cells following infection with *Mycobacterium tuberculosis*.** *The Journal of Immunology* 1997, **159**(2):635-643.
401. Tonon S, Goriely S, Aksoy E, Pradier O, Del Giudice G, Trannoy E, Willems F, Goldman M, De Wit D: ***Bordetella pertussis* toxin induces the release of inflammatory cytokines and dendritic cell activation in whole blood: impaired responses in human newborns.** *European journal of immunology* 2002, **32**(11):3118-3125.
402. D'Alessandro U: **A phase 3 trial of RTS, S/AS01 malaria vaccine in African infants.** *Name: New England Journal of Medicine* 2012, **367**:2284-2295.
403. Rts S: **Efficacy and safety of the RTS, S/AS01 malaria vaccine during 18 months after vaccination: a phase 3 randomized, controlled trial in children and young infants at 11 African sites.** *PLoS medicine* 2014, **11**(7):e1001685.
404. Tiono AB, Kangoye DT, Rehman AM, Kargougou DG, Kaboré Y, Diarra A, Ouedraogo E, Nébélé I, Ouédraogo A, Okech B: **Malaria incidence in children in South-West Burkina Faso: comparison of active and passive case detection methods.** *PLoS One* 2014, **9**(1):e86936.
405. Ouédraogo A, Tiono AB, Diarra A, Sanon S, Yaro JB, Ouedraogo E, Bougouma EC, Soulama I, Gansané A, Ouedraogo A: **Malaria morbidity in high and seasonal malaria transmission area of Burkina Faso.** *PLoS One* 2013, **8**(1):e50036.

406. Hanekom WA, Hughes J, Mavinkurve M, Mendillo M, Watkins M, Gamielien H, Gelderbloem SJ, Sidibana M, Mansoor N, Davids V: **Novel application of a whole blood intracellular cytokine detection assay to quantitate specific T-cell frequency in field studies.** *Journal of immunological methods* 2004, **291**(1):185-195.
407. Ndhlovu ZM, Angenendt M, Heckel D, Schneck JP, Griffin DE, Oelke M: **Development of an Artificial-Antigen-Presenting-Cell-Based Assay for the Detection of Low-Frequency Virus-Specific CD8+ T Cells in Whole Blood, with Application for Measles Virus.** *Clinical and Vaccine Immunology* 2009, **16**(7):1066-1073.
408. Meddows-Taylor S, Shalekoff S, Kuhn L, Gray GE, Tiemessen CT: **Development of a whole blood intracellular cytokine staining assay for mapping CD4+ and CD8+ T-cell responses across the HIV-1 genome.** *Journal of virological methods* 2007, **144**(1):115-121.
409. Maecker H, Rinfret A, D'Souza P, Darden J, Roig E, Landry C, Hayes P, Birungi J, Anzala O, Garcia M: **Standardization of cytokine flow cytometry assays.** *BMC immunology* 2005, **6**(1):13.
410. Shearer WT, Rosenblatt HM, Gelman RS, Oyomopito R, Plaeger S, Stiehm ER, Wara DW, Douglas SD, Luzuriaga K, McFarland EJ: **Lymphocyte subsets in healthy children from birth through 18 years of age: the Pediatric AIDS Clinical Trials Group P1009 study.** *Journal of Allergy and Clinical Immunology* 2003, **112**(5):973-980.
411. Halsey N, Galazka A: **The efficacy of DPT and oral poliomyelitis immunization schedules initiated from birth to 12 weeks of age.** *Bulletin of the World Health Organization* 1985, **63**(6):1151.
412. Gans HA, Arvin AM, Galinus J, Logan L, DeHovitz R, Maldonado Y: **Deficiency of the humoral immune response to measles vaccine in infants immunized at age 6 months.** *Jama* 1998, **280**(6):527-532.
413. Hermann E, Truyens C, Alonso-Vega C, Even J, Rodriguez P, Berthe A, Gonzalez-Merino E, Torrico F, Carlier Y: **Human fetuses are able to mount an adultlike CD8 T-cell response.** *Blood* 2002, **100**(6):2153-2158.
414. Ceesay SJ, Casals-Pascual C, Nwakanma DC, Walther M, Gomez-Escobar N, Fulford AJ, Takem EN, Nogaro S, Bojang KA, Corrah T: **Continued decline of malaria in The Gambia with implications for elimination.** *PloS one* 2010, **5**(8):e12242.
415. Ho M, Webster HK, Looareesuwan S, Supanaranond W, Phillips RE, Chanthavanich P, Warrell DA: **Antigen-Specific Immunosuppression in Human Malaria Due to *Plasmodium falciparum*.** *The Journal of Infectious Diseases* 1986, **153**(4):763-771.
416. Millington OR, Gibson VB, Rush CM, Zinselmeyer BH, Phillips RS, Garside P, Brewer JM: **Malaria impairs T cell clustering and immune priming despite normal signal 1 from dendritic cells.** *PLoS Pathog* 2007, **3**(10):e143.
417. Bliss CM, Drammeh A, Bowyer G, Sanou GS, Jagne YJ, Ouedraogo O, Edwards NJ, Tarama C, Ouedraogo N, Ouedraogo M *et al*: **Viral Vector Malaria Vaccines Induce High-Level T Cell and Antibody Responses in West African Children and Infants.** *Molecular Therapy*.
418. Ford T: **Cryopreservation-related loss of antigen-specific IFN γ producing CD4+ T-cells can skew immunogenicity data in vaccine trials: lessons from a Malaria vaccine trial substudy.** *Vaccine* 2017, **In press**.
419. Ansong D, Asante KP, Vekemans J, Owusu SK, Owusu R, Brobby NA, Dosoo D, Osei-Akoto A, Osei-Kwakye K, Asafo-Adjei E: **T cell responses to the RTS, S/AS01 E and RTS, S/AS02 D malaria candidate vaccines administered according to different schedules to Ghanaian children.** *PLoS One* 2011, **6**(4):e18891.
420. Hawkrigde T, Scriba TJ, Gelderbloem S, Smit E, Tameris M, Moyo S, Lang T, Veldsman A, Hatherill M, van der Merwe L: **Safety and immunogenicity of a new tuberculosis vaccine, MVA85A, in healthy adults in South Africa.** *Journal of Infectious Diseases* 2008, **198**(4):544-552.
421. Tameris MD, Hatherill M, Landry BS, Scriba TJ, Snowden MA, Lockhart S, Shea JE, McClain JB, Hussey GD, Hanekom WA: **Safety and efficacy of MVA85A, a new tuberculosis vaccine, in infants previously vaccinated with BCG: a randomised, placebo-controlled phase 2b trial.** *The Lancet* 2013, **381**(9871):1021-1028.
422. Moormann AM: **How might infant and paediatric immune responses influence malaria vaccine efficacy?** *Parasite immunology* 2009, **31**(9):547-559.
423. Howie SR: **Blood sample volumes in child health research: review of safe limits.** *Bulletin of the World Health Organization* 2011, **89**(1):46-53.
424. Bejon P, Mwacharo J, Kai O, Mwangi T, Milligan P, Todryk S, Keating S, Lang T, Lowe B, Gikonyo C: **A phase 2b randomised trial of the candidate malaria vaccines FP9 ME-TRAP and MVA ME-TRAP among children in Kenya.** *PLOS Clin Trial* 2006, **1**(6):e29.

425. Bejon P, Ogada E, Mwangi T, Milligan P, Lang T, Fegan G, Gilbert SC, Peshu N, Marsh K, Hill AV: **Extended follow-up following a phase 2b randomized trial of the candidate malaria vaccines FP9 ME-TRAP and MVA ME-TRAP among children in Kenya.** *PLoS One* 2007, **2**(8):e707.
426. Mensah VA, Gueye A, Ndiaye M, Edwards NJ, Wright D, Anagnostou NA, Syll M, Ndaw A, Abiola A, Bliss C: **Safety, Immunogenicity and Efficacy of Prime-Boost Vaccination with ChAd63 and MVA Encoding ME-TRAP against *Plasmodium falciparum* Infection in Adults in Senegal.** *PLoS One* 2016, **11**(12):e0167951.
427. Bliss CM, Drammeh A, Bowyer G, Sanou GS, Jagne YJ, Ouedraogo O, Edwards NJ, Tarama C, Ouedraogo N, Ouedraogo M: **Viral Vector Malaria Vaccines Induce High-Level T Cell and Antibody Responses in West African Children and Infants.** *Molecular Therapy*.
428. Masopust D, Ha S-J, Vezys V, Ahmed R: **Stimulation history dictates memory CD8 T cell phenotype: implications for prime-boost vaccination.** *The Journal of Immunology* 2006, **177**(2):831-839.
429. Barnes E, Folgori A, Capone S, Swadling L, Aston S, Kurioka A, Meyer J, Huddart R, Smith K, Townsend R: **Novel adenovirus-based vaccines induce broad and sustained T cell responses to HCV in man.** *Science translational medicine* 2012, **4**(115):115ra111-115ra111.
430. Reyes-Sandoval A, Fitzgerald J, Grant R, Roy S, Xiang Z, Li Y, Gao G, Wilson J, Ertl H: **Human immunodeficiency virus type 1-specific immune responses in primates upon sequential immunization with adenoviral vaccine carriers of human and simian serotypes.** *Journal of virology* 2004, **78**(14):7392-7399.
431. Capone S, Reyes-Sandoval A, Naddeo M, Siani L, Ammendola V, Rollier CS, Nicosia A, Colloca S, Cortese R, Folgori A: **Immune responses against a liver-stage malaria antigen induced by simian adenoviral vector AdCh63 and MVA prime-boost immunisation in non-human primates.** *Vaccine* 2010, **29**(2):256-265.
432. Darrah PA, Patel DT, De Luca PM, Lindsay RW, Davey DF, Flynn BJ, Hoff ST, Andersen P, Reed SG, Morris SL: **Multifunctional TH1 cells define a correlate of vaccine-mediated protection against *Leishmania major*.** *Nature medicine* 2007, **13**(7):843-850.
433. Makedonas G, Betts MR: **Polyfunctional analysis of human t cell responses: importance in vaccine immunogenicity and natural infection.** In: *Springer seminars in immunopathology: 2006*: Springer; 2006: 209-219.
434. Hermanson G, Chun S, Felgner J, Tan X, Pablo J, Nakajima-Sasaki R, Molina DM, Felgner PL, Liang X, Davies DH: **Measurement of antibody responses to modified vaccinia virus Ankara (MVA) and Dryvax® using proteome microarrays and development of recombinant protein ELISAs.** *Vaccine* 2012, **30**(3):614-625.
435. Betts MR, Brenchley JM, Price DA, De Rosa SC, Douek DC, Roederer M, Koup RA: **Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation.** *Journal of Immunological Methods* 2003, **281**(1-2):65-78.
436. Cooper AM, Dalton DK, Stewart TA, Griffin J, Russell D, Orme I: **Disseminated tuberculosis in interferon gamma gene-disrupted mice.** *The Journal of experimental medicine* 1993, **178**(6):2243-2247.
437. Stahl P, Ruppert V, Schwarz RT, Meyer T: **Trypanosoma cruzi evades the protective role of interferon-gamma-signaling in parasite-infected cells.** *PloS one* 2014, **9**(10):e110512.
438. Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart TA, Bloom BR: **An essential role for interferon gamma in resistance to *Mycobacterium tuberculosis* infection.** *The Journal of experimental medicine* 1993, **178**(6):2249-2254.
439. Pied S, Roland J, Louise A, Voegtli D, Soulard V, Mazier D, Cazenave P-A: **Liver CD4- CD8- NK1. 1+ TCRαβ intermediate cells increase during experimental malaria infection and are able to exhibit inhibitory activity against the parasite liver stage *in vitro*.** *The Journal of Immunology* 2000, **164**(3):1463-1469.
440. Blackwell J: **Genetic susceptibility to leishmanial infections: studies in mice and man.** *Parasitology* 1996, **112**(S1):S67-S74.
441. Bloom BR, Flynn J, McDonough K, Kress Y, Chan J: **Experimental approaches to mechanisms of protection and pathogenesis in *M. tuberculosis* infection.** *Immunobiology* 1994, **191**(4-5):526-536.
442. Bogdan C, Moll H, Solbach W, Röllinghoff M: **Tumor necrosis factor-α in combination with interferon-γ, but not with interleukin 4 activates murine macrophages for elimination of *Leishmania major* amastigotes.** *European journal of immunology* 1990, **20**(5):1131-1135.
443. Liew F, Li Y, Millott S: **Tumor necrosis factor-alpha synergizes with IFN-gamma in mediating killing of *Leishmania major* through the induction of nitric oxide.** *The Journal of Immunology* 1990, **145**(12):4306-4310.

444. Chan J, Xing Y, Magliozzo RS, Bloom BR: **Killing of virulent *Mycobacterium tuberculosis* by reactive nitrogen intermediates produced by activated murine macrophages.** *The Journal of experimental medicine* 1992, **175**(4):1111-1122.
445. Stanley DA, Honko AN, Asiedu C, Trefry JC, Lau-Kilby AW, Johnson JC, Hensley L, Ammendola V, Abbate A, Grazioli F: **Chimpanzee adenovirus vaccine generates acute and durable protective immunity against ebolavirus challenge.** *Nature medicine* 2014, **20**(10):1126-1129.
446. Nookala S, Srinivasan S, Kaliraj P, Narayanan RB, Nutman TB: **Impairment of Tetanus-Specific Cellular and Humoral Responses following Tetanus Vaccination in Human Lymphatic Filariasis.** *Infection and Immunity* 2004, **72**(5):2598-2604.
447. Cooper P, Espinel I, Paredes W, Guderian R, Nutman T: **Impaired tetanus-specific cellular and humoral responses following tetanus vaccination in human onchocerciasis: a possible role for interleukin-10.** *Journal of Infectious Diseases* 1998, **178**(4):1133-1138.
448. Nacher M: **Malaria vaccine trials in a wormy world.** *Trends in parasitology* 2001, **17**(12):563-565.
449. Yin J, Vahey MT, Dai A, Lewis MG, Arango T, Yalley-Ogunro J, Greenhouse J, Mendoza K, Khan A, Sardesai NY: ***Plasmodium inui* infection reduces the efficacy of a simian immunodeficiency virus DNA vaccine in a rhesus macaque model through alteration of the vaccine-induced immune response.** *Journal of Infectious Diseases* 2012, **206**(4):523-533.
450. Moorthy VS, Imoukhuede EB, Keating S, Pinder M, Webster D, Skinner MA, Gilbert SC, Walraven G, Hill AV: **Phase 1 evaluation of 3 highly immunogenic prime-boost regimens, including a 12-month reboosting vaccination, for malaria vaccination in Gambian men.** *Journal of Infectious Diseases* 2004, **189**(12):2213-2219.
451. Manuel ER, Yeh WW, Seaman MS, Furr K, Lifton MA, Hulot SL, Autissier P, Letvin NL: **Dominant CD8+ T-lymphocyte responses suppress expansion of vaccine-elicited subdominant T lymphocytes in rhesus monkeys challenged with pathogenic simian-human immunodeficiency virus.** *Journal of virology* 2009, **83**(19):10028-10035.
452. McElrath MJ, De Rosa SC, Moodie Z, Dubey S, Kierstead L, Janes H, Defawe OD, Carter DK, Hural J, Akondy R: **HIV-1 vaccine-induced immunity in the test-of-concept Step Study: a case-cohort analysis.** *The Lancet* 2008, **372**(9653):1894-1905.
453. Wizel B HR, Parker KC, Coligan JE, Church P, Gordon DM, Ballou WR, Hoffman SL: **Irradiated sporozoite vaccine induces HLA-B8-restricted cytotoxic T lymphocyte responses against two overlapping epitopes of the *Plasmodium falciparum* sporozoite surface protein 2.** *The Journal of Experimental Medicine* 1995, **182**(5):1435-1445.
454. Davis AG, Huff CG, Palmer TT: **Procedures for maximum production of exoerythrocytic stages of *Plasmodium fallax* in tissue culture.** *Experimental parasitology* 1966, **19**(1):1-8.
455. Strome C, De Santis P, Beaudoin R: **The cultivation of the exoerythrocytic stages of *Plasmodium berghei* from sporozoites.** *In vitro* 1979, **15**(7):531-536.
456. Sinden R, Smith J: **Culture of the liver stages (exoerythrocytic schizonts) of rodent malaria parasites from sporozoites in vitro.** *Transactions of the Royal Society of Tropical Medicine and Hygiene* 1980, **74**(1):134-136.
457. Lambiotte M, Landau I, Thierry N, Miltgen F: **Development of schizonts in cultured hepatocytes of adult rats after in vitro infection with *Plasmodium yoelii* sporozoites.** *Comptes rendus des séances de l'Académie des sciences Série III, Sciences de la vie* 1981, **293**(8):431.
458. Pirson P: **Culture of the exoerythrocytic liver stages of *Plasmodium berghei* sporozoites in rat hepatocytes.** *Transactions of the Royal Society of Tropical Medicine and Hygiene* 1982, **76**(3):422.
459. Mazier D, Landau I, Miltgen F, Druilhe P, Lambiotte M, Baccam D, Gentilini M: **[In vitro infestation of adult thamnomy's hepatocytes with *Plasmodium yoelii* sporozoites; schizogony and release of infecting merozoites].** *Comptes rendus des seances de l'Academie des sciences Serie III, Sciences de la vie* 1982, **294**(19):963-965.
460. Hollingdale MR, Leland P, Schwartz AL: **In vitro cultivation of the exoerythrocytic stage of *Plasmodium berghei* in a hepatoma cell line.** *The American journal of tropical medicine and hygiene* 1983, **32**(4):682.
461. Calvocalle J, Moreno A, Eling W, Nardin E: **In vitro development of infectious liver stages of *P. yoelii* and *P. berghei* malaria in human cell lines.** *Experimental parasitology* 1994, **79**(3):362-373.
462. Long GW, Leath S, Schuman R, Hollingdale MR, Ballou WR, Sim BKL, Hoffman SL: **Cultivation of the exoerythrocytic stage of *Plasmodium berghei* in primary cultures of mouse hepatocytes and continuous mouse cell lines.** *In vitro cellular & developmental biology* 1989, **25**(9):857-862.

463. Mazier D, Beaudoin R, Mellouk S, Druilhe P, Texier B, Trosper J, Miltgen F, Landau I, Paul C, Brandicourt O: **Complete development of hepatic stages of *Plasmodium falciparum* in vitro**. *Science* 1985, **227**(4685):440-442.
464. Karnasuta C, Pavanand K, Chantakulkij S, Luttiwongsakorn N, Rassamesoraj M, Laohathai K, Webster HK, Watt G: **Complete development of the liver stage of *Plasmodium falciparum* in a human hepatoma cell line**. *The American journal of tropical medicine and hygiene* 1995, **53**(6):607.
465. Sattabongkot J, Yimamnuaychoke N, Leelaudomlipi S, Rasameesoraj M, Jenwithisuk R, Coleman RE, Udomsangpetch R, Cui L, Brewer TG: **Establishment of a human hepatocyte line that supports in vitro development of the exo-erythrocytic stages of the malaria parasites *Plasmodium falciparum* and *P. vivax***. *The American journal of tropical medicine and hygiene* 2006, **74**(5):708-715.
466. Prudêncio M, Mota MM, Mendes AM: **A toolbox to study liver stage malaria**. *Trends in parasitology* 2011, **27**(12):565-574.
467. Trager W, Jensen JB: **Human malaria parasites in continuous culture**. *Science* 1976, **193**(4254):673-675.
468. Kennedy MC, Wang J, Zhang Y, Miles AP, Chitsaz F, Saul A, Long CA, Miller LH, Stowers AW: **In vitro studies with recombinant *Plasmodium falciparum* apical membrane antigen 1 (AMA1): production and activity of an AMA1 vaccine and generation of a multiallelic response**. *Infection and immunity* 2002, **70**(12):6948-6960.
469. Duncan CJ, Hill AV, Ellis RD: **Can growth inhibition assays (GIA) predict blood-stage malaria vaccine efficacy?** *Human vaccines & immunotherapeutics* 2012, **8**(6):706-714.
470. Mellouk S, Berbiguier N, Druilhe P, Sedegah M, Galey B, Yuan L, Leef M, Charoenvit Y, Paul C, Hoffman S: **Evaluation of an in vitro assay aimed at measuring protective antibodies against sporozoites**. *Bulletin of the World Health Organization* 1990, **68**(Suppl):52.
471. House BL, Hollingdale MR, Sacchi Jr JB, Richie TL: **Functional immunoassays using a in vitro malaria liver-stage infection model: where do we go from here?** *Trends in parasitology* 2009, **25**(11):525-533.
472. Rénia L, Grillot D, Marussig M, Corradin G, Miltgen F, Lambert P, Mazier D, Del Giudice G: **Effector functions of circumsporozoite peptide-primed CD4+ T cell clones against *Plasmodium yoelii* liver stages**. *The Journal of Immunology* 1993, **150**(4):1471-1478.
473. Weiss W, Mellouk S, Houghten RA, Sedegah M, Kumar S, Good M, Berzofsky J, Miller L, Hoffman S: **Cytotoxic T cells recognize a peptide from the circumsporozoite protein on malaria-infected hepatocytes**. *The Journal of experimental medicine* 1990, **171**(3):763-773.
474. Trimnell A, Takagi A, Gupta M, Richie TL, Kappe SH, Wang R: **Genetically attenuated parasite vaccines induce contact-dependent CD8+ T cell killing of *Plasmodium yoelii* liver stage-infected hepatocytes**. *The Journal of Immunology* 2009, **183**(9):5870-5878.
475. Hügél F-U, Pradel G, Frevert U: **Release of malaria circumsporozoite protein into the host cell cytoplasm and interaction with ribosomes**. *Molecular and biochemical parasitology* 1996, **81**(2):151-170.
476. Desai SA, Rosenberg RL: **Pore size of the malaria parasite's nutrient channel**. *Proceedings of the National Academy of Sciences* 1997, **94**(5):2045-2049.
477. Hoffman SL, Doolan DL: **Malaria vaccines—targeting infected hepatocytes**. *Nature medicine* 2000, **6**(11):1218-1219.
478. Robson K, Frevert U, Reckmann I, Cowan G, Beier J, Scragg I, Takehara K, Bishop D, Pradel G, Sinden R: **Thrombospondin-related adhesive protein (TRAP) of *Plasmodium falciparum*: expression during sporozoite ontogeny and binding to human hepatocytes**. *The EMBO journal* 1995, **14**(16):3883.
479. Ejigiri I, Ragheb DR, Pino P, Coppi A, Bennett BL, Soldati-Favre D, Sinnis P: **Shedding of TRAP by a rhomboid protease from the malaria sporozoite surface is essential for gliding motility and sporozoite infectivity**. *PLoS Pathog* 2012, **8**(7):e1002725.
480. Kurokohchi K, Carrington M, Mann DL, Simonis TB, Alexander-Miller MA, Feinstone SM, Akatsuka T, Berzofsky JA: **Expression of HLA class I molecules and the transporter associated with antigen processing in hepatocellular carcinoma**. *Hepatology* 1996, **23**(5):1181-1188.
481. Boegel S, Löwer M, Bukur T, Sahin U, Castle JC: **A catalog of HLA type, HLA expression, and neo-epitope candidates in human cancer cell lines**. *Oncolimmunology* 2014, **3**(8):e954893.
482. Prudêncio M, Rodrigues CD, Ataíde R, Mota MM: **Dissecting in vitro host cell infection by *Plasmodium* sporozoites using flow cytometry**. *Cellular microbiology* 2008, **10**(1):218-224.
483. Rodrigues T, Prudêncio M, Moreira R, Mota MM, Lopes F: **Targeting the liver stage of malaria parasites: a yet unmet goal**. *Journal of medicinal chemistry* 2011, **55**(3):995-1012.

484. Parvanova I, Epiphany S, Fauq A, Golde TE, Prudêncio M, Mota MM: **A small molecule inhibitor of signal peptide peptidase inhibits *Plasmodium* development in the liver and decreases malaria severity.** *PLoS one* 2009, **4**(4):e5078.
485. Lupton EJ, Roth A, Patrapuvich R, Maher SP, Singh N, Sattabongkot J, Adams JH: **Enhancing longevity of *Plasmodium vivax* and *P. falciparum* sporozoites after dissection from mosquito salivary glands.** *Parasitology international* 2015, **64**(2):211-218.
486. Sheldon S, Poulton K: **HLA typing and its influence on organ transplantation.** In: *Transplantation Immunology*. Springer; 2006: 157-174.
487. Abi-Rached L, Jobin MJ, Kulkarni S, McWhinnie A, Dalva K, Gragert L, Babrzadeh F, Gharizadeh B, Luo M, Plummer FA: **The shaping of modern human immune systems by multiregional admixture with archaic humans.** *Science* 2011, **334**(6052):89-94.
488. Calarota SA, Baldanti F: **Enumeration and characterization of human memory T cells by enzyme-linked immunospot assays.** *Clinical and Developmental Immunology* 2013, **2013**.
489. FREDERICK M, GRIMM E, KROHN E, SMID C, YU T-K: **Cytokine-induced cytotoxic function expressed by lymphocytes of the innate immune system: distinguishing characteristics of NK and LAK based on functional and molecular markers.** *Journal of interferon & cytokine research* 1997, **17**(8):435-447.
490. Naume B, Nonstad U, Steinkjer B, Funderud S, Smeland E, Espevik T: **Immunomagnetic isolation of NK and LAK cells.** *Journal of immunological methods* 1991, **136**(1):1-9.
491. He X-S, Mahmood K, Maecker HT, Holmes TH, Kemble GW, Arvin AM, Greenberg HB: **Analysis of the frequencies and of the memory T cell phenotypes of human CD8+ T cells specific for influenza A viruses.** *Journal of Infectious Diseases* 2003, **187**(7):1075-1084.
492. Bednarek MA, Sauma S, Gammon M, Porter G, Tamhankar S, Williamson A, Zweerink H: **The minimum peptide epitope from the influenza virus matrix protein. Extra and intracellular loading of HLA-A2.** *The Journal of Immunology* 1991, **147**(12):4047-4053.
493. Morrison J, Elvin J, Latron F, Gotch F, Moots R, Strominger JL, McMichael A: **Identification of the nonamer peptide from influenza A matrix protein and the role of pockets of HLA-A2 in its recognition by cytotoxic T lymphocytes.** *European journal of immunology* 1992, **22**(4):903-907.
494. Ma J, Trop S, Baer S, Rakhmanaliev E, Arany Z, Dumoulin P, Zhang H, Romano J, Coppens I, Levitsky V: **Dynamics of the major histocompatibility complex class I processing and presentation pathway in the course of malaria parasite development in human hepatocytes: implications for vaccine development.** *PLoS one* 2013, **8**(9):e75321.
495. Austin LS, Kaushansky A, Kappe SH: **Susceptibility to *Plasmodium* liver stage infection is altered by hepatocyte polyploidy.** *Cellular microbiology* 2014, **16**(5):784-795.
496. Geary TG, Jensen JB: **Effects of antibiotics on *Plasmodium falciparum* in vitro.** *The American journal of tropical medicine and hygiene* 1983, **32**(2):221-225.
497. Hegge S, Kudryashev M, Barniol L, Frischknecht F: **Key factors regulating *Plasmodium berghei* sporozoite survival and transformation revealed by an automated visual assay.** *The FASEB Journal* 2010, **24**(12):5003-5012.
498. O'flaherty E, Wong WK, Pettit S, Seymour K, Ali S, Kirby J: **Regulation of T-cell apoptosis: a mixed lymphocyte reaction model.** *Immunology* 2000, **100**(3):289-299.
499. Adams AB, Williams MA, Jones TR, Shirasugi N, Durham MM, Kaech SM, Wherry EJ, Onami T, Lanier JG, Kokko KE: **Heterologous immunity provides a potent barrier to transplantation tolerance.** *The Journal of clinical investigation* 2003, **111**(12):1887-1895.
500. Burrows S, Khanna R, Burrows J, Moss D: **An alloresponse in humans is dominated by cytotoxic T lymphocytes (CTL) cross-reactive with a single Epstein-Barr virus CTL epitope: implications for graft-versus-host disease.** *The Journal of experimental medicine* 1994, **179**(4):1155-1161.
501. Gamadia LE, Remmerswaal EB, Surachno S, Lardy NM, Wertheim-van Dillen PM, van Lier RA, ten Berge IJ: **Cross-reactivity of cytomegalovirus-specific CD8+ T cells to allo-major histocompatibility complex class I molecules.** *Transplantation* 2004, **77**(12):1879-1885.
502. Weissler JC, Yarbrough W, Toews GB, Nicod LP: **Human natural killer cells enhance a mixed leukocyte reaction.** *Journal of leukocyte biology* 1988, **43**(4):291-298.
503. Kim S, Poursine-Laurent J, Truscott SM, Lybarger L, Song Y-J, Yang L, French AR, Sunwoo JB, Lemieux S, Hansen TH: **Licensing of natural killer cells by host major histocompatibility complex class I molecules.** *Nature* 2005, **436**(7051):709-713.
504. Janetzki S, Britten CM, Kalos M, Levitsky HI, Maecker HT, Melief CJ, Old LJ, Romero P, Hoos A, Davis MM: **"MIATA"—minimal information about T cell assays.** *Immunity* 2009, **31**(4):527-528.

505. Schmidt NW, Butler NS, Badovinac VP, Harty JT: **Extreme CD8 T cell requirements for anti-malarial liver-stage immunity following immunization with radiation attenuated sporozoites.** *PLoS Pathog* 2010, **6**(7):e1000998.
506. Morrot A, Zavala F: **Effector and memory CD8+ T cells as seen in immunity to malaria.** *Immunological reviews* 2004, **201**(1):291-303.
507. Renggli J, Hahne M, Matile H, Betschart B, Tschopp J, Corradin G: **Elimination of *P. berghei* liver stages is independent of Fas (CD95/Apo-I) or perforin-mediated cytotoxicity.** *Parasite immunology* 1997, **19**(3):145-148.
508. Butler NS, Schmidt NW, Harty JT: **Differential effector pathways regulate memory CD8 T cell immunity against *Plasmodium berghei* versus *P. yoelii* sporozoites.** *The Journal of Immunology* 2010, **184**(5):2528-2538.
509. Mellouk S, Green SJ, Nacy CA, Hoffman SL: **IFN-gamma inhibits development of *Plasmodium berghei* exoerythrocytic stages in hepatocytes by an L-arginine-dependent effector mechanism.** *The Journal of immunology* 1991, **146**(11):3971-3976.
510. Weiss WR, Berzofsky J, Houghten RA, Sedegah M, Hollindale M, Hoffman SL: **AT cell clone directed at the circumsporozoite protein which protects mice against both *Plasmodium yoelii* and *Plasmodium berghei*.** *The Journal of Immunology* 1992, **149**(6):2103-2109.
511. Mueller A-K, Deckert M, Heiss K, Goetz K, Matuschewski K, Schlüter D: **Genetically attenuated *Plasmodium berghei* liver stages persist and elicit sterile protection primarily via CD8 T cells.** *The American journal of pathology* 2007, **171**(1):107-115.
512. Mellouk S, HOUGHTEN RA, SEDEGAH M, KUMAR S, GOOD SMF, MILLER JABLH: **Cytotoxic T cells recognize a peptide from the circumsporozoite protein on malaria-infected hepatocytes.** *The Journal of experimental medicine* 1990, **171**:763-773.
513. Doolan DL, Sedegah M, Hedstrom RC, Hobart P, Charoenvit Y, Hoffman SL: **Circumventing genetic restriction of protection against malaria with multigene DNA immunization: CD8+ T cell-, interferon gamma-, and nitric oxide-dependent immunity.** *Journal of Experimental Medicine* 1996, **183**(4):1739-1746.
514. Vreden SG, Blok WL, Sauerwein RW, Oettinger MC, Verhave J-P, Meuwissen JE, Meer JWVD, Broek MFVD: **Inhibition of *Plasmodium berghei* liver schizont development and reduction of cytokine production capacity in rats by dietary fish oil supplementation.** *American Journal of Tropical Medicine and Hygiene* 1995, **53**:206-206.
515. Vreden SG, Van den Broek MF, Oettinger MC, Verhave JP, Meuwissen JH, Sauerwein RW: **Cytokines inhibit the development of liver schizonts of the malaria parasite *Plasmodium berghei* in vivo.** *European journal of immunology* 1992, **22**(9):2271-2275.
516. Nussler A, Pied S, Goma J, Réhia L, Miltgen F, Grau GE, Mazier D: **TNF inhibits malaria hepatic stages *in vitro* via synthesis of IL-6.** *International immunology* 1991, **3**(4):317-321.
517. Depinay N, Franetich JF, Grüner AC, Mauduit M, Chavatte J-M, Luty AJ, van Gemert G-J, Sauerwein RW, Siksik J-M, Hannoun L: **Inhibitory effect of TNF-α on malaria pre-erythrocytic stage development: influence of host hepatocyte/parasite combinations.** *PLoS One* 2011, **6**(3):e17464.
518. Schofield L, Ferreira A, Altszuler R, Nussenzweig V, Nussenzweig R: **Interferon-gamma inhibits the intrahepatocytic development of malaria parasites *in vitro*.** *The Journal of Immunology* 1987, **139**(6):2020-2025.
519. Davies C, Pudney M, Nicholas J, Sinden R: **The novel hydroxynaphthoquinone 566C80 inhibits the development of liver stages of *Plasmodium berghei* cultured *in vitro*.** *Parasitology* 1993, **106**(01):1-6.
520. Davies C, Pudney M, Matthews P, Sinden R: **The causal prophylactic activity of the novel hydroxynaphthoquinone 566C80 against *Plasmodium berghei* infections in rats.** *Acta Leidensia* 1988, **58**(2):115-128.
521. Shapiro TA, Ranasinha CD, Kumar N, Barditch-Crovo P: **Prophylactic activity of atovaquone against *Plasmodium falciparum* in humans.** *The American journal of tropical medicine and hygiene* 1999, **60**(5):831-836.
522. Radloff PD, Philips J, Nkeyi M, Kremsner P, Hutchinson D: **Atovaquone and proguanil for *Plasmodium falciparum* malaria.** *The Lancet* 1996, **347**(9014):1511-1514.
523. LaCrue AN, Sáenz FE, Cross RM, Udenze KO, Monastyrskyi A, Stein S, Mutka TS, Manetsch R, Kyle DE: **4 (1H)-Quinolones with liver stage activity against *Plasmodium berghei*.** *Antimicrobial agents and chemotherapy* 2013, **57**(1):417-424.
524. Bates MD, Meshnick SR, Sigler CI, Leland P, Hollingdale MR: ***In vitro* effects of primaquine and primaquine metabolites on exoerythrocytic stages of *Plasmodium berghei*.** *The American journal of tropical medicine and hygiene* 1990, **42**(6):532-537.

525. Horras CJ, Lamb CL, Mitchell KA: **Regulation of hepatocyte fate by interferon- γ .** *Cytokine & growth factor reviews* 2011, **22**(1):35-43.
526. Schoenborn JR, Wilson CB: **Regulation of interferon- γ during innate and adaptive immune responses.** *Advances in immunology* 2007, **96**:41-101.
527. Crispe IN: **The liver as a lymphoid organ.** *Annual review of immunology* 2009, **27**:147-163.
528. Nemeth E, Baird AW, O'Farrelly C: **Microanatomy of the liver immune system.** In: *Seminars in immunopathology: 2009*: Springer; 2009: 333.
529. Doolan DL, Hoffman SL: **IL-12 and NK cells are required for antigen-specific adaptive immunity against malaria initiated by CD8+ T cells in the *Plasmodium yoelii* model.** *The Journal of Immunology* 1999, **163**(2):884-892.
530. Itoh AM, K. Nishioji, H. Fujii, H. Nakamura, T. Kirishima, T. Toyama, N. Yamauchi, Y. Nagao, S. Narumi, T. Okanoue, Y: **Time course profile and cell-type-specific production of monokine induced by interferon- γ in concanavalin A-induced hepatic injury in mice: comparative study with interferon-inducible protein-10.** *Scandinavian journal of gastroenterology* 2001, **36**(12):1344-1351.
531. Hirano S, Iwashita Y, Sasaki A, Kai S, Ohta M, Kitano S: **Increased mRNA expression of chemokines in hepatocellular carcinoma with tumor-infiltrating lymphocytes.** *Journal of gastroenterology and hepatology* 2007, **22**(5):690-696.
532. Nagao M, Nakajima Y, Hisanaga M, Kayagaki N, Kanehiro H, Aomatsu Y, Ko S, Yagita H, Yamada T, Okumura K: **The alteration of Fas receptor and ligand system in hepatocellular carcinomas: how do hepatoma cells escape from the host immune surveillance in vivo?** *Hepatology* 1999, **30**(2):413-421.
533. Owen-Schaub LB, Radinsky R, Kruzel E, Berry K, Yonehara S: **Anti-Fas on nonhematopoietic tumors: levels of Fas/APO-1 and bcl-2 are not predictive of biological responsiveness.** *Cancer Research* 1994, **54**(6):1580-1586.
534. Leithäuser F, Dhein J, Mechtersheimer G, Koretz K, Brüderlein S, Henne C, Schmidt A, Debatin K, Krammer P, Möller P: **Constitutive and induced expression of APO-1, a new member of the nerve growth factor/tumor necrosis factor receptor superfamily, in normal and neoplastic cells.** *Laboratory investigation; a journal of technical methods and pathology* 1993, **69**(4):415-429.
535. Chowdhury D, Lieberman J: **Death by a thousand cuts: granzyme pathways of programmed cell death.** *Annual review of immunology* 2008, **26**:389.
536. Stalder T, Hahn S, Erb P: **Fas antigen is the major target molecule for CD4+ T cell-mediated cytotoxicity.** *The Journal of Immunology* 1994, **152**(3):1127-1133.
537. Marshall NB, Swain SL: **Cytotoxic CD4 T cells in antiviral immunity.** *BioMed Research International* 2011, **2011**.
538. Mazingue C, Cottrez-Detoeuf F, Louis J, Kweider M, Auriault C, Capron A: **In vitro and in vivo effects of interleukin 2 on the protozoan parasite leishmania.** *European journal of immunology* 1989, **19**(3):487-492.
539. Adib-Conquy M, Scott-Algara D, Cavaillon J-M, Souza-Fonseca-Guimaraes F: **TLR-mediated activation of NK cells and their role in bacterial/viral immune responses in mammals.** *Immunology and cell biology* 2014, **92**(3):256-262.
540. Souza-Fonseca-Guimaraes F, Adib-Conquy M, Cavaillon J-M: **Natural killer (NK) cells in antibacterial innate immunity: angels or devils?** *Molecular Medicine* 2012, **18**(2):270.
541. Bluman EM, Bartynski KJ, Avalos BR, Caligiuri MA: **Human natural killer cells produce abundant macrophage inflammatory protein-1 alpha in response to monocyte-derived cytokines.** *Journal of Clinical Investigation* 1996, **97**(12):2722.
542. Yonehara S, Ishii A, Yonehara M: **A cell-killing monoclonal antibody (anti-Fas) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor.** *The Journal of experimental medicine* 1989, **169**(5):1747-1756.
543. Itoh N, Yonehara S, Ishii A, Yonehara M, Mizushima S-I, Sameshima M, Hase A, Seto Y, Nagata S: **The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis.** *Cell* 1991, **66**(2):233-243.
544. Suda T, Takahashi T, Golstein P, Nagata S: **Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family.** *Cell* 1993, **75**(6):1169-1178.
545. Ogasawara J, Watanabe-Fukunaga R, Adachi M, Matsuzawa A, Kasugai T, Kitamura Y, Itoh N, Suda T, Nagata S: **Lethal effect of the anti-Fas antibody in mice.** *Nature* 1993, **364**(6440):806-809.
546. Holz LE, Fernandez-Ruiz D, Heath WR: **Protective immunity to liver-stage malaria.** *Clinical & Translational Immunology* 2016, **5**(10):e105.

547. Fernandez-Ruiz D, Ng WY, Holz LE, Ma JZ, Zaid A, Wong YC, Lau LS, Mollard V, Cozijnsen A, Collins N: **Liver-resident memory CD8+ T cells form a front-line defense against malaria liver-stage infection.** *Immunity* 2016, **45**(4):889-902.
548. Niehaus JZ, Miedel MT, Good M, Wyatt AN, Pak SC, Silverman GA, Luke CJ: **SERPINB12 is a slow-binding inhibitor of granzyme A and hepsin.** *Biochemistry* 2015, **54**(45):6756-6759.
549. Zaritskaya L, Shurin MR, Sayers TJ, Malyguine AM: **New flow cytometric assays for monitoring cell-mediated cytotoxicity.** *Expert review of vaccines* 2010, **9**(6):601-616.
550. Bryan NS, Grisham MB: **Methods to detect nitric oxide and its metabolites in biological samples.** *Free Radical Biology and Medicine* 2007, **43**(5):645-657.
551. Misko TP, Moore WM, Kasten TP, Nickols GA, Corbett JA, Tilton RG, McDaniel ML, Williamson JR, Currie MG: **Selective inhibition of the inducible nitric oxide synthase by aminoguanidine.** *European journal of pharmacology* 1993, **233**(1):119-125.
552. Sant'Angelo DB, Robinson E, Janeway Jr CA, Denzin LK: **Recognition of core and flanking amino acids of MHC class II-bound peptides by the T cell receptor.** *European journal of immunology* 2002, **32**(9):2510-2520.
553. Mohan JF, Unanue ER: **Unconventional recognition of peptides by T cells and the implications for autoimmunity.** *Nature Reviews Immunology* 2012, **12**(10):721-728.
554. Ertl HC, Dietzschold B, Otvos L: **T helper cell epitope of rabies virus nucleoprotein defined by tri- and tetrapeptides.** *European journal of immunology* 1991, **21**(1):1-10.
555. Hemmer B, Kondo T, Gran B, Pinilla C, Cortese I, Pascal J, Tzou A, McFarland HF, Houghten R, Martin R: **Minimal peptide length requirements for CD4+ T cell clones—implications for molecular mimicry and T cell survival.** *International immunology* 2000, **12**(3):375-383.
556. Salazar-Mather TP, Orange JS, Biron CA: **Early murine cytomegalovirus (MCMV) infection induces liver natural killer (NK) cell inflammation and protection through macrophage inflammatory protein 1 α (MIP-1 α)—dependent pathways.** *Journal of Experimental Medicine* 1998, **187**(1):1-14.
557. Morrison C: **Landmark green light for Mosquirix malaria vaccine.** In.: Nature Publishing Group; 2015.
558. Olotu A, Fegan G, Wambua J, Nyangwesio G, Leach A, Lievens M, Kaslow DC, Njuguna P, Marsh K, Bejon P: **Seven-year efficacy of RTS, S/AS01 malaria vaccine among young African children.** *New England Journal of Medicine* 2016, **374**(26):2519-2529.
559. Casimiro DR, Chen L, Fu T-M, Evans RK, Caulfield MJ, Davies M-E, Tang A, Chen M, Huang L, Harris V: **Comparative immunogenicity in rhesus monkeys of DNA plasmid, recombinant vaccinia virus, and replication-defective adenovirus vectors expressing a human immunodeficiency virus type 1 gag gene.** *Journal of virology* 2003, **77**(11):6305-6313.