

The role of antigen in the maintenance and localisation of CD8⁺ T-cells in the context of liver stage malaria



*A thesis submitted in fulfilment of the requirements for the degree
of Doctor of Philosophy*

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**University of Oxford, St Cross College
The Jenner Institute, Nuffield Department of Medicine
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Abstract

A highly effective vaccine against malaria is urgently needed, with leading vaccination strategies involving the induction of protective antigen-specific CD8⁺ T-cells via heterologous prime-boost viral vector immunization, targeting primarily the pre-erythrocytic liver stages of the *Plasmodium falciparum* lifecycle. To date, the greatest immunogenicity has been obtained through a heterologous prime boost regimen, where vaccination with an Adenoviral vector is followed 8 weeks later by a Modified Vaccinia Ankara virus (MVA) boost. Experimental work directed at providing a greater understanding of CD8⁺ T-cell memory responses induced by Ad-MVA vaccinations lead to the development of a novel vaccine strategy aimed at priming CD8⁺ T-cells in the periphery and subsequently targeting them to hepatic tissue with protein loaded poly(lactic-co-glycolic acid) nanoparticles or recombinant viral vectors. Durable Ag-specific CD8⁺ T-cells exhibiting a phenotype of tissue-resident memory T-cells were generated in the liver, with a ten-fold increase over the conventional heterologous vector regimen. Importantly, in *P. berghei* sporozoite challenge models of liver-stage malaria, this strategy was found to result in unprecedented levels of sterile protection across multiple clinically relevant antigens and mouse strains. This prime and target immunization strategy for liver-stage malaria may provide a novel general approach for prevention or immunotherapy against other hepato-trophic pathogens.

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The scientific process does not happen in a vacuum, but is instead a collaborative product of talented people and the environment they create; with that, I would like to thank those who have helped me along the way.

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in a greater contextual picture. Working with him has been an amazing experience, both challenging and incredibly rewarding, I am very grateful for this amazing opportunity I have been given.

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List of Acronyms and Abbreviations

AAV: Adeno-associated virus

Ab: Antibody

Ad: Adenovirus

AdHu5: Human Adenovirus serotype 5

Ag: Antigen

AhR: Aryl hydrocarbon receptor

APC: Antigen Presenting Cell

CD: Cluster of differentiation

ChAd63: Chimpanzee Adenovirus serotype 63

CHMI: Controlled human malaria infection

CNS: Central Nervous System

DAMPs: Damage Associated Molecular Patterns

dLN: draining Lymph Node

DMEM: Dulbecco's modified Eagle medium

DNA: Deoxyribonucleic acid

EMP: Erythrocyte Membrane Protein Family

EPhA2: EPH receptor A2 (ephrin type-A receptor 2

FRC: Fibroblastic Reticular Cell

GAP: Genetically-Attenuated Parasites

GDP: Gross Domestic Product

GIA: Growth inhibitory activity

GrzB: Granzyme B

HBV: Hepatitis B Virus

HCV: Hepatitis C Virus

HEPES: (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HEV: High Endothelial Venules

HIV: Human immunodeficiency virus

HSV: Herpes Simplex Virus

ICAM-1: Intercellular Adhesion Molecule 1

IFN: Interferon

IL: Interleukin

iRBC: Infected red blood cell

ISG20: Interferon-stimulated gene 20 kDa protein

kDa: Kilodalton

KLF2: Krüppel-like Factor 2

Ko: Knock-out

LCMV: Lymphocytic Choriomeningitis Virus

LFA-1: Lymphocyte function-associated antigen 1

LN: Lymph Node

LSA1: Liver Stage Antigen-1

LSAP2: Liver Associated Antigen-2

mAb: Monoclonal antibody

MAC: Membrane attack complex (of complement)

MAIT: Mucosal Associated Invariant T-cells

ME-TRAP: Multiple epitope-thrombospondin-related adhesion protein

MHC: Major histocompatibility complex

MLC: Memory Lymphoid Clusters

MOMP: Mitochondrial Outer Membrane Permeabilization

MPL: 3-O-desacyl-4'-monophosphoryl lipid A

mRNA: messenger ribonucleic acid

MVA: Modified vaccinia Ankara

ndLN: non-draining Lymph Node

NIH: National Institute of Health

NK: Natural killer (cell)

NKT: Natural Killer T-cell

PAMPs: Pathogen Associated Molecular

Pb9: SYIPSAEKI, immune-dominant peptide on H-2K^d background

PBMC: Peripheral blood mononuclear cell

PBS: (Dulbecco's) phosphate-buffered saline

PCR: Polymerase chain reaction

PECAM-1: Platelet endothelial cell adhesion molecule (CD31)

PfAARP: *Plasmodium falciparum* apical asparagine-rich protein

PfAMA-1: *Plasmodium falciparum* apical membrane antigen 1

PfCSP: *Plasmodium falciparum* circumsporozoite protein P

PfGLURP: *Plasmodium falciparum* glutamate-rich protein

PfMSP-1: *Plasmodium falciparum* merozoite surface protein 1

PfRH5: *Plasmodium falciparum* reticulocyte-binding protein homologue 5

PKG: cGMP-dependent protein kinase

PRR: Pattern Recognition Receptors

PVM: Parasitophorous Vacuole Membrane

RAS: Radiation-Attenuated Parasites

RBC: Red blood cell

RNA: Ribonucleic acid

RPMI medium: Roswell Park Memorial Institute medium

RTS/S: Mosquirix

S1P: Sphingosine-1-Phosphate

S1PR1: Sphingosine-1-Phosphate Receptor 1

SHIP-1: Phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 1

SLAM: Signaling lymphocytic activation molecule

SPECT-2: sporozoite microneme protein essential for cell traversal 2

SPZ: sporozoite

SR-1: Stem Regenin 1

TAP: Transporter associated with antigen processing

Tcm: Central Memory T-cell

TCR: T-cell receptor

Teff: Effector T-cell

Tem: Effector Memory T-cell

TGF: Transforming growth factor

TLR: Toll-like Receptor

TNF: Tumor necrosis factor

Trm: Tissue Resident Memory T-cell

VCAM-1: Vascular cell adhesion protein 1

VLA-4: Very Late Antigen-4

VLP: Virus-like Particle

WHO: World health organization

WT: Wild-Type

Chapter I

Introduction

Chapter I

Introduction

I.a – Background and history of malaria: a global problem

Malaria is one of the deadliest parasitic human diseases. Although economic development and eradication campaigns (especially draining of swamp lands), has eliminated malaria in many areas of the world, certain regions, namely Sub-Saharan Africa, South and South-East Asia and parts of Central and South-America are still affected. Indeed, despite the focus of the WHO eradication campaigns from the mid-1950s, latest reports show that there are still 212 million cases of malaria a year, resulting in 429,000 deaths [2]. The cause of malaria is a unicellular protozoan parasite *Plasmodium* of which several species exist, transmitted by infectious bites of *Anopheles* mosquitoes to a mammalian host. Of most importance, *Plasmodium falciparum* is by in large the most lethal to humans, resulting in high incidences of severe forms of the disease principally due to its ability to change the surface properties of infected erythrocytes, causing them to stick to blood vessel walls (a mechanism called sequestration) [1]. Aside from the high morbidity and mortality associated with the disease (affecting primarily young children under the age of five), the economic and societal impact is staggering, estimated to be approximately 1% of GDP a year for certain countries due in part to costs of drugs, sick leave and other related complications [3].

Early descriptions of malaria reach as far back as 2700 BC, where symptomatic descriptions can be found in the ancient Chinese medical textbook Nei Ching. The parasite, however, was not described until 1880 by the French army surgeon Laveran

who was on duty in Algeria, and named the parasite *Oscillaria malariae* [4]. Indeed, his seminal work on *Plasmodium*, and *Trypanosoma*, would result in his Nobel Prize in 1907. Later work by Grassi and Ross in the late 1890s described the role of transmission by mosquitos, as well as the presence of multiple malaria species [5, 6]. Of importance to malaria research, the rodent-infecting *Plasmodium berghei* and *Plasmodium yoelii* would be later discovered in the mid-1900 and would become powerful laboratory tools to study the basic biology of *Plasmodium* species.

It is clear that there is an absolute need for an effective vaccine against malaria. In the subsequent sections, the uniqueness of the malaria life-cycle and the challenges the immune system faces to clear the pathogen will be discussed. Over the years, extensive work has been done to develop an effective, translatable vaccine product to take to the field: the challenges faced by the malaria research community cannot be understated. Several vaccine strategies have been proposed, against either the pre-erythrocytic stages, blood-stages or mosquito stages of *Plasmodium* and will be described. The experimental work described in this thesis focuses primarily on the liver-stages of the disease, hence a detailed introduction will be provided on the immune response mediating liver-stage protection and the important role of CD8⁺ T-cells in clearing infected cells. Furthermore, the mechanisms of naïve CD8⁺ T-cell activation and memory formation will be discussed. Lastly, this chapter will focus on a novel subset of memory CD8⁺ T-cells and its newly identified importance in mediating protection against invading pathogens.

I.b – Malaria life-cycle, pathogenesis and current malaria vaccine strategies

I.b.i – Malaria life-cycle

The malaria life-cycle is very complex, involving stages both in the mammalian host and in the mosquito vector. The number of transformations within incredibly diverse anatomical environments is truly staggering. The malaria life-cycle can be sub-divided into three, natural stages: the invasion of and growth within the liver (also known as the pre-erythrocytic) stage, the blood- (erythrocytic) and finally the sexual-stage (the last of which occurs within the mosquito host) (Fig1.1). Out of the three stages, only the erythrocytic-stage is symptomatic in the mammalian host, whereby parasites multiply exponentially inside red blood cells. Cyclical bursting of infected RBCs (iRBCs) releases waves of blood-stage parasites (merozoites) which result in anemia and triggering of cytokine storms giving rise to the systemic symptoms of disease (fever, sweats, chills etc). In addition, as later discussed, untreated blood-stage malaria can result in parasites crossing the blood-brain barrier and the development of cerebral malaria, which is often fatal.

Malaria begins with the bite from an infectious female *Anopheles* mosquito, where only a few parasites sporozoites (spz) (predicted around 100 spz in experimental situations, fewer than 50 spz/bite in natural infections [7, 8]) are released predominantly into the dermis of the host as the mosquito takes a blood meal [9]. Once in the skin, sporozoites display high-speed gliding motility of about 1-2 μ m/sec [10, 11], and have the ability to traverse host cells by piercing through cell membranes [12], before parasites can locate a blood vessel and travel to the liver. Importantly, experiments injecting *P. berghei* spz into the mouse ear, have revealed that approximately 60% of spz remain in

the bite site, with only 10-15% leaving the skin via blood or lymphatic vessels and reaching the liver within minutes from injection [13].

Once in the liver, spz traverse hepatocytes and liver-resident macrophages (known as Kupffer cells after Karl Wilhelm von Kupffer who first described them in 1876), before invading a hepatocyte and undergoing asexual replication. Indeed, the importance of cell traversal has long been debated. It was initially believed to be important for the increased local production of hepatocyte-growth factor, suggested to aid spz infectivity in cell culture conditions [14]. More recent evidence would suggest that cell traversal, mediated largely by the protein SPECT2, is required to avoid capture by phagocytic Kupffer cells [15]. However, the actual invasion mechanism in the final hepatic cell is still not completely understood, with no conclusive evidence showing what causes spz to switch from gliding and cell traversal behavior, to final cell invasion [1]. Invasion is thought to involve several proteins such as the circumsporozoite protein (CSP) and Pf36 binding to particularly highly-sulfated heparan sulfate proteoglycans (such as SR-1, CD81, and EphA2) on hepatocytes and hepatic stellate cells [16, 17]. Following cell invasion, the parasite undergoes a remarkable phenotypic change: developing inside an immunologically-silent protective parasitophorous vacuole membrane (PVM), escaping for the most part immune detection [18]. Inside the vacuole, schizogony results in the generation of thousands of *P. berghei* merozoites within just two days (or 6-7 days in the case of human *P. falciparum* infections), resulting in a completely transformed parasite with novel antigenic proteins, escaping once again the detection of the adaptive arm of the immune system (such that the clonally expanded T-

cells generated from the primary encounter in the skin no longer recognize the parasite-infected cells due to peptide-MHC:TCR mismatches).

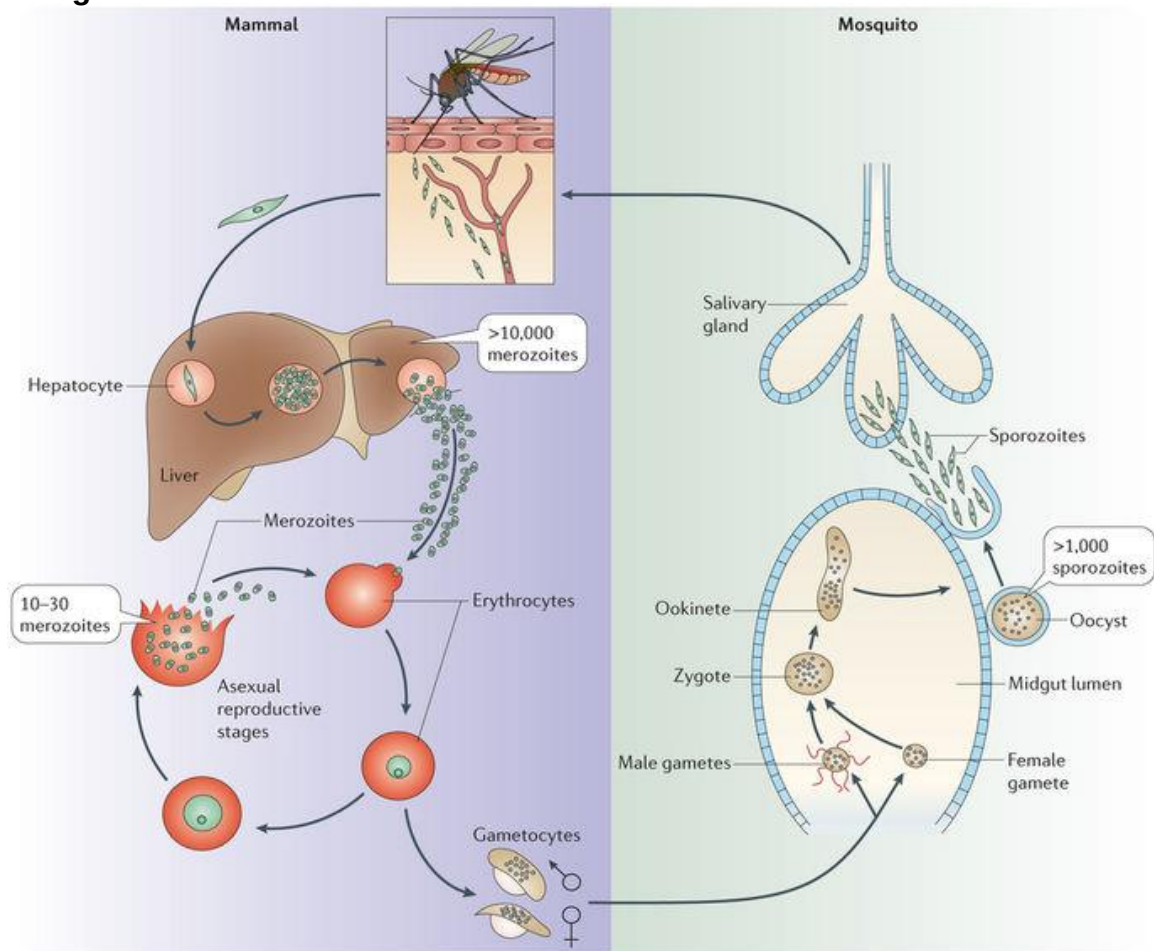
Following the development of merozoites, blood-stage malaria begins.

Merozoite egress from hepatocytes in the form of merozoites without causing necrosis or apoptosis of the host cell, carefully using the host's cell membrane to bud off, evading once again immune recognition by impeding the flipping of phosphatidylserine on their membrane surface (a classic "eat-me-signal" crucial for the clearance of apoptotic cells) [19]. Each merozoite contains thousands of merozoites, which once into the bloodstream invade erythrocytes at incredible speed. Inside red blood cells, merozoites undergo three morphologically distinct transformations: the ring stage, trophozoite stage, and following exponential mitotic replication, the schizont stage which terminates with the rupture of the RBC. Red blood cell invasion occurs in cyclical 48h waves, associated with the characteristic spike in malarial fever caused by the erythrocytic lysis. In fact, the rate of parasite multiplication is directly linked to disease severity [20], with multiple factors involved in the process such as number of merozoites released from the liver-stages of the disease, efficiency of invasion, the rate and number of mitosis cycles. Lastly, to complete the plasmodium life-cycle, approximately 0.1% of schizonts develop into sexual gametocytes rather than infectious merozoites.

This allows for the completion of the final segment of the malaria life-cycle, which occurs once a mosquito takes a blood meal from an infected individual. Following an increase in pH, and a decrease in temperature (crucial signals for the parasite to detect presence inside the mosquito), male and female gametocytes exflagellate (removing the RBC membrane) and develop into mature sexual gametes able to undergo fertilization

[1]. The resulting zygote then matures into a flagellated ookinete, which attaches to the mosquito midgut, and forms a number of oocysts, each producing thousands of sporozoites [1]. Once formed, sporozoites are then able to migrate from the midgut to the mosquito's salivary gland, ready to re-start the infectious cycle with the next mosquito bite [1].

Figure 1.1



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Fig. 1.1: Complete life-cycle of the *Plasmodium* parasite.

A schematic representation of the malaria life-cycle between the mammalian host and mosquito vector. Outlined, are the numbers indicating progeny formed after replication into merozoites from the liver-stages of the disease to the blood-stages. During the blood-stages of the disease, each merozoite invades an erythrocyte and undergoes 3-4 nuclear division that result in the formation of 10-30 new merozoites (each cycle lasting 48-72hrs in humans) which eventually burst out of the cell resulting in the characteristic spike in fever and other associated symptoms. Some parasites transform into male or female gametocytes, which are subsequently taken up by a mosquito bite to complete the full *Plasmodium* life-cycle generating more sporozoites. (Figure taken from Menard et al. [1]).

I.b.ii– Malaria pathogenesis

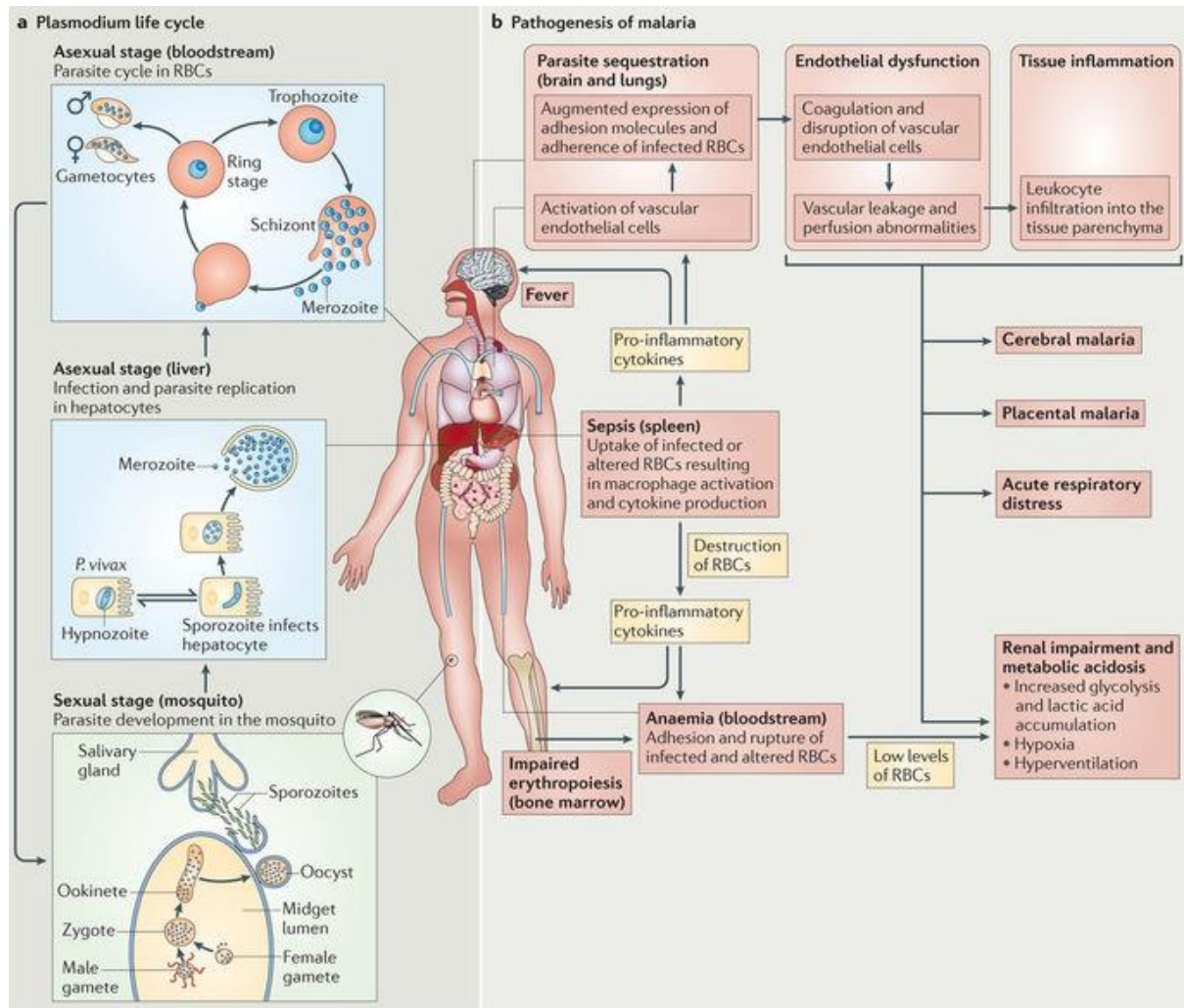
The pathogenesis of malaria is highly variable, ranging from asymptomatic to fatal in a short time-frame: indeed, part of the mortality associated with the disease is due to missed early diagnosis specifically in young children. It is worth to re-emphasize, that all symptoms associated with the disease coincide with the erythrocytic-stages of the disease (hence approximately 6-7 days post infectious bite in humans), with the liver-stages of the disease completely asymptomatic. In the case of *P. falciparum*, the most common clinical symptoms associated with the disease are recurrent spikes in fever, headache, severe fatigue and chills, nausea and vomiting (Fig. 1.2) [21]. Primarily, these are caused by high-levels of circulating cytokines and inflammatory mediators, predominantly caused by a tremendous activation of the innate immune system. Mortality is typically caused by severe anemia, cerebral malaria, and respiratory distress (caused by metabolic acidosis).

The described symptoms are mostly due to three main pathophysiological events: release of large quantities of cytokines by splenic macrophages and monocytes, the rupture and active removal of infected red blood cells by splenic macrophages, and adhesion of infected erythrocytes to the vasculature wall [21]. Malaria Pathogen Associated Molecular Patterns (PAMPs) and Damage Associated Molecular Patterns (DAMPs) release (such as *Plasmodium* glycosylphosphatidylinositol anchors, haemozoin, and nucleic acid release [22-24]) result in Pattern Recognition Receptor (PRR) activation on a multitude of immune and non-immune cells, resulting in the considerable production of cytokines such as IL-6, IL-1 β , IL-12, TNF α and IFN γ responsible for the severe malaise [25, 26]. High-levels of pro-inflammatory cytokines

further augments systemic inflammation by inducing macrophage priming (resulting in an increased robust expression of proinflammatory mediators in response to a second LPS challenge), TLR hyper-responsiveness, and inflammasome assembly [27]. Severe anaemia is directly caused by the rupture and clearance of infected iRBC, but also caused by high levels of circulating IL-6 and TNF α , which augment the anaemia symptoms due to the downregulation of erythropoiesis [28, 29].

The other key pathological consequence of the disease is the sequestration of infected red blood cells on the vascular wall of several organs especially the lungs, brain, and liver. Erythrocyte sequestration occurs in part due to activation of the vascular endothelium caused by the increased cytokine exposure (resulting in upregulation of adhesion molecules) [21]. At the same time, *Plasmodium* iRBCs produce a diverse range of adhesion proteins such as the erythrocyte membrane protein family (EMP-1), which bind a multitude of vascular receptors such as CD36, ICAM1 and PECAM1, complement receptor 1, heparan sulphate and chondroitin sulphate A [30]. Vascular sequestration allows for the *Plasmodium* parasite to actively escape from clearance by splenic macrophages. However, it causes severe pathology in the host as erythrocytic adherence to the vascular wall results in the triggering of the coagulation cascade, fibrin deposition, increased local inflammation and disruption of endothelial barrier integrity [25, 31]. The obstruction of capillaries and severe vascular inflammation are an important contribution to the acute respiratory distress and cerebral malaria observed in patients.

Figure 1.2



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Fig. 1.2: The life-cycle of the *Plasmodium falciparum* parasite and its associated pathogenesis.

(a) The malaria life-cycle of *Plasmodium* between the mammalian host and mosquito vector showing the asexual blood and liver stages and the sexual stage in the mosquito. (b) A diagrammatic representation of the complex pathophysiology of malaria in the human host. (Figure taken from Gazzinelli et al. [21])

I.b.iii – Overview of current vaccine strategies against malaria

Some infections, such as smallpox, polio, or varicella lead to life-long immunity. However, in the case of malaria, *Plasmodium* exposure in endemic regions leads to incomplete protection even after multiple infections often coinciding with the wet season. Although complete protection does not develop, it has been observed that individuals living in endemic regions over time develop enough immunity to modulate disease

severity and blood parasite-load [32]. In fact, it is uncommon for adults to develop symptoms of severe malaria past childhood, with adults in endemic regions undergoing bouts of asymptomatic infection [33]. It is however believed that such naturally acquired immunity is predominantly to the blood-stages of the disease, with seminal work by Cohen et al., showing that passively-transferred antibody from naturally immune individuals is sufficient to mediate protection against severe infection [34]. However, it must be noted that children remain very susceptible to malaria with high levels of morbidity and mortality, as well as pregnant women.

Developing a malaria vaccine is therefore highly challenging, with the difficulty being compounded by no clear protective natural immune correlates and the antigenic and phenotypic changes occurring during the parasite's life-cycle. Due to the marked, multi-stage complexity of *Plasmodium*, no current vaccination strategy is protective at all the different stages of the parasite life-cycle. Indeed, most vaccine regimens focus on one of the three stages, with the possibility of one day combining vaccines against pre-erythrocytic, erythrocytic and mosquito stages, in a multi-stage vaccine regimen. Indeed, several vaccination strategies currently exist mostly in experimental research phases, which involve whole sporozoites vaccines, or subunit vaccines for different stages of the life-cycle.

Vaccine development targeting liver-stage malaria has shown some success over the years, with the currently most advanced malaria vaccine strategy targeting spz hepatic invasion [35]. Indeed, the liver-stages of the disease provide a natural bottleneck to the malaria cycle; the elimination of hepatic stages would result in no symptomatic blood-stages and no subsequent transmission to the mosquito vector. Furthermore, antigenic

polymorphism is significantly more limited in the liver-stages than the blood-stages of the disease due to lack of immune selective pressures, lending itself to the possibility of multi-strain protection [36]. Liver-stage vaccine immune correlates of protection will be discussed in detail in the following section, here we will briefly list some of the recent vaccine developments.

For a multitude of pathogens, a highly successful vaccination strategy has been the administration of whole pathogens, either inactivated or live-attenuated. Importantly, heat-killed spz were found not to be protective in mice [37], hence significant efforts have been placed on live-attenuated spz development, still capable of infecting hepatocytes. Specifically, radiation-attenuated spz (RAS), genetically-attenuated parasites (GAP), or infection-treatment vaccination (such as spz administration under chemical prophylaxis cover) are being explored, with RAS the most advanced whole-spz vaccine strategy currently under investigation [38]. The advantage of whole-spz vaccination regimens is the antigenic breadth to which the recipient is exposed to, allowing for improved protection. The greatest disadvantage results from the complex manufacturing process (as discussed later).

Alternatively, subunit vaccines for the pre-erythrocytic stages of malaria have principally involved RTS,S to induce antibody responses to block spz invasion, and heterologous, viral vector prime-boost regimens which induce high numbers of CD8⁺ T-cells (discussed in greater detail in the following section). RTS,S/AS01, a vaccine produced by GlaxoSmithKline, is a protein-in-adjuvant formulation involving a hepatitis B virus surface antigen core (assembled into a virus-like particle (VLP)) fused to parts of the most abundant spz surface proteins CSP. The VLP is administered together with an

adjuvant, the liposome-based AS01, containing 3-O-desacyl-4'-monophosphoryl lipid A (MPL) and the saponin QS-21 [39]. Following antibody binding, spz become opsonized targets rapidly cleared by macrophages and monocytes [40]. RTS,S/AS01 has completed a Phase III with vaccine efficacy ranging from 20-50% depending on the participant's age, waning significant after the first months of vaccination [41]. Although this approach is promising, future development must be continued to improve the low vaccine efficacy, with future developments focused on improving antibodies titers following vaccination [35].

Aside from pre-erythrocytic malaria vaccine development, significant efforts have also taken place to develop blood-stage and transmission-blocking malaria vaccines. Blood-stage malaria vaccination strategies especially, should have the advantage of generating an immune response that resembles the naturally-acquired blood-stage protection [32, 33]. This would be advantageous, with recurrent exposure acting as a natural boost to the vaccine. On the other hand, antigenic proteins from the blood-stages of disease are highly polymorphic, and can undergo extensive mutations resulting in constant evasion under immune pressure [42, 43]. Furthermore, blood-stage vaccines may not result in sterile protection of recipients, functioning by reducing morbidity and mortality associated to the disease. Whether vaccine-induced antibodies can effectively mediate protection in an infection setting is unclear, especially as merozoites are already highly targeted by antibodies generated in natural infections [44]. Probably the biggest challenge faced by blood stage malaria vaccines is the requirement of unusually high antibody titers to achieve efficacy [45], with a variety of formulations based on viral vectors, VLP, or protein-in-adjuvant formulations attempting to achieve this. Anti-

merozoites antibodies predominantly function either by inhibiting directly merozoite entry into erythrocytes or by inducing opsonization and/or complement activation [46].

The choice of malaria antigens used is crucial, with probably not a single antibody epitope alone mediating full vaccine efficacy. Early studies focused on merozoite surface antigens such as *Pf*MSP-1, *Pf*MSP-3, *Pf*GLURP, and *Pf*AMA-1 known to induce antibody responses in the field [47, 48]. However, they have shown to have very low efficacy in Phase IIb clinical trials [49, 50]. More recently, through the use of effective-screening strategies [51] and standardized merozoite Growth Inhibition Assays (GIA) [52], novel promising blood-stage candidates involving targets to the apical complex (required for merozoite invasion) such as *Pf*RH5 [45, 51], and to a lesser extent *Pf*AARP [53], are the focus of present and future research in the field, showing more promising efficacy in the context of pre-clinical work.

Lastly, vaccines are also in development to block the transmission of malaria within the mosquito vector. Transmission-blocking malaria vaccines have a unique functional mechanism as, rather than providing direct protection against malaria for the human host, transmission-blocking vaccines involve the generation of neutralizing antibodies that target the sexual stages in the mosquito mid-gut [54]. Indeed, the prediction by Ross that below a certain transmission rate, malaria transmission cannot be sustained, goes as far back as 1911 [55]. Specifically, antibody targets against gametocytes, gametes or ookinetes stages have shown promise in mosquito membrane-feeding assays: which allows the assessment of vaccine efficacy [54]. Hence, transmission blocking vaccine efficacy in the field would require high-coverage of the endemic population during at least one-wet season and would be assessed by a decline in

the incidence rates of malaria during the following malaria cycle, which can at times be logistically challenging to determine. Although transmission-blocking vaccines have the advantage of low polymorphism due to lack of immune pressure on the parasite, high-antibody titers would need to be generated and probably maintained for at least one transmission season, as it is unlikely natural exposure would boost vaccine induced responses [56]. Again, the choice of malaria antigen has involved numerous candidates, particularly of relevance *Pfs25*, *Pfs230* and *Pfs48/45*, known to play key roles either in the pre-fertilization or post-fertilization states essential for the parasite to colonise the midgut of the mosquito [54]. As of date, small Phase I clinical studies are currently taking place to assess safety and immunogenicity [57], as well as assessing novel candidates such as *Pfs230* [58].

I.c – Understanding the immune correlates of protection against the pre-erythrocytic stage of the disease

Irradiated sporozoites, mentioned in the previous section, were shown in seminal work to result in sterile protection in mouse and human challenge studies around the early 1970s, often described as the “gold-standard” for the field [59, 60]. The question that then arose was, what is the immunological mechanism resulting in sterile protection behind RAS vaccination? Indeed, depending on the radiation dose used, RAS-spz arrest at the time of DNA replication (20-24 hrs after infection, half-way through liver-stage development) [1], providing clear evidence that sterile immunity could be generated from the pre-erythrocytic stages of the malaria life-cycle. Initial work in the field had suggested that neutralizing antibodies against the surface protein Pb44 were predominantly involved in mediating protection in RAS-induced vaccination models [61,

62]. Indeed, as discussed earlier in the context of RTS,S, antibodies against CSP can mediate protection in mice and human. However, seminal subsequent work in the field involving depletion studies and adoptive-transfer experiments of CSP-specific CD8⁺ T-cells, clearly demonstrated that CD8⁺ T-cells played a non-redundant role in mediating protection following irradiated-spz administration [63-66]. These CD8⁺ T-cells were shown to be specific for the 34immune-dominant peptide sequence SYIPSAEKI, also known as Pb9, part of the *P. berghei* CSP protein in a BALB/c background (H-2K^d). In fact, SYIPSAEKI recognizing CD8⁺ T-cells are able to clear liver-stage malaria, with other strains of mice being significantly more susceptible to severe disease and harder to protect. Further evidence potentially relevant to protection also arises from human correlative studies of individuals living in endemic areas. Indeed, low-levels of CSP-specific CD8⁺ T-cells can be found in the circulation in endemic populations, suggesting that natural exposure can stimulate the expansion of CD8⁺ T-cells [67]. Furthermore, a positive correlation has been suggested between resistance to severe malaria and CD8⁺ T-cell responses to CSP epitopes induced by natural exposure (a subset of cells which can also be identified in individuals vaccinated with RAS-spz [68]) [69]. Altogether, experimental mouse studies and human correlative-data suggest that CD8⁺ T-cells may be relevant in liver-stage malaria protection.

I.c.i – Mechanisms of CD8⁺ T-cell mediated protection

Although the liver stages of malaria are brief (2 days in mice, and 6-7 days in human), there is enough time for CD8⁺ T-cells to recognize peptide antigens presented on MHC class I molecules, in a cell contact-dependent fashion. In the context of liver stage malaria, they recognize hepatocytes infected with *Plasmodium* spz, which present spz

antigens on MHC-I in a predominantly TAP-dependent manner [70]. In essence, this means that cytosolic antigen proteins are degraded by the proteasome into peptides, which can then be transported to the ER via TAP, allowing loading on MHC-I complexes. CSP is cross-presented via endosomes in DCs, while in hepatocytes, the protein must be present directly in the cytosol. Furthermore studies utilizing bone-marrow chimeras have shown that hepatocytes or other parenchymal cells are indeed required to present antigen to CD8⁺ T-cells, with Teff and MHC miss-matching abrogating CD8⁺ T-cell mediated protection [71]. Lastly, prolonged antigen exposure appears to be crucial for the induction of high-numbers of CD8⁺ T-cells in the liver following RAS immunization [72], potentially suggesting that RAS immunization provides limiting amounts of antigens in the hepatic site. Although shown through adoptive transfer of transgenic Ag-specific CD8⁺ T-cells, the direct mechanisms of persisting Ag are still unclear.

In vivo imaging has shown that Antigen-specific (Ag-specific) CD8⁺ T-cells cluster around infected hepatocytes in large numbers (a phenotype associated with activation and degranulation in a multitude of infectious diseases) [73, 74]. Indeed, this report confirms an early report showing clusters of T-cells around infected hepatocytes [75]. Protection was shown to require Ag-specific CD8⁺ T-cells, with no bystander activation of co-transferred T-cells of an irrelevant specificity. Importantly, cluster formation required G-protein coupled receptor active signaling (potentially chemokine signaling) as the administration of Pertussis Toxin greatly reduces protective capacity of CD8⁺ T-cells [73].

The killing mechanisms of CD8⁺ T-cells at the sites of infection are still partially unclear. It is known that perforin, Granzyme B and Fas/Fas-L interactions are probably all crucial to mediate the protection. However, single knock-out experiments have shown that killing mechanisms of CD8⁺ T-cells are probably redundant, with no single knock-out model resulting in impaired protection [76]. Significant effort regarding investigating the role of IFN γ has been made. Indeed, work *in vitro* has suggested that IFN γ can inhibit parasite development in hepatic cell lines, speculated to be via a non-canonical form of autophagy, where autophagic proteins are deposited directly onto the PVM and result in degradation [77]. Furthermore, subsequent *in vivo* work showed that animals immunized and later challenged resulted in decreased protection when the anti-IFN γ antibody (DB-1) was administered [63]. However, subsequent work clearly showed that transfers of IFN γ ko Ag-specific CD8⁺ T-cells in animals resulted in protection equal to WT Ag-specific cells [78]. The apparent discrepancies in these experimental systems could be in part due to the vaccination strategies and mouse genetic background used, as well as the presence of endogenous production of IFN γ by other cells (such as NKT cells). We can however conclude that IFN γ produced by CD8⁺ T-cells, although maybe not playing a direct effect, correlates positively with T-cell activation and protection.

I.c.ii – Quality and Quantity of Liver CD8⁺ T-cells

Due to the limitations of clinical studies, pre-clinical work in mice has been fundamental in understanding in greater detail the role of CD8⁺ T-cells in the context of malaria. Through the use of antigen-specific CD8⁺ T-cell transfers, it was elucidated that under natural exposure to *Plasmodium* spz administered via skin injection, dendritic cells

(specifically of the CD8 α^+ subtype [79]) prime CD8 $^+$ T-cells [71]. Activated CD8 $^+$ T-cell effectors will then travel systemically, where they can aid in inducing an immune response against the liver-stages of the disease (Fig. 1.3). These cells however probably are not directly involved in mediating protection, with liver-resident CD8 $^+$ T-cells required to clear all infected hepatocytes. In fact, a sufficiently high number of anti-parasite CD8 $^+$ T-cells in the liver is probably needed to find and clear all the infected hepatocytes in a contact dependent manner [80].

The crucial importance on the numbers of Ag-specific CD8 $^+$ T-cells required for protection was initially shown by work from the Harty lab. Indeed, through the use of adoptive transfers of Pb9-specific CD8 $^+$ T-cells generated from a transgenic *Listeria monocytogenes* infectious model expressing the Pb9 epitope, surprisingly high-numbers of Ag-specific CD8 $^+$ T-cells were required to mediate protection in challenge experiments [80]. Subsequent work using irradiated-spz as a vaccination model to generate parasite-specific CD8 $^+$ T-cells showed similar quantitative requirements: with C57BL/6 mice being harder to protect due to a lack of the immune-dominant, protective, Pb9 epitope [81]. Lastly, careful work by Spencer et al., revealed there is a crucial balance between the number of infected hepatocytes and circulating liver effector CD8 $^+$ T-cells [82]. Here they show that Pb9 specific CD8 $^+$ T-cells do not actively migrate into the liver during a spz infection and that protection is dependent on a fine balance between numbers of infected hepatocytes and Ag-specific CD8 $^+$ T-cells. Hence, administration of fewer-spz, resulted in decreased numbers of Ag-specific CD8 $^+$ T-cells recruited into division and significantly higher blood parasitemia. Although these studies show a clear importance in

the number of CD8⁺ T-cells required to clear the infection, they do not investigate the phenotypic and quantitative aspects of liver-resident CD8⁺ T-cells.

Characterization and mechanistic understandings of the requirements of liver-residency by CD8⁺ T-cells in malaria was shown in seminal work by the Zavala lab. Firstly, by performing a careful transcriptional analysis comparing liver and spleen memory CD8⁺ T-cells, a multitude of differences were seen involving the cell cycle, chemokine receptor expression (in part allowing for unique trafficking), and transcription

Figure 1.3

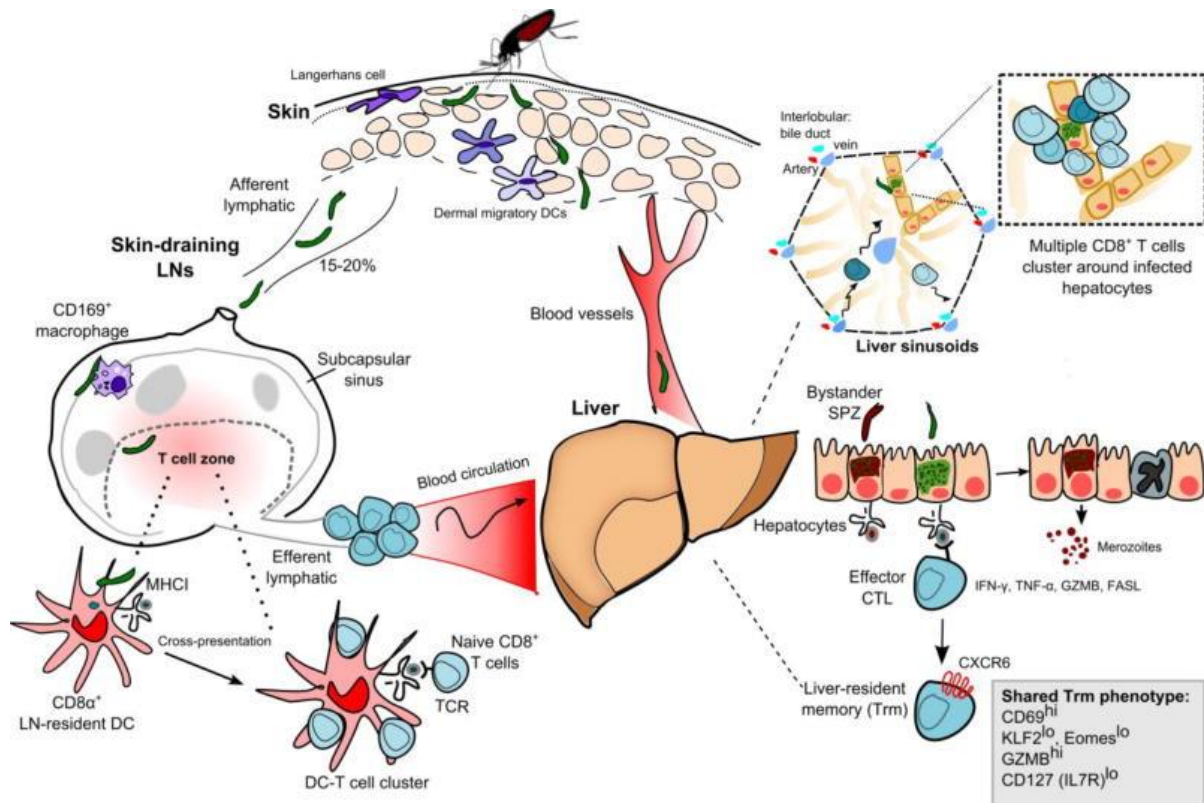


Fig. 1.3: Immune response triggered post spz administration via infectious bite.

Following an infectious mosquito bite, 15-20% spz are found to drain to the skin-draining lymph node (LN), are actively taken-up by sub-capsular sinus macrophages and presented to CD8⁺ T-cells via CD8α⁺ DC, activating an effector CD8⁺ T-cell response. The remainder of the spz instead find their way to the liver and are directly presented by hepatocytes. If effector or Trm cognate Ag-specific CD8⁺ T-cells are present, CD8⁺ T-cells can form large clusters and release effector molecules (such as IFNγ, TNFα, GrmB, FasL), killing the infected hepatocytes. In the absence of Ag-specific CD8⁺ T-cells, merozoites form and blood-stage malaria begins. (Figure taken from Radtke et al., [76].)

factor expression [83]. Specifically, liver-resident CD8⁺ T-cells were found to be CD69^{hi} and expressed low levels of transcription factors Eomes and KLF2. KLF2 has been shown to regulate the expression of S1PR1 and CD62L and, together with CD69, allows for retention outside lymph nodes [84]. Furthermore, liver CD8⁺ T-cells were found to be Granzyme B^{hi}, CD127^{lo}, and expressing high-levels of the chemokine receptor CXCR6. Indeed, such a phenotypic program (CD69⁺ CXCR6⁺ Eomes^{lo} KLF2^{lo} Granzyme B^{hi} CD127^{lo}) was recently further confirmed in another study, in which the true tissue-residency nature of this subset of cells was shown through parabiotic studies [85].

Importantly, through the use of animal knock-out models, Tse et al., further showed that CXCR6 was found to be crucially important in the retention of liver CD8⁺ T-cells [86]. In fact, although numbers of memory CD8⁺ T-cells were normal in tissues such as the spleen, liver-resident CD8⁺ T-cells were reduced, and mice showed decreased protection. These findings were in fact in accordance with the earlier mentioned quantitative requirements of circulating effector CD8⁺ T-cells for protection.

On a last note, although CD8⁺ T-cell have been highly characterized in the context of liver-stage malaria, one would speculate that they must be functioning in a highly-complex immune environment. Indeed, although not necessary to mediate protection, CD4⁺ T-cells, NK and NKT are probably actively recruited and involved in protecting against spz infection. Indeed, NKT are known to cooperate with Kupffer cells and produce large quantities of cytokine in other liver infections [87]. Furthermore, work in non-human primates, has shown that NK, $\gamma\delta$ T-cells and MAIT-cells were also found to be increased in numbers following RAS-vaccination [88]; whether these cells contribute

to the clearance of infected hepatocytes potentially by altering the immune microenvironment through the release of cytokines is still an area of future investigation.

I.c.iii – Vaccine induction of protective liver-stage malaria immunity

Since the 1970s, iv administration of RAS, live attenuated-spz, have been known to result in sterile protection. This concept has in recent years been re-visited by the private company Sanaria, in collaboration with Bob Seder at the NIH. Indeed, the current vaccine regimen requiring four intravenous vaccinations of irradiated-spz (2.7×10^5 /dose) has shown long-term efficacy, with 55% protection 21-weeks post vaccination [88]. Previous work from the group had highlighted intravenous administration as particularly important to generate protective immune responses, required to induce anti-parasite liver CD8⁺ T-cells in non-human primate experiments [89, 90]. Although such results are promising, a successful means of culturing the spz has not yet been developed, requiring manual dissection from mosquito salivary glands (a process not yet automated), limiting large scale production. Furthermore, irradiated-spz require cryopreservation for storage, making them an unlikely and costly option for vaccination programs in developing countries, the main target population of a malaria vaccine.

The other approach to attenuate spz is to genetically knock-out genes that are crucial for complete liver-stage development. Indeed, genes such as UIS3 and P36p were some of the first genetically modified *P. berghei* spz unable to progress to blood-stages of the disease and conferred complete protection against spz challenges in mice [1, 91]. As of today, knockouts of genes involved in the formation of the PVM, apicoplast, fatty acid synthesis or hepatocyte egress have all shown to be successful [92, 93]. Indeed,

genetically attenuated parasites arrest at different stages of the parasite life-cycle depending on the knockout, with late-blocked and very late-blocked parasites (such as PKG^{-/-} spz which are unable to egress into fully formed merozoites) resulting in improved protection in re-challenge models [94]. Although the use of genetically-modified spz encounters the same challenges as with irradiated-spz, the generation of spz knock-outs can be very informative in determining which stages and antigens are crucial for the induction of a protective immune response [92].

Lastly, subunit-vaccination involving the use of viral vectored vaccines has been found to be a promising avenue, albeit lacking particularly high vaccine efficacy in clinical human trials. The advantages of this platform lies in the induction of particularly high-numbers of Ag-specific CD8⁺ T-cells combined with effective antibody production both in pre-clinical mouse work and in human studies [95]. Indeed, multiple recombinant viruses have recently been tested such as alpha-viruses, Adeno-associated viruses, vesicular stomatitis virus, measles virus just to name a few. Over the years, prime-boost vaccination strategies involving Adenoviruses followed by Modified Vaccinia Ankara viral vectors (expressing the same antigen), have been found to be particularly effective at inducing high-numbers of Ag-specific CD8⁺ T-cells. Specifically, Chimpanzee Adenoviruses (ChAd) have been used in multiple clinical settings, with excellent immunogenicity and safety profiles in humans, importantly avoiding the problem associated with human Adenoviruses of potentially strain neutralizing antibodies rendering the vaccine inefficacious [96]. The use of Chimpanzee Ads was further solidified as it avoided the use in the field of a Human Adenovirus Type 5 (AdHu5) which had disappointingly low efficacy and some probably unfounded safety concerns in

HIV-infected subjects due to pre-existing immunity from natural exposure to human Adenovirus species [97].

To date, the most advanced vaccine regimen involving viral-vectors in the context of liver stage malaria has been the use of ChAd63 in combination with MVA, expressing the malaria antigen *Pf*TRAP. Human clinical trials in naïve individuals have shown around 25% efficacy in challenge studies [98], and 70% in adults living in endemic areas [99]. Such a difference is speculated to be due to pre-exposure to malaria, potentially suggesting the vaccine is boosting pre-existing memory responses. Although promising, several developments are ongoing to improve both vector immunogenicity through the use of the invariant chain (believed to improve MHC-I presentation) [100] and antigen choice [101]. On this note, a recent screen has found that other liver-stage malaria candidates could be used well in mice, LSA-1, and LSPA-2 have appeared to be particularly promising, with optimistic pre-clinical work showing they could be used in combination [102, 103]. Sub-unit vaccines have the disadvantage of missing the antigenic “breadth” of whole-spz vaccines, hence optimal antigen choice is crucial. However, they are substantially easier and cheaper to manufacture, as well as distribute in the field, key qualities of vaccines (especially for target populations residing mostly in developing countries). With improved antigen choice and CD8⁺ T-cell induction, heterologous viral vectors vaccines appear to be promising candidates in the malaria field.

I.d – Mechanisms of CD8⁺ T-cell induction and memory differentiation

I.d.i – Brief background on CD8⁺ T-cell activation

CD8⁺ T-cells are a critical component of the immune system important for the control of a broad range of intracellular pathogens (namely viruses, bacteria, protozoans)

as well as cancerous cells. Indeed, upon CD8⁺ T-cell activation, antigen specific (Ag-specific) T-cells undergo extensive clonal expansion required to eliminate the ongoing pathogen challenge. Initial activation of naïve T-cells occurs primarily in secondary lymphoid tissues where T-cells get activated upon encountering cognate antigen presented on MHC complexes. Indeed, the migration of lymphocytes

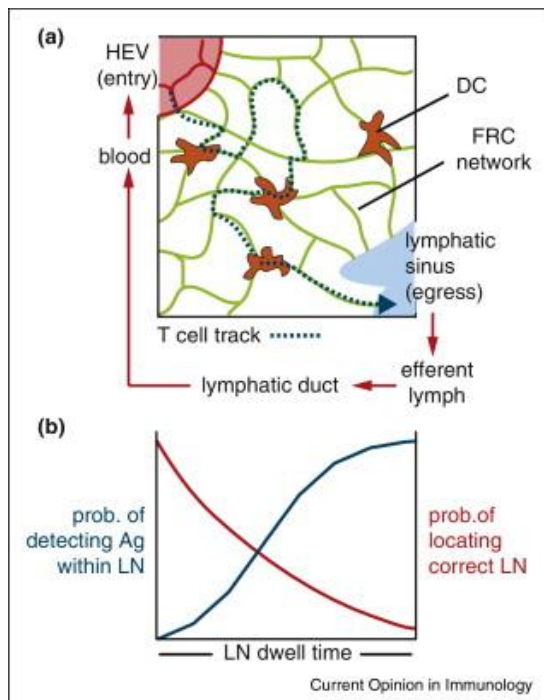


Fig. 1.4: Antigen surveillance by T-cells in lymph nodes. (a) Naïve T-cells enter via HEVs and migrate along a Fibroblastic Reticular cell (FRC) network, performing a random-walk at approximately 11 $\mu\text{m}/\text{min}$, looking for cognate antigen. When no DC presenting cognate peptide is found, T-cells egress via efferent lymphatics and re-enter the circulation. (b) A trade-off exists between the probability of locating the correct LN and detecting cognate Ag, depending on the dwell time each T-cell spends within one LN. (Figure taken from Mandl et al., [118]).

between tissues was hypothesized to be a “productive system for the organisms” as far back as in 1936 by Sjövall [104]. In fact, with the advent of static and intravital microscopy techniques, the dynamic behavior, cellular interactions, and localization of T-cell interactions within physiological anatomical tissues have shed light on several crucial aspects of CD8⁺ T-cell induction [105].

The T-lymphocyte surveillance

system begins with the generation of a naïve T-cell pool, whereby T-cells expressing a diverse array of T-cell receptors (TCR) are selected within the thymus. Thymocyte differentiation involves a test of TCR recognition to self-peptide/MHC molecule ligands (a crucial step for thymocyte survival, cell fate

commitment to CD4⁺ or CD8⁺ lineages, and maturation) [106-109]. Somewhat paradoxically, full T-cell activation against the same self-peptide/MHC molecules results in clonal deletion [110, 111]. In part, this paradox is resolved by the classical affinity model of thymocyte selection whereby the strength of the interaction of the TCR with ligand plays a crucial role in determining cell fate [112]. Of note, TCR signaling strength and its role in determining cell fate is potentially a recurring theme in T-cell biology speculated to be involved in processes such as T-helper cell differentiation, and memory T-cell generation.

Following thymic selection, Ag-specific naïve CD8⁺ T-cells enter the peripheral T-cell pool where they scan the environment for antigen. Upon a pathogenic stimulus, they are recruited into the immune response with surprising speed and efficiency [113, 114]. In fact, this crucial concept was first postulated by the Australian immunologist Frank Burnet as the clonal selection theory [115]. Such ability to find cognate antigen is remarkable as estimates suggest that the frequency of Ag-specific CD8⁺ T-cell is approximately 1-100 cell/million for any one antigen [116, 117]. To increase the odds of antigen encounters, secondary lymphoid organs efficiently capture draining antigens from lymph fluid (in the context of lymph nodes (LN)) and blood (in the context of the spleen), and present it on antigen presenting cells (APC). In fact, a sophisticated surveillance system has evolved, whereby naïve T-cells continuously circulate rapidly between blood, lymph and lymph node (in which they spend approximately 12-hours to several days [118]), scanning APCs by migrating along the fibroblastic reticular cell (FRC) network in the T-cell zone (Fig. 1.4) [119]. Upon pathogen entry, naïve T-cells enter the LNs through high endothelial venules (HEV) by upregulating CCR7, CD62L and integrins LFA-1 and

VLA-4 [104, 120]. Importantly, pathogen introduction results in increased LN size and cellularity, angiogenesis, lymphatic expansion, and increased DC migration from peripheral sites to the LN, all resulting in increased T-cell retention [121, 122].

Specifically, the upregulation of C-type lectin superfamily protein CD69 on activated CD8⁺ T-cells (either by antigen or by sensing of IFN α) directly inhibits S1PR1, resulting in impaired T-cell egress [123].

Following entry into LN, CD8⁺ T-cell activation has been described to occur in three sequential stages via careful two-photon intravital imaging [124]. During the first 8 hours after entering the LN, T-cells undergo multiple short encounters with DCs, over time decreasing their motility, engaging TCR and co-stimulatory molecules such as CD28, culminating with the upregulation of activation markers such as CD69, CD25 (IL-2R) and CD44 (Fig. 1.5a) [104]. During the next 12 hours, long-lasting stable conjugates with DCs are made and secretion of IL-2 and IFN γ begins. In the last phase at around 48 hours, T-cell proliferation begins as well as increased dissociation from DCs and T-cell motility. Hence, T-cell clonal expansion is mediated by a combination of signals from the TCR, co-stimulators and IL-2 sensing (required to induce the anti-apoptotic protein Bcl-2 and stimulate the progression through the cell cycle by increasing cyclin production and degrading p27 [125, 126]). TCR-signaling, co-stimulation and cytokine binding have been predicted to sum linearly, amplifying T-cell expansion [127]. Importantly, the result of CD8⁺ T-cell activation leads to an incredible >50,000-fold increase in the number of Ag-specific T-cells, the majority of which are short-lived effector T-cells (Teff) [128]. With the upregulation of transcription factors Blimp-1, T-bet and eomesodermin (suggested to act as a molecular threshold for effector CD8⁺ differentiation) [129, 130],

CD8⁺ T-cells develop cytoplasmic granules containing Granzyme and perforin, upregulate Fas Ligand, and secrete increasing quantities of cytokines such as TNF α and IFN γ [130]. However, approximately 90% of Teff CD8⁺ T-cells will subsequently undergo programmed apoptosis via a decrease in Bcl-2 and hence activation of the Bim pathway and the formation of Mitochondrial Outer Membrane Permeabilization Pore (MOMP), (Fig. 1.5b) [131]. The mechanisms behind this organized contraction phase are not yet clear. In part, this could be due to cell-intrinsic mechanisms: recent models suggest that dilutions of c-Myc upon multiple rounds of division might play a crucial role in

Figure 1.5

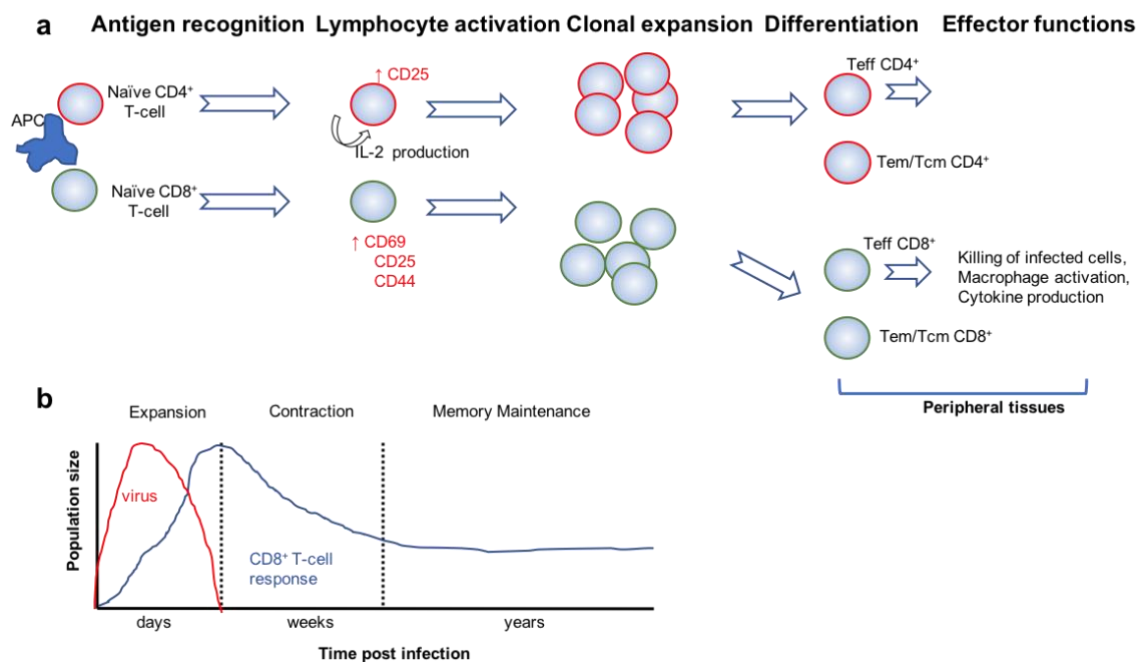


Fig. 1.5: Phases of T-cell response. (a) T-cell activation requires several stages including T-cell arrest and antigen recognition (presented on either MHC class I or II). Following antigen recognition, lymphocyte activation occurs (involving upregulation of CD69, CD25 and CD44) and subsequently disengagement from the APC. This then allows for clonal expansion and effector differentiation (*Figure in part adapted from Abbas Immunology*). (b) Kinetics of CD8⁺ T-cell response: showing the expansion, contraction and memory generation phases of the immune response. (*Figure in part adapted from Russ et al., [128]*).

determining the number of cell division Teff undergo before cell death [132]. Others evidence suggests that CD8⁺ T-cell contraction is controlled by early inflammation (although in this model, treatment with antibiotics following *Listeria monocytogenes* to decrease PRR activation would also decrease antigenic load) and TCR engagement [133]. Although the mechanisms governing the contraction phases are not yet clear with probably a combination of cell intrinsic and environmental factors involved, the resolution of the immune response results in the generation of a long-lived population of memory CD8⁺ T-cell, crucial in mediating long-term protection against pathogen re-encounter.

In the classical model of CD8⁺ T-cells activation, all initiation events occur rapidly in draining lymph nodes, as described above. Indeed, following the administration of heterologous prime-boost viral vectors, following intramuscular administration, CD8⁺ T-cell activation does indeed occur in the draining lymph nodes (dLN). Interestingly however, in the context of RAS intravenous administrations this is probably not the case. Indeed, antigen presentation in RAS vaccination is still a question of debate involving in the part the spleen and in part the liver, a unique organ where direct CD8⁺ T-cell priming can occur in the hepatic sinusoids by a range of cells (hepatocytes, Kupffer cells, liver sinusoidal endothelial cells and Hepatic Stellate cells) [134]. Work done by Chakravarty et al., have shown that removal of spleen prior to immunization, dramatically reduces the activated T-cell pool in the liver [71]. However, some evidence that direct priming in the hepatic tissue can still occur comes from adoptive transfers of Ag-specific T-cell together with infected hepatocytes (bearing matched MHC haplotypes) into TAP-deficient MHC-class I mismatched recipient mice [135]. Although direct priming was shown through

activation of CD8⁺ T-cells, the experimental set-up was potentially questionable as it is unclear where adoptively transferred hepatocytes presenting antigen after intravenous administration would be similar to an endogenous infection setting. In conclusion, further work in the field involving RAS administration and careful confocal imaging in the short term could elucidate the location of CD8⁺ T-cell priming and nature of the APC. The use of imaging techniques would be beneficial in this setting as early priming events could be imaged, allowing for a characterization of the cells directly involved, the organ(s) at play and the temporal kinetics of the response.

I.d.ii – Phenotype of long-lived memory CD8⁺ T-cell

Following the naïve T-cell expansion and contraction phases of the immune response, the resulting 100-1000 fold increase of Ag-specific T-cells sets the basis for the quantitative post-exposure differences in adaptive T-cell immunity. Indeed, memory CD8⁺ T-cells are maintained within the host in a quiescent steady state (acquiring stem-cell like qualities), undergoing slow proliferation over time [136]. Maintenance of memory CD8⁺ T-cells does not need direct antigen recognition, as work has shown that adoptive transfers into MHC deficient animals showed no population decline, requiring mostly IL-7 (for expression of anti-apoptotic molecules Bcl-2 and Bcl-X_L [137, 138]) and IL-15 (for cell proliferation [139-141]) for long-term maintenance. The majority of these experiments have the notable caveat that many of the transgenic animals (such as MHC deficient animals) into which these memory adoptive transfers have been performed in are lymphopenic, resulting in a massive and highly unphysiological T-cell expansion.

Aside from such quantitative differences, there are important qualitative changes memory T-cell display relative to naïve CD8⁺ T-cells. In fact, memory CD8⁺ T-cells have been shown to have unique migratory patterns and immediate effector cytokine and granule production upon TCR triggering, contributing to their ability to rapidly respond in secondary infections. Early seminal work by Sallusto and Lanzavecchia was some of the first to describe the heterogeneity of immunological memory, showing that memory T-cells can be sub-divided into central-memory (T_{cm}) or effector-memory (T_{em}) CD8⁺ T-cell depending on the expression of adhesion molecules and cytokine receptors such as CD62L and CD127 in mice [142]. Later work went on to further characterize these subsets revealing differences in re-call expansion kinetics, sensitivity to antigen re-stimulation, cytokine production, and effector molecule expression (perforin, Granzyme B) [143]. Another previously un-appreciated memory T-cell subset, tissue-resident memory (T_{rm}) T-cells has recently been described [144-146], which can be further subdivided depending on the organ they reside in. T_{rm} are uniquely defined by their inability to migrate outside of the tissue they reside in, with the most stringent characterization of this subset of cells requiring parabiotic studies to fully appreciate the lack of cell migratory behaviors.

Over the years, the field has focused on a number of issues regarding memory T-cells, predominantly centered around three main questions: How is T-cell memory diversification regulated? Are the mechanisms cell intrinsic or determined by the microenvironment? Which of the emerging subsets provide the basis for long-lived T-cell memory? [147]. It is important to state that although a plethora of subsets and mechanisms have been proposed to be involved in regulating the memory phases of the

response, there does not appear one solidified model. Ultimately, probably no single model is able to address all the aspects of memory formation, with a multitude of models accurately describing partial aspects of the response.

I.d.iii– Generation of long-lived memory CD8⁺ T-cell

The mechanisms of long-lived memory CD8⁺ T-cells generation is a highly complex field, and a short conceptual framework to the proposed mechanistic models, rather than a detailed description of the literature, will be provided. In fact, over the years, different infection models used have resulted in the generation of slightly different memory CD8⁺ T-cell populations (both in terms of quantity and quality), with each report defining the memory population with often varying markers of preference (resulting in increased challenges when comparing different bodies of work). Experimental model differences can briefly be listed as resulting from antigenic differences (pathogen-used, delivery, dose, Ag-perseverance, location, MHC-loading, and immuno-dominance), inflammatory components (shaping cytokine environment, antigen-presentation, APC maturation, recruitment/activation of other innate/innate-like cells), and naïve cell population used (transgenic vs endogenous T-cells, TCR affinity, pre-cursor frequency, cell state) [148-154]. This is by no means a complete list, but it is worth to note the large numbers of variables that can impact memory formation. The take-home message is that defined inputs generated by infection or vaccination models result in robust, reproducible immune responses, with distinct kinetics and phenotypes; however, a large number of variables can influence the output, resulting in heterogeneity in the memory population.

Several models have been put forward regarding memory T-cell differentiation. Of particular note: the “one-cell, one-fate” and the “one-cell, multiple-fates” hypotheses are most notable in the field (Fig. 1.6) [155]. Initially proposed models suggested that memory T cells originate from linear differentiation from effectors either after [156] or before [157] the peak of T-cell expansion. However, through the use of single-cell transfers of congenitally-marked Ag-specific naïve cells and barcoding technologies, it has been possible to track the expansion of single T-cell clones during *Listeria monocytogenes* infections, as well as others [158-161]. The results from these elegant experiments have provided strong support for the “one-cell, multiple-fates hypothesis”, with heterogeneous clonal family trees detected in recipient host. Furthermore, as elegantly described by Buchholz, Schumacher and Busch, starting out from individual precursors, the population diversity could be the result of fractal expansion (such that the response derived from one T-cell re-capitulates the whole population) or in a composite fashion (such that only by combining all individual responses does one obtain the population average) (Fig. 1.7) [147]. As mentioned by the authors, the use of single-cell technologies would then be paramount if single-cell behaviors cannot be deduced by analyzing the entire population.

The next question has been to understand when during the expansion phases does such differentiation occur? No final consensus has yet been reached; however, we would like to highlight here that *all* models in the field will be a combination of cell-intrinsic and cell-extrinsic factors, that will influence memory formation in the priming phases of the response, throughout the expansion and subsequent contraction. For example, it is

Figure 1.6

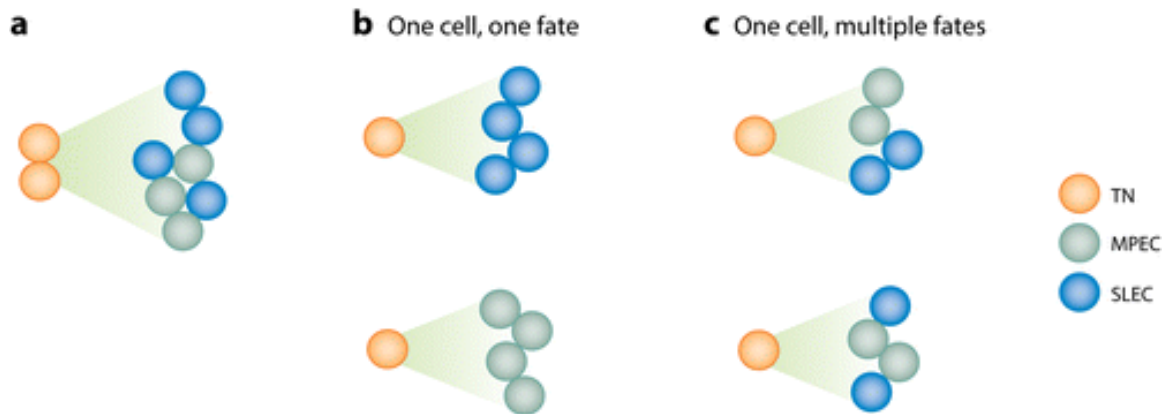


Fig. 1.6: Generation of memory CD8⁺ T-cells. (a) Polyclonal naïve CD8⁺ T-cells (TN), proliferate upon T-cell activation and result in the formation of an effector response involving Short Lived Effector Cells (SLEC), which eventually undergo apoptosis upon contraction and Memory Precursor Effector Cells (MPEC), later forming long-lived memory cells. When analyzing cell expansion, this heterogeneity could arise from (b) a “one-cell, one-fate” model whereby each TN is recruited to one fate or (c) “one-cell, multiple-fates” model, with each TN generating diverse progeny. (Figure taken from Buchholz *et al.*, [121]).

known that TCR affinity can have vast implications for CD8⁺ T-cell proliferation and differentiation, with low-affinity clones resulting in complete activation but curtailed clonal expansion [162]. Aside from the unique TCR specifications of each clone, other cell intrinsic properties such as the amount of signaling components present in a given T-cell (for example, expression of the co-receptor CD8, or SHIP-), can potentially result in heterogeneous responses [163]. The immune system has however evolved to respond

against an incredibly diverse range of pathogens, matching a dynamic and specific T-cell response to each. It would be incredibly detrimental if the whole immune response were to hinge only on T-cell intrinsic mechanisms. Instead, there are numerous cell-extrinsic mechanisms that will influence memory $CD8^+$ T-cell differentiation, allowing for a collective and cooperative immune response to occur with multiple cell players. For example, TLR activation via PAMPs results in the production of $IFN-\alpha/\beta$ which in turn

Figure 1.7

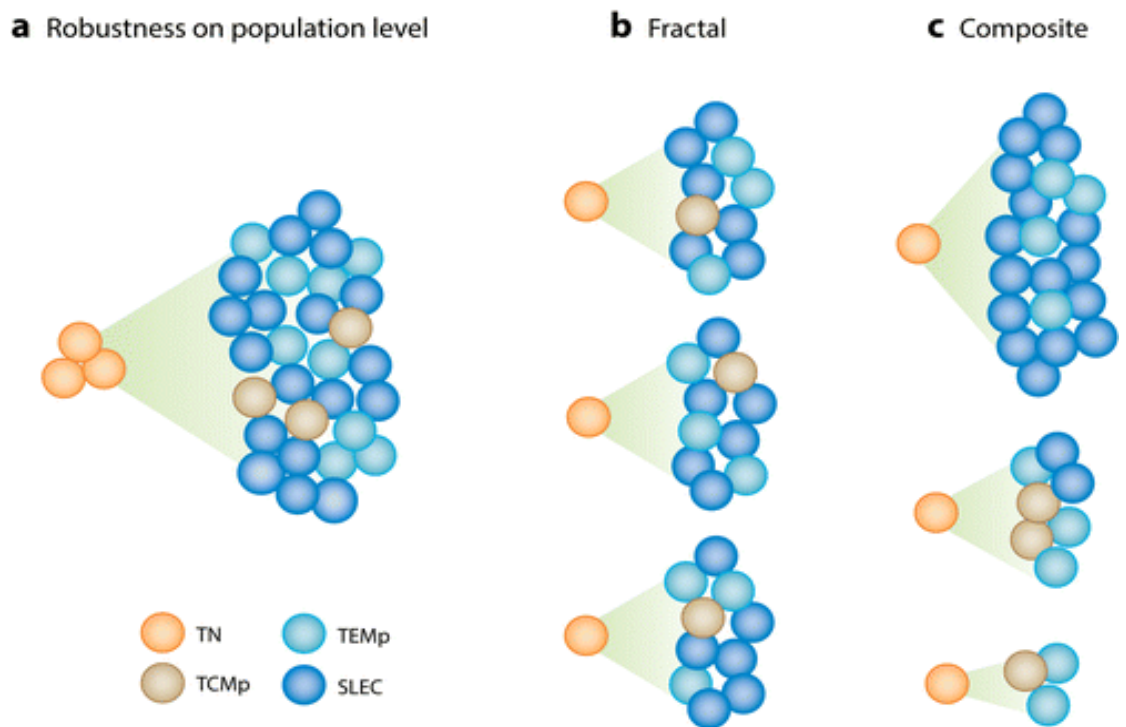


Fig. 1.7: Robustness at the population level results from population averaging of single cell heterogeneity. (a) Each pathogen/vaccination model results in the expansion of a T-cell immune response from a naïve repertoire (TN) which results in the generation of a heterogeneous population which includes Short Lived Effector Cells (SLEC), and long-lived Memory Precursor Effector Cells (MPEC), which can be further subdivided into Tem precursors (TEMp) or Tcm precursors (TCMp). The response to each pathogen can be described as being robust (i.e. predictable) at the whole population level (in terms of Teff and Tem/Tcm cell numbers, and phenotypes). Such a robust response pattern could be generated from either (b) a fractal response, with each single TN being a representative of the whole population or (c) a composite response, with each single TN showing great offspring heterogeneity and only by pooling all single-cell expansion can one observe the whole population. (Figure taken from Buchholz et al., [147]).

can result in DC production of IL-15 (and maybe in part IL-2), which can help shape the developing CD8⁺ memory response [164-166].

Together these models suggest that generation of memory T-cells is a complex, multi-component differentiation process, that can be robustly induced in the host, resulting in a tailored response against each pathogen.

I.e – Tissue-Resident Memory T-cells: A new memory subtype

Over the last decade, seminal work in the field has changed the ongoing paradigm of Tem and Tcm memory CD8⁺ T-cell with the appreciation that another subset of non-migratory memory T-cell called tissue-resident (Trm) T-cells exists. Two laboratories then showed that in the skin and dorsal root ganglia (following HSV infection) and in the small intestines (after LCMV infection), that memory CD8⁺ T-cells can remain in tissues after infection [144, 167]. It appears that each organ has a unique immune tissue-signature program both in terms of transcription factors as well as cell phenotype (in part confined by the unique anatomical architecture of each organ), whereby T-lymphocytes that reside within the tissue anatomy often express markers of other tissue-resident cells (such as innate-lymphoid cells, $\gamma\delta$ T-cells, or NKT cells).

I.e.i – Trm location and phenotype

One of the defining features of Trm cells is their inability to re-circulate and their unique tissue localization. Indeed through parabiotic studies it has become apparent that a fraction of memory T-cells are able to circulate between the vascular system of two

conjoint animals, with a substantial proportion being retained within each animal [144]. Specifically, Trm are found in greater frequencies at barrier sites such as the skin, lungs, gastro-intestinal track, and reproductive-tract (Fig. 1.8) [144, 168, 169]. However, there have also been reports of Trm in internal organs such as the kidney, liver, thymus and pancreas [144]. Trm in general are enriched in certain anatomical compartments of each tissue, often not distributed in a random fashion within the organ. In fact, the specific spatial distribution of Trm plays a crucial role on Trm survival mechanisms, population density, and ability to migrate (or remain sessile) and hence survey the tissue compartment. For example, skin Trm are found predominantly in the epithelial layers and hair follicle epithelium where they receive unique developmental signals crucial for their survival [170, 171]. In contrast to T-cells within the dermis that are amoeboid in shape and are capable of fast migration (6 mm/min), Trm in the epidermis adopt a dendritic morphology and show severely decreased motility (1–2 mm/min) [145]. In other tissues, such as the liver, Trm have been found to remain in blood vessels and patrol the sinusoidal vasculature [85, 168, 172], in a similar fashion to NKT cells [173]. Such diversity is probably dictated by “tissue-specific” rules.

Phenotypically, two of the defining cell markers for Trm have been CD69, and the α E integrin (CD103) for the majority of the cells [174]. Of specific note, however, Trm in the liver, intestines and secondary lymphoid organs have been found to be CD103⁻ [174]. One may speculate that rather than variability dependent on different induction models, expression of these markers is determined more by the specific requirements of tissue itself. In addition, other markers that have been associated with Trm have been the expression of high levels of CD44, and low levels of CD62L, CCR7 and S1PR1 (required

for LN entry) [174]. Lastly, Trm have been described to have a unique transcriptional signature, characterized in part by their tissue of residence [171]. For example, work done on skin, lung and small intestines has revealed that several TCR-signaling modulators are upregulated in Trm (specifically CTLA-4, ICOS, KLRE1 and members of the SLAM family), as well as transcription factors such as Nr4a, which has been implicated in

Figure 1.8 limiting T-cell pro-inflammatory functions [175].

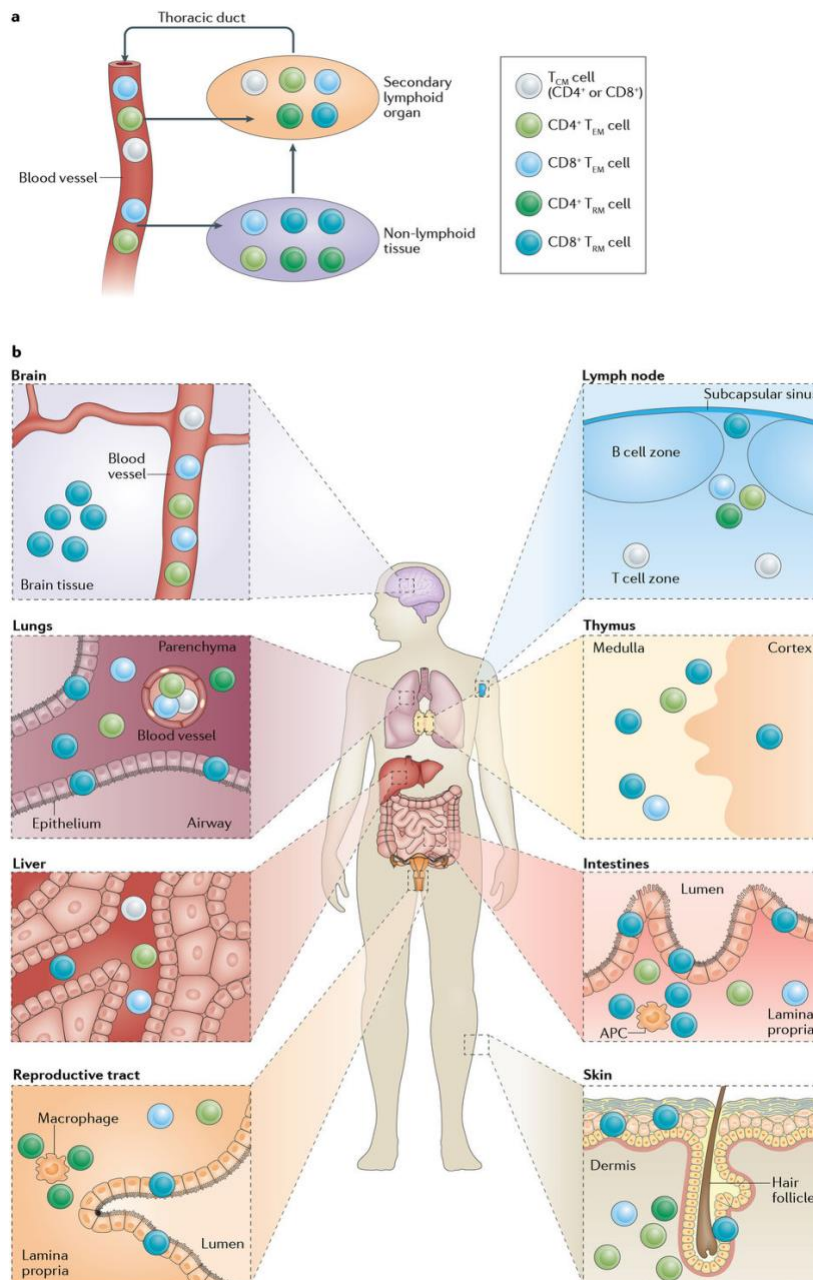


Fig. 1.8: Memory CD8⁺ T-cell location and classification.

(a) CD8⁺ and CD4⁺ T-cells of different phenotypes (broadly classified as T_{CM}, T_{EM} and T_{RM}) can be found at high frequencies in secondary lymphoid organs, non-lymphoid tissues or in the circulation at depicted frequencies. (b) T_{RM}, T_{CM} and T_{EM} have been described in various organs, either forming small clusters together with macrophages or DC (ex: in the reproductive tract) or patrolling through the vasculature (ex: in the liver). Importantly shown, T-cells can occupy different positions within the same tissue (ex: the epidermal or dermal skin layers) and hence might have unique migration, morphology and cell phenotypes in each of these sub-compartments. The result is that T_{RM} are uniquely tailored to the tissue microenvironment they reside in (Figure taken from Mueller&MacKay [174]).

I.e.ii – Trm induction and retention

The mechanisms behind the induction of Trm in different tissues are still being determined. Trm development and regulation probably occurs via specific checkpoints such as entry into the tissue, retention and long-term maintenance. It has long been shown that Teff entering the tissue at a site of peripheral infection undergo the classical adhesion cascade involving, tethering, rolling and then adherence to activated vasculature via chemokine sensing, and integrin elongation on the cell surface [176]. Once inside the tissue, Teff migrate towards an infected cell following a chemokine gradient [177].

By taking into account the Trm literature published in the last years, distinct tissue classes can be observed. These are determined on the degree of access by Teff into each tissue at steady state. In fact, tissues can be described as being either permissive or restrictive, with all tissues allowing for Teff entry during the inflammatory process but not at steady state. Restrictive tissues are organs like the skin epidermis, genital-urinary tract, lungs, salivary glands and ganglia [178]. On the other hand, permissive tissues are seeded by Trm in the absence of local inflammation and Teff can easily enter the tissue without a permissive vascular endothelium. Examples of these are the intestinal epithelium, peritoneal cavity and liver tissue. Importantly, permissive tissues constitutively express homing molecules and chemokines. For example, the liver endothelium has been described to express CXCL16 at steady state, with CXCR6⁺ CD8⁺ T-cells being retained in the vasculature [85, 173]. Another example is the tissue-homing molecule MAdCAM-1 and CCL25, expressed by the endothelium of the intestinal mucosa, bound by the integrin Lymphocyte Peyer patch adhesion molecule (LPAM, $\alpha_4\beta_7$), and CCR9 respectively [146]. Tissue permissiveness probably also shapes the

requirements for Trm induction. In certain tissues, such as the skin and vaginal mucosa, nonspecific inflammation or chemokine administration (such as CXCL9 and CXCL10) results in CD103 upregulation and formation of Trm [179, 180]. However, in organs such as the lungs and CNS, local antigen recognition is required for upregulation of CD103 [179, 181]. Specifically, in an elegant set of experiments by Lee et al., when anti-MHC-peptide antibodies were administered, CD69 and CD103 expression was reduced [181].

As mentioned earlier, the differentiation programs required for memory formation are not yet clear. Various non-lymphoid organs are seeded by Teff during an infection and once there, further differentiate to acquire distinct phenotypes [146]. Some have suggested that a transcriptional program exists for Trm inductions, with the regulation of successive transcription factors such as Eomes, TCF1, T-bet, and Hobit [171, 182, 183]. Furthermore, others have shown that Trm are generated from the Teff pool and share common ancestry with Tem and Tcm cells. Evidence for this also arises from the fact that Tcm and Trm were found to share common clonal origins following primary skin infections [184]. This data however must be taken with caution, as kinship cannot be traced via TCR clonality alone, as approximately 200-1000 T-cells share the same TCR (in part further expanded upon by clonal homeostatic proliferation), and Trm generation could result from encountering separate T-cell differentiation programs. Future work in the field is still required to address these questions in greater detail.

The mechanisms of tissue-retention and survival have crucially implicated several molecules such as TGF- β , IL-15, formation of clusters within the tissue parenchyma and downregulation of lymph-node entry receptors. In fact, active signaling via the TGF- β pathway results in the downregulation of T-bet, which in turn releases the CD103 locus

[171, 185, 186]. Such reports are however limited to skin, gut and lungs tissues where CD103 is needed to bind to E-cadherin on the epithelium [187], and requirements in other tissues such as the liver are predictably different (as mentioned earlier, active signaling via CXCR6-CXCL16 interaction is required for retention in the liver sinusoids [86]). Tissue-retention has also been attributed to the presence of memory lymphoid clusters (MLCs): formed by the association of CD4⁺ T-cells and tissue-resident macrophages producing chemokines [188]. Although no replicating pathogens have been found within MLCs, reports have shown that low levels of antigenic peptides can be found, allowing for long-term retention into peripheral tissues via TCR engagement and CD69 upregulation [188, 189]. Furthermore, retention is also dependent on the inhibition of re-entry into the lymph nodes via the S1P gradient sensing. Indeed, upregulation of CD69 has been shown to bind to the transmembrane domain of the S1P1 receptor, promoting its degradation [123]. The downregulation of the transcription factor KLF2 upon entry into peripheral tissues, further augments tissue retention, resulting in a decreased expression of both S1P1 and CCR7, both required for lymph-node re-entry [83, 84].

Lastly, reports have implicated IL-15 in Trm survival in certain tissues such as skin, lung and gastro-intestinal tract, as have flavonoids, polyphenolics and indoles bound via the Aryl hydrocarbon receptor (AhR) in the skin [171, 190]. The complete mechanisms behind Trm retention and survival however are still unclear: what governs cytokine/chemokine production and by what cells? Is there a limiting number of Trm that can enter the tissue? Are the Trm niches competitive? What survival factors are necessary for Trm maintenance? These are just some of the crucial questions still to be answered in

the field. The complexity in the system is furthermore compounded by the tissue-specificity: it is clear from the literature that certain Trm properties are comparable across organs, while others are not.

I.e.iii – What are the mechanisms of protection by Trm CD8⁺ T-cells?

Multiple mechanisms of Trm protection have been described upon pathogen re-encounter, involving temporal, quantitative and qualitative advantages over Tem/Tcm memory populations. Importantly, they have been described to be crucial in mediating protection to invading pathogens due to their high proliferative potential and rapid effector cytokine production. Furthermore, increased host protection results in part from the increased number of Ag-specific CD8⁺ T-cells at anatomical sites close to pathogen entry, as well as having direct effects by rapidly clearing infected cells, and inhibiting pathogen proliferation. In part, these functions occur via cell-cell direct mechanisms, but also Trms play crucial roles in altering the localized tissue-environment via production of cytokines, significantly enhancing the tissue-specific immune response by creating a “state-of-alarm” [191].

Of most importance for Trm functioning is the location of residence. By being situated close to invading pathogen entry, Trm have a significant temporal advantage over circulating and lymph-node resident Tem and Tcm, and have been shown to be crucial in mediating protection against local skin vaccinia infections, HSV and respiratory lung infections to name a few [144, 169, 192, 193]. Depending on the tissue of residence, they are either sessile (such as in the skin) or motile (as have been found in

the liver sinusoids): migration and movement within the tissue is strictly confined by the tissue architecture [194]. In the context of liver Trm, they have been shown to patrol the liver sinusoids at 3-7µm/min with high meandering indexes [172]. Due to the large liver search area, Trm migration probably becomes a crucial factor for finding few antigenic events.

Aside from their positional and quantitative differences, Trm have been described to have several phenotypic differences from other memory T-cells. Importantly, they have been shown to secrete rapidly large quantities of cytokines such as IFN γ and TNF α following pathogen encounter, acting in a “sentinel-like” fashion, and augmenting the local immune response both by activating other innate cells (such as NK, NKT and $\gamma\delta$ -T-cells) and recruiting further Teff cell into the immune site [195]. Through the release of pro-inflammatory cytokines, Trm can induce vascular endothelium activation (upregulation of VCAM-1 and ICAMs-1 molecules), allowing fast recruitment of Teff into the site of action and upregulation of antiviral and antibacterial genes [191, 193]. Furthermore, they have been shown to express higher quantities of mRNA transcripts of Granzyme B and perforin, as well as having higher levels of co-stimulatory molecules such as ICOS, making them effective fast responders [174]. Lastly, aside from killing the infected cells themselves, potentially causing tissue damage and immunopathology, some of these molecules can act on the pathogen directly. This is indeed the case for CD8⁺ T-cell secretion of Granzyme B which was been shown to degrade the HSV-1 immediate early protein ICP4, crucial for viral replication [196, 197], as are cytokines such as IFN γ , critical for clearing *L. major*, HSV-2 and HBV infections [195, 198].

On a last note, Trm have been speculated to be potentially better “equipped” at surveying peripheral tissues. For example, they have been shown to express receptors making them difficult targets of virus-invasion. Lung Trm have been shown to express IFN-induced transmembrane protein IFITM3 which can restrict alphavirus and influenza infection, by inhibiting viral fusion with cellular membranes [199, 200]. Other proteins, such as ISG20 (an Interferon-induced RNase specific for viral single-stranded RNA) and IFI44 (found to correlate with inhibited viral replication) have also been found to be highly expressed by Trm [183, 201].

In conclusion, Trm have multiple location, temporal and phenotypic qualities allowing them to boost localized immune responses and play a crucial role in pathogen clearance. In fact, a recent report comparing specific-pathogen free and pet-shop mice has found Trm populations to be highly different between these two strains of mice, with pet-shop mice having a significant immunogenic advantage in a multitude of challenge models involving *Listeria*, LCMV, and although more questionable, *P. berghei* malaria [202].

I.f – Thesis Outline and Aims

Liver-stage malaria vaccines require high-numbers of Ag-specific CD8⁺ T-cells to mediate protection, with liver-CD8⁺ T-cells numbers shown to be particularly critical.

The aims of this thesis were to investigate some of the properties involving CD8⁺ T-cell memory formation and localization, with the hope to improve sub-unit viral vector malaria vaccines. The following topics will be covered:

- **Chapter III:** The local memory CD8⁺ T-cell responses following Ad5 intramuscular vaccination will be investigated, with some preliminary evidence suggesting that prolonged antigen presentation following a recombinant Ad5 injection resulted in changes in CD8⁺ T-cells memory phenotype and location.
- **Chapter IV:** A novel liver-stage malaria vaccine strategy: Prime and Target, with the specific aim to localize long-term memory CD8⁺ T-cell responses within the liver tissue using either protein-loaded nanoparticles or viral vectors as targeting agents was developed. Subsequently, prime and target vaccination was shown to result in 100% sterile immunity in a variety of different challenge models relevant to clinical liver-stage malaria.
- **Chapter V:** The immune response induced by prime and target using advance multi-parameter confocal imaging techniques and flow-cytometry was characterized. CD8⁺ Trm were found to be the protective subset, and subsequently shown to be sufficient in mediating protection in *P. berghei* challenge model of disease.
- **Chapter VI:** Lastly, this thesis is concluded with a summary of the experimental outputs, specifically focusing on interesting future directions and possible research avenues.

- **Experimental limitations:** There are several experimental limitations in the current thesis what should be taken in consideration when interpreting the data in the following chapters. Firstly, developing a vaccine for human use against *P. falciparum* using *P. berghei* and in-bred mice with limited MHC repertoires has multiple caveats. As *P. falciparum* does not infect small rodents, pre-clinical mouse work has focused on using other *Plasmodium* species as malaria models: *P. berghei*, *P. yoelii*, *P. chabaudi* and *P. vinckei*. None of these murine parasites are able to completely mimick the infection and pathogenesis seen in humans: with certain significant differences in terms of antigenic repertoire, antigen expression kinetics and abundancy, and parasite-life cycle. Among these, *P. berghei* spz production was better established and more commonly used in our laboratory, and hence was chosen as the murine model in this thesis to allow comparison with previous studies. The life-cycles of *P. berghei* is very similar to that of *P. falciparum*, with the major difference being the duration of the liver-stage: lasting only approximately 48 to 52 hours in mice, and expression of several liver-stage proteins such as LSA1, LSAP2, and TRAP. Furthermore, another limitation of this thesis is the use of transgenic murine malaria parasites that express specific *P. falciparum* antigens or the model antigen Ovalbumin (OVA). Transgenic parasites can be generated by either allelic replacement of the *P. berghei* gene with the *P. falciparum* homolog (an optimal scenario), or integration of the *P. falciparum* gene at a disposable point in the *P. berghei* genome by double homologous recombination. In the context of spz with *P. falciparum* gene additions, these foreign antigens are expressed under the UIS4 promoter, which changes the expression profile of the antigens and may alter the expression level compared to the native promoter. Although all spz are confirmed to be fit (hence

infectious), their use as immune tools is somewhat limited until tested in human clinical trials. Lastly, the use of OVA as a study model is a major limitation in this study.

Ovalbumin is a T-cell dependent antigen commonly used a model protein for studying Ag-specific immune responses in mice on a C57BL/6 background. In the context of CD8⁺ T-cell responses, SIINFEKL-MHC complexes are highly immunodominant, generating strong T-cell responses that might not be comparable to T-cell responses in against human *P. falciparum* p-MHC complexes.

I.g – Chapter Figure Acknowledgements

I would like to acknowledge the following authors who kindly gave consent to allow the inclusion of their figures found this chapter: *Menard et al.*, *Gazzinelli et al.*, *Mueller et al.*, *Radtke et al.*, *Mandl and Germain*, *Buchholz et al.*

Chapter II

Materials and Methods

Chapter II

Materials and Methods

II.a – List of Materials and Reagents

Details of all commercially available reagents used in this thesis are provided.

Table II.a- List of reagents used in thesis

Material	Supplier	Catalogue Number
0.2µm syringe filter	Sartorius	16534
12-well tissue culture plates	Appleton Woods	BC011
15ml centrifuge tubes	Fisher Scientific	FB55950
18-gauge catheter 1.3x45mm	VWR International	TERUSR-DM1845PX
2-β Mercaptoethanol [50µm]	Gibco	31350-010
2ml syringes	Nu-Care Products	M0185
50ml centrifuge tubes	Greiner	227261
50ml syringes	Nu-Care Products	M0865
5ml polystyrene tubes	VWR International	734-0001
5ml syringes	Nu-Care Products	M2187
60mm petri dishes	Corning	430166
6-well tissue culture plates	Fisher Scientific	TKT-520-030T

70µm cell strainers	BD Biosciences	352350
8-well microscope slides	VWR International	631-0451
96-well collagen coated plates	BD Biosciences	356407
96-well U bottom plates	VWR International	734-0027
96-well flat bottom plates	Fisher Scientific	TKT-180-070U
Bovine Serum Albumin (BSA)	PAA Laboratories	K41-001
Carbonate-bicarbonate buffer	Sigma Aldrich	C3041
CD4 (L3T4) MicroBeads	Miltenyi Biotec	130-049-201
CD8 T cell Isolation Kit, mouse	Miltenyi Biotec	130-095-236
CellTrace™ CFSE Cell Proliferation Kit, for flow cytometry	Thermo Fisher	C34554
Cluster tubes	Corning	4401
Collagenase (Type IV)	Sigma Aldrich	C5138
Cryogenic vials	Star Lab	E3110-6122
Cytofix/Cytoperm™ fixation/permeabilization kit	BD Biosciences	555028
DAPI (4',6-diamidino-2-phenylindole)	Sigma Aldrich	101101353
Dimethyl sulfoxide (DMSO)	Sigma Aldrich	D2650
D-luciferin potassium salt	PerkinElmer	122796

Dulbecco's Modified Eagle Medium (DMEM)	Sigma Aldrich	D6546
Dulbecco's Phosphate Buffered Saline (D- PBS)	Sigma Aldrich	D8537
Endotoxin free water	Sigma Aldrich	W3500
Ethanol	Sigma Aldrich	32221
Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich	E7889
Fetal calf serum (FCS)	Sigma Aldrich	F2442
Formalin	Sigma Aldrich	HT501128
Fructose	Sigma Aldrich	F0127
Giemsa	Sigma Aldrich	GS1L
Glycerol	Sigma Aldrich	G7893
Goat anti-mouse whole IgG alkaline phosphatase conjugate	Sigma Aldrich	A3562
Golgi Plug	BD Biosciences	555028
Hank's Balanced Salt Solution	Sigma Aldrich	H9394
Heparin	Sigma Aldrich	H4784
HEPES	Sigma Aldrich	H3375
IgG from normal rat serum	Sigma Aldrich	14131

Immersion oil	Sigma Aldrich	10890
Isoflurane	Oxford University Veterinary Services	n/a
L-Lysine	Sigma Aldrich	56-87-1
Live/dead Aqua	Invitrogen	L34957
LS Columns	Miltenyi Biotec	130-042-401
Methanol	Fisher Scientific	M/3900/17
Microcentrifuge tubes	Fisher Scientific	FB74031
Micro-fine Insulin Syringe with needle 0.5ml 29g x 12.7mm	Nu-Care Products	M4892
Micro-fine Insulin Syringe with needle 1ml 29g x 12.7mm	Nu-Care Products	M4891
Microlance needle 26g x 5/8inch	Nu-Care Products	M4300
Microscope slides (Plus, charged)	Fisher Scientific	MNJ-200-010H
Microvette tubes	Sarstedt	16.444
Minimum Essential Medium Eagle (MEM) α - modification	Sigma Aldrich	M4526
Mr. Frosty cryocontainer	Fisher Scientific	CRY-120-010T
Neutral buffered formalin	Sigma Aldrich	HT501128

OCT freezing media	Sakura Finetek	25608-930
Ovalbumin protein (Albumin from chicken egg white)	Sigma Aldrich	A5503-1G
PBS	Sigma Aldrich	D8537
Peptide: IPQSLDSWWTSL	ProImmune	na
Peptide: ISQAVHAAHAEINEAGR	ProImmune	na
Peptide: SIINFEKL	ProImmune	na
Peptide: SYIPSAEKI	ProImmune	na
Percoll	Sigma Aldrich	P1644
Phenylhydrazine	Sigma Aldrich	P26252
<i>Renilla</i> Luciferase Assay System	Promega	E2820
RPMI-1640	Sigma Aldrich	R0883
Scalpel (surgical blade)	Swann Morton	0302
Sodium azide (NaN ₃)	Fluka Analytical	08591
Sodium bicarbonate (NaHCO ₃)	Sigma Aldrich	S8761
Sodium hydrogen phosphate	Sigma Aldrich	7558-79-4
Sodium dihydrogen phosphate	Sigma Aldrich	7558-80-7
Sodium chloride (NaCl)	Sigma Aldrich	S5886
Sodium periodate	Sigma Aldrich	7790-28-5
Sucrose	Sigma Aldrich	57-50-1

Transfer pipettes	Fisher Scientific	FB55348
Trehalose	Sigma Aldrich	6138-23-4
Triton™ X-100	Sigma Aldrich	X100
Trizma® HCl buffered solution (Ph8)	Sigma Aldrich	T2694
Trypan Blue	Sigma Aldrich	T8154
Tubing	Fisher Scientific	PYH-700-020L

Table II.b- Antibodies used in thesis

Antibody	Supplier	Catalogue Number
Alexa Fluor 488 conjugated goat anti-mouse IgG	Life Technologies	A11008
Anti-mouse CD11b-Biotin clone M1/70	BioLegend	101204
Anti-mouse CD11c-Biotin clone N418	BioLegend	117304
Anti-mouse CD19-Biotin clone MB19-1	BioLegend	101504
Anti-mouse CD16-32 Purified (Fc block) clone 93	eBioscience	14-0161-81
Anti-mouse CD3ε-APC clone 145-2C11	eBioscience	17-0031

Anti-mouse CD45R (B220)-Biotin clone RA3-6B2	BioLegend	103204
Anti-mouse CD49b-Biotin clone DX5	BioLegend	108904
Anti-mouse CD4-eFluor 450 clone RM4-5	eBioscience	48-0042-80
Anti-mouse CD4-eFluor 650 clone GK1.5	eBioscience	95-0041-41
Anti-mouse CD4-FITC clone RM4-4	eBioscience	11-0043-81
Anti-mouse CD62L-PeCy7 clone MEL-14	eBioscience	25-0621-81
Anti-mouse CD8 α -FITC clone 53-6.7	eBioscience	11-0081
Anti-mouse CD8 α -PerCPCy5.5 clone 53-6.7	BD Biosciences	551162
Anti-mouse H-2K ^b Biotin clone AF6-88.5	BD Biosciences	553568
Anti-mouse IFN γ -APC clone XMG1.2	eBioscience	17-7311
Anti-mouse IFN γ -eFluor® 450 clone XMG1.2	eBioscience	48-7311
Anti-mouse MHC Class II (I-A/I-E)-PE clone M5/114.15.2	eBioscience	12-5321-82
Anti-mouse MHC Class II (I-A/I-E)-PE clone M5/114.15.2	eBioscience	12-5321-82
Anti-mouse TNF α -FITC clone MP6-XT22	eBioscience	11-7321
Anti-mouse CD45.1; (BV421, A20)	BioLegend	110731

Anti-mouse CD45.2; (e594, 104)	BioLegend	109850
Anti-mouse B220 (BV421)	BD Biosciences	562922
Anti-mouse Collagen IV	abcam	
Anti-mouse pMHC-SIINFEKL; Pe clone 25-D1.16	BioLegend	141604
Anti-mouse CD69 (Pe-Cy7, H1.2F3)	BioLegend	104512
Anti-mouse CXCR6 (FITC, SA051D1)	BioLegend	151108
Anti-mouse TCRva2 (APC, B20.1)	BioLegend	127820
Anti-mouse TCRvb5 (Pe, MR9-4)	BioLegend	316704
Anti-SIINFEKL Pentamer (Pe, 093)	Proimmune	093
Qdot 655-Streptavidin Conjugate	Thermo Fisher	Q10121MP
Anti-mouse CD44 (IM7), BV480	BD Biosciences	746710
Anti-mouse CD127 (Biotin)	BD Biosciences	555288
Fc-block (anti-mouse CD16/32)	ThermoFisher	14-0161-82
Anti-mouse F4/80 (647; 6F12)	BD Biosciences	564226
Anti-mouse CD31 (MEC13.3)	BioLegend	102501
Anti-mouse Ki67 (647, B56)	BioLegend	151206
Anti-CSP (3D11-FITC): <i>Kindly provided by A. Radtke</i>	na	na
Jojo (nucleic acid stain); JOJO™-1 iodide	Thermo Fisher	J11372 (Discontinued)
Anti-mouse Granzyme B	abcam	ab4059

Anti-mouse CD69 (AF2386)	abcam	ab202909
Anti-mouse IFN γ (XMG1.2, Pe)	BioLegend	505807

II.b – Solutions and Buffers

1. **ACK Lysis Buffer:** 8.29g NH₄Cl (0.15M), 1g KHCO₃ (1mM), 37.2mg Na₂EDTA in 800ml dH₂O. pH adjusted to 7.2-7.4 with HCl (1M) before making a final solution up to 1L with dH₂O.
2. **FACS Buffer:** 1% FCS and 0.1% sodium azide in PBS.
3. **Giemsa:** 5% Giemsa in dH₂O.
4. **Perm/Wash:** 10x Perm/Wash buffer was diluted in dH₂O prior to use.
5. ***Plasmodium berghei* Freezing Medium:** 11ml FCS, 4.2ml 5% NaHCO₃ and 5.5mg neomycin were added to 96ml RPMI-1640.
6. **Fixing PLP Buffer:** 12.5ml of PFA (4%), 0.106g of NaIO₄ (sodium periodate), 18.75ml of L-Lysine (0.2M), 18.75ml of P-buffer at Ph7.4, 100ml of H₂O (composed of 81ml of Na₂HPO₄ with 19ml of NaH₂PO₄).
7. **Complete α -MEM Medium:** 500ml MEM α -modification was supplemented with 5ml L-glutamine (2mM), 5ml pen/strep (100U penicillin, 100 μ g streptomycin), 500 μ l 2mercaptoethanol (50 μ m) and 50ml of heat inactivated FCS (10%).
8. **Immunohistochemistry Blocking Buffer:** 1% normal mouse serum and bovine serum albumin block solution containing 0.3% Triton X-100, Fc-block (anti-CD16/CD32) was also added (0.01%).
9. **MACS Buffer:** 2.5g BSA (0.5%) and 2ml 0.5M EDTA (2mM) were added to 500ml D-PBS. The buffer was sterile filtered prior to use.

II.c – Methods

All methods used in this thesis will be divided on a chapter by chapter basis.

II.c.i – Chapter III Methods

Animals and Immunizations

All mice were used in accordance with the UK Animals (Scientific Procedures) Act under project license number 30/2886 granted by the UK Home Office. Animals were group housed in individually vented cages under specific pathogen-free conditions, with constant temperature and humidity and with lighting on a 12:12 light/dark cycle (8 am to 8 pm). For induction of short-term anesthesia, animals were anesthetized using vaporized isoflurane (IsoFlo) for all procedures. All animals were humanely sacrificed at the end of each experiment by an approved schedule 1 method. Female or male mice (at least six weeks of age) were used in the following strains: C57BL/6 (Harlan, UK), B6 CD45.1 (Ly5.1) (Biomedical Services, Oxford UK). C57BL/6-Tg(TcraTcrb) (OT I, Charles River, France), and C57BL/6-Tg(TcraTcrb)425Cbn/Crl (OT II, kindly provided by A. Reyes-Sandoval). HAdV5-OVA (Ad.OVA), HAdV5-eGFP (Ad.eGFP), HAdV5-mcherry (Ad.mcherry), and MVA-OVA (MVA.OVA) were all produced by Jenner Institute Vector Core Facility. Intramuscular (im) immunizations were performed into the *Musculus tibialis* (50µl volume of viral vector in sterile PBS) using a 0.5mL insulin syringe (BD). Mice received 1×10^8 infectious units (iu) of Ad.OVA or 1×10^6 plaque forming units (pfu) of MVA.OVA unless otherwise stated.

Intracellular Cytokine Staining (ICS)

Spleens, lymph nodes, livers were harvested at the relevant time-point post immunization. Briefly, single cell suspensions were prepared by grinding the tissue through a 70µm cell strainer; liver homogenates were similarly prepared and subsequently passed over a 33% percoll gradient. Following erythrocyte lysis with ACK, cells were re-suspended in complete MEM Eagle α -modification (Sigma) media and re-stimulated for 6hrs with 1µg/mL of SIINFEKL and/or ISQAVHAAHAEINEAGR peptides (Proimmune) at 37°C in the presence of a Golgi Plug (Protein transport inhibitor, BD). Blood peptide re-stimulation: 5 to 6 drops of peripheral blood were collected into tubes containing 200µl of 10mM EDTA. The blood samples were incubated twice with 1ml of ACK RBC Lysis Buffer. The remaining white pellet was then re-suspended in 500µl of complete media. Cells are added in a 96-well U-bottom plate along with stimulatory or un-stimulatory mixtures, and incubated at 37°C 10%CO₂ for 5-6hrs as described above.

Cells were washed in PBS-BSA (0.5% BSA, 0.05% Sodium Azide in Dulbecco's Phosphate Buffered Saline solution, Sigma). Surface staining for CD4, CD8, CD62L, CD127, CD45.1 (or CD45.2) and Live/Dead Fixable Aqua Dead cell stain (Invitrogen) was performed in the dark on ice for at least 45min. Subsequently, cells were fixed with 10% neutral buffered formalin solution (containing 4% PFA; Sigma-Aldrich) for 5min on ice, prior to intracellular cytokine staining for cytokines (mainly IFN γ) with Abs diluted in Perm/Wash buffer (BD Biosciences). Cells were acquired on a LSRII flow cytometer (BD) and analyzed using FlowJo (Treestar). Murine antigen specific cells were

determined by gating on live cells, size, singlet and either CD4⁺CD8⁻ or CD4⁻CD8⁺. All statistical analysis was performed using Prism v6.0 (Graphpad).

Cell Adoptive Transfers and Labeling

Spleens and lymph node single cell suspensions were prepared as previously described for ICS procedure either from vaccinated or naïve transgenic animals. CFSE powder (Cell Trace CFSE, Invitrogen) was re-suspended in DMSO and diluted at a final concentration of 5 μ M in pre-warmed RPMI (Sigma). Cells were incubated for 10min in a 37°C water bath and frequently inverted to ensure homogenous labeling. The reaction was stopped with cold RPMI-10% FCS (Sigma). Cells were under-layered with FSC prior to counting. Tail intravenous (iv) injections were performed for cell adoptive transfers in 100 μ L volumes using a 0.5mL insulin syringe (BD); 1x10⁶ cells were transferred per mouse unless otherwise stated. Recruitment into division was calculated by dividing the number of cells in each division by 2ⁿ, where n is equal to the division number. This value indicates the number of original cells that went through at least one cycle of cell division, and is expressed as a percentage of the calculated number of original cells [203, 204].

Immunohistochemistry

CD11c-YFP animals were vaccinated with Ad.mcherry (euthanized at day 3 post-administration) or alternatively C57BL/6 animals were vaccinated with Ad.OVA and one month post-vaccination, 2x10⁶ CFSE⁺ OTI-T-cells were subsequently adoptively transferred and animals were euthanized one two-days post transfer.

Popliteal dLN and ndLN were harvested and fixed with PLP buffer (0.05 M phosphate buffer containing 0.1 M L-lysine (pH 7.4), 2 mg/ml NaIO₄, and 10mg/ml paraformaldehyde) over night at 4° Celsius and then equilibrated in 30% sucrose solution for another 24 hours. Tissues were then frozen in OCT and stored at -80° Celsius. Multiple LN sagittal 25µm sections were then made with a Leica cryostat. Sections were blocked for 1-2hr with a 1% normal mouse serum and bovine serum albumin block solution containing 0.3% Triton X-100. Sections were then stained with directly conjugated antibodies or two-step staining process was used. Specifically, anti-B220 (BV421), anti-Collagen IV (used with goat anti-rabbit 647 secondary antibody, Invitrogen), anti-CD8 (647) and anti-pMHC-SIINFEKL-Ab (Pe, clone 25-D1.16). Each antibody incubation step was conducted for a minimum of 8 hours in a dark, humidifier chamber at room temperature. A Zeiss 710, confocal microscope with a motorized stage for tiled imaging. Images were analyzed using Imaris software.

II.c.ii – Chapter IV Methods

PLGA Np generation

PLGA Np encapsulating OVA, BSA, APC-OVA, APC (Sigma) were made using a double emulsion method. PLGA Np adjuvanted with R848 and MPL, containing OVA were also made, as described [205]. In brief, protein (5 mg) was dissolved in water (200 µl) and sonicated (intensity 2, 30s) with 200 mg PLGA (Resomer RG502) in 2 ml dichloromethane to form the primary emulsion. Primary emulsion was then rapidly added to 15ml 1% Poly-vinyl alcohol solution and sonicated (intensity 8, 30s). The solvent was then rapidly removed by evaporation at a reduced pressure. Hardened particles were

collected by centrifugation and washed with DI water three times. Finally, particles were lyophilized with 15% cryoprotectant trehalose and stored at 4°C until ready to use. Pb9 (SYIPSAEKI) peptide (ProImmune) loaded particles were produced as described except a single emulsion was used in which 2mg peptide were dissolved directly into the solvent. Adjuvant particles were made as per protocol described elsewhere[205]. Particles were sized by dynamic light scattering analysis using a Zetasizer nano (Malvern instruments). Total protein loading was determined by hydrolysis of the PLGA with 0.1M NaOH 1% SDS followed by neutralization with 0.1M HCl. Encapsulated protein was subsequently quantified using BCA assay against a standard of relevant protein. On average, protein loading was found to be 1.655 ± 0.53 µg/ml of pellet with a size of 380 ± 30 nm.

Experimental animals and immunisations

All animal work was approved by the University of Oxford Animal Care and Ethical Review Committee and performed under Project License 30/2889 or approved by the NIAID Animal Care and Use Committee (NIH), and performed under the LSB-4E animal license. Male and female C57BL/6 CD45.2 and CD45.1, BALB/c, and ICR (CD-1) mice (Envigo, UK) and B6.SJL-Cd45a(Ly5a)/Nai, C57BL/6Nai-[Tg]TCR OT-1-[KO]RAG1[206] (Taconic) and Ubiquitin-tdTomato transgenic mice (generous gift from R. Tsien, University of California, SD), of at least six weeks of age were used. Mice were immunized im into the *Musculus tibialis* with a dose of 1×10^8 infectious units (iu) for Ad5 and ChAd63, or 1×10^6 plaque forming units (pfu) for MVA. For tail vein iv, the following doses were administered: 1×10^9 iu for Ad vectors or 1×10^6 pfu for MVA

vectors unless stated. Viral vectors were all produced by Jenner Institute viral vector core facility, Oxford UK. The following constructs were used expressing either model or leading liver-stage malaria antigens[101]: HAd5-OVA, HAd5-HBsAg, HAd5.ME-TRAP, ChAd63.ME-TRAP, ChAd63-*Pf*LSA1, ChAd63-*Pf*LSAP2, ChAd63-*Pf*TRAP, MVA-OVA, MVA-HBsAg, MVA.ME-TRAP, MVA-*Pf*LSA1, MVA-*Pf*LSAP2, MVA-*Pf*TRAP. PLGA-Np containing 25µg of protein were administered by tail vein iv, unless otherwise stated.

ICS assay

Intracellular cytokine staining (ICS) was performed as previously described above [100]. In short, liver lymphocytes were isolated following *in situ* PBS perfusion of the liver via the portal vein. Tissues were subsequently passed through a 70 µm cell strainer, and single cell suspensions added to a 33% isotonic Percoll solution (Sigma) and centrifuged at 693g for 12 minutes [207]. Erythrocytes were lysed via treatment with ACK lysis buffer for 5min before final resuspension of isolated lymphocytes in complete media (complete MEM Eagle olated lymphocy(Sigma)). Splenocytes were similarly passed through cell strainer, erythrocytes lysed, and subsequently added to complete media. Lymphocytes were stimulated for six hours at 37°C with appropriate peptides at a final concentration of 1 µg/ml (OVA peptide used were SIINFEKL and/or ISQAVHAAHAEINEAGR, HBsAg H2-L^d dominant peptide IPQSLDSWWTSL, all from ProImmune, Oxford, UK), 1 µg/ml GolgiPlug (BD) or left unstimulated in complete media and GolgiPlug for control samples.

Cells were subsequently surface stained on ice with anti-CD4 (e650, GK1.5), anti-CD8 α (e450, 53-6,7), anti-CD69 (Pe-Cy7, H1.2F3), anti-CD127 (Biotin, A7R34), anti-CD62L (APC-Cy7, MEL-14), anti-CXCR6 (FITC, 221002), anti-TCRV α 2 (APC, B20.1), anti-TCRV β 5 (Pe, MR9-4), or anti-SIINFEKL-Pentamer (Pe, 093), Qdot 565 Streptavidin Conjugate (Invitrogen) and LIVE/DEAD Fixable Aqua Dead cell staining (Invitrogen). Following surface staining, cells were fixed with 4% formaldehyde solution (Buffered Formalin solution (10%), Sigma) for 5 min on ice. Intracellular staining was performed using anti-IFN γ (APC, XMG1.2) in Perm-Wash buffer. All staining reagents were purchased from BD and eBioscience, except for anti-CXCR6 from RD Systems, and SIINFEKL-Pentamer from ProImmune (Oxford, UK), Samples were acquired using an LSR II flow cytometer (BD) or LSR Fortessa (BD) and analysed using FlowJo v9 or v10.1 (Tree Star Inc.). Lymphocytes were gated on live, size, singlet and subsequently gated on CD8⁺ T-cells.

Challenge studies

Vaccinated mice were challenged using sporozoites of previously described transgenic *P. berghei* lines. Two lines express OVA; either under the control of the *Pbhsp70* promoter (*OVA::mcherry_{Pbhsp70}*; line 2027cl1), resulting in cytoplasmic expression in sporozoites and intrahepatic parasites, or as a fusion protein with the *Pbhsp17* (*OVA::Hep17_{Pbhsp17}*; line 2030cl1), resulting in OVA locating to the parasitophorous vacuole (PVM) in the infected hepatocyte[208]. As control, *mcherry_{hsp70}* (*mcherry_{Pbhsp70}* (line 1804cl1) spz were also used. Three transgenic lines express the *P. falciparum* antigens in sporozoite and liver-stages *PfLSA1_{Pbuis4}* (2230cl1), *PfLSAP2_{Pbuis4}*

(2239cl1), and *Pf*TRAP *Pbuis4* (2281cl1)[101]. Spz were isolated by dissecting out the salivary glands of *P. berghei* infected *A. stephensi* mosquitoes around day 21 post-feed. Salivary glands were homogenized, spz counted and mice were challenged with 1000 spz via tail vein iv 21 days post final vaccination, unless otherwise stated. An unvaccinated group of mice (naïve) served as an infection control in each experiment. Mice were monitored from four days post-challenge by thin film blood smears stained with 5% Giemsa, and assessed by light microscopy for the presence of blood-stage parasites (schizonts). Once positive blood-films were confirmed on three consecutive days, or mice reached the end-point of 15 days parasite free (sterilely protected), mice were culled. The time to 1% parasitaemia was subsequently calculated using linear regression based on the three consecutive thin blood films, dependent on the spread of data collected.

***In vivo* luminescence**

BALB/c mice were vaccinated via tail vein iv administration with HAd5-Luc (1×10^9 iu), ChAd63-Luc (1×10^9 iu), and MVA-Luc (1×10^6 pfu). All vectors express *Photinus* Firefly Luciferase. Before imaging, animals were anaesthetized with Isoflurane and subcutaneous injection in the scruff of the neck with 0.75mg D-Luciferin (Caliper Life Sciences, UK). Image capture (exposure time 10s) was performed using IBIS200 imaging system (Caliper Life Sciences, UK) and data was analyzed using Living Image 4.0 software (Perkin Elmer, USA) [209, 210].

Localization of particles post iv administration

C57BL/6 animals were vaccinated with Np.APC-OVA-iv or im. Seven days post administration, flow-cytometry was performed in liver, spleen, lung, dLN and muscle tissue. APC intensity is shown for each tissue. C57BL/6 animals were vaccinated with Np.APC-OVA-iv or im. At day 4 post-injection, 2×10^6 OTI and TdTomato wild-type polyclonal CD8⁺ T-cells were co-adoptively transferred. Livers were perfused with ice-cold PBS and fixed using 1% PFA for 12h, dehydrated in 30% sucrose, and subsequently embedded in OCT freezing media (Sakura Finetek). Subsequently, liver sections were stained for CD45.2 (OTI), CD69, CD31, and F4/80.

Statistical analysis

Prism v5.0c (Graphpad, USA) was used for all analyses. Median value with interquartile range are shown in all graphs, unless otherwise stated. Differences between two groups were compared using a Mann-Whitney test for non-normal distributions. Alternatively a non-parametric One-Way or Two-way ANOVA were otherwise used, corrected for multiple comparisons using a Bonferroni's *post hoc* test. Survival in challenge experiments is presented using Kaplan-Meier curves and significance tested using the Log-Rank (Mantel-Cox) Test.

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Immunofluorescence

Livers were perfused with ice-cold PBS and fixed using 1% PFA for 12h, dehydrated in 30% sucrose, and subsequently embedded in OCT freezing media (Sakura

Finetek). 30µm sections were made with a Leica cryostat and blocked with a blocking buffer containing 1% Fc-Block (anti-CD16/32), 1% BSA and 0.3% Triton X-100 for 3 h. Sections were stained with directly conjugated antibodies or appropriate primary and secondary antibodies for 5 h at room temperature or overnight at 4 °C, in humidified chamber. The following antibodies were used for staining: anti-F4.80 (BM8), anti-CD31 (MEC13.3), anti-MHCII (M5/114.1.2), anti-CD3 (17A2), anti-CD8 (53-6.7), anti-CD4 (RM4-5), CXCR6 (SA051D1), Ki67 (B56), anti-CS (3D11-FITC), CD44 (IM7) and Jojo (nucleic acid stain, ThermoFisher). The following antibodies were used as primaries: anti-Granzyme B (ab4059), anti-CD69 (AF2386), anti-IFN γ (XMG1.2, conjugated to Pe). The following secondaries were used: goat anti-Rat, goat anti-rabbit, and donkey anti-goat IgG (all Invitrogen). Images were acquired on a Leica SP8 confocal microscope (40x and 60x objectives), and subsequent analysis performed using Imaris software (Bitplane). Histo-cytometry analysis was performed as previously described[211]. In short, confocal images were corrected using the Leica Channel Dye Separation module. Surface creation for F4/80 and CD3⁺ T-cells was created and Mean Fluorescence intensity for all channels was obtained. Objects were then exported into FlowJo X (TreeStar Inc.) and later analysed with Prism (Graphpad). Specifically, Suppl Fig1b: histograms show APC-OVA signal on gated F4/80 cells from mice vaccinated with either Np.APC-OVA-iv or Np.APC-OVA-im. Fig2: mean number of cells were quantified around a 100µm radius around infected hepatocytes (IS, infectious site). Equal numbers of IS (specifically 20 IS) were quantified for each condition. Fig3: Trm and Tem were normalized to volume imaged; clusters were defined by at least 10 cells in 20µm radius.

Depletion studies

For depletion studies, mice were vaccinated as previously described. Three weeks post vaccination anti-CD4 (GK1.5, 600µg, BioXCell), anti-CD8 (YTS169.4, 300µg, BioXCell) or Rat IgG2b Isotype control (LTF-2, 600µg, BioXCell) depletion antibodies were administered in 150µl *i.p.* two days prior to challenge. Mice were subsequently challenged using sporozoites expressing OVA (OVA::*Hep17^{Pbhep17}*; line 2030cl1). Infected animals were monitored every day, and bled at day 1 post-challenge to confirm antibody depletion. Acute depletion of lymphocytes requires two-week for complete reconstitution, well above the two-day window for liver-stage malaria.

FTY720 and splenectomy studies

B6.SJL-Cd45a(Ly5a)/Nai mice were immunized im Ad5-OVA 1×10^8 infectious units (iu). Two weeks later, tail vein iv injections of PLGA-OVA (25µg) were administered. Three weeks later, FTY720 (Sigma) (0.2 mg/ml, dissolved in saline solution) or vehicle control was administered ip at 1 mg/kg (three daily injections prior to spz challenge). Alternatively, mice were anesthetized via isoflourane and spleens were removed following double ligation of the splenic vessels or the animals were sham operated. Underlying muscles and skin incisions with were sutured and mice were allowed to recover from the surgery for 2.5 weeks. Subsequently, FTY720 (Sigma) at 1 mg/kg was administered as previously described and mice were subsequently spz challenged. Alternatively, mice were vaccinated with Ad.OVA-im/Np.OVA-iv and three weeks later FTY720 (1 mg/kg), FTY720 and anti-CD8 (YTS169.4, 300µg, BioXCell),

anti-CD8 antibody alone, or vehicle and IgG2b Isotype (LTF-2, 600µg, BioXCell) controls were administered ip prior to challenge.

In an alternative set of experiments, B6.SJL-Cd45a(Ly5a)/Nai mice were primed with Ad5-OVA im and two weeks later received three consecutive ip injections of FTY720 (1 mg/kg) or vehicle control. Subsequently, mice were iv injected with PLGA-OVA Np (Np iv, 25µg) and four days later ip injected with BrdU (2mg). After 24hrs, livers were harvested and stained. BrdU staining was performed as described per protocol (APC BrdU Flow Kit, BD). Cells were surface stained prior to fixation and permeabilization with Cytofix/Cytoperm and incubated with Cytoperm Permeabilization Buffer Plus (all BD) for 10min on ice. Cells were treated with DNase (30µg/10⁶ cells) for 1h at 37°C and stained with anti-BrdU for 45min on ice, and samples were acquired on Fortessa flow cytometer.

Adoptive transfers

Lymphocytes from spleens and livers from Ad.OVA-im/Np.OVA-iv vaccinated C57BL/6 CD45.1 mice were obtained as described above three weeks post last vaccination. CD8⁺ T-cells lymphocytes were subsequently negatively selected using Miltenyi negative selection CD8⁺ T-cell isolation kits (Miltenyi Biotec). Following negative selection, Cell Trace APC or CFSE (5µM) (Thermo Fisher) was added for 10 minutes at 37°C to either spleen (APC) or liver tissue (CFSE). In repeat experiments, dyes were inverted labelling spleens with CFSE and livers Cell Trace APC. A small sample of cells was stained with anti-SIINFELK Pentamer Pe, CD8-e450 and CD4-e650 to determine the frequency of Ag-specific CD8⁺ T-cells prior to cell transfer and purity of

the sample (cells were found to be 95% polyclonal CD8⁺ T cells). CD8⁺ T-cells from livers and spleens were mixed in a 1:1 ratio, and transferred to recipient naïve C57BL/6 CD45.2 mice via tail vein iv (with each mouse receiving 1x10⁶ Pen⁺ CD8⁺ from liver and 1x10⁶ Pen⁺ CD8⁺ from spleen). Spleens and liver of recipient animals were obtained one week later for flow cytometry analysis.

Similarly, B6.SJL-Cd45a(Ly5a)/Nai were iv vaccinated with Np.APC-OVA or Np.APC. Four days post vaccination, 2x10⁶ OTI (CD45.2) and 2x10⁶ polyclonal wild-type CD8⁺ TdTomato T-cells (both negatively selected using Miltenyi negative selection CD8⁺ T-cell isolation kits) were injected into recipient mice. Livers were subsequently harvested three days post transfer, tissue was subsequently sectioned and stained as described below.

Statistical analysis

As for the previous chapter, Prism v5.0c (Graphpad, USA) was used for all analyses. Median value with interquartile range are shown in all graphs. Differences between groups were compared using a Mann-Whitney test for non-normal distributions, a non-parametric One-Way or Two-way ANOVA as appropriate (all multiple testing was corrected using a Bonferroni's *post hoc* test). Survival in challenge experiments is presented using Kaplan-Meier curves and significance tested using the Log-Rank (Mantel-Cox) Test.

Chapter III

Local memory CD8⁺ T-cell responses following Ad5 intramuscular vaccination

Chapter III

Local memory CD8⁺ T-cell responses following Ad5 intramuscular vaccination

III.a – Introduction

CD8⁺ T-cells have been shown to be critical in the control of a wide range of intracellular pathogens such as viruses, intracellular bacteria and protozoan parasites. Understanding the mechanisms regulating immune cell expansion following activation, formation and maintenance of memory cells, as well as memory CD8⁺ T-cell phenotype and anatomical localization has been the focus of extensive immunological research [143, 194]. Indeed, seminal work by Sallusto and Lanzavecchia proposed two different subsets of memory T-cells: T-effector (Tem) or T-central (Tcm) Memory T-cells, which differed in immediate effector function (such as perforin or cytokine production) and the expression of homing receptors allowing them to migrate to peripheral or secondary lymphoid organs [142, 212]. Indeed, several markers have been used to demark Tem and Tcm in mice, such as the expression of the L-selectin (CD62L) which binds GlyCAM-1 expressed abundantly on HEVs, and IL-7 receptor- α chain (CD127). Tcm were found to be CD62L⁺ CD127⁺, while Tem have been described to be as CD62L⁻ CD127⁺ [213]. Understanding how these populations of cells are generated from the priming, expansion, and contraction phases of the immune response, maintained long-term in the host, and how they can be experimentally modified are crucial questions in the field. Of most importance, a greater understanding of these processes would have important implication from both a basic science prospective (cell fate decisions in immunology are highly

complex and largely not understood) and translational prospective, (with impacts on rational vaccine design against a wide range of pathogens and cancer-immunotherapy) [214].

In the context of liver-stage malaria, protection is dependent to a significant extent on the induction of CD8⁺ T-cells against *Plasmodium* antigens. As mentioned in Chapter I, seminal work performed in the early 1970s demonstrated that inoculation with irradiated sporozoites was able to induce complete protection in malaria challenges in both mouse and human studies, and that this protection was largely dependent on spz-specific CD8⁺ T-cells [60, 63]. Having identified the cellular immune response crucial in mediating sterile protection, multiple vaccine approaches have attempted to induce high numbers of memory CD8⁺ specifically by using recombinant viral vector vaccines, irradiated sporozoites (RAS) [88], and genetically attenuated parasites (GAP) [92]. To date, a leading vaccine strategy has been heterologous prime boost regimen, where vaccination with an Adenoviral vector is followed 8 weeks later by a Modified Vaccinia Ankara virus (MVA) boost [215].

Replication-defective recombinant Adenoviral vectors induce potent and sustained CD8⁺ T-cell immune responses upon administration, making them an attractive option for vaccine development. In fact, vectored vaccines have been widely used in early stages of clinical trials against a range of diseases including malaria, HIV, influenza, tuberculosis, and more recently Ebola [95, 216-218]. However, despite the large number of prime-boost Adeno-MVA pre-clinical and clinical studies, the mechanisms behind their unique ability to induce unusually high levels of memory CD8⁺ T-cells are still unclear.

Non-replicating Adenovirus-vectors have been known to generate a unique cellular T-cell response with a protracted contraction phase, inducing particularly high numbers of Ag-specific effector and memory CD8⁺ T-cells, and a memory CD8⁺ T-cell response skewed towards a Tem phenotype [219, 220]. In fact, Ads are arguably the most potent inducers of protective CD8⁺ T-cells in comparison to other leading vector technologies such as MVA, Fowlpox, and DNA based vectors [221]. Ad vectors, made non-replicative via the deletion of E1, E3 and E4 regions, induce robust transgene expression, and are able to infect dividing and post-mitotic cells without chromosome insertions [221].

In part, this has been attributed to the property of Adenoviral vectors for long-term persistence in a multitude of different tissues both in mice and macaques, detected for up to a year-post administration by nested RT-PCR [222]. The long-term persistence of Ag-expression by Adeno viral vectors was also observed using Ad5-Luciferase constructs tracked over time [223]. Work using Ad5-SIINFEKL Tet-off constructs, further showed that CD8⁺ T-cell expansion is decreased following Doxycycline administration between 3-13 days post-vaccination [219]. The experiments described in this chapter aimed to characterize the key properties of heterologous prime-boost vaccination and in so doing, provide a better understanding of the factors that regulate CD8⁺ T-cell expansion and subsequent T-cell memory localization in prime-boost vaccination regimens.

III.b – Results

III.b.i – Kinetics and cell expansion of memory CD8⁺ T-cells following prime-boost vaccination

Heterologous prime-boost vaccination involving a combination of Adenovirus and MVA viral vectors is a leading vaccination strategy against liver stage malaria, showing effective CD8⁺ T-cell induction both in mice [224], and in humans [98]. Previous work has shown that prime-boost vaccination results in incredibly high numbers of Ag-specific CD8⁺ T-cells, exhibiting predominantly an effector-memory (CD127⁺ CD62L⁻) CD8⁺ T-cell phenotype [220, 225, 226]. To investigate the factors governing memory CD8⁺ T-cell expansion and localization in the context of an Ad-MVA vaccination regimen, the kinetics of Ag-specific CD8⁺ and CD4⁺ T-cell responses induced by Ad or MVA vectors were first characterized. Mice were vaccinated intramuscularly (im) with either Ad.OVA, or MVA.OVA. A third group of mice were boosted with MVA.OVA 12-weeks after an Ad.OVA prime; allowing the comparison of the recall response induced in this heterologous prime-boost strategy simultaneously to the priming responses.

Lymphocytes were isolated from the spleens of mice at different time points, and tested for the frequency of OVA specific CD8⁺ and CD4⁺ T-cells, enumerated after *ex vivo* peptide stimulation (Fig. 3.1A-B). High frequencies of IFN γ secreting CD8⁺ and CD4⁺ T-cells were observed at D14 post vaccination following an Ad.OVA vaccination, while both the MVA prime and boost responses peaked one week earlier. IFN γ production following *ex-vivo* stimulation was used as a surrogate to measure cells of cells that are Ag-specific. Similar kinetics were observed in the MVA boosted animals, resulting in a higher frequency of IFN γ ⁺ Ag-specific T-cells at D7 after boost. In contrast,

a single administration of MVA resulted in very low numbers of Ag-specific CD8⁺ and CD4⁺ T-cells when compared to responses in Ad prime and MVA boosted mice. The total number of Ag-specific CD8⁺ and CD4⁺ T-cells at D42 post vaccination in various organs was also determined, with the highest numbers of antigen specific cells observed in the spleen, liver and draining LN (dLN) (Fig. 3.1C-F). Consistent with published data [225], vaccination with a heterologous Ad-MVA viral vector regimen induced particularly high numbers of CD8⁺ Ag-specific T-cells in the relevant tissues.

To determine whether this high-frequency of Ag-specific CD8⁺ T-cells was dependent on Ad-priming or due to MVA recall responses, an adoptive transfer of Ag-specific CD8⁺ T-cells into recipient mice and subsequent tracking of their proliferative responses was performed. Spleen and dLN were harvested from CD45.1 C57BL/6 mice 30 days post Ad.OVA vaccination and labelled with CFSE. After labeling, increasing numbers (10⁴, 10⁵, and 10⁶) of CD45.1 Ag-specific CD8⁺ T-cells were adoptively transferred via intravenous (iv) injection into recipient CD45.2 C57BL/6 hosts (Fig. 3.2A). Antigen specificity of cells transferred was determined using TCR-V α ₂ and TCR-V β ₅ staining (here called TCR Va/b⁺ in short), identifying TCR variable regions specific for OVA-specific T-cells [206, 227]. Recipient mice were subsequently boosted with the same dose of MVA.OVA, and Intracellular Cytokine Staining (ICS) on blood and spleen samples was performed at D7 and D20 respectively. Following the MVA boost, both at D7 in peripheral blood and D20 in the spleen, higher numbers of Ag-specific CD8⁺ T-cells observed correlated with higher numbers of Ag-specific CD8⁺ T-cells transferred (Fig. 3.3A, C). However, higher frequencies of transferred Ag-specific CD8⁺ T-cells recruited into division were observed in mice that had received lower numbers of T-cells

(Fig. 3.3B, D, E). Next, the phenotype of CD8⁺ T-cells was investigated and using CD127 and CD62L expression, CD8⁺ T-cells were classified into either Tcm or Tem cells. Although higher frequencies of Tem and Tcm cells were maintained when 10⁶ Ag-specific memory CD8⁺ T-cells were transferred in comparison to 10⁴ cells (Fig 3.3F), the ratio of Tem:Tcm was comparable across all groups.

To further investigate the role of boosting in shaping the number and phenotype of memory cell production, the above experiment was repeated with the same number (1x10⁵ CFSE⁺ TCR Va/b⁺) of Ag-specific CD8⁺ T-cells, but in this instance, different doses of MVA.OVA were administered (10⁷, 10⁶, 10⁵ pfu or none) (Fig. 3.2B). Although at D7 in the peripheral blood there appeared to be a correlation between a higher MVA dose and a higher frequency of recalled transferred Ag-specific cell (Fig. 3.4A-B), by D20 there was no difference between the groups both in the total number of transferred cells, and in the frequency of lymphocytes recruited into division (Fig. 3.4C-D). Administering an MVA boost did increase the total number of Ag-specific CD8⁺ T-cells, compared to the non-boosted group (Fig. 3.4A-E). Lastly, increasing the dosing of the MVA boost had little impact on the frequencies of Tem and Tcm cells generated (Fig. 3.4F). Having obtained some preliminary data that memory CD8⁺ T-cell phenotype appeared to be highly influenced by the initial T-cell expansion during the priming phases of prime-boost response (with little effects observed on numbers or phenotype by altering MVA.OVA dosing), the next experiment was to determine how memory CD8⁺ T-cells were distributed in peripheral tissues following Ad.OVA vaccination.

III.b.ii – Antigen persistence following Ad.OVA im administration

Previous literature had shown that non-replicating Adenovirus viral vectors persisted long term after im administration [222], and it was speculated that memory CD8⁺ T-cell localization and phenotype may be influenced by such persistent antigen expression. Hence, the presence and location of Adenovirus vector persistence of antigen in this model was investigated using transferred OVA specific naïve OTI T-cell proliferation as surrogate for OVA antigen processing and presentation by MHC class I molecules [124, 228, 229]. Mice were vaccinated im with Ad.OVA or Ad.eGFP and at D30 post vaccination, 1×10^6 CFSE labelled CD45.1 OTI were adoptively transferred into vaccinated mice. Three days post cell transfer, spleen and LNs were harvested and lymphocytes were isolated for flow-cytometric analysis. In animals that received an OTI adoptive transfer one month after Ad5 vaccination, three days post- cell transfer, OTI cells had undergone extensive cell division both in the spleen, and in the dLN in OVA vaccinated recipient mice (Fig. 3.5A, C). Furthermore, OTI cells up-regulated CD69 and produced IFN γ (Fig. 3.5B). In contrast, little to no cell division was observed in the contralateral ndLN. OTII CD4⁺ T-cells were transferred in the same experimental model (Fig. 3.6A-B), and some cell division was also observed in the context of MHC-II presentation.

Future work will determine the APC involved in long-term Adenovirus antigen expression and antigen presentation in the dLN using detailed histo-cytometry. This will be investigated by im administering Ad.mcherry, and one month post-vaccination, staining the dLN with anti-mcherry Ab and a DC panel involving CD11c, MHC II, CD11b, and DEC205 (allowing DC characterization between resident cDC1 and cDC2

subtypes, as well as migratory DC [211, 230]) will be pursued. Indeed, preliminary confocal imaging shows that three days post Ad.mcherry vaccination, sub-capsular sinus CD169⁺ macrophages (characterized by their location) are predominantly mcherry⁺, as well as many CD11c-YFP⁺ DCs in the T-cell zone (Fig. 3.7). Previous work in the field would suggest these to be CD8 α ⁺ DC known to present Ad5 viral antigens, albeit non-heamatopoietic cells are also thought to be involved in presenting Ad5 viral antigen [231, 232]. Furthermore, through the adoptive transfer of large numbers of OTI T-cells one month post Ad.OVA vaccination, T-cell clustering can be used as a surrogate to antigen-presentation. Initial immunohistochemistry clearly shows higher OTI T-cells clustering around SIINFEKL⁺ cells, with subsequent work required to carefully characterize their phenotype (Fig. 3.8).

To determine the effect of long term Ag-persistence for memory CD8⁺ T-cell phenotype and location, C57BL/6 mice were vaccinated with Ad.OVA and at D30 post-vaccination, wild-type Ag-specific CD8⁺ T-cells were stained for CFSE and transferred into recipient mice previously vaccinated with Ad.eGFP, Ad.OVA, or left naïve (Fig. 3.9A). At D15 post-transfer, spleen and LNs were harvested and ICS was performed. It must be noted that, in this experimental design, there was the potential transfer of a small amount of antigen from the spleen and LN of harvested donor tissue, as CD8⁺ T cells were not isolated using a negative-selection kit. Whether this was enough to have physiological effect is to be determined with future experiments.

In this experiment, Ag-specific T-cells were found to be comparable in the spleen and ndLN across all recipient mice groups (Fig. 3.10A). However, when comparing the frequency of Ag-specific memory CD8⁺ T-cells in the dLN, an enrichment of Ag-specific

CD8⁺ T-cells were found in recipient mice that had been vaccinated with Ad.OVA rather than Ad.eGFP, or left naïve. Interestingly, this trend was not observed in splenic tissue, either because circulating cells confounded differences in recruitment of Ag-specific T-cells, or because low-level persistent antigen injected during the adoptive transfer was captured preferentially in the spleen in all recipient groups. Looking at the phenotype of Ag-specific memory cells transferred using CD62L and CD127 as memory markers, the data would suggest that in the presence of persistent antigen in dLN, a higher frequency of Tem cells than Tcm were promoted (Fig. 3.10B). This ratio was inverted when cells were transferred in the naïve or Ad.eGFP recipient host, suggesting peripheral persistence of Ag either recruits, stimulates or maintains a Tem memory phenotype. Future experiments will address whether the differences observed in the two populations also correlated to functional characteristics of the known two sub-sets, namely cytokine production, long-term survival, and proliferative capacity [143, 233].

Lastly, to determine the effect MVA boosting on CD8⁺ T-cell memory localization and phenotype, the same experimental set up was performed and after 15 days, mice were boosted with MVA.OVA and flow-cytometry performed 15 days post-boost (Fig. 3.9B). Similarly to previous observations, the frequency of Ag-specific CD8⁺ T-cells was enriched in the dLN of recipient animals vaccinated with Ad.OVA rather than Ad.eGFP, exhibiting a Tem phenotype with higher frequency than in Ad.eGFP recipient animals (Fig. 3.10C-D). Given the same phenotype before and after MVA boost was observed (Fig 3.10B vs Fig 3.10D), it would suggest that the MVA is not altering the memory phenotype, merely enhancing the response initiated by the Adenovirus prime.

III.c – Figures

Figure 3.1

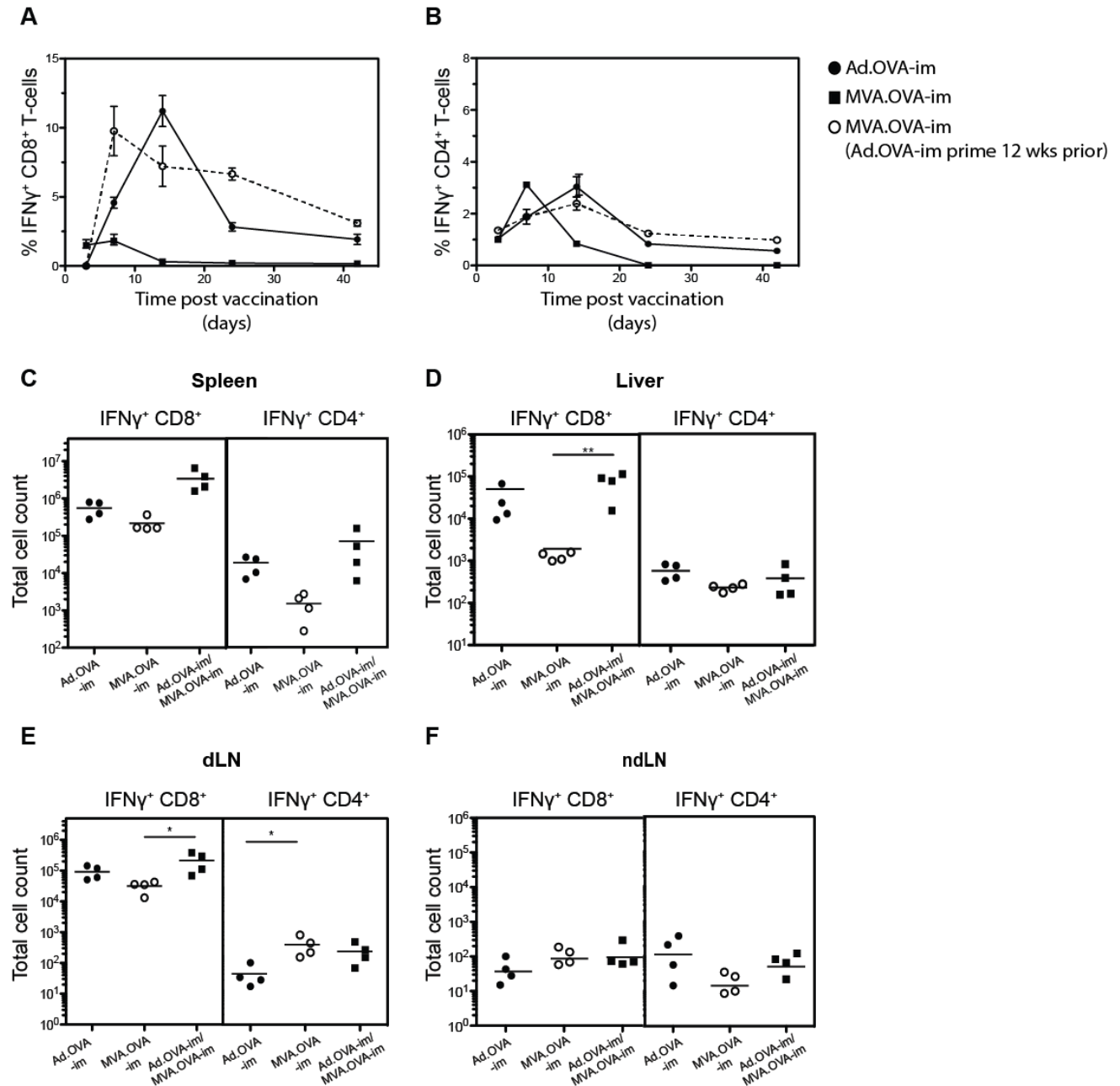
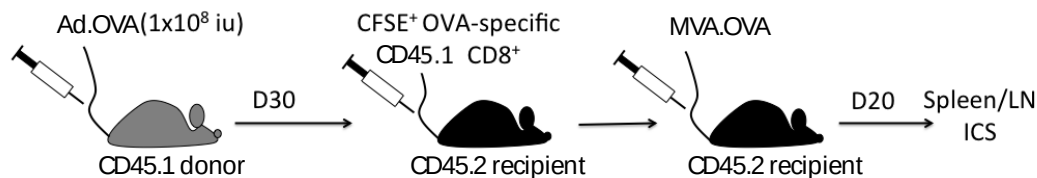


Fig. 3.1: Frequency of IFN γ ⁺ CD8⁺ or CD4⁺ T-cells following vaccination with different viral vectors. C57BL/6 mice were primed with Ad.OVA-im (1×10^8 iu), MVA.OVA-im (1×10^6 pfu), or boosted with MVA.OVA-im (1×10^6 pfu) after receiving Ad.OVA-im (1×10^8 iu) 12 weeks prior. ICS was performed at various time points using spleen, liver, dLN and ndLN tissues. (A) Spleen Ag-specific CD8⁺ and (B) CD4⁺ T-cells were identified by positive staining for IFN- γ after a 6hrs stimulation with SIINFEKL and ISQAVHAAHAEINEAGR peptides. Data points represent a mean of 4 mice per group. Statistical significant difference in the frequency of SIINFEKL specific CD8⁺ was observed at D14 between Ad and MVA (**** $p < 0.0001$), and Ad .OVA-im and Ad.OVA-im/MVA.OVA-im. $p < 0.01$ (**); tested with Two-Way Anova and corrected for multiple comparisons. Total cell counts of IFN γ ⁺ CD8⁺ and CD4⁺ T-cells in (C) spleen, (D) liver, (E) dLN and (F) ndLN at D42 post vaccination. $p < 0.05$ (*); $p < 0.001$ (**).

Figure 3.2



A

Table1	# CFSE ⁺ CD45.1 ⁺ TCR $\alpha\beta$ ⁺ CD8 ⁺ transferred	MVA.OVA boost (pfu)
Group1	1x10 ⁴	1x10 ⁶
Group2	1x10 ⁵	1x10 ⁶
Group3	1x10 ⁶	1x10 ⁶

B

Table2	# CFSE ⁺ CD45.1 ⁺ TCR $\alpha\beta$ ⁺ CD8 ⁺ transferred	MVA.OVA boost (pfu)
Group1	1x10 ⁵	no boost
Group2	1x10 ⁵	1x10 ⁵
Group3	1x10 ⁵	1x10 ⁶
Group4	1x10 ⁵	1x10 ⁷

Fig. 3.2: Schematic outline of experimental adoptive transfers.

(A) C57BL/6 mice were vaccinated with Ad.OVA-im (1x10⁸ iu). At D30 post-vaccination 10⁶, 10⁵ or 10⁴ CD45.1 CFSE⁺ TCR $\alpha\beta$ ⁺ CD8⁺ T-cells were transferred and subsequently boosted with MVA.OVA-im (1x10⁶ pfu). At D20 post-transfer, Spleen and LN ICS was performed.

(B) Similarly, C57BL/6 mice were vaccinated with Ad.OVA-im (1x10⁸ iu). At D30 post-vaccination 10⁵ CD45.1 CFSE⁺ TCR $\alpha\beta$ ⁺ CD8⁺ T-cells were transferred and subsequently boosted with various doses of MVA.OVA-im (1x10⁵, 1x10⁶, 1x10⁷ pfu) or left untreated. At D20 post-transfer, Spleen and LN ICS was performed.

Figure 3.3

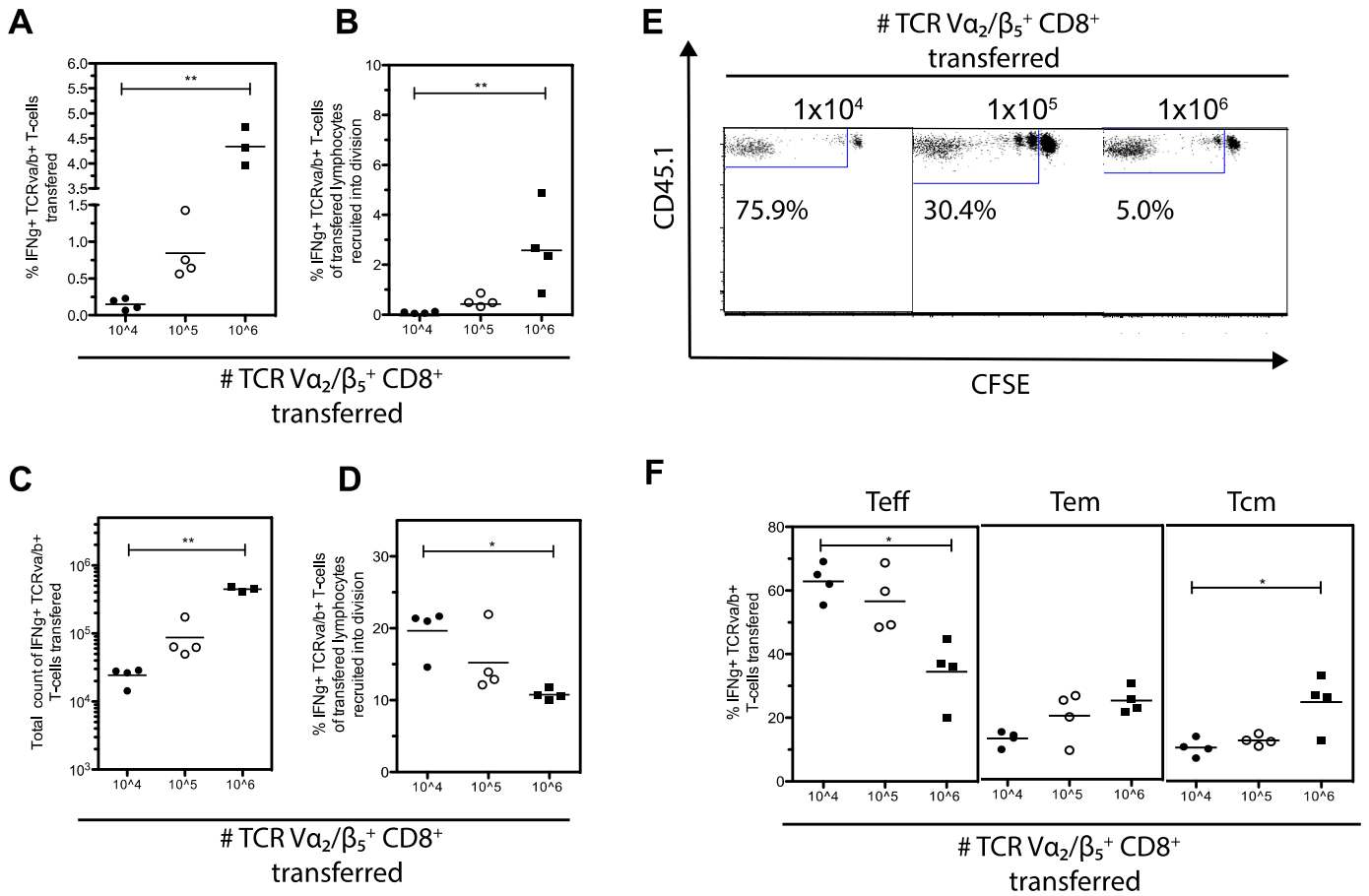


Fig. 3.3: A higher numbers of adoptively transferred Ag-specific CD8⁺ T-cells are maintained post MVA boost.

C57BL/6 mice were vaccinated with Ad.OVA-im (1x10⁸ iu). At D30 post-vaccination 10⁶, 10⁵ or 10⁴ Ly5.1⁺ CFSE⁺ TCR V α ₂/β₅+ CD8⁺ T-cells were transferred and subsequently boosted with MVA.OVA-im (1x10⁶ pfu). (A) Frequency of transferred IFN γ + TCR V α ₂/β₅+ CD8⁺ T-cells and (B) percent recruited into division following vaccination at D7 in peripheral blood determined via ICS. (C) Total cell count of transferred IFN γ + TCR V α ₂/β₅+ CD8⁺ T-cells and (D) percent recruited into division following vaccination at D20 in spleen determined via ICS. (E) Representative flow plots showing cell division of transferred cells from each group. (F) Frequency of Ag-specific Teff, Tem, and Tcm of transferred T-cells in spleen at D20 post MVA-OVA vaccination using CD62L and CD127 as memory markers. Data was analyzed with a Kruskal-Wallis test and a post-hoc Dunn's test; p<0.05 (*), p<0.005 (**).

Figure 3.4

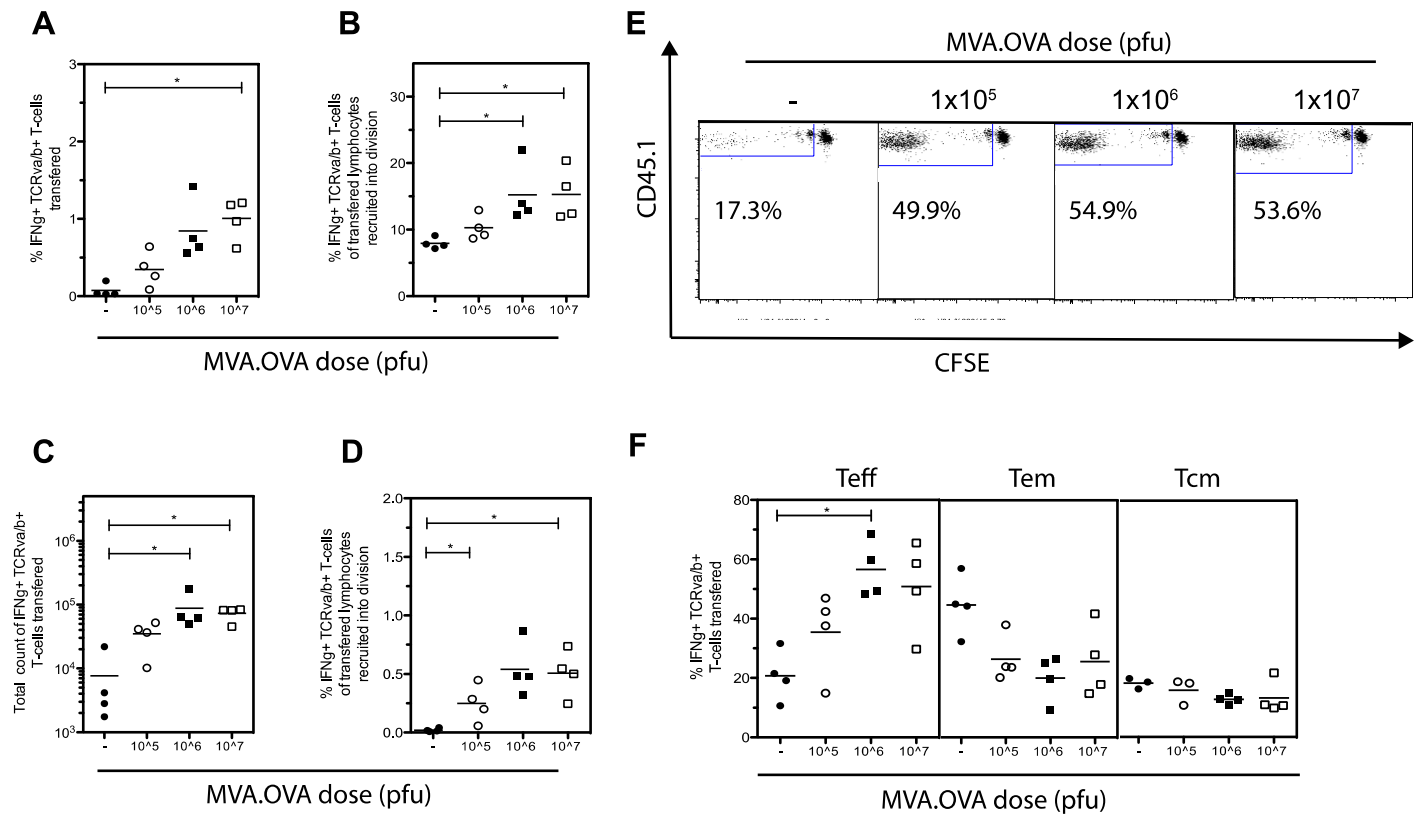


Fig. 3.4: Increasing the dose of MVA-OVA has little effect on the number of adoptively transferred Ag-specific CD8⁺ T-cells maintained post boost. C57BL/6 mice were vaccinated with Ad.OVA-im (1x10⁸ iu). At D30 post-vaccination 10⁵ CD45.1⁺ CFSE⁺ TCR $\nu\alpha_2/\beta_5$ ⁺ CD8⁺ T-cells were transferred and subsequently boosted with 10⁷, 10⁶, and 10⁵ (pfu) MVA.OVA-im or not boosted at all (-). **(A)** Frequency of transferred IFN γ ⁺ TCR $\nu\alpha_2/\beta_5$ ⁺ CD8⁺ T-cells and **(B)** percent recruited into division following vaccination at D7 in peripheral blood determined via ICS. **(C)** Total cell count of transferred IFN γ ⁺ TCR $\nu\alpha_2/\beta_5$ ⁺ CD8⁺ T-cells and **(D)** percent recruited into division following vaccination at D20 in spleen was determined by ICS. **(E)** Representative flow plots showing cell division of transferred cells from each group. **(F)** Frequency of Ag-specific Teff, Tem, and Tcm of transferred T-cells in spleen at D20 post MVA.OVA-im vaccination using CD62L and CD127 as memory markers. Data was analyzed with a Kruskal-Wallis test and a post-hoc Dunn's test; p<0.05 (*).

Figure 3.5

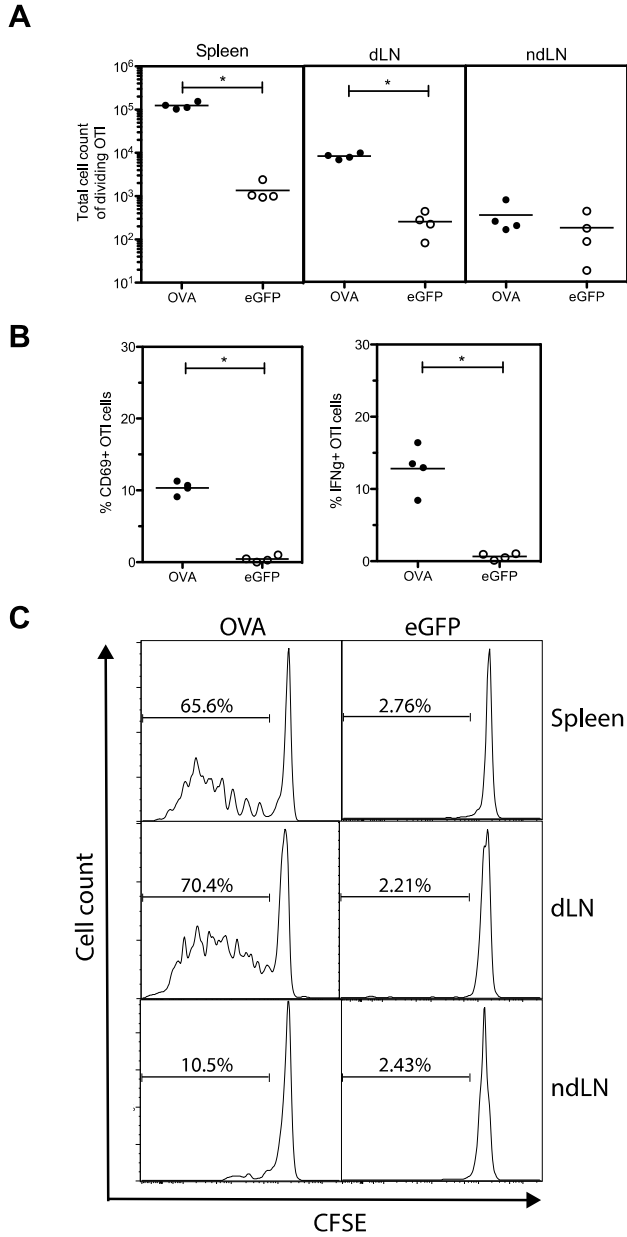


Fig. 3.5: Adoptively transferred OTI T-cells divide extensively in spleen, and dLN in a one-month prior previously vaccinated recipient host with Ad.OVA-im.

C57BL/6 mice were vaccinated with Ad.OVA-im or Ad.eGFP-im (1×10^8 iu), at D30 post Ad vaccination 1×10^6 CFSE⁺ CD45.1⁺ OTI cells were transferred, and spleen, dLN, ndLN were harvested 3-days post transfer. **(A)** Total cell counts of dividing cells in spleen, dLN, and ndLN are shown. **(B)** CD69 up-regulation and IFN γ production of OTI cells in dLN. **(C)** Representative CFSE staining histograms showing OTI cell division. Data was analyzed with an unpaired Mann-Whitney test (* $p < 0.05$).

Figure 3.6

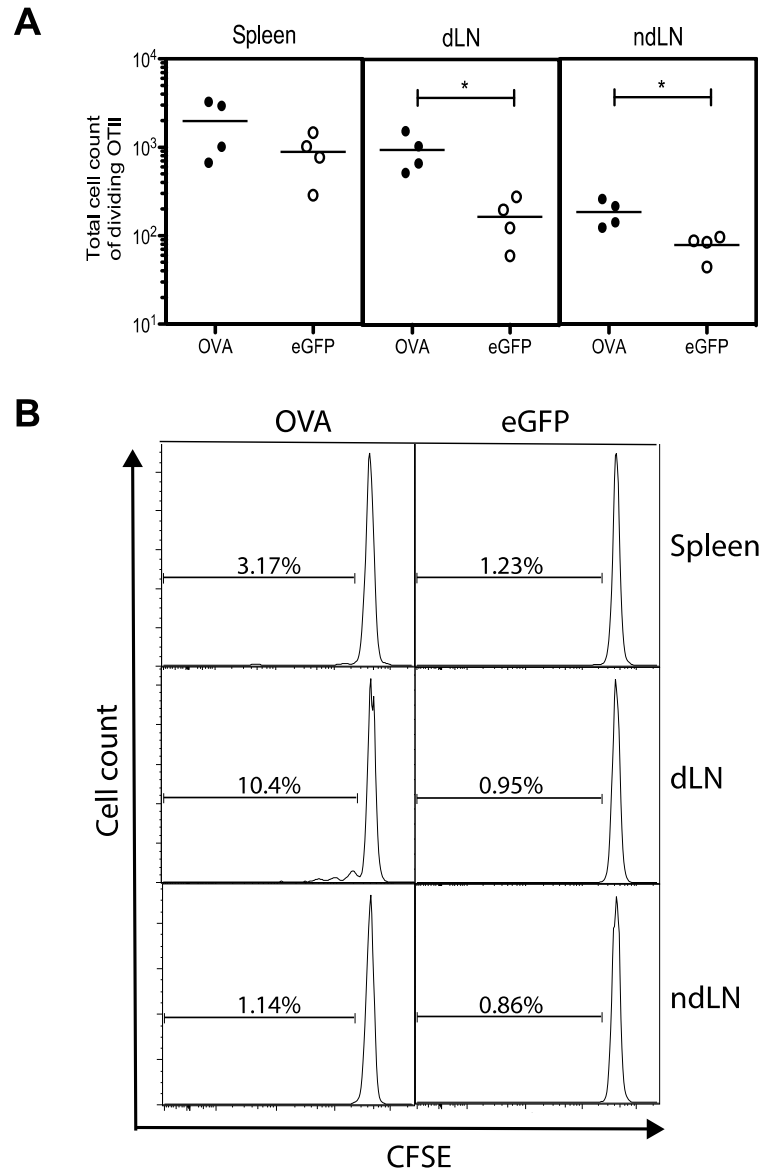


Fig. 3.6: Adoptive transferred OTII T-cells when transferred into mice previously vaccinated with Ad.OVA-im.

C57BL/6 mice were vaccinated with Ad.OVA-im or Ad.eGFP-im (1×10^8 iu), at D30 post Ad vaccination 1×10^6 CFSE⁺ CD45.1⁺ OTII cells were transferred, and spleen, dLN, ndLN were harvested 3-days post transfer. **(A)** Total cell counts of dividing cells in spleen, dLN, and ndLN are shown. **(B)** Representative CFSE staining histograms showing OTII cell division. Data was analyzed with an unpaired Mann-Whitney test (* $p < 0.05$).

Figure 3.7

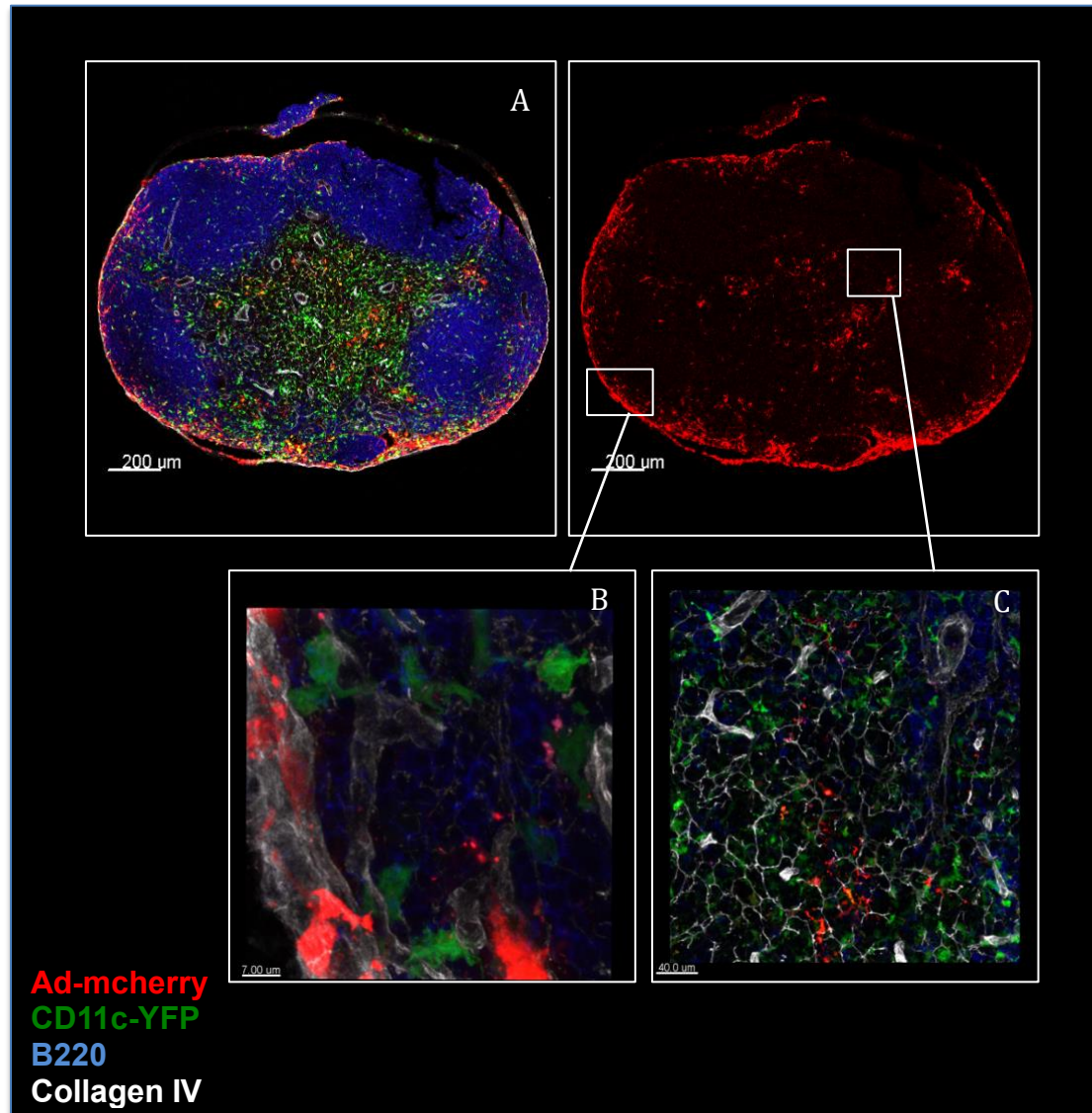


Fig. 3.7: Histology of LN following Ad.mcherry at D3 post vaccination.

(A-C) CD11c-YFP mice were vaccinated with Ad5-mcherry (1×10^8 iu). At D3 post vaccination, dLNs were harvested, fixed and stained. (A) Overall viral localization in the dLN (red). LN staining with B220 (blue) to demark B-cells zone and Collagen IV (white) to provide architectural anatomy of the LN. (B) Higher magnification of sub-capsular sinus and (C) paracortex.

Figure 3.8

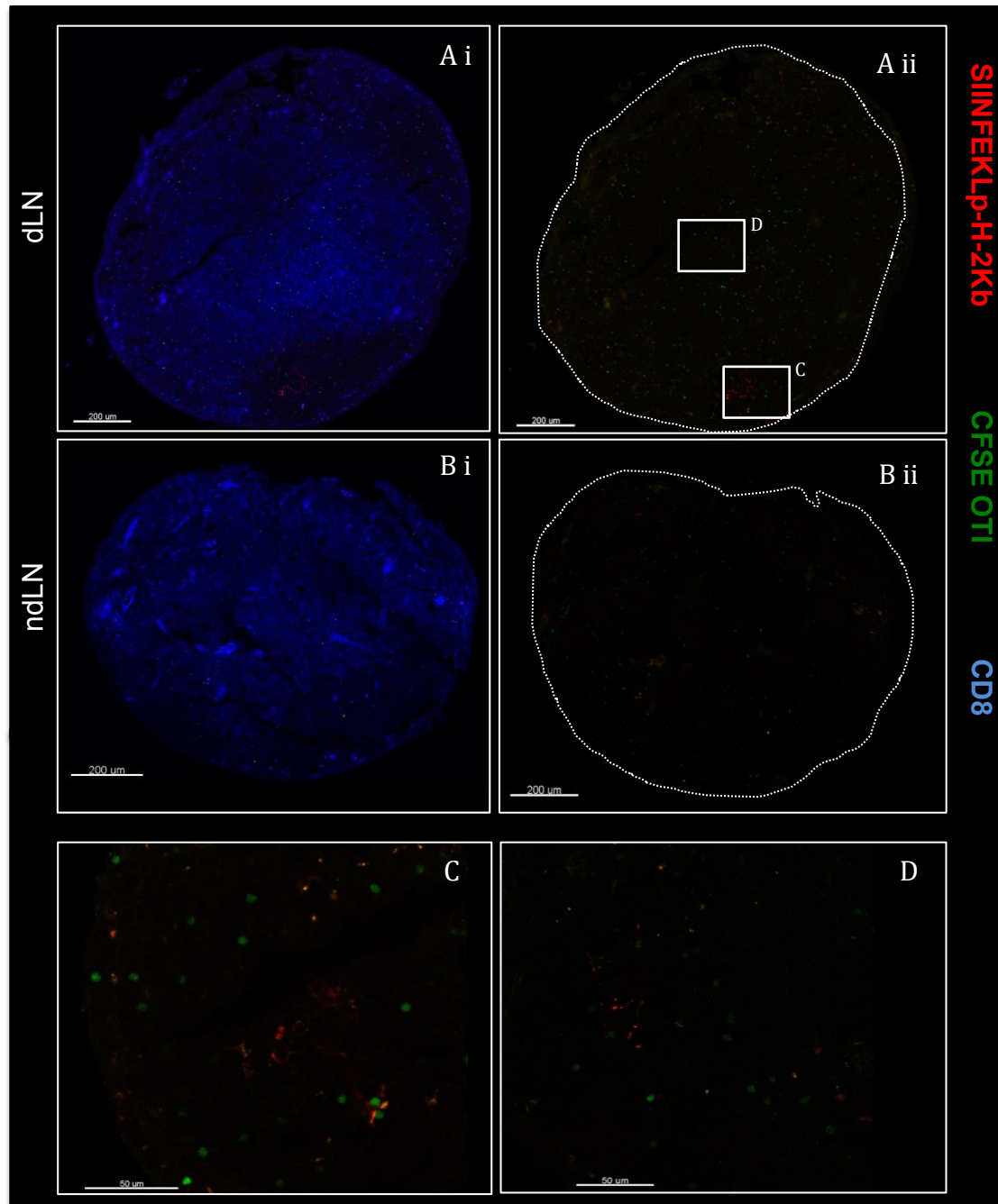


Fig. 3.8: Histology of LN following at D30 post Ad.OVA vaccination and OTI adoptive transfer. C57BL/6 mice were vaccinated with HAdV5-OVA (1×10^6 iu); at D30 post vaccination 10^6 CFSE⁺ OTI⁺ cells were transferred and three days later, dLN and ndLN were harvested, fixed and stained. **(Ai)** Composite image: Draining LN (dLN) showing SIINFEKL⁺ cells (red, stained with SIINFEKL peptide bound to H-2Kb mAB 25-D1) and transferred CFSE⁺ OTI⁺ cells (green) and **(Bi)** Composite image of same panel in Non-draining (ndLN). **(Aii)** Same image of dLN showing only SIINFEKL⁺ cells (red) and transferred CFSE⁺ OTI⁺ cells (green). **(Bii)** Same image of ndLN showing only SIINFEKL⁺ cells (red) and transferred CFSE⁺ OTI⁺ cells (green). **(C)** Higher magnification details from dLN **(Aii)** of sub-capsular sinus, and **(D)** paracortex.

Figure 3.9

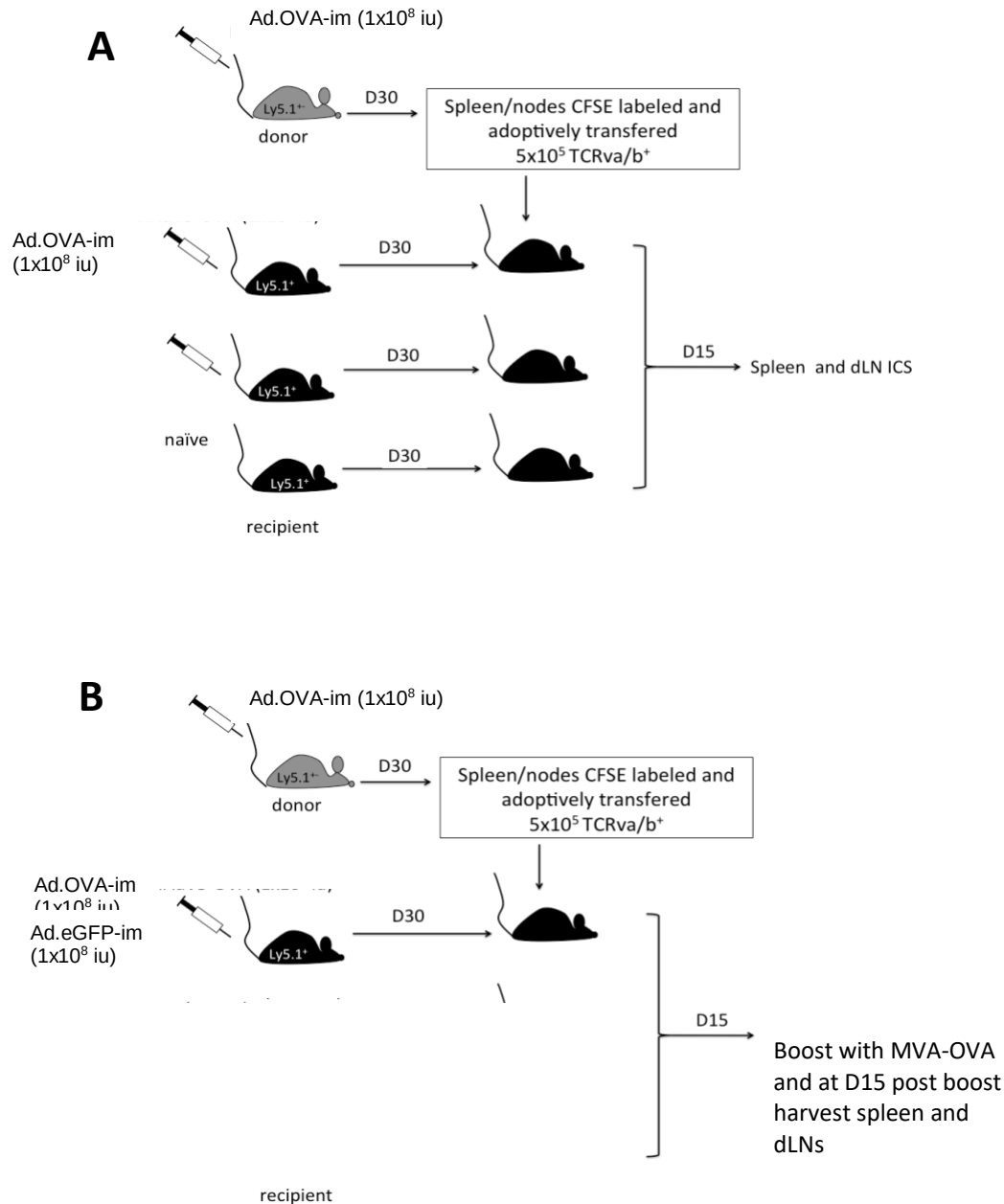


Fig. 3.9: Experimental design diagram of adoptive transfer experiment of memory CD8⁺ T-cells. Donor CD45.1 (Ly5.1⁺) were vaccinated with Ad.OVA-im (1×10^8 iu) and at D30 post infection, spleens and LN were labeled with CFSE and subsequently 5×10^5 TCRva/b⁺ CD8⁺ memory T-cells were adoptively transferred into recipient mice. Recipient mice C57BL/6 mice were vaccinated with Ad.OVA-im or Ad.eGFP-im (1×10^8 iu), or left untouched. **(A)** Spleen and dLN and ndLN were obtained for flow cytometry at D15 post-transfer. **(B)** Mice were subsequently boosted at D15 post transfer. Subsequently, spleen and dLN and ndLN were obtained for flow cytometry at D15 post MVA vaccination.

Figure 3.10

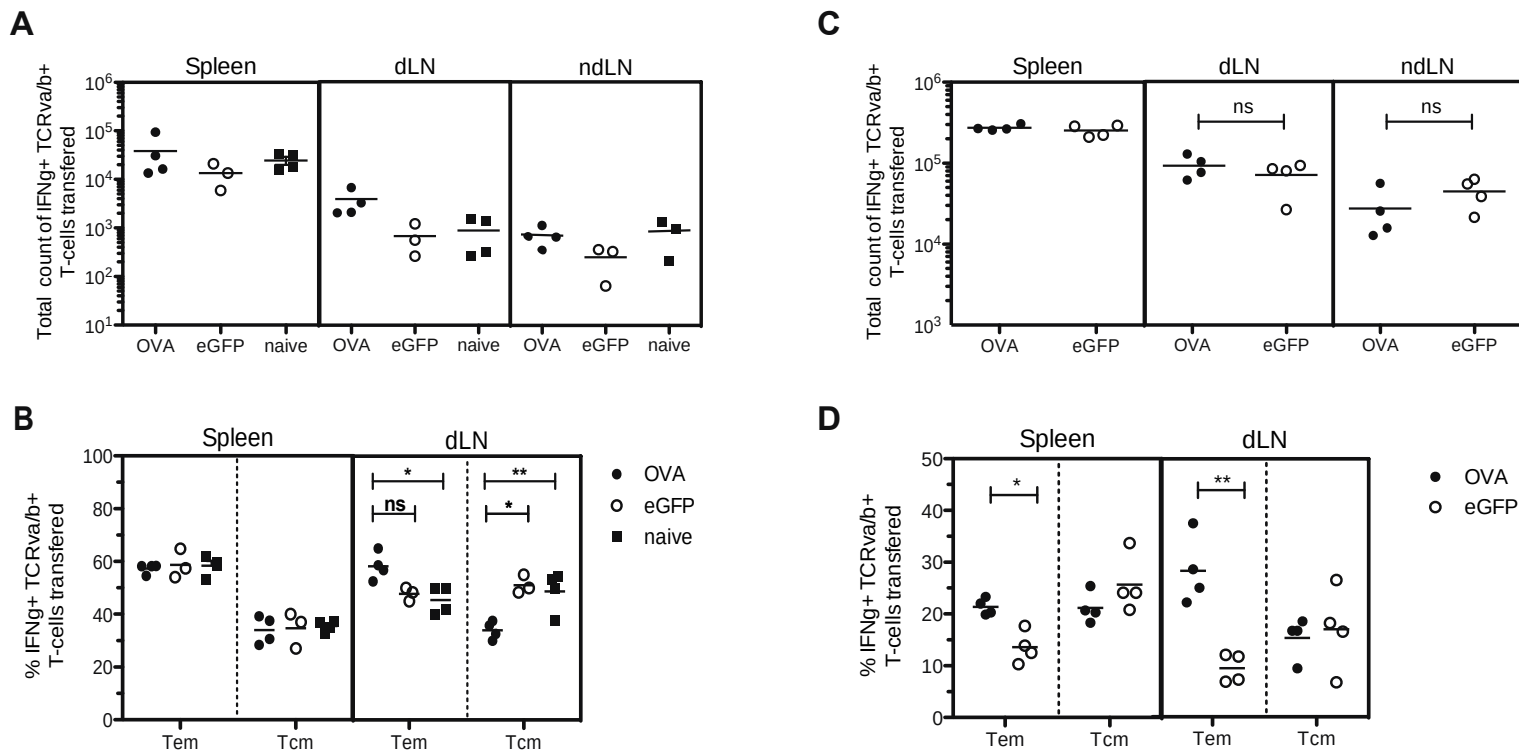


Fig. 3.10: Adoptive transfer of memory CD8⁺ T-cells in previously vaccinated hosts. CD45.1 (Ly5.1⁺) were vaccinated with Ad.OVA-im (1x10⁸ iu) and at D30 post infection, spleens and LN were labeled with CFSE and subsequently 5x10⁵ TCRva/b⁺ CD8⁺ memory T-cells were adoptively transferred into recipient mice. Recipient mice C57BL/6 mice were vaccinated with Ad.OVA-im or Ad.eGFP-im (1x10⁸ iu), or left untouched. **(A)** Total cell count of CD45.1⁺ adoptively transferred Ag-specific CD8⁺ memory T-cells in spleen, dLN and ndLN. **(B)** Frequency of CD45.1⁺ Ag-specific transferred cells expressing markers of Tem (CD62L⁻ and CD127⁺) or Tcm (CD62L⁻ and CD127⁺) in spleen and dLN in different recipients. In a separate experiment, a similar adoptive transfer was performed, but mice boosted with MVA expressing OVA 15 days after adoptive cell transfer. **(C)** Total cell count of CD45.1⁺ adoptively transferred Ag-specific CD8⁺ memory T-cells in spleen, dLN and ndLN at D15 post MVA administration. **(D)** Frequency of CD45.1⁺ Ag-specific transferred cells expressing markers of Tem (CD62L⁻ and CD127⁺) or Tcm (CD62L⁻ and CD127⁺) in spleen and dLN in different recipients at D15 post-MVA administration. Data analyzed with a One-Way Anova and Corrected with Bonferroni multiple comparison test. p<0.05 (*), p<0.005 (**).

III.d – Discussion

Protection against many pathogens requires high numbers of Ag-specific CD8⁺ memory T-cells and non-replicating viral vectored vaccines have shown a remarkable capacity to induce them. Heterologous Ad-MVA prime-boost vaccination induces particularly high numbers of Ag-specific CD8⁺ and CD4⁺ T-cells. Indeed, by examining the kinetics of the response following intramuscular administration of viral vectors, Ad vaccinated animals showed a protracted contraction phase, with enhanced CD8⁺ T-cell induction when compared to MVA [219, 220, 225]. We speculate that this is in part due to the uniqueness of recombinant Adenovirus vectors. Our findings are in accordance with the hypothesis that Ag-experience during the priming and later phases of the response, shapes the size of the memory population following re-activation [234]. In fact, work by von Andrian's lab suggests that the concentration of antigen on the individual APC is crucial in determining the differentiation to either a memory or effector phenotype [235].

To understand if the high frequency of Ag-specific CD8⁺ T-cells induced by the heterologous prime-boost were due to the priming or boosting phases of the response, Ag-specific memory CD8⁺ T-cells were adoptively transferred to naïve recipient mice and subsequently boosted with MVA. In this experimental setting, boosting a high number of Ag-specific memory CD8⁺ T-cells resulted in similarly high numbers of Ag-specific transferred T-cells post MVA-boost contraction. Furthermore, by transferring different numbers of Ag-specific CD8⁺ T-cells, the resulting memory phenotype post-boost was altered with higher adoptive T-cell transfer doses resulting in greater frequencies of T_{cm} and to some extent T_{em}. Interestingly however, increasing the MVA

boost dose by a two-log fold had no outcome in terms of generating higher numbers of Ag-specific memory CD8⁺ T-cells in the long term. Hence, although the dose of MVA boosting might not correlate with greater numbers of Ag-specific CD8⁺ T-cells post boost-contraction, a low dose of MVA certainly increased the number of Ag-specific CD8⁺ T-cells generated overall. A major caveat to this experiment is that high-numbers of Ag-specific CD8⁺ T-cells might directly shape the re-call response (rather than just the MVA dose used), and that only cell-intrinsic effects were studied, with extrinsic-tissue effects on local priming responses also potentially influencing the response.

Together, these data would suggest that the uniqueness of the heterologous Adeno-MVA prime-boost vaccination strategy lies in part in the Adeno response phase. Indeed, it has been described in several studies that memory T-cell populations, once established, do not readily expand further, maintaining the overall number of memory T-cells constant over re-call responses [236]. The order of viral vectors in heterologous vaccines strategies has been shown to matter, from data suggesting that Ad-MVA responses are significantly better at expanding large numbers of CD8⁺ T-cells than MVA-Ad vaccination regimens [237]. Similarly, this was further illustrated in a study using recombinant influenza and vaccinia vectors, where it was found that robust CD8⁺ T-cell responses could be generated after an influenza-prime/vaccinia-boost combination; however, when the order of viral vectors was switched, a much smaller CD8⁺ T-cell response was observed [238]. From a vaccine perspective, these findings are important as they suggest that in vaccine regimens involving multiple viral vector administrations, optimal CD8⁺ T-cell priming is a critical step in long-term memory CD8⁺ T-cell

generation. Further work looking at different priming vaccines will help tease out how priming phases of an immune response shape long-term memory development.

What aspect of an Adenoviral response allows for such increased cell expansion is yet to be determined. T-cell activation and memory formation probably results from the integration of a multitude of signals from dendritic cells and the surrounding environment, which can shape T-cell expansion and differentiation [147]. In part, an early strong TCR stimulation (“Signal 1”) is required for sustained T-cell expansion, and has been shown to play a role in determining memory generation [239, 240]. Furthermore, several papers (reviewed in detail by Krishnamurty and Pepper) have also shown that higher levels of inflammatory mediators favor the generation of Teff rather than memory cells [241]. Hence, in the context of Adenoviral vectors as priming viral vectors, high CD8⁺ T-cell memory generation could be in part due to antigen availability, with Adenoviral vectors being able to express high levels of antigen for a protected period of time [222, 223]; alternatively, Adenoviral vectors could induce a unique microenvironment (such as upregulation of co-stimulatory molecules on DCs, viral tropism, and cytokine signature) that particularly favors the expansion and maintenance of memory T-cells.

Having obtained some preliminary data that memory CD8⁺ T-cell generation appeared to be highly influenced by the initial T-cell expansion during the priming phases of prime-boost Ad-MVA response, we next wanted to explore what determined memory CD8⁺ T-cell localization following Ad.OVA vaccination. Using recruitment and cell proliferation of adoptively transferred OTI and OTII T-cells, the presence and location of the Ag-persistence was confirmed, and found to be primarily localized in

draining secondary lymphoid organs, especially spleen and dLNs. There was little recruitment or division of transferred OTII T-cells, which could be due to either little Ag presented on MHC-II complexes, or because TCR sensitivity is significantly lower with OTII CD4⁺ T-cells.

Next, we speculated that antigen persistence from an Adenoviral intramuscular administration could influence the localization of Ag-specific memory CD8⁺ T-cells similarly to what was observed with transgenic naïve T-cells. To assess this, adoptively transferred memory CD8⁺ T-cells were found to be enriched in dLN of Ad.OVA vaccinated recipient mice rather ndLN of the same animal one month post-vaccination. Such preferential enrichment at the dLN site was not seen when recipient animals were vaccinated with Ad.eGFP or when left untouched. Furthermore, the frequency of Tem is increased when memory CD8⁺ T-cells were adoptively transferred into Ad.OVA vaccinated hosts rather than Ad.eGFP recipients or naïve control animals, suggesting that Ad.OVA vaccinated recipients not only altered the location of memory CD8⁺ T-cells, but also their phenotype. Lastly, a similar experiment was performed whereby MVA.OVA vaccination was performed post-adoptive transfer. Following MVA.OVA boost, a similar number of adoptively transferred memory Ag-specific CD8⁺ T-cells were observed at day 15 post-administration irrespective of the recipient host. Similar to previous experiment, when OVA-specific memory CD8⁺ T-cells were transferred into Ad.OVA recipient animals and then boosted, they preferentially adopted a Tem memory phenotype rather than when transferred to Ad.eGFP vaccinated recipient animals.

The importance of residual antigen presentation has been implicated in a multitude of different viral infections. For example, in the context of influenza lung

infections, previous work had shown that prolonged presentation of residual antigen in the lung-draining LN (with respiratory dendritic cells found to be essential for both sampling and presenting residual viral antigen), is required to maintain large numbers of antigen-specific memory CD8⁺ T cells in the lung airways [242] [243]. Although a certain body of literature speculates that early priming events (such as the amount of antigen present on each DC, cytokine environment and co-stimulatory molecules [235]) can have important implications in determining memory cell formation (as mentioned earlier), it appears that memory CD8⁺ T-cell localization and phenotype can also be highly influenced by persistent antigen presentation, throughout the course of an immune response.

As mentioned in Chapter I, two important parameters are crucial in the context of liver stage malaria sterile protection: memory CD8⁺ T-cell numbers and location. Significant work has been undertaken to improve Ad-MVA viral vector heterologous prime-boost administration. Importantly, utilization of strong long-CMV promoters, incorporation of an MHC-II invariant chain, viral tropism alteration or adjuvant co-administration seems to have some improvements in the total number of long term CD8⁺ memory T-cells generated [100, 221, 241, 244]. However, little work in the malaria field has focused on altering CD8⁺ T-cell memory location. In the context of liver-stage malaria, it would be beneficial to localize CD8⁺ T-cells in the hepatic tissue. The data presented in this chapter has shown that transferred effector CD8⁺ T cells can be recruited to a site of antigen persistence (Fig 3.7) generated by Adenovirus administration. Given high numbers of CD8⁺ T-cells needed in the liver at the time of sporozoite exposure to protect from liver stage malaria, the immediate question is whether high doses of antigen

can be used to recruit effector T-cells to the liver and whether they are able to mediate sterile protection.

Chapter IV

Utilizing Antigen Deposits in the Context of Liver-stage Malaria: a Prime and Target Vaccine Approach

Chapter IV

Utilizing Antigen Deposits in the Context of Liver-stage Malaria: a Prime and Target Vaccine Approach

IV.a – Introduction

Complete protection from malaria spz challenge was demonstrated in both humans [59], and in mice [60], by vaccination with irradiated spz. Later seminal work by Ruth and Victor Nussenzweig, as well as others, identified that CD8⁺ T-cells generated after irradiated spz vaccination were crucial players in mediating liver-stage malaria protection in mice; this paved the way to alternative vaccine strategies aimed at inducing high-levels of liver-stage specific CD8⁺ T-cells [63, 245]. To this end, substantial effort has been invested in optimizing a viral vector, heterologous prime-boost strategy able to generate high frequencies of Ag-specific CD8⁺ T-cells [246, 247]. As mentioned in Chapter I, a leading vaccine approach comprises a simian Adenovirus (Ad) viral vector prime, subsequently boosted with Modified Vaccinia Ankara (MVA) [248-250]. Vaccination with a heterologous prime-boost Ad and MVA expressing TRAP (Thrombospondin-Related Adhesive Protein: implicated in spz recognition and entry into hepatocytes) can induce high numbers of CD8⁺ T-cells with partial clinical efficacy in both malaria naïve and pre-exposed individuals [248, 251]. The induced CD8⁺ T-cell numbers correlate with efficacy in humans, suggesting that increased numbers of circulating CD8⁺ T-cells would be required for higher protection. Despite the substantial progress that has been made to generate improved viral vector vaccine strategies (for example, utilizing the fusion of antigens to MHC-II invariant chain [100]), vaccines

aimed at inducing cell-mediated immunity against malaria have been hindered by a number of challenging spatio-temporal factors pertaining to parasite biology and liver microanatomy.

Firstly, using only peripheral vaccination regimens, it is hard to produce and maintain the high numbers of *Plasmodium*-specific circulating T-cells necessary to confer liver-stage protection [252]. In fact, Spencer et al., have shown that Ag-specific CD8⁺ T-cells do not actively migrate into the liver during a malaria infection and protection is dependent on CD8⁺ T-cells able to locate the small number of infected cells in the liver [82]. Furthermore, it is known that mice with reduced numbers of liver-resident memory CD8⁺ T-cells were less protected against spz challenge, despite having normal numbers of memory CD8⁺ T-cells in the spleen [86]. Secondly, the immune response required to kill infected hepatocytes has a temporal constraint: the liver stages of *Plasmodium* are very short (5-7 days for *P. falciparum* infections in humans, 2 days for *P. berghei* infections in mice) [253-256]. Thirdly, the malaria liver-stage infection generates a “needle in a haystack” situation, whereby under natural conditions, only about 100 spz infect the liver naturally following an *Anopheles* mosquito bite (or in challenge models: 1000 spz are injected iv) [76]. This is particularly challenging for the immune system, as sufficient numbers of CD8⁺ T-cells are required to search all infected hepatocytes probably by probing through the liver sinusoidal fenestrations [257]. Lastly, the parasite biology places an incredible challenge on the immune system: spz migrate through the hepatic vasculature [13, 258], shed large quantities of decoy proteins and following cell transversal [259, 260], invade a hepatocyte eventually forming a quiescent parasitophorous vacuole, altering their antigenic repertoire almost completely [1]. Hence

any adaptive immune response generated will quickly result ineficacious as no longer antigen specific.

A novel vaccine platform was designed to attempt to address some of the challenges faced by heterologous prime-boost viral vector vaccination, with several specific goals in mind:

1. Recruiting large numbers of Ag-specific CD8⁺ T-cells to hepatic tissue while avoiding exhaustion or tolerance phenotypes often associated with hepatic priming and hepatic viral infections [261-264].
2. Utilizing vaccine agents that were known to be safe for clinical use.
3. Utilizing vaccine agents that were deployable on a large scale, ideally cheap to manufacture, and required a regime of no more than two-three vaccine injections.
4. Resulted in long-lasting sterile immunity to *P. berghei* spz-challenges.

For this, a novel vaccine strategy that would utilize antigen deposits to target CD8⁺ T-cells to the tissue needed was developed. Having speculated that a resident population of CD8⁺ T-cells might be crucial in mediating protection against liver-stage malaria, a vaccine platform was designed with the primary focus of generating this population of cells, while at the same time being safe, cost-effective, easily manufacturable and deployable. In an effort to direct CD8⁺ T-cells to the liver, protein-loaded nanoparticles (Np) in combination with viral vectors were initially chosen. The choice of nanoparticles occurred for primarily three reasons: firstly, they had been shown in bioavailability studies to be highly concentrated within the liver tissue (our organ target) following intravenous (iv) administration [265-267]. Secondly, they would allow to administer directly to the liver large quantities of encapsulated Ag (with or without the

addition of adjuvants). Thirdly, Nps are easily and cheaply manufacturable, allowing for a multitude of different proteins and adjuvant combinations to be potentially tested [268]. Use of Nps has the potential of becoming a powerful tool to dissect the requirements for optimal hepatic targeting and homing of CD8⁺ T-cells.

In this thesis, poly(lactic-co-glycolic acid) (PLGA) was chosen as the Np polymer primarily for its human safety profile, increased Ag uptake and presentation by APCs and macrophages, and prolonged release of Ag creating a depot effect [269]. PLGA copolymer has been approved by the Food and Drug Administration (FDA) for several therapeutic devices, owing to its biodegradability and biocompatibility. OVA was chosen as the model antigen for several reasons: OVA protein is cheap and easy to encapsulate in PLGA-Np. Secondly, OVA is a T-cell dependent antigen widely used as a model protein for studying antigen-specific immune responses in mice. This allowed access to a large body of literature, as well as readily available tools such as tetramers and antibodies [270], transgenic OVA-specific lymphocytes [206], as well as transgenic OVA-sporozoites [208]. These tools allowed for enhanced evaluation of immune responses to the vaccine, as well as potentially opening interesting future avenues of research.

IV.b – Results

IV.b.i – Prime and target immunization utilizing protein-loaded nanoparticles

The distribution of Np following im or iv administration was first investigated. In accordance with the literature, following iv injection of PLGA Np containing ovalbumin conjugated to APC (Np.APC-OVA), APC signal was found to be

concentrated primarily within the hepatic tissue and found to be concentrated in sinusoidal lining F4/80⁺ Kupffer cells as assessed both by flow-cytometry and by confocal imaging (Fig. 4.1A-C). In contrast, im injection of Np did not show any liver-specific concentration (Fig. 4.1A, C). To assess if Ag was being actively presented on MHC-I molecules, experiments with adoptively co-transferred naïve OVA-specific CD8⁺ OTI T-cells, together with WT polyclonal ubiquitin Td-Tomato T-cells were performed. In fact, OTI T-cells but not naïve polyclonal CD8⁺ T-cells, clustered and increased CD69 expression at sites of Np.APC-OVA uptake by Kupffer cells, indicating that the associated OVA Ag had been processed and presented by MHC class I molecules within the tissue (Fig. 4.1D) [124, 228, 229].

Having determined that iv administration of Np are able to deliver Ag to hepatic tissue which can be processed and presented directly by liver resident Kupffer cells; the ability of Np to promote the localization of primed effector/memory CD8⁺ T-cells to the liver and hence potentially mediate protection against liver-stage malaria was addressed. CD8⁺ T-cell were primed via im route for multiple reasons. Firstly, hepatic priming has been described often to be sub-optimal resulting in exhausted or tolerogenic CD8⁺ T-cell phenotypes [256, 261]; while im priming is successfully able to generate strong immune responses especially when using viral vectors as priming agents [215]. Secondly, PLGA-Np without adjuvants have been described to be poor priming immunogens, often used in the induction of immune tolerance [271, 272]. Hence, it was hypothesized that CD8⁺ T-cell expansion should be mediated with a more potent vaccination regimen [225]. In summary, it was speculated that primed effector/memory CD8⁺ primed from an im injection would be circulating throughout the body looking for potential infected target

cells, and PLGA-Np loaded with cognate antigen would be up-taken by Kupffer cells and would act as a “target” into the hepatic tissue. Hence, this vaccination strategy was called “prime and target”.

Mice were primed with human Adenovirus-5 expressing ovalbumin (Ad.OVA-im) and two weeks later Np.OVA were administered iv (Ad.OVA-im/Np.OVA-iv; Fig. 4.2A). Three weeks later, the level of protection against liver-stage malaria conferred by Ad.OVA-im/Np.OVA-iv was assessed via injection of infectious sporozoites (spz) iv. Vaccinated mice were challenged with two different transgenic (Tg) *P. berghei* spz expressing OVA (OVA-spz): either under the control of the *Pbhsp70* promoter (OVA::*mcherry*_{*Pbhsp70*}), resulting in cytoplasmic expression in sporozoites and intrahepatic stages of the life-cycle, or as a fusion protein with the *Pbhep17* (OVA::*Hep17*_{*Pbhep17*}), resulting in OVA locating to the parasitophorous vacuole (PVM) in the infected hepatocyte [208]. Both lines were used to assess if antigen availability and antigen presentation, predicted to be different between the two strains, would impact vaccine protection. Vaccine efficacy was then determined as the time to reach an endpoint of 1% blood parasitemia. This endpoint was chosen as it is in line with previous literature, humane regarding animal welfare, and past 1% parasitemia disease outcome results in death within just few days.

Prime and target (Ad.OVA-im/Np.OVA-iv) vaccination was found to be highly efficacious, with 100% of mice displaying sterile protection in both challenge models, whereas Ad.OVA-im or Ad.OVA-im/Np.OVA-im failed to protect mice from malaria (Fig. 4.2B; Fig. 4.3A, B). Having shown such surprising efficacy, the immunological phenotype of the vaccine was next determined by flow-cytometry. Ad.OVA-

im/Np.OVA-iv immunization but not Ad.OVA-im/Np.OVA-im or Ad.OVA-im resulted in a ten-fold increase in frequency and total number of OVA-specific (SIINFEKL-Pentamer binding (Pen⁺)) CD8⁺ T-cells in the liver (Fig. 4.2C), with little to no increase in other tissues such as the spleen or muscle dLN (Fig. 4.3C-E). Im injection of Np (Ad.OVA-im/Np.OVA-im) increased the number of Ag-specific CD8⁺ T-cells in the muscle dLN, suggesting that Np containing large amounts of OVA protein can act as a targeting tool to other tissues depending on the route of vaccine administration. In a similar fashion to CD8⁺ T-cells, CD4⁺ T-cells were also shown to increase in number in hepatic tissue albeit in a more modest fashion (Fig. 4.3F). The timing of administration was found to be optimal at two weeks post Ad.OVA-im administration, during the priming phases of the response (Fig. 4.4A-D); although no later time points were investigated. An attempt was made to investigate whether the Np iv boosting dose increased Ag-specific T-cells upon Np administration iv within the liver and/or in peripheral secondary lymphoid sites, or if cells were simply recruited there from the peripheral circulation. To do this, an experimental set-up involving a combination of FTY720 and BrdU pulsation was used (Fig. 4.5A). FTY720 administration limits egress of activated T-cells from lymphoid sites via the internalization of S1P receptors [273], and pulsed-BrdU tracks cell proliferation by intercalating into the DNA of rapidly dividing cells during the S-phases of the cell cycle. Following administration, the data would suggest that both *in situ* proliferation in secondary lymphoid tissues and a migration of Pen⁺ CD8⁺ T-cells to the liver is occurring (Fig. 4.5A-C). Although not completely conclusive, it would be plausible that a combination of local proliferation and local recruitment to the tissue could be occurring following Np-iv injection. Although

Ad.OVA-im and Ad.OVA-iv injection results in similar numbers of Ag-specific CD8⁺ T-cells, Ad.OVA-iv resulted in a greater frequency of cells expressing PD-1, Tim-3, and Lag-3 (known markers of T-cell exhaustion) [261, 264, 274], suggesting that prime and target might be a preferential vaccine strategy to induce more effector/effector-memory CD8⁺ T-cells able to clear infected hepatocytes (Fig. 4.6 A, B).

Lastly, an attempt was made to increase Ag-specific CD8⁺ T-cell numbers in the liver by altering the dose and/or the formulation of PLGA-Np used [271]. For this, OVA loaded Np were conjugated to two adjuvants (R837-MPL) as described previously by Kasturi et al [205]. This unique combination of adjuvants had been shown previously to increase T-cell responses via coupling antigen delivery with a TLR4 and TLR7 ligand. Hence, following Ad.OVA-im injection, different PLGA doses with or without R837-MPL coupling (adj) were administered; however, adjuvant addition was found to have only minor effects in increasing liver Ag-specific CD8⁺ T-cells, having an effect only when low doses of Ag were administered (Fig. 4.7A). Furthermore, different doses of Ad.OVA-im prime: 10⁴, 10⁶, or 10⁸ iu, were investigated (Fig. 4.7B). Although not significant, there appeared to be a positive trend between dose of Ad.OVA-im administered and number of Ag-specific CD8⁺ T-cell found in the liver three weeks post vaccination. Although further experimentation would be required, this would be suggestive that Ag availability played a crucial role in determining the number of Ag-specific CD8⁺ T-cells in the hepatic tissue.

IV.b.ii – Prime and target immunization utilizing viral vectors

Having determined that prime-target vaccination involving Np.OVA-iv, results in sterile protection from spz challenge and generates particularly high numbers of Ag-specific CD8⁺ T-cells in the liver tissue, other clinically translational methods of hepatic targeting were examined. Specifically, from a translation prospective, although Np have been demonstrated to be safe in clinical studies, they would require large quantities of GMP grade *Plasmodium* protein to be encapsulated within the PLGA shell. *Plasmodium* proteins often times have unique protein folds and have been shown to be particularly challenging to purify and expand [274-276]. As an alternative to PLGA proteins we chose to investigate antigen delivery to the liver utilizing viral vectors. These would have the advantage not to require protein production and purification, are a cheap and easily modifiable platform allowing multiple malaria antigen to be easily tested [51, 101]. Furthermore, specifically in the case of Adenoviruses, these viral vectors have a known high viral tropism for hepatic tissues, accumulating within the liver tissue long term by infecting both Kupffer cells and hepatocytes directly [277, 278]. For these reasons, the ability to obtain a similar protective result by substituting a recombinant viral boost for the Np boost was investigated (Fig. 4.8).

Human and simian Adenoviral and MVA vectors backbones were initially assessed as a first pass; as these viral vectors are available for clinical use, allowing for faster bench-to-bedside testing. To assess viral localization following intravenous administration, luciferase expressing viral vectors were imaged over time. Bioluminescent imaging revealed that Adeno- as well as MVA-vectors were highly liver-tropic after iv administration (Fig. 4.9A, B), and thus delivered antigen to hepatic tissue.

Of note, Adenoviral vectors showed a significant long-term luciferin expression over time, and seemed to highly accumulate in liver tissue.

Next, we assessed if prime-target vaccine regimens involving combinations of Ad.OVA-im/Ad.OVA-iv or Ad.OVA-im/MVA.OVA-iv (Fig. 4.8), also resulted in sterile protection. Following vaccination, both Ad.OVA-im/Ad.OVA-iv or Ad.OVA-im/MVA.OVA-iv were able to generate substantial numbers and frequency of Ag-specific CD8⁺ T-cells in the liver, comparable to that achieved by Np.OVA-iv boosting after priming (Fig. 4.9C, 4.10A), with a high percentage of these cells also producing IFN γ . Vaccine efficacy was next assessed via a challenge with OVA-spz (OVA::*Hep17_{Pbhep17}*). As expected, boosting using Ad.OVA-im or MVA.OVA-im vectors failed to induce strong protection. However, boosting with Ad.OVA-iv or MVA.OVA iv resulted in 80% sterile protection durable for at least 2-months post vaccination (Fig. 4.9D; Fig. 4.10C). Furthermore, the protective response mediated by prime-target vaccination with viral iv boost was found to be dose-dependent (Fig. 4.10B).

IV.b.iii – Prime and target immunization results in sterile protection with a variety of clinically relevant antigens

Protective efficacy of a prime and target strategy with clinically relevant antigens and viral vectors was investigated, having performed most of the work with a model antigen: OVA. For this, the following antigens were examined: Pb9 (an immune-dominant *P.berghei* epitope [66] from the liver-stage circumsporozoite (CSP) protein, used extensively in pre-clinical mouse malaria work), and the three leading *P. falciparum* liver-stage malaria antigens: Thrombospondin-Related Anonymous Protein (*Pf*TRAP),

Liver-stage Antigen 1 (*Pf*LSA1) and Liver-stage Associated Protein 2 (*Pf*LSAP2), all now in clinical development [101, 103]. Each antigen was then expressed individually in widely tested ChAd63 Chimp-Adenovirus vectors, also used extensively in clinical development. Following an im injection of ChAd63 viral vector, two weeks later mice were administered either ChAd63-iv or MVA-im, each vector expressing the same cognate antigen as the primary immunogen. MVA-im was chosen as the comparison as Ad-MVA heterologous prime-boost vaccination was as of then the leading viral vector vaccine strategy. Animals were then challenged 3-weeks after the targeting vaccination with wild-type (WT) or Tg-spz expressing the cognate *P. falciparum* antigen under the *P. berghei* UIS4 liver-stage promoter in *P.berghei* [279]. Vaccination using a single recombinant ChAd63-im/ChAd63-iv regimen, with vectors expressing the relevant cognate antigen, conferred 100% sterile protection using all Tg spz tested: specifically, for *Pb*CSP, *Pf*TRAP, *Pf*LSA1 and *Pf*LSAP2 Adenoviral vectors, contrasting with much lower efficacy observed for the traditional im heterologous prime-boost regimes (Fig. 4.11A-D). Moreover, an attempt was made to target with peptide loaded Np (Np.P9-iv) and we were able to obtain comparable levels of sterile protection between Np.Pb9-iv or ChAd63.ME-TRAP-iv (Pb9 immuno-dominant epitope is contained within a multi-epitope string (ME) in this construct).

Collectively, these results demonstrated that effective protection through prime-target immunization could be achieved via im Adenoviral priming and a target iv administration of either Np or a recombinant viral vector, and most simply by two administrations of an Adenoviral vector using a heterologous iv route for the target injection. Lastly, because viral vectors are able to express a wide range of antigens, we

wanted to test the ability to produce high numbers of liver Ag-specific T-cells against other liver pathogens. As proof of principle, Hepatitis B surface antigen (Fig. 4.12A-D) was chosen. Again, either MVA.HBsAg-iv or Ad5.HBsAg-iv viral vectors results in a marked increase in antigen-specific CD8⁺ T-cells in the liver tissue, further demonstrating the versatility of this vaccine platform.

IV.c - Figures

Figure 4.1

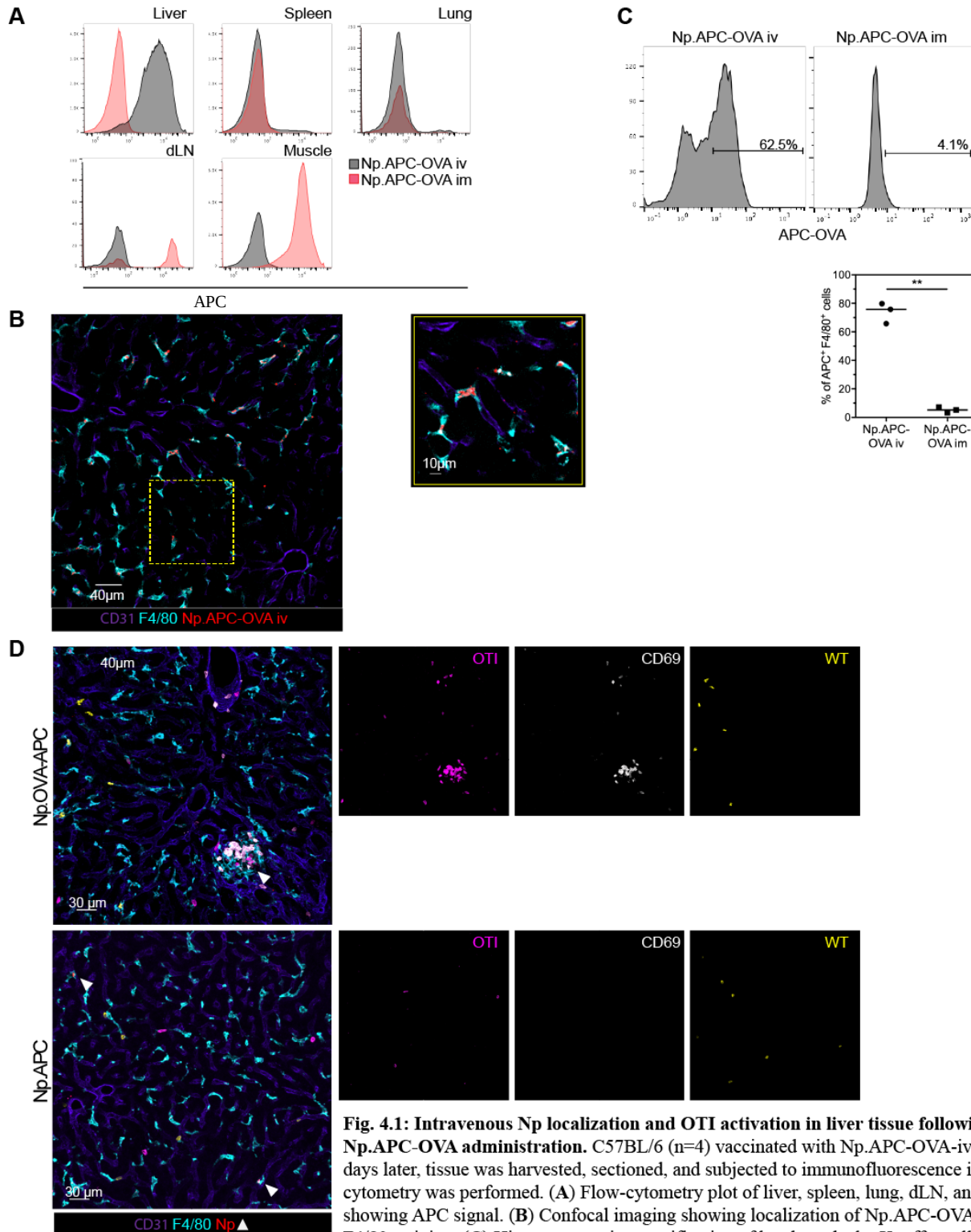


Fig. 4.1: Intravenous Np localization and OTI activation in liver tissue following iv Np.APC-OVA administration. C57BL/6 (n=4) vaccinated with Np.APC-OVA-iv or im. Seven days later, tissue was harvested, sectioned, and subjected to immunofluorescence imaging or flow cytometry was performed. **(A)** Flow-cytometry plot of liver, spleen, lung, dLN, and muscle showing APC signal. **(B)** Confocal imaging showing localization of Np.APC-OVA-iv, CD31 and F4/80 staining. **(C)** Histo-cytometric quantification of bead uptake by Kupffer cells: a surface was created on the F4/80⁺ channel in Imaris and Mean Fluorescence Intensity channel parameters were imported into FlowJo. A representative sample showing APC-OVA intensity for Np.APC-OVA iv or Np.APC-OVA im vaccinated animals. Quantification of the frequency of APC⁺ F4/80⁺ cells of total F4/80⁺ following Np.APC-OVA iv or Np.APC-OVA im. **(D)** C57BL/6 (n=4) were iv vaccinated with Np.APC-OVA or Np.APC. At day 4 post injection, 2x10⁶ OTI and TdTomato wild-type polyclonal (WT) CD8⁺ T-cells were co-adoptively transferred. IF images of liver sections obtained at day 3 post adoptive transfer and stained for CD45.2 (OTI), CD69, CD31, and F4/80. Arrowhead denotes Np. Maximum projection of a 20µm z-stack. p<0.05 (*).

Figure 4.2

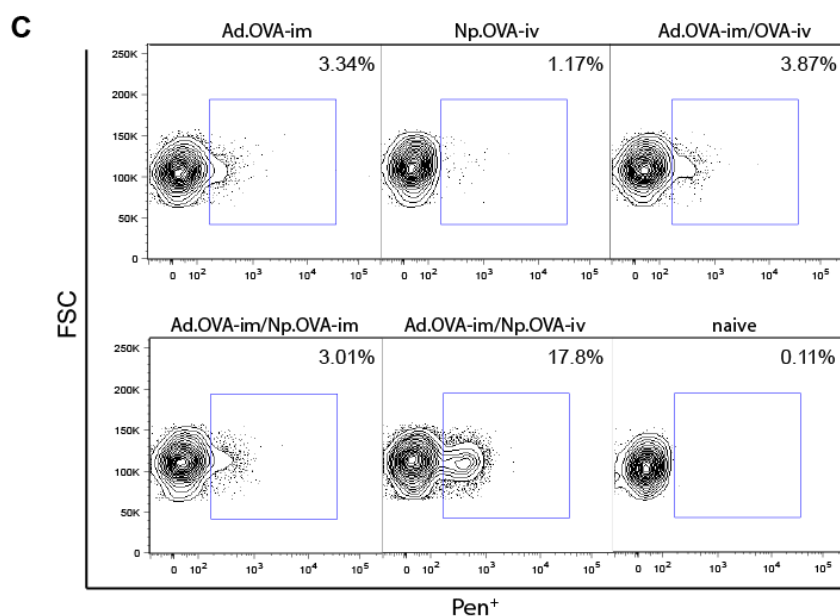
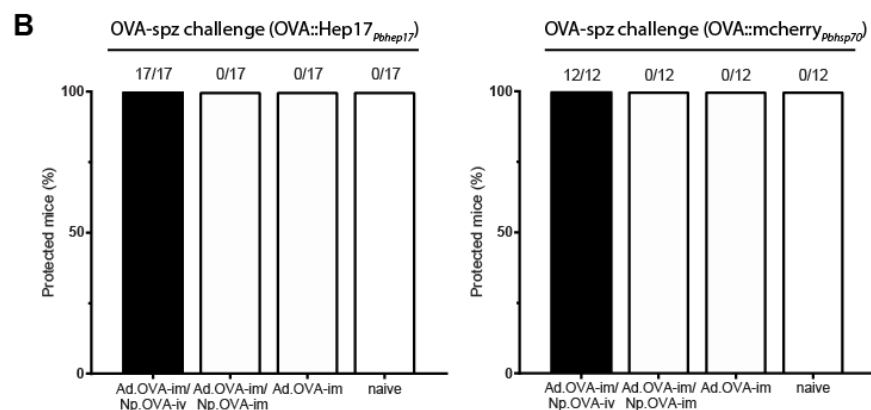
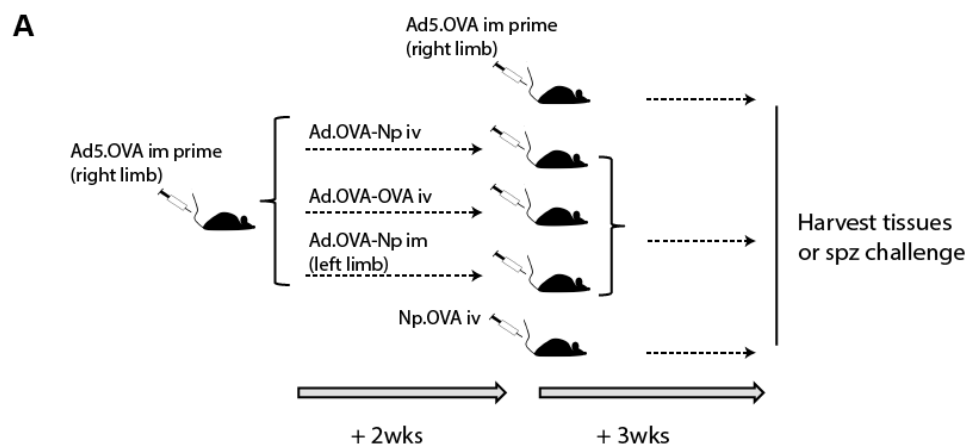


Figure 4.2: Schematic representation of prime and target vaccination regimen
(A) Experimental plan: C57BL/6 mice were primed with Ad.OVA-im (right limb) or not (Np.OVA-iv). Two weeks later, mice received an iv administration of Np.OVA (Ad.OVA-im/Np.OVA-iv), un-encapsulated OVA protein (Ad.OVA-im/OVA-iv) or received im administration of Np.OVA (Ad.OVA-im/Np.OVA-im). Three weeks later, tissues were either harvested for flow cytometric analysis or, alternately, mice were challenged with spz to determine protection. **(B)** Pooled challenge data from C57BL/6 mice (n=17) primed with Ad.OVA-im followed by Np.OVA-iv (Ad.OVA-im/Np.OVA-iv), Np.OVA-im (Ad.OVA-im/Np.OVA-im), or no Np.OVA (Ad.OVA-im). Mice were challenged with either OVA::Hep17 *P. berghei* spz; or OVA::mCherryPbhs70 *P. berghei* spz three weeks post vaccination. Data shown is pooled from 3-2 independent experiments. **(C)** Representative Pentamer (Pen⁺) gating strategy: lymphocytes were gated on live, size and singlet population and subsequently gated on CD8⁺ T-cells. Frequency of Pen⁺ CD8⁺ T-cells of total CD8⁺ T-cells in liver is shown.

Figure 4.3

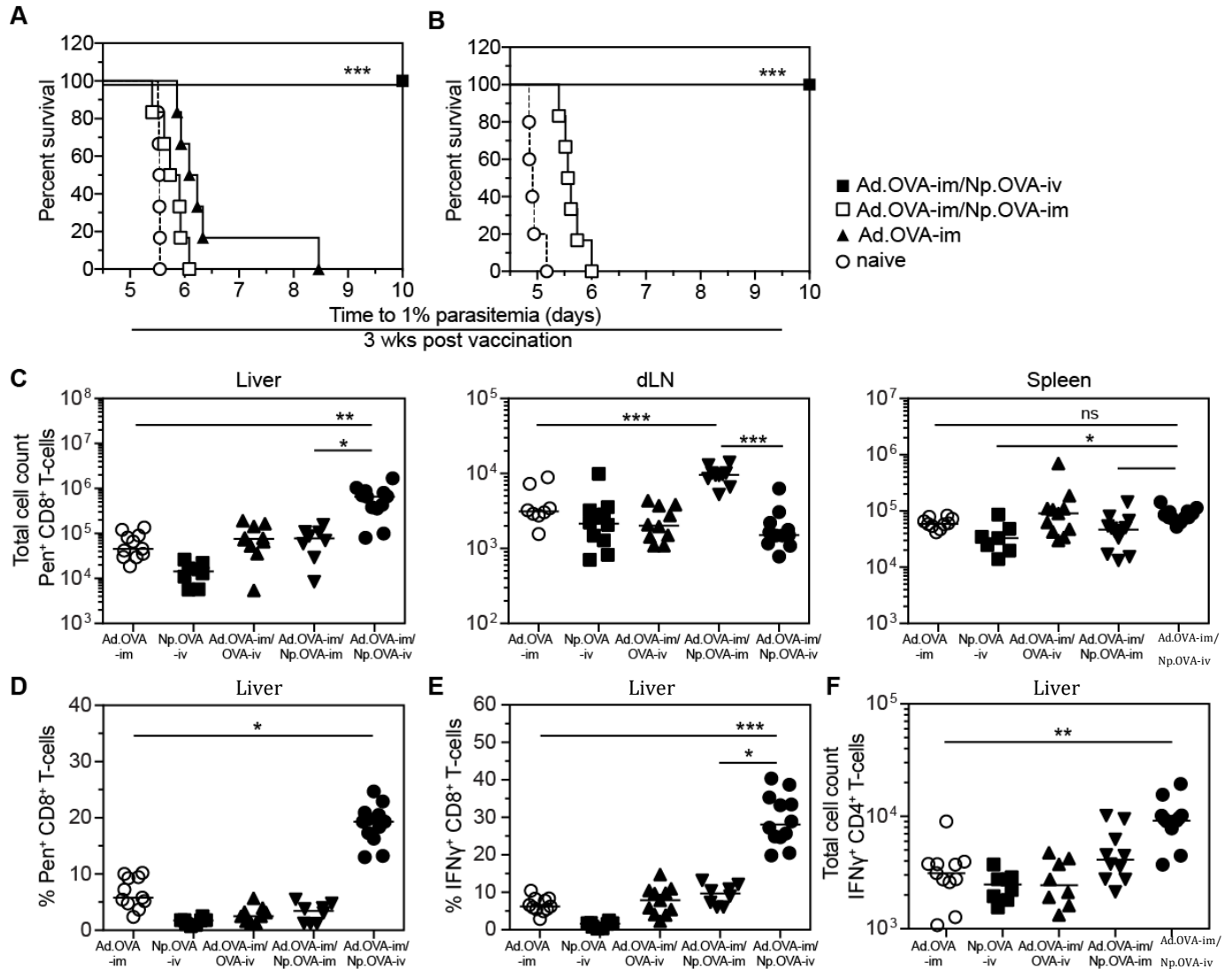


Fig. 4.3: Prime and target immunization protects mice against challenge with Tg OVA-spz

C57BL/6 mice (n=6) were primed with Ad.OVA-im followed by Np.OVA-iv (Ad.OVA-im/Np.OVA-iv), Np.OVA-im (Ad.OVA-im/Np.OVA-im), or no Np.OVA (Ad.OVA-im). Mice were challenged with (A) OVA::Hep17 *P. berghei* spz or (B) OVA::mCherry_{PbHsp70} *P. berghei* spz. Data shown are representative of 3-2 independent experiments; Log-rank Mantel-Cox Test, $p < 0.001$ (***). C57BL/6 mice were primed with either Ad.OVA-im or Np.OVA-iv (Np.OVA-iv). Ad.OVA-im primed animals were subsequently vaccinated with either OVA protein (Ad.OVA-im/OVA-iv), Np.OVA-im (Ad.OVA-im/Np.OVA-im) or Np.OVA-iv (Ad.OVA-im/Np.OVA-iv), or were left untreated (Ad.OVA-im). Three weeks post Np or Ad.OVA-im vaccination, animals were examined by flow cytometry for (C) total Pen⁺ CD8⁺ T-cells in liver, dLN, and spleen; frequency of (D) Pen⁺ T-cells and (E) IFN γ ⁺ CD8⁺ T-cells of total CD8⁺ T-cells in liver; (F) total cell count of IFN γ ⁺ CD4⁺ T-cells in liver. Data shown is pooled from 2-4 independent experiments, each symbol representative of individual mouse (n=9-12). Median shown: Kruskal Wallis test corrected with Dunn's multiple comparison test, $p < 0.001$ (***); $p < 0.01$ (**); $p < 0.05$ (*); not significant (ns).

Figure 4.4

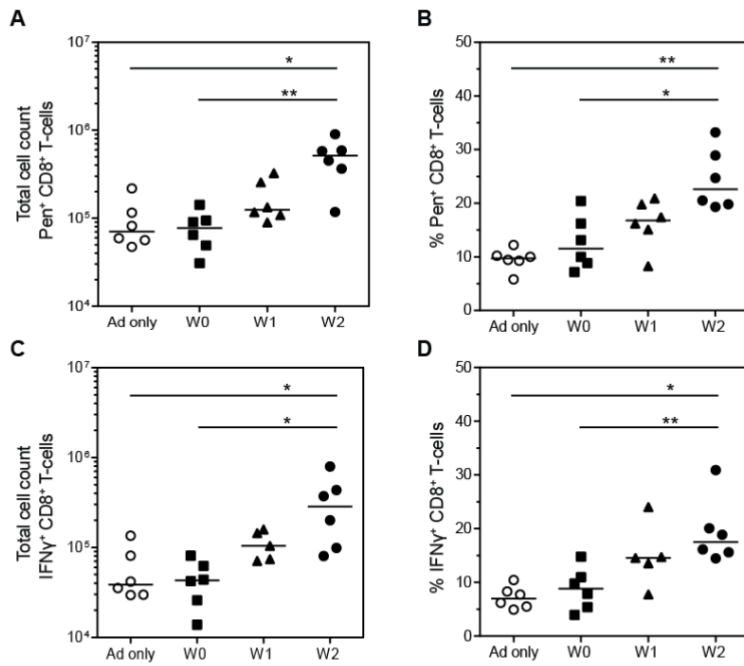


Fig. 4.4: Optimisation of immunisation regimen timings for targeting Np dose C57BL/6 mice (n=6) were primed with Ad5-OVA im and then received Np.OVA-iv either on the day of prime (W0), one week post prime (W1) or two weeks post prime (W2), or they did not receive particles (Ad only). Three weeks after receiving the final immunisation, livers were harvested and the total number of Ag-specific CD8⁺ T-cells enumerated using Pen⁺ staining (A), and frequency (B) or intracellular IFN γ staining following *ex vivo* restimulation with SIINFEKL peptide (C), and frequency (D) was enumerated. p<0.05 (*); p<0.005 (**); not significant (ns).

Figure 4.5

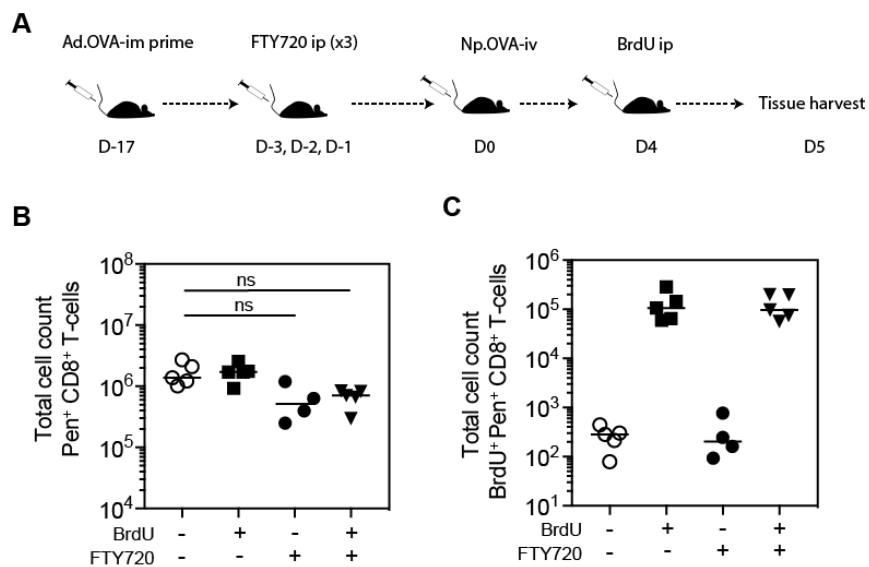


Fig. 4.5: Total Pen⁺ CD8⁺ T-cell numbers in the liver are not affected by FTY720 administration.

(A) Schematic representation: C57BL/6 mice (n=5) were primed with Ad.OVA-im and two weeks later received three consecutive ip injections of FTY720 or vehicle control. Subsequently, mice were iv injected with Np.OVA (Np.OVA-iv) and four days later ip injected with BrdU. After 24hrs, livers were harvested and stained. (B) Total cell count of Pen⁺ CD8⁺ T-cells; (C) BrdU⁺ Pen⁺ CD8⁺ T-cells. Not significant (ns).

Figure 4.6

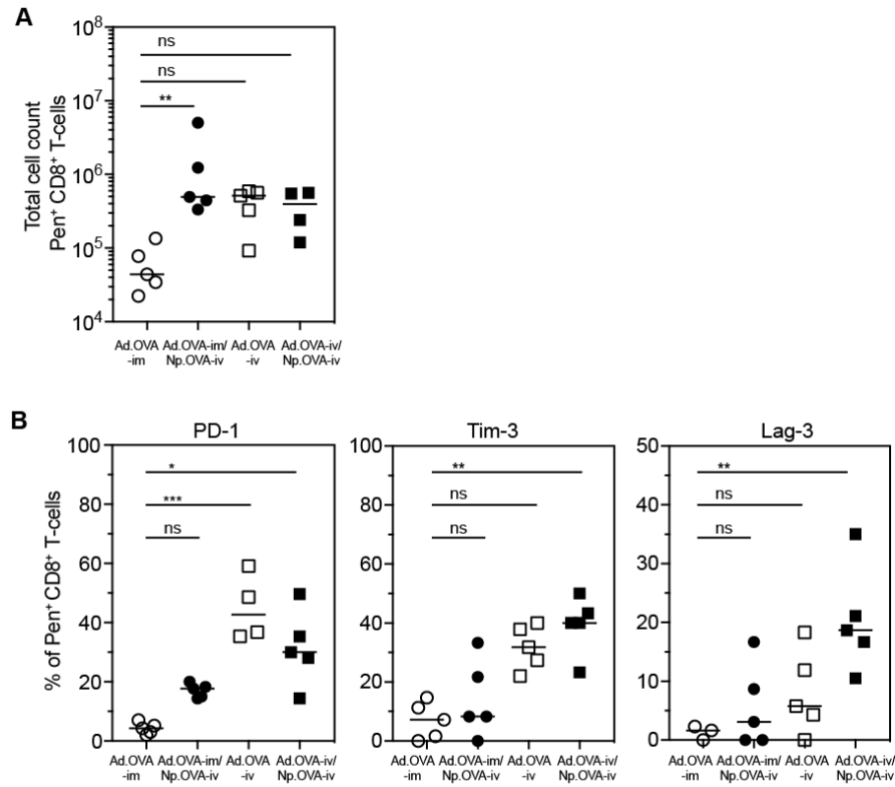


Fig. 4.6: PD-1, Tim-3, and Lag-3 expression on Ag-specific CD8⁺ T-cells was found to increase following hepatic priming.

C57BL/6 mice (n=5) were primed with Ad.OVA-im or Ad.OVA-iv. Two weeks later, mice were either left unvaccinated or Np.OVA-iv was administered. Total liver antigen specific CD8⁺ T-cells were assessed using Pen⁺ (A) with frequency of PD-1, Tim-3, and Lag-3 expression shown (B). p<0.001 (**); p<0.005 (**); p<0.05 (*); not significant (ns).

Figure 4.7

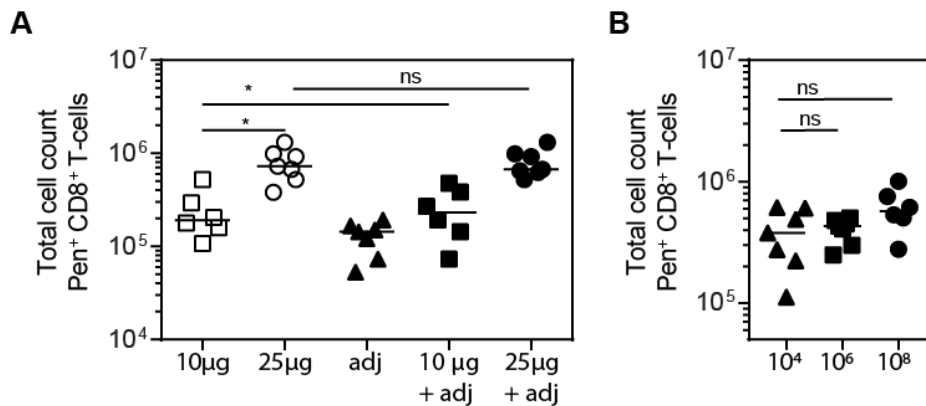


Fig. 4.7: T-cell recruitment to the liver is dependent in part on the dose of PLGA-OVA administered.

(A) C57BL/6 mice were primed with Ad.OVA (1x10⁸ iu) im and two weeks later given either 10μg (10μg) or 25μg (25μg) Np.OVA-iv either with or without adjuvant (R848-MPL)-containing Np (adj). Livers were harvested three weeks later. The total number of antigen specific CD8⁺ T-cells was quantified by Kb-SIINFELK Pentamer staining. (B) C57BL/6 mice (n=6) were primed with different doses of Ad.OVA-im (1x10⁴ iu (10⁴), 1x10⁶ iu (10⁶), and 1x10⁸ iu (10⁸)) and two weeks later, Np.OVA-iv (25μg) were administered to all groups, livers were harvested and stained. The total number of antigen specific CD8⁺ T-cells was quantified by Kb-SIINFELK Pentamer staining. p<0.05 (*); not significant (ns). Data shown is pooled from two independent experiments, each symbol representative of individual mouse (n=6-7). Median shown, Kruskal Wallis test corrected with Dunn's multiple comparison test, p<0.05(*); not significant (ns).

Figure 4.8

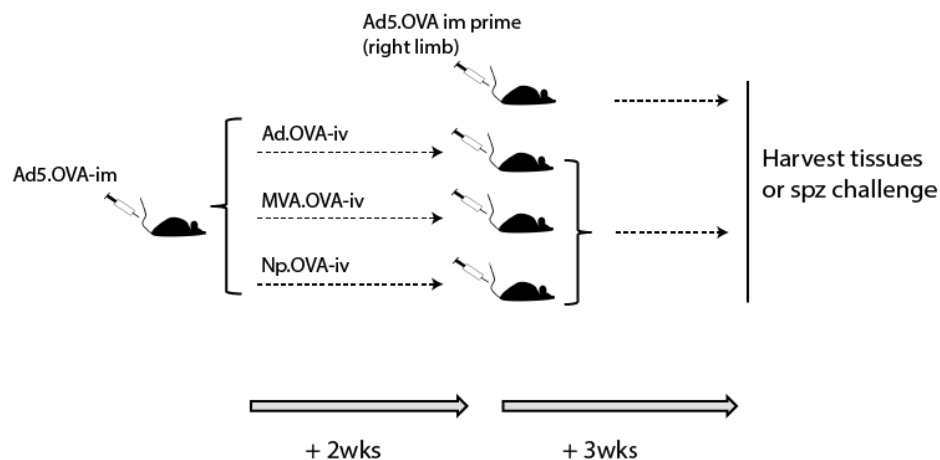


Fig. 4.8: Schematic representation of prime and target vaccination regimen

Experimental plan: C57BL/6 mice were primed with Ad.OVA-im. Two weeks later, mice received an iv administration of Np.OVA (Ad.OVA-im/Np.OVA-iv), Ad.OVA (Ad.OVA-im/Ad.OVA-iv) or MVA.OVA (Ad.OVA-im/MVA.OVA-iv) or were left unvaccinated (Ad.OVA-im). Three weeks later, tissues were either harvested for flow cytometric analysis or, alternately, mice were challenged with spz to determine protection.

Figure 4.9

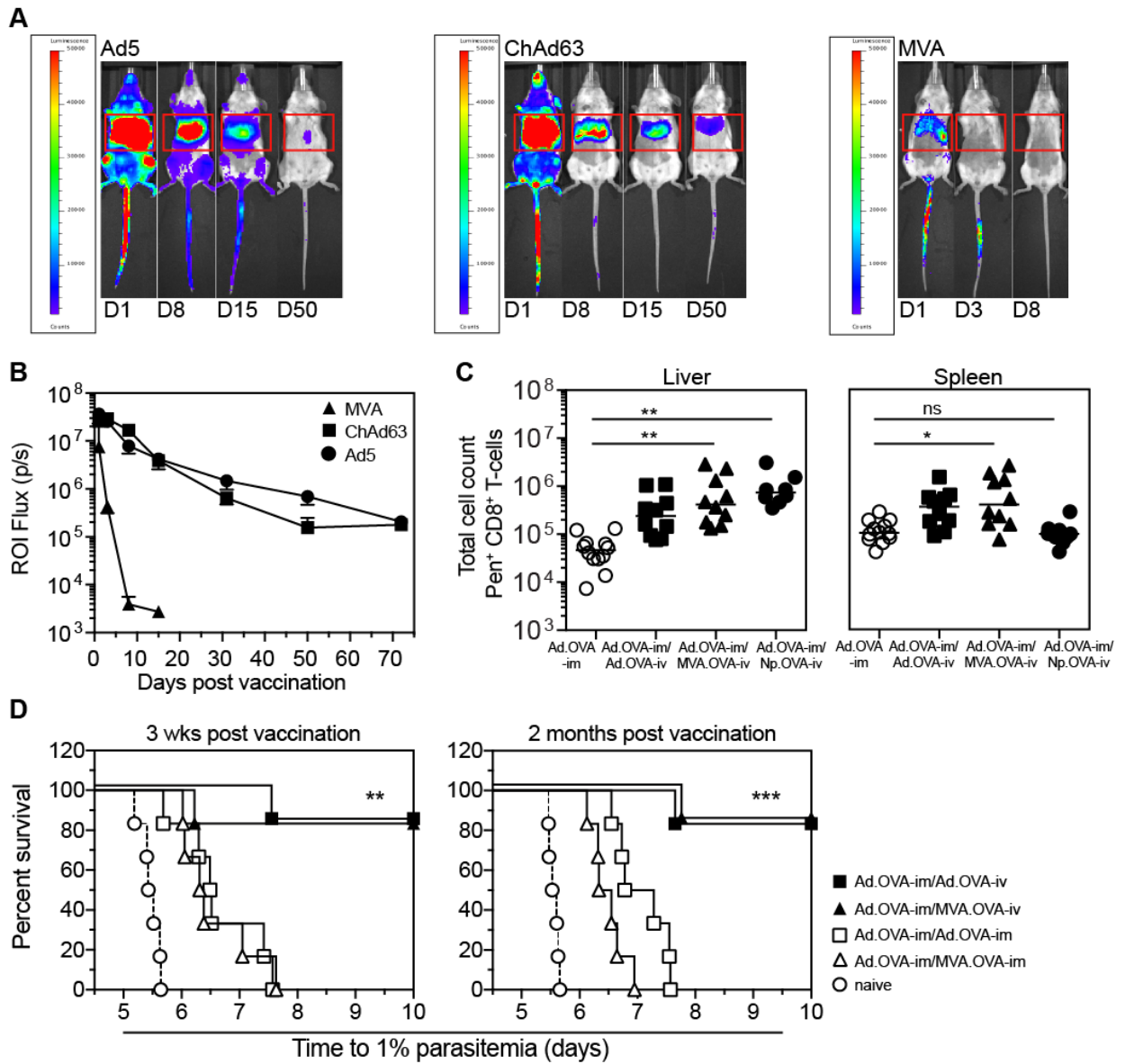


Fig. 4.9: Viral vectors as targeting agents induce strong protective immunity against challenge with Tg OVA-spz. BALB/c mice (n=5) were vaccinated iv with Ad5, ChAd63 or MVA expressing luciferase and imaged at different days (D) post vaccination. (A) Overlay image indicating the location of bioluminescence. In red, Region of Interest (ROI) is shown, with (B) ROI Flux analysis over time. (C) C57BL/6 mice were all primed with Ad.OVA-im followed two weeks later by Ad.OVA iv (Ad.OVA-im/Ad.OVA-iv), MVA-OVA-iv (Ad.OVA-im/MVA.OVA-iv), Np.OVA-iv (Ad.OVA-im/Np.OVA-iv) or untreated (Ad.OVA-im). Total Pen⁺ T-cells in liver or spleen. Data shown is pooled from two independent experiments, each symbol representative of individual mouse (n= 8-12). Median shown, Kruskal Wallis test corrected with Dunn's multiple comparison test, $p < 0.01$ (**); $p < 0.05$ (*); not significant (ns). (D) C57BL/6 mice (n=6) were vaccinated with Ad.OVA-im/Ad.OVA-im, Ad.OVA-im/MVA.OVA-im, Ad.OVA-im/Ad.OVA-iv, or Ad.OVA-im/MVA.OVA-iv. Mice were challenged 3 weeks after final immunization or 2 months after final immunization with OVA::Hep17 *P. berghei* spz. Data shown are representative of two independent experiments; Log-rank Mantel-Cox Test, $p < 0.001$ (***); $p < 0.01$ (**).

Figure 4.10

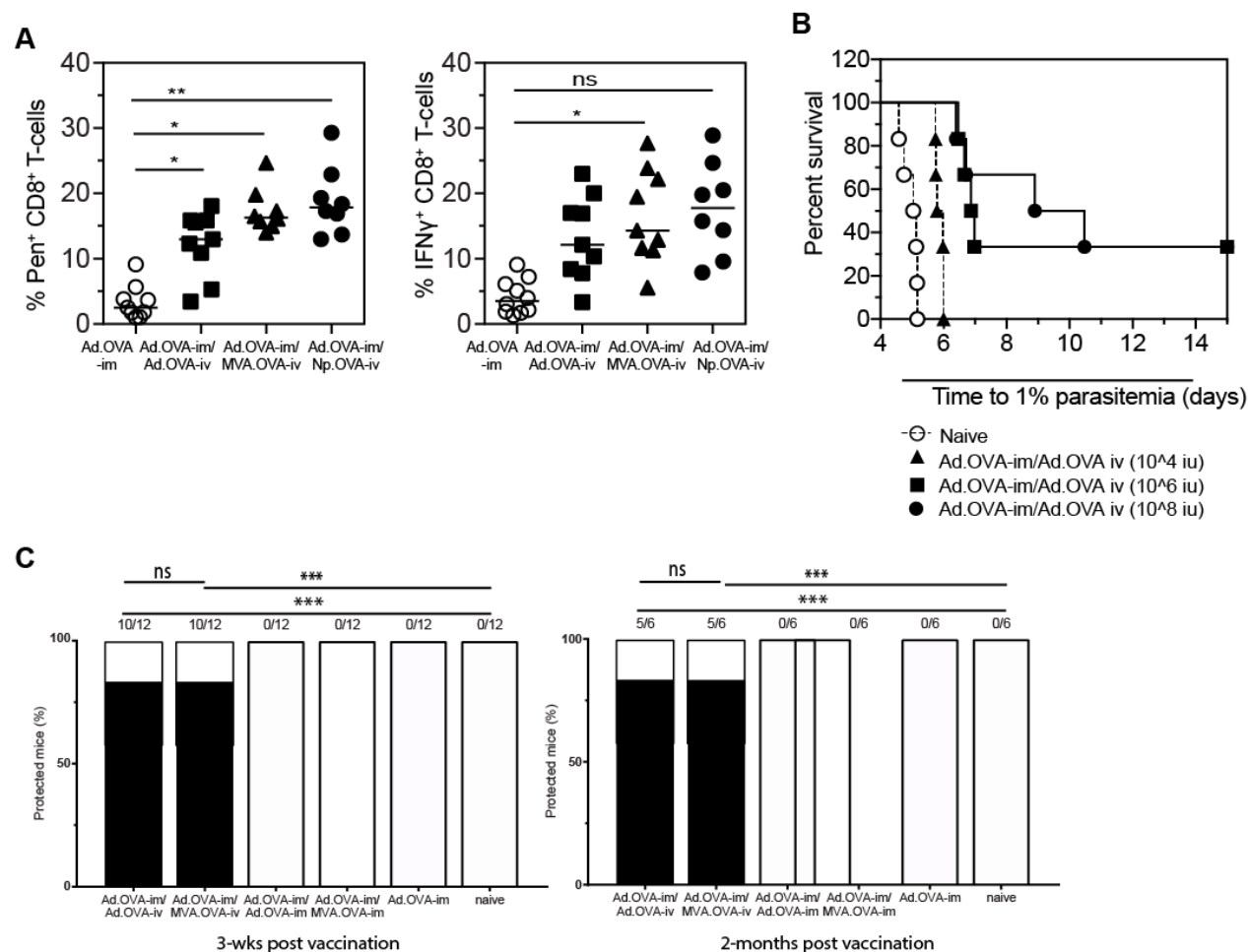


Fig. 4.10: A prime and target approach using viral vectors as targeting agents shows similar immunization profile to PLGA Np targeting.

(A) C57BL/6 mice were primed with Ad.OVA (1x10⁸ iu) im and two weeks later mice received either Ad.OVA iv (Ad.OVA-im/Ad.OVA-iv), MVA.OVA iv (Ad.OVA-im/MVA.OVA-iv), or Np.OVA iv (Ad.OVA-im/Np.OVA-iv), or were left untouched (Ad.OVA-im). Three weeks later, livers were harvested and frequency of (A, left) Pen⁺ CD8⁺ T-cells, (A, right) IFN γ ⁺ CD8⁺. Data shown is pooled from two independent experiments, each symbol representative of individual mouse (n=8-10). Median shown, Kruskal Wallis test corrected with Dunn's multiple comparison test, p<0.05 (*), p<0.01 (**), not significant (ns). (B) Two weeks after priming of C57BL/6 mice (n=6) with Ad.OVA-im, the animals received Ad.OVA-iv at different doses: 1x10⁴ iu, 1x10⁶ iu, or 1x10⁸ iu. Three weeks later all mice were challenged with OVA::Hep17 *P. berghei* spz and time to 1% parasitemia determined by thin blood films. (C) Pooled challenge data from C57BL/6 mice (n=6-12) primed with Ad.OVA-im followed by Ad.OVA-iv (Ad.OVA-im/Ad.OVA-iv), MVA.OVA-iv (Ad.OVA-im/MVA.OVA-iv), Ad.OVA-im (Ad.OVA-im/Ad.OVA-im), MVA.OVA-im (Ad.OVA-im/MVA.OVA-im), or no target dose (Ad.OVA-im). Mice were challenged with OVA::Hep17 *P. berghei* spz three weeks or 2-months post vaccination. Kruskal Wallis test corrected with Dunn's multiple comparison Test, p<0.001(***), not significant (ns).

Figure 4.11

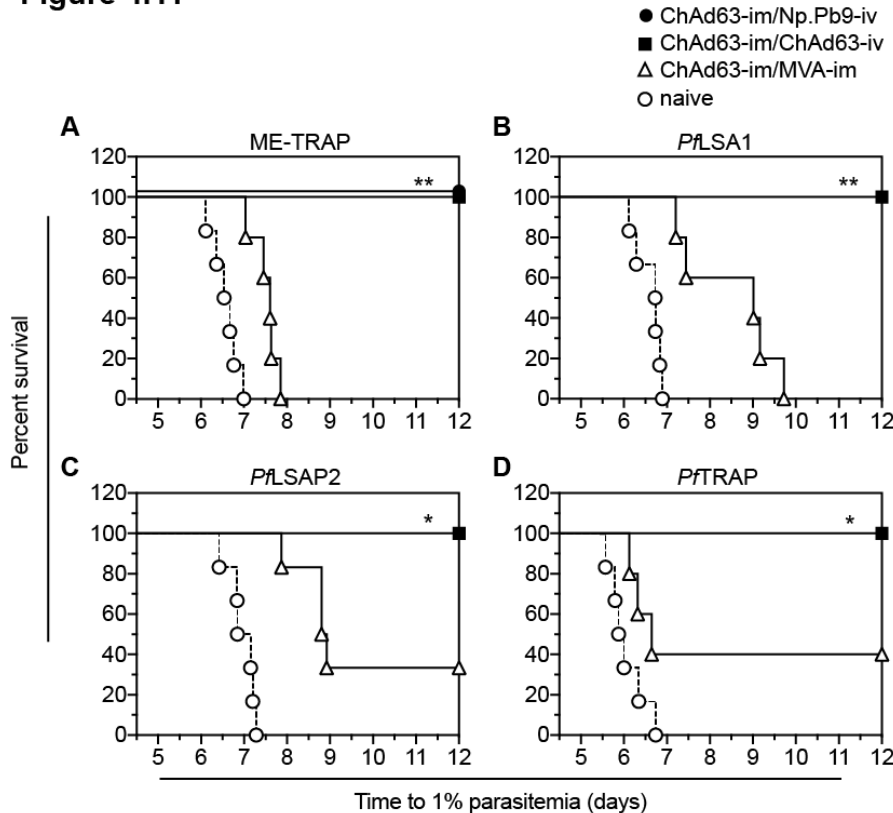


Fig. 4.11: Prime and target approach sterily protects mice against challenge with transgenic spz expressing prominent clinical candidate *P. falciparum* vaccine antigens.

BALB/c mice ($n=6$) were primed im with ChAd63 viral vectors expressing the following antigens (A) ME-TRAP, (B) *PfLSA1* or (C) *PfLSAP2*. Mice were subsequently vaccinated with either MVA-im (ChAd63-im/MVA-im), or ChAd63 iv (ChAd63-im/ChAd63-iv) each expressing the respective cognate antigen. For the ME-TRAP vaccinated group (A) an additional group of BALB/c mice ($n=6$) received ChAd63.ME-TRAP-im followed by Np.Pb9-iv (ChAd63-im/Np.Pb9-iv). (D) CD-1 mice ($n=6$) were primed with ChAd63 viral vectors expressing *PfTRAP* im. Mice received a subsequent immunization of either MVA im, or ChAd63 iv vectors each expressing *PfTRAP* antigen. Protective efficacy of immunization was determined after challenge with WT or Tg *P. berghei* spz expressing the cognate *P. falciparum* antigen. Data shown are representative of two independent experiments. Statistical comparison between Ad-Ad iv and Ad-MVA im vaccinations: $p<0.05$ (*); $p<0.005$ (**).

Figure 4.12

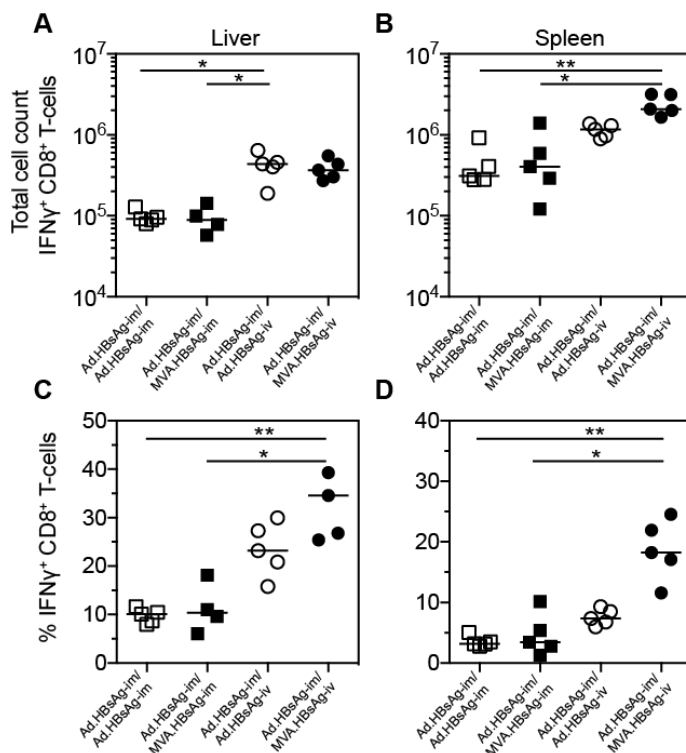


Fig. 4.12: A prime and target approach using viral vectors generates high numbers of HBsAg CD8⁺ T-cells in the liver.

BALB/c mice ($n=5$) were primed im with Ad.HBsAg (1x10⁸ iu). After two weeks, mice received Ad.HBsAg-im or Ad.HBsAg-iv or alternatively MVA.HBsAg-im or MVA.HBsAg-iv. Three weeks later, liver and spleen tissues were harvested and (A) total number of IFN γ ⁺ CD8⁺ T-cell or (B) frequency of IFN γ ⁺ CD8⁺ T-cells was determined following ex-vivo peptide stimulation. $p<0.005$ (**); $p<0.05$ (*).

IV. d – Discussion

CD8⁺ T-cells are known to be critical for the control of intracellular pathogens, and have been shown to play a critical role in the elimination of hepatocytes infected by *Plasmodium* parasites. As previously discussed in Chapter I, several vaccine strategies aim to generate this cell type using a wide variety of platforms such as vectored vaccines, irradiated spz (RAS), or genetically attenuated parasites (GAP) [280]. Specifically, in the context of viral vectors, there has been a significant effort has been placed over the years to improve the leading vaccine candidate (a heterologous prime-boost strategy involving Adeno and MVA viral vectors). Although promising clinical trials have resulted in partial protection both in mice, non-human primates and in humans [98, 99, 225, 250], sterile protection is often difficult to achieve and maintain long term. Hence, an attempt was made to devise a vaccine regimen that would specifically localize CD8⁺ T-cells in the tissue where they are most needed, predicting that a local population of CD8⁺ T-cells might result in improved efficacy. To achieve this, a prime-target vaccine strategy was devised that would generate high numbers of CD8⁺ T-cells following Ad.OVA-im administration and target effector/effector-memory CD8⁺ T-cells to the liver utilizing Np.OVA or viral vector intravenous administration.

Prime and target vaccination induces a log-fold increase in liver Ag-specific CD8⁺ T-cells compared to Ad.OVA-im only, resulting in sterile protection three weeks post challenge utilizing two different OVA Tg-spz lines. Intramuscular rather than intravenous priming injection of Ad.OVA resulted in fewer Ag-specific CD8⁺ T-cells expressing known markers of T-cell exhaustion or anergy (such as PD-1, Tim-3 and Lag-3) [263, 264]. Although a direct challenge comparing the long-term efficacy between im

vs iv priming is still required, one may speculate that T-cell exhaustion might result in decreased efficacy over protracted periods of time. Liver CD8⁺ T-cell retention appears to be dose dependent and requires little TLR engagement for optimal Trm formation in hepatic tissue. Viral vectors were also investigated as alternative to PLGA-Np, with both Adeno and MVA viral vectors showing 80% sterile protection up to two-months post challenge.

Lastly, it was shown that this platform is highly flexible, allowing for easy screening of multiple *P. falciparum* malaria liver stage candidates (such as leading vaccine candidates *Pf*TRAP, *Pf*LSA1 and *Pf*LSAP2) using the clinically tested ChAd63 backbone [101, 103]. Interestingly, Pb9 peptide-loaded nanoparticles were also shown to induce sterile protection. Peptides can have unique half-lives and are often easily degraded; however, PLGA-encapsulation would suggest that antigen presentation on cognate MHC-I molecules is also highly effective. This could be of clinical interest as peptides are often easy to produce and manufacture, and might be also an interesting screening tool for further investigation specifically in the context of cancer immune-therapy where Np formulation could be tailored to unique HLA-haplotypes.

Regarding the use of more translational antigens such as *Pf*TRAP, *Pf*LSAP, and *Pf*LSAP2, there are some potential caveats that might result in difficulties in assessing translational vaccine efficacy potential. In the case of all of these Tg-spz models, the protein of interest is added into the *P. berghei* genome under the strong promoters UIS4 [101]. This could result in increased antigen abundance and/or duration of antigen expression during the hepatic stages of the life-cycle, in comparison to a natural infection. For example, heterologous viral vector prime-boost regimen historically

expressed the pre-erythrocytic antigen *Pf*TRAP [98]. TRAP has known important functional roles in spz motility, attachment to hepatocytes and liver-stage development [281]; however, this vaccine resulted in approximately 25% efficacy in human clinical trials. In part, this may be due to low numbers of TRAP-specific CD8⁺ T-cells in the liver. Alternatively, TRAP might also be weakly presented during the spz stages and might result in low protection. Here, we show that prime-target expressing ChAd63.ME-TRAP is able to mediate protection in CD1 mice challenged with transgenic *P. berghei*-*Pf*TRAP, three weeks post vaccination. However, further experiments using a *Pf*TRAP replacement line (when the antigen is expressed under the endogenous *P. berghei* promoter) will be telling.

In conclusion, prime and target vaccination is a great improvement over current vaccination strategies, showing surprisingly high vaccine efficacy, as well as potential for clinical translatability. Next, some crucial questions regarding the mechanism of vaccine protection, the phenotype and location of the induced cellular immune response, and long-term efficacy of prime-target vaccine regime were addressed.

Chapter V

Characterizing the tissue-specific Prime and Target Response

Chapter V

Characterizing the tissue-specific Prime and Target Response

V.a – Introduction

Given the crucial role of CD8⁺ T cell responses in protection against malaria parasites, it is important to understand the nature and longevity of the CD8⁺ T-cells induced via prime-target vaccination *in situ*. As mentioned in Chapter I, *Plasmodium* sporozoites invade and replicate within hepatocytes which present parasite-derived antigens on MHC-I and are susceptible to CD8⁺ T-cell killing both *in vitro* and *in vivo* [76]. Therefore, the elimination of target hepatocytes has been generally considered to result from a CD8⁺ T-cell direct contact-dependent processes. In fact, mouse bone-marrow chimeras revealed that direct peptide recognition between effector CD8⁺ T-cells and hepatocytes required cognate MHC-I [71]. Because parasites remain in the liver for such a short time window, intrahepatic CD8⁺ T-cells are probably an essential population in mediating protection. In support of this, work done utilizing adoptive transfers of Ag-specific CD8⁺ T-cells during a spz challenge, no active migration of large numbers of effector CD8⁺ T-cells was observed at a macroscopic whole-organ level [82]. Hence, having shown that prime-target vaccination can induce sterile protection from spz challenges, one could speculate that the CD8⁺ T-cells induced could be tissue-resident CD8⁺ T-cells, mediating their effector functions locally when encountering infected hepatocytes.

Malaria-specific CD8⁺ T-cells have been described to mediate protection through the production of several effector functions. Of note, the production of INF γ and TNF α [63,

282], as well as the perforin and Granzyme B have been shown to be crucial to clear infected hepatocytes through various knock-out mouse models [283, 284]. However, it is likely that these pathways work redundantly because CD8⁺ T-cells lacking IFN γ could still limit parasite expansion, and mice lacking perforin and FasL also mounted a protective immune response against *Plasmodium* parasites [78]. With the advancement of intra-vital imaging techniques, the elimination of infected hepatocytes by effector CD8⁺ T-cells has been visualized. Induction of Ag-specific CD8⁺ T-cells from irradiated spz vaccination have been shown to produce large clusters of CD8⁺ T-cells which have been speculated to be crucial for clearing all infected hepatocytes [73]. However, *in situ* imaging of CD8⁺ T-cell effector functions has not yet been yet visualized. Prime-target vaccination might also induce large CD8⁺ T-cell clusters producing effector functions; hence, multi-parameter confocal microscopy was utilized to visualize the protective recall immune response. Having characterized the local immune response following reactivation of CD8⁺ T-cells at the site of infection, the longevity of the prime-target immune response was further investigated.

Successful vaccine strategies must induce long-lived memory responses. In recent years, tissue-resident (Trm) T-cells have been shown to be important in a multitude of disease settings [174]. In the context of liver-stage malaria, while the initial trafficking appears to be an indiscriminate process, the retention in the tissue seems to be dependent on specific environmental cues. Specifically, the transmembrane chemokine CXCL16 has been implicated in the maintenance of CD8⁺ T-cells and CXCR6⁺ NKT cells [83, 86, 173]. In fact, adoptive transfers of CXCR6^{-/-} CD8⁺ T-cells are able to traffic in the infected organ, but do not get retained in the liver, suggesting that CXCR6 is crucial for

either retention or survival in the hepatic tissue [86]. Aside from CXCR6, other markers that have been associated with liver tissue residency are CD69⁺, Granzyme B^{hi}, CD127^{lo}, and the downregulation of the transcription factors Eomes and KLF2 [83]. Lastly, through parabiosis studies, liver tissue-resident CD8⁺ T-cells were confirmed to be a long-lived, non-migratory population of CD8⁺ T-cells, and were found to be a crucial population to mediate protection against spz challenge [85]. Therefore, it was experimentally investigated if prime-target vaccination was able to induce Ag-specific Trm CD8⁺ T-cells and if these Trm could be maintained long term over time. A detailed understanding of the re-call responses involved in mediating local protection against spz challenge, as well as the life-span of the memory cells involved in such processes would be essential for further vaccine improvement.

V.b – Results

V.b.i – CD8⁺ T-cell cluster around infected hepatocytes resulting in effector responses

Having determined that prime and target vaccination induces sterile protection, an assessment was made of the cellular mechanisms underlying the protective immune response generated. For this, multi-parameter confocal imaging was used to quantitatively characterize cell positioning and cell phenotype *in situ* [211]. Hence, mice were immunized with either Ad.OVA-im/Np.OVA-iv or Ad.OVA-im only. Three weeks after Np.OVA-iv administration, mice were challenged with transgenic OVA-spz (*OVA::mcherry^{Pbhsp70}*) or non-transgenic spz (*mcherry^{Pbhsp70}*) and liver tissue was then harvested 12 hours post challenge. Twelve hours post spz-injection was chosen as it would probably allow enough time for CD8⁺ T-cells to find infected hepatocytes and

begin producing effector molecules. A super-physiological dose of spz was administered (10,000 spz/mouse) due to the imaging constraints of finding enough infected hepatocytes to quantify. Such high spz injections might result in higher-recruitment of immune cells to the infected hepatocytes due to increased local tissue inflammation.

Following spz challenge, liver tissue from Ad.OVA-im/Np.OVA-iv, but not Ad.OVA-im animals, showed OVA-spz encircled by large clusters of Kupffer cells (F4/80⁺) and polyclonal CD8⁺ T-cells producing IFN γ and Granzyme B *in situ*, markers of known effector cell function (Fig. 5.1A-C; 5.2A). Additionally, some of the clustered cells were Ki67⁺, indicating recent cell proliferation upon Ag-recognition (Fig. 5.1B, D) [285]. In contrast, mice given only Ad.OVA-im showed no cell accumulation or effector staining around infected hepatocytes suggesting that at 12 hours post spz infection few cells were actively recruited to the site of infection (Fig. 5.1A-C; 5.2A). As previously observed [73, 79], these T-cell aggregates were only present in animals challenged with spz-OVA parasites while only smaller in size clusters (with no IFN γ and Granzyme B production) were found in non-transgenic spz challenged animals (Fig. 5.2A). Importantly, CD8⁺ T-cell numbers present in the clusters greatly increased following prime-target vaccination. The low number of lymphocytes present around non-transgenic spz only challenged animals suggests that large cluster formation required cognate antigen recognition to recruit activated T-cells and clear infected hepatocytes. In fact, immunizing animals with an irrelevant encapsulated protein (Np.BSA-iv) and subsequently challenging them with OVA-spz failed to confer protection (Fig. 5.2B). Similarly, mice vaccinated with a combination of viral vectors expressing ME-TRAP as prime and target or Ad.OVA-im subsequently followed by Ad.ME-TRAP-iv or

MVA.ME-TRAP-iv also did not result in protection (Fig. 5.2C), further supporting the hypothesis that the cluster formation observed is associated with protective immunity and dependent on cognate antigen recognition.

To confirm that the cell population critical in mediating liver-stage protection were the CD8⁺ T-cells, a cell depletion experiment was performed after prime-target immunization (Ad.OVA-im/Np.OVA-iv). Utilizing depleting antibodies (Fig. 5.3), administration of anti-CD8 antibody prior to OVA-spz challenge completely ablated vaccine efficacy (Fig. 5.1F). CD4⁺ T-cells, on the other hand, although also present in large numbers around infected hepatocytes (Fig. 5.1B, C), were largely dispensable for protection against OVA-spz challenge (Fig. 5.1F). Lastly, as mentioned in Chapter IV, protection was found to scale in a dose-dependent manner. In fact, higher levels of liver Ag-specific CD8⁺ T-cells (generated by decreasing Np.OVA vaccine dose administered) positively correlated with increased protection (Fig. 5.4). Together, the imaging and depletion studies emphasized a critical role for CD8⁺ T-cells visualized in antigen-dependent clusters in mediating protection against liver-stage malaria.

V.b.ii – CD8⁺ T-cell expressing markers of tissue residency are found to be sufficient at mediating protection

Recent data suggested that an important contribution to local immunity is provided by Trm T-cells [85, 180, 188]. It was postulated that protection from OVA-spz challenge by prime-target immunization could be the result of such CD8⁺ liver-resident T-cells, rather than the protection being due to acute recruitment of memory effector cells from lymphoid sites. To examine whether Trm cells were present in the liver after vaccination with Ad.OVA-im/Np.OVA-iv or Ad.OVA-im, we employed quantitative

histocytometric analysis of liver tissue 5-weeks after Np.OVA-iv administration or Ad.OVA-im only. Following prime-target immunization, a significant increase in Trm (CXCR6⁺ CD69⁺ CD44⁺) [76, 85, 211] and T-effector memory (Tem, CXCR6⁻ CD69⁻ CD44⁺) cells were observed predominantly in the liver sinusoids (Fig. 5.5A, B; Fig. 5.6A-C) when compared to Ad.OVA-im vaccinated animals. Similarly, increased small cellular clusters containing Tem, Trm, CD4⁺ T-cells, and F4/80 MHC-II⁺ Kupffer cells, were also observed in the tissue parenchyma of Ad.OVA-im/Np.OVA-iv vaccinated animals, appearing similar to previously described Memory Lymphocyte Clusters (MLC) (Fig. 5.5A, C, D) [188]. We further confirmed the Trm phenotype observed with flow-cytometry, and a similar increase in Pen⁺ CD8⁺ T-cells expressing CXCR6 and CD69 was seen following Np.OVA administration (Fig. 5.7A, B). Moreover, prime-target vaccination utilizing viral vectors similarly induced a high frequency of Pen⁺ CXCR6⁺ CD69⁺ CD8 T-cells, with Ad.OVA-iv showing a slightly greater increase than MVA.OVA-iv (Fig. 5.7C).

To provide further evidence that liver Trm are induced following prime-target activation, liver and spleen CD8⁺ T-cells were isolated, labelled and transferred to naïve recipient hosts (Fig. 5.8A). One week later, spleens and livers of recipient animals were harvested, and flow cytometry was performed. Liver CD8⁺ T-cells were found to preferentially home back to the liver tissue rather than the spleen (Fig. 5.8B-C), further characterizing them a liver-resident T-cell population as has been observed with other cell types.

To determine the durability of the prime-target vaccine regimen, mice were immunised and lymphocytes were isolated at different time-points post-vaccination for

flow-cytometry analysis. In accordance with the Trm phenotype of the cells previously imaged, a population of liver-localized Ag-specific CD8⁺ T-cells, expressing markers of tissue residency, could be detected for at least 36 days following Ad.OVA-im/Np.OVA-iv immunization (Fig. 5.5E, F). Kinetically, this population of cells appeared to be relatively stable, with no drastic decrease in cell numbers over time. Most importantly, protection in directly vaccinated animals was observed to be 100% in mice challenged 2-months post-vaccination, and exhibited only a 30% reduction when the animals were challenged after 6-months, emphasizing the longevity of the vaccine-induced immune response (Fig. 5.5G; Fig. 5.7D).

Although many of the T-cells found in the liver 5-weeks after Np boost had a Trm phenotype, it was still possible that the functionally relevant protective cells were acutely recruited from peripheral pools upon OVA-spz challenge. To assess if immunity resulted from Tem drawn into the liver upon OVA-spz infection or if liver Trm were sufficient to mediate protection, mice were challenged under two conditions. Firstly, vaccinated animals were administered FTY720 (to inhibit lymphocyte egress from lymph nodes and splenic white pulp, the impact on memory populations was determined via flow cytometry, Fig. 5.9A) and splenectomised (removing the remaining splenic red pulp T-effector pool) [286] (Fig. 5.5H). Secondly, FTY720 alone or in combination with anti-CD8 depleting antibodies was administered (Fig. 5.9B). In either model, Trm were found to be sufficient for protection in liver-stage malaria.

In summary, these data suggest that prime-target vaccination regimen induces a high frequency of Trm that are stably maintained over time and sufficient for protection

against liver-stage malaria by generating large clusters around infected hepatocytes and locally releasing effector molecules.

Figure 5.1

V.c – Figures

Fig. 5.1 Localization and quantification of immune response to spz challenge. Immunofluorescence (IF) images depicting spz in liver 12 hours after challenge (ch.) with an iv injection of 5x10⁴ OVA::mCherry_{P_{phag70}} *P. berghei* spz (OVA-spz) or mCherry_{P_{phag70}} (spz). Mice had been previously vaccinated (v.) with Ad.OVA-im/Np.OVA-iv or Ad.OVA-im only, and challenged three weeks post Np.OVA-iv injection. (A) IF images of liver sections (maximum projection of a 20µm z-stack) stained for CS protein, CD8, CD4, and F4/80; arrowheads denote spz in the tissue. (B) IF image depicting liver section from Ad.OVA-im/Np.OVA-iv challenged with OVA-spz. Section stained for CD8, CD4, Ki67, GrzB, IFNγ and Jojo (purple-nucleus). In small, single colour panels, maximum projection of a 20µm z-stack. Quantification of IF images: (C) Mean cell count per Infectious Site (IS) of CD8⁺ and CD4⁺ T-cells (equal numbers of IS imaged per condition). In situ frequency of (D) CD8⁺ (E) CD4⁺ T-cell expressing Ki67, GrzB and IFNγ from mice vaccinated with Ad.OVA-im/Np.OVA-iv and challenged with OVA-spz. IF images are representative of two independent experiments, for a total of n=4 animals per condition (2 liver sections imaged/animal). Median shown from all pooled IF data, Kruskal Wallis test corrected with Dunn's multiple comparison test, p<0.001(***); p<0.01(**); p<0.05(*). (F) C57BL/6 mice (n=5) were vaccinated with Ad.OVA-im/Np.OVA-iv. Three weeks post Np.OVA-iv vaccination mice were administered anti-CD4, anti-CD8 depleting antibodies (Ab) or a combination of both antibodies. Alternatively, IgG2b isotype control antibody was administered (Iso Ctrl). Mice were subsequently challenged with OVA::Hep17 P. berghei spz. Data shown are representative of 2 independent experiments; Log-rank Mantel-Cox Test, not significant (ns).

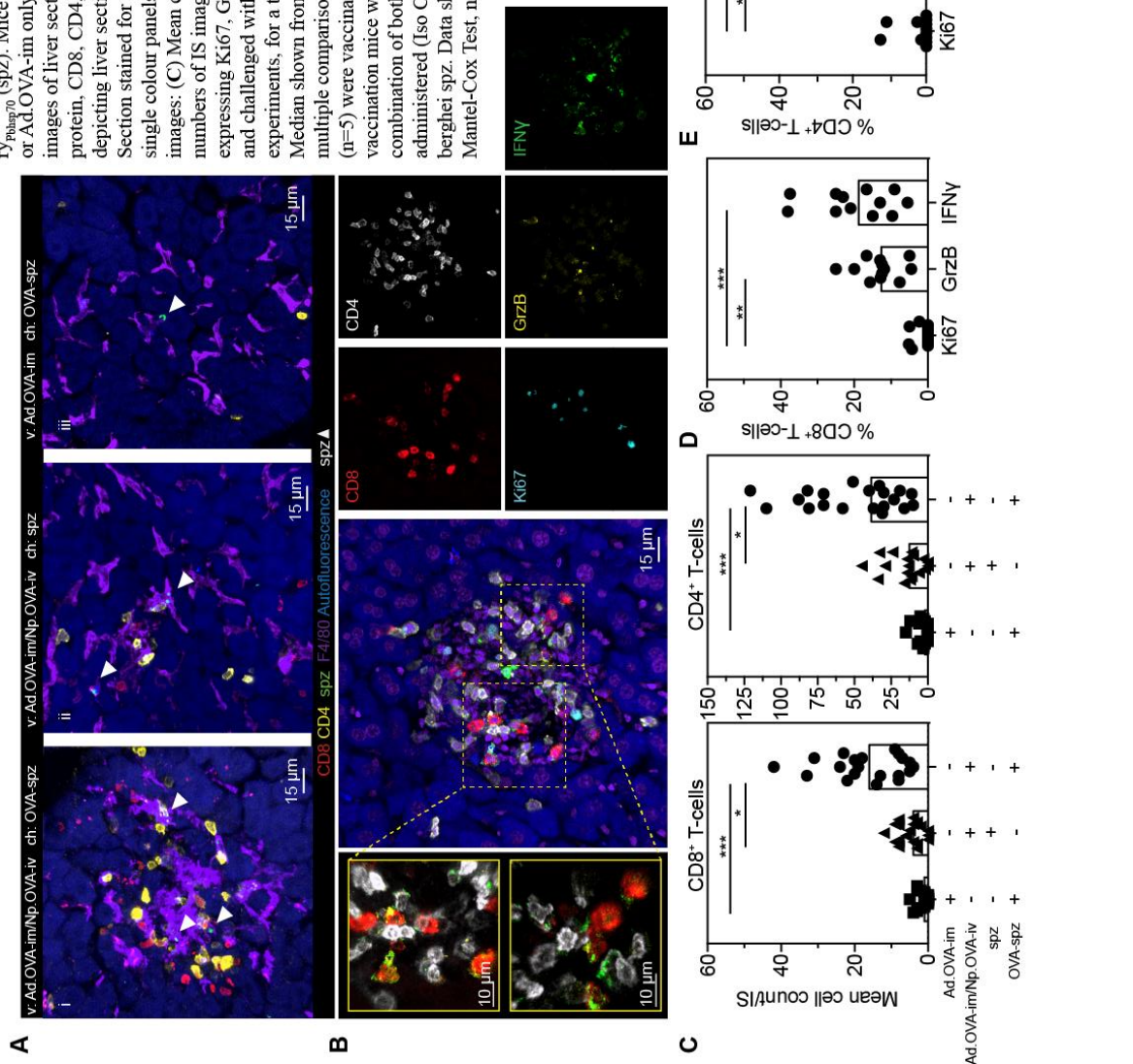


Figure 5.2

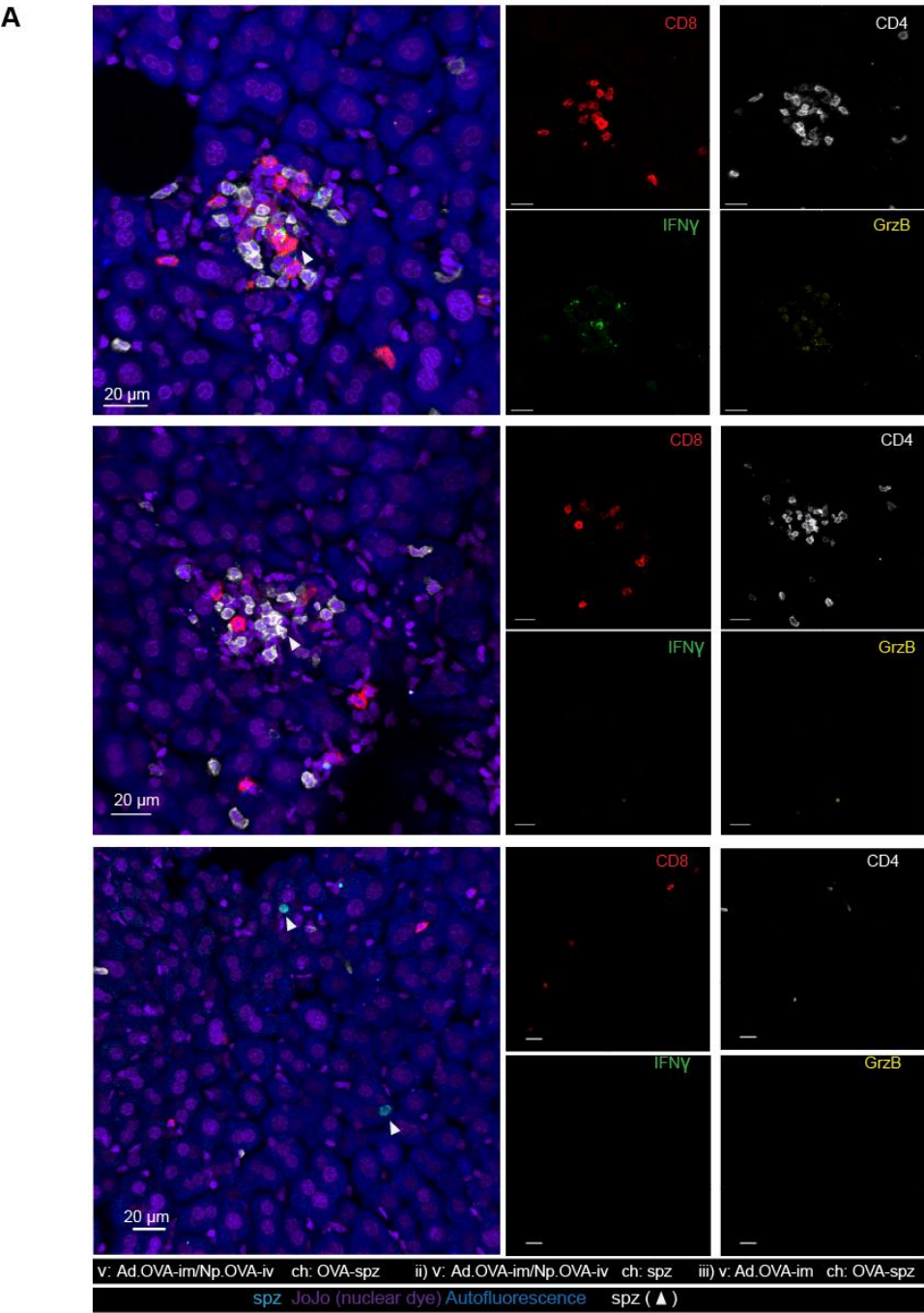


Fig 5.2: A prime and target approach protects mice against challenge with transgenic *P. berghei* OVA-spz only when mice were vaccinated against OVA.

(A) IF images depicting spz in liver 24 hours after challenge (ch:) iv injection of 5×10^4 OVA::mCherry_{Pbbsp70} *P. berghei* spz (OVA-spz, Panel i, iii) or mcherry_{Pbbsp70} (spz, Panel ii) in C57BL/6. 3 weeks earlier mice had been vaccinated (v:) with Ad.OVA-im followed by Np.OVA-iv (Ad.OVA-im/Np.OVA-iv) (Panel i-ii) or Ad.OVA-im only (Panel iii). Section stained for spz, CD8, CD4, GrzB, and IFN γ as well as with JoJo (purple-nucleus). In small, single colour panels, arrowheads denote spz in the tissue; maximum projection of a 20 μ m z-stack. (B) C57BL/6 mice (n=6) were primed with Ad.OVA-im, after two weeks mice received iv Np.OVA or Np.BSA (25 μ g protein). An additional unvaccinated group served as an infection control. Three weeks after Np administration all mice were challenged with OVA::Hep17 *P. berghei* spz, and time to 1% parasitemia assessed by thin blood film; $p < 0.001$ (***) . (C) C57BL/6 mice (n=6) were primed with Ad.OVA-im, and two weeks later received Ad.OVA-iv or MVA.OVA-iv. As control, mice received Ad.ME-TRAP-iv or MVA.ME-TRAP-iv. Alternatively, mice primed and later received an iv vaccination with Ad5.ME-TRAP. An additional unvaccinated group served as an infection control. Three weeks later all mice were challenged with OVA::Hep17 *P. berghei* spz. $p < 0.001$ (***) . Data shown are representative of 2 independent experiments; IF images are representative of 2 independent experiments, for a total of n=4 animals per condition.

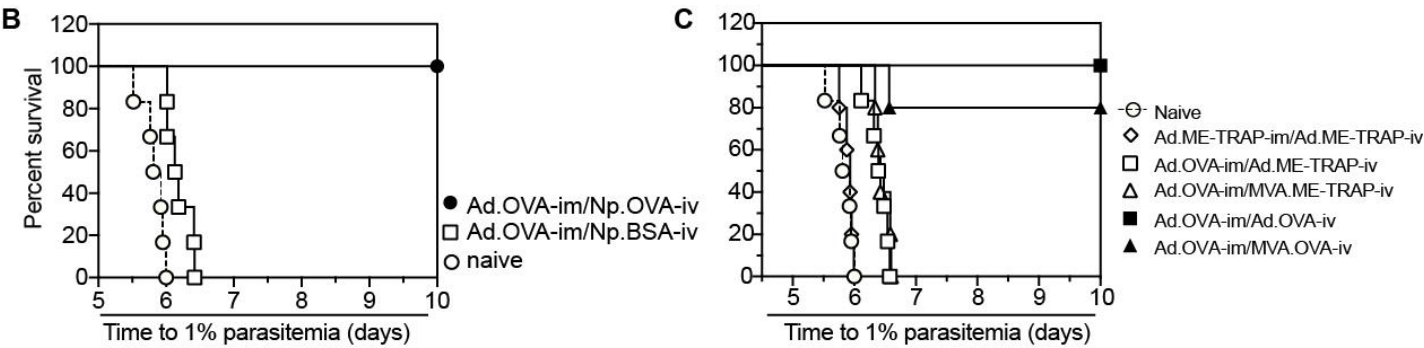


Figure 5.3

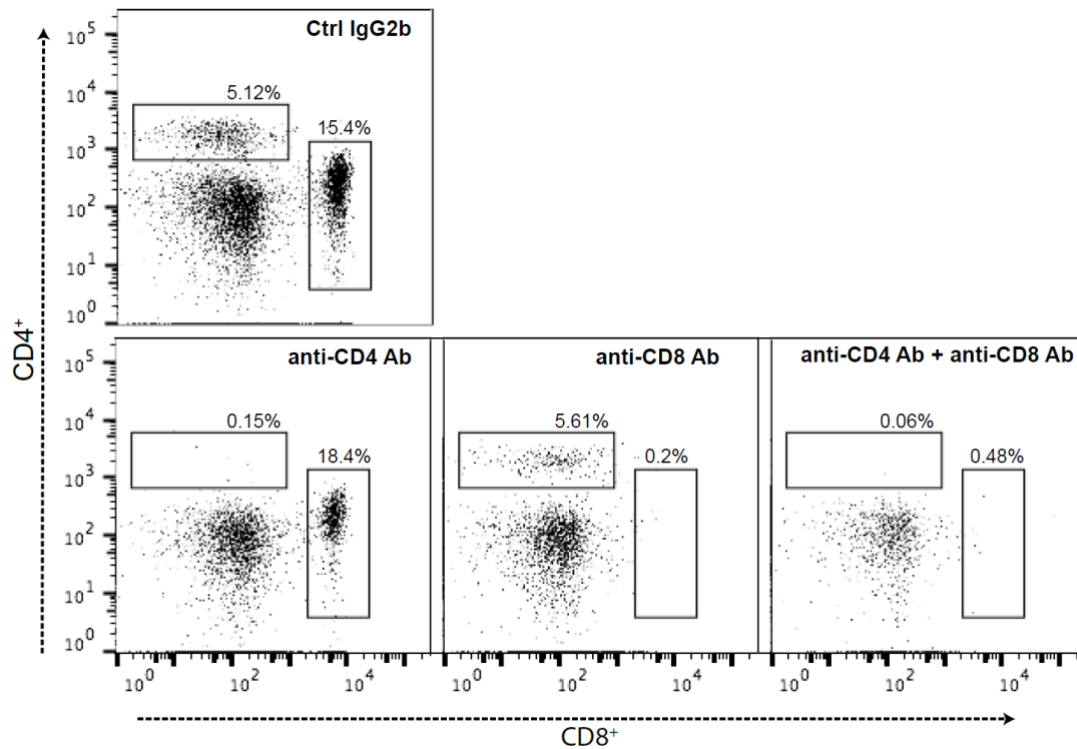


Fig. 5.3: Flow plots showing representative lymphocyte depletion following administration of depleting antibodies.

C57BL/6 mice (n=5) were vaccinated with Ad.OVA-im/Np.OVA-iv. Three weeks after, mice were treated with ip injections of anti-CD4, anti-CD8, anti-CD4 and anti-CD8 depleting antibodies, or with isotype control (Ctrl IgG2b). Peripheral blood was obtained prior to spz challenge, and lymphocytes were analyzed by flow cytometry. Representative flow plots showing successful lymphocyte depletion.

Figure 5.4

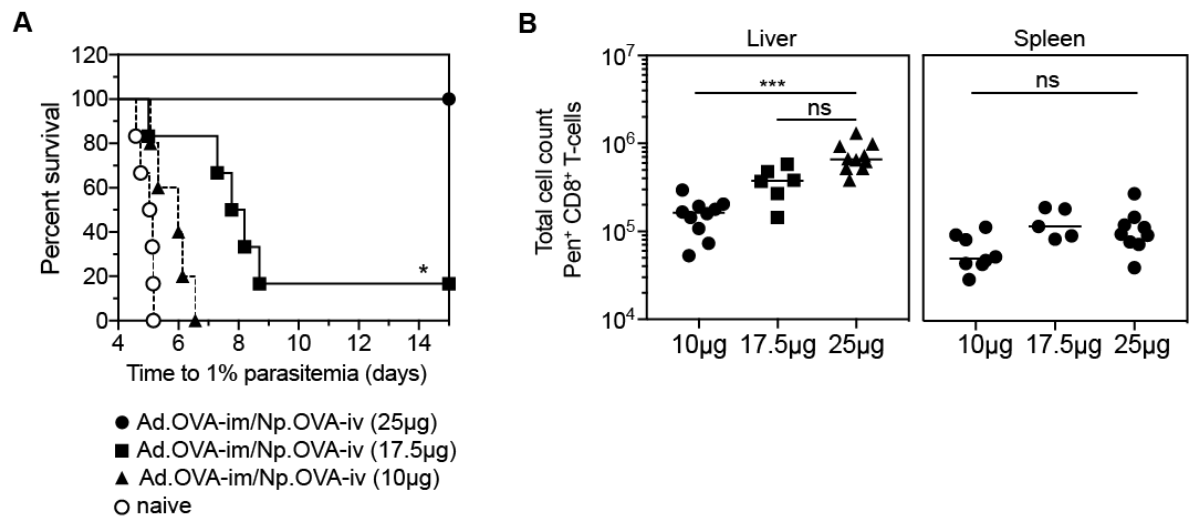


Fig. 5.4: Higher numbers of liver Pen⁺ CD8⁺ T-cells correlated with greater protection following spz challenge. C57BL/6 mice (n=5) were primed with Ad.OVA (1x10⁸ iu) im and two weeks later given iv either 10μg (10μg), 17.5μg (17.5μg) or 25μg (25μg) Np.OVA. **(A)** Three weeks later all mice were challenged with OVA::Hep17 *P. berghei* spz and time to 1% parasitemia determined by thin blood films. **(B)** Total cell count of Pen⁺ CD8⁺ T-cells in liver or spleen. p<0.001(***); p<0.05 (*); not significant (ns).

(Next page) Fig. 5.5: Prime and target vaccination induces liver Trm stably maintained in the tissue and sufficient in mediating protection.

(A) IF images depicting liver tissue of mice vaccinated with Ad.OVA-im/Np.OVA-iv, 5-weeks post Np.OVA-iv vaccination. Panel i: CD8, CXCR6, CD4, CD69, CD44, CD3, CD31 (staining vasculature endothelium), and JoJo (nucleus). Panel ii: F4/80, MHC-II, CD8, CD4 and JoJo. In small, single colour panels, maximum projection of a 20μm z-stack. Arrowhead denotes examples of Trm (CD8⁺ CXCR6⁺ CD69⁺ CD44⁺ T-cell), Star-Arrowhead denotes Tem (CD8⁺ CXCR6⁺ CD69⁻ CD44⁺ T-cell). Quantification of IF images: **(B)** Total cell count/100μm³ of Trm or Tem in Ad.OVA-im or Ad.OVA-im/Np.OVA-iv vaccinated animals. **(C)** Total number of clusters/100μm³ in Ad.OVA-im or Ad.OVA-im/Np.OVA-iv vaccinated animals. **(D)** Frequency of Tem (CD8⁺ CXCR6⁺ CD69⁻ CD44⁺ T-cell), Trm (CD8⁺ CXCR6⁺ CD69⁺ CD44⁺ T-cell) and CD4⁺ T-cells of total CD3⁺ cells in clusters from mice vaccinated with Ad.OVA-im/Np.OVA-iv. **(E)** C57BL/6 mice (n=6) were vaccinated Ad.OVA-im/Np.OVA-iv or Ad.OVA-im only. Total number of TCRv⁺ (Vα₂/Vβ₅) liver CD8⁺ T-cells over time, with dashed line representing Np injection. At day 36 post Np.OVA-iv administration **(F)** total Pen⁺ T-cells in liver and spleen. **(G)** C57BL/6 mice (n=6) were vaccinated with Ad.OVA-im/Np.OVA-iv or Ad.OVA-im. Mice were challenged with OVA::Hep17 *P. berghei* spz 2-months, or 6-months post Np.OVA-iv vaccination. **(H)** C57BL/6 mice (n=6) were vaccinated with Ad.OVA-im/Np.OVA-iv. Three weeks after Np.OVA administration, mice were either splenectomized or sham operated, and FTY720 was administered. Alternatively, mice were treated with FTY720 and/or anti-CD8 depleting Ab. Mice were subsequently challenged with OVA::Hep17 *P. berghei* spz. IF images are representative of 2 independent experiments, for a total of n=4-6 animals per condition. p<0.001(***); p<0.005(**); p<0.05(*); not significant (ns).

Figure 5.5

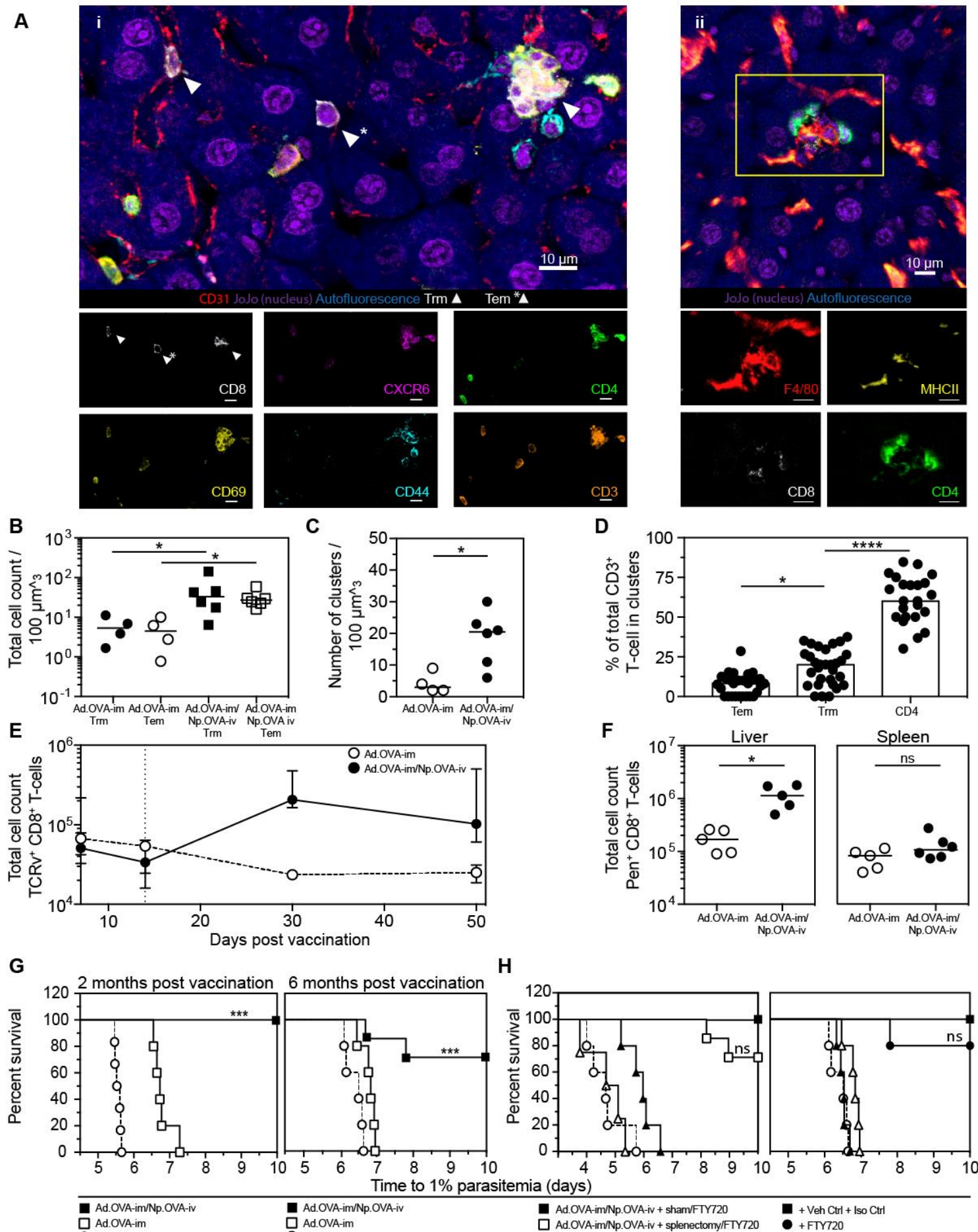
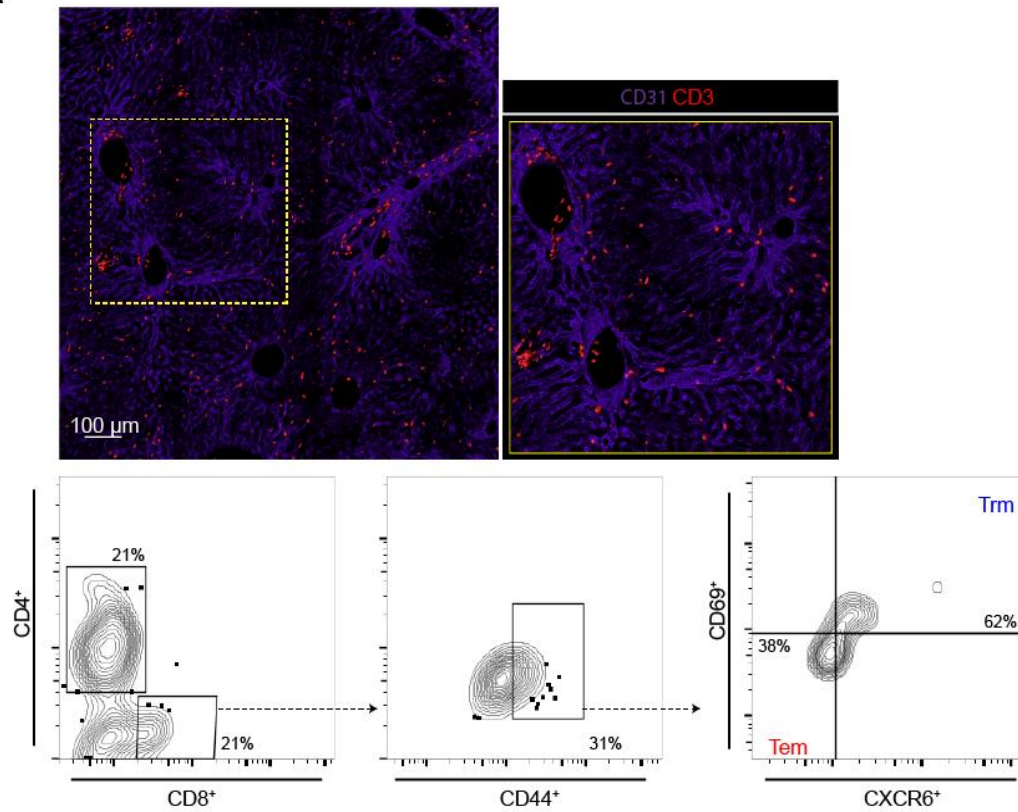
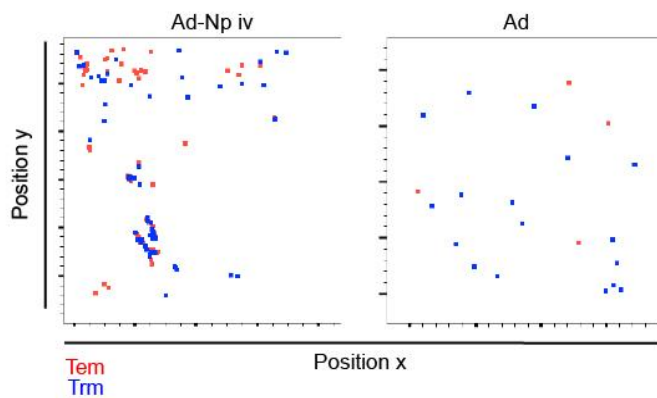


Figure 5.6

A



B



C

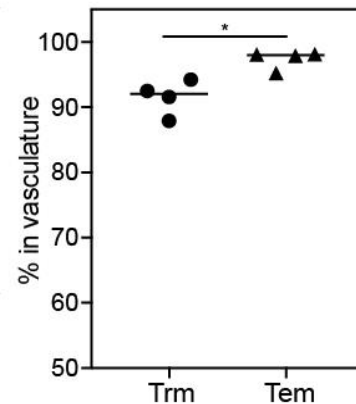


Fig. 5.6: Histocytometric analysis of liver sections.

(A) Representative histocytometric analysis of IF images from Fig 3: sections were masked on CD3 using the surface tool in Imaris software. Following export of representative channels into FlowJo, lymphocytes were gated on CD4 and CD8 expression. CD8⁺ T-cells were subsequently gated on CD44⁺ expression and subsequently gated on CD69⁺ and CXCR6⁺ expression (Trm: CD69⁺ CXCR6⁺, Tem: CD69⁺ CXCR6⁺). (B) Spatial distribution in x, y plane of Trm (blue) and Tem (red) cells in liver sections of representative Ad.OVA-im/Np.OVA-iv and Ad.OVA-im vaccinated animals. (C) Frequency of Trm and Tem of total CD8⁺ T-cells in vasculature in Ad.OVA-im/Np.OVA-iv vaccinated animals (IF images are representative of 2 independent experiments, for a total of n=4-6 animals per condition). p<0.05 (*).

Figure 5.7

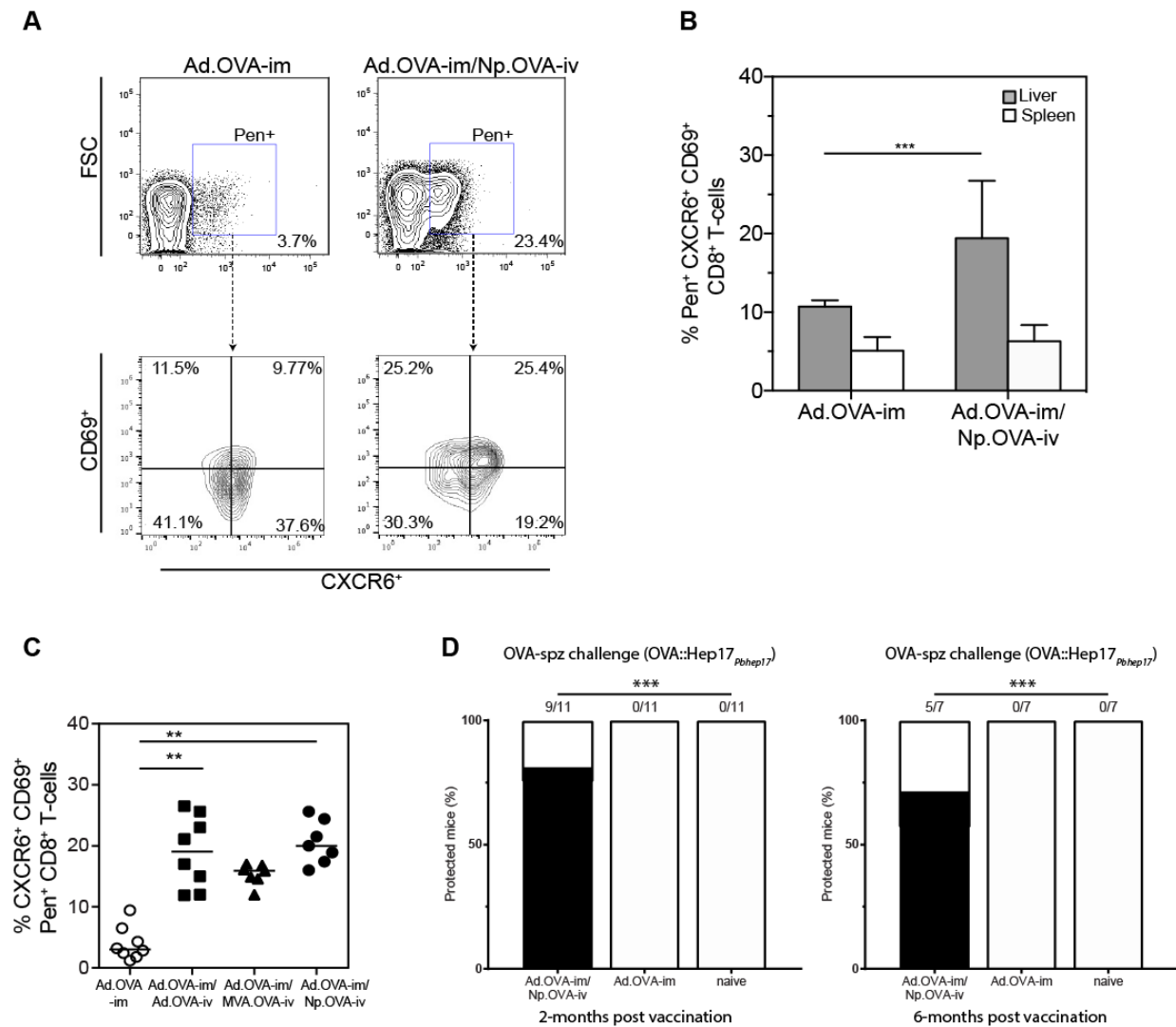


Fig. 5.7: Gating strategy showing Trm phenotype of Pen⁺ T-cells in liver following Np administration.

(A) Flow-cytometry gating strategy of representative Ad.OVA-im and Ad.OVA-im/Np.OVA-iv vaccination regime in liver at day 36 for CXCR6 and CD69 T-cell expression. Lymphocytes were gated on live cells, size and singlets and subsequently on CD8⁺ T-cells. In quadrants, CD8⁺ T-cell population gated on frequency for total Pen⁺ T-cells, and subsequently gated on CXCR6 and CD69. (B) Frequency of Pen⁺ CXCR6⁺ CD69⁺ T-cells in liver and spleen of total CD8⁺ T-cells at day 36 post Ad.OVA-im and Ad.OVA-im/Np.OVA-iv vaccination. (C) Frequency of Pen⁺ CXCR6⁺ CD69⁺ T-cells in liver of total CD8⁺ T-cells at day 36 post Ad.OVA-im and Ad.OVA-im/Ad.OVA-iv, Ad.OVA-im/MVA.OVA-iv, or Ad.OVA-im/Np.OVA-iv vaccination. Data shown is pooled from two independent experiments, each symbol representative of individual mouse (n=8-9). Median shown, Kruskal Wallis test corrected with Dunn's multiple comparison test. p<0.001(***). (D) Pooled challenge data from C57BL/6 mice (n=7-11) primed with Ad.OVA-im followed by Np.OVA-iv (Ad.OVA-im/Np.OVA-iv), or no Np.OVA (Ad.OVA-im). Mice were challenged with OVA::Hep17 P. berghei spz two or six months post-vaccination. Kruskal Wallis test corrected with Dunn's multiple comparison Test, p<0.001(***).

Figure 5.8

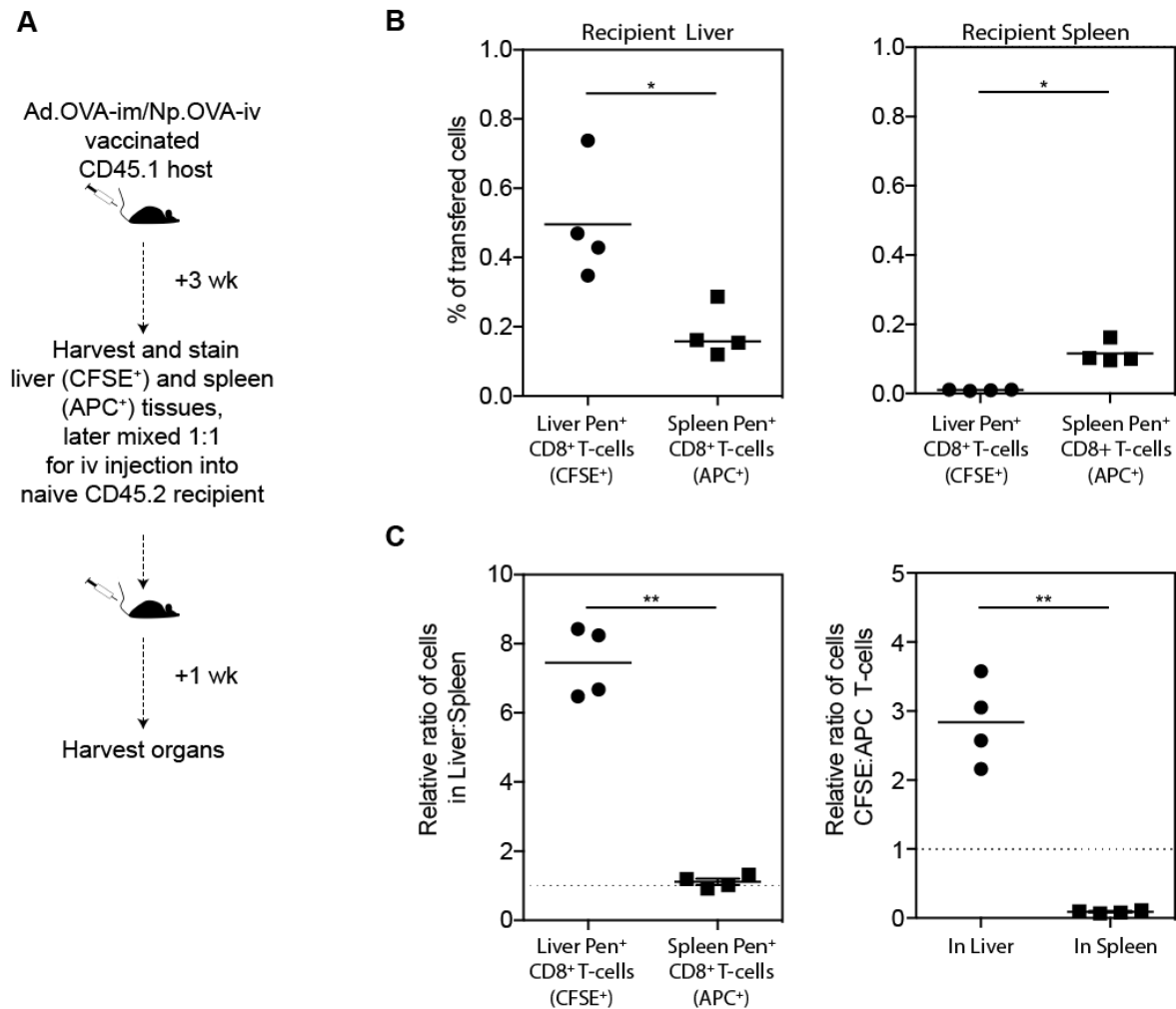
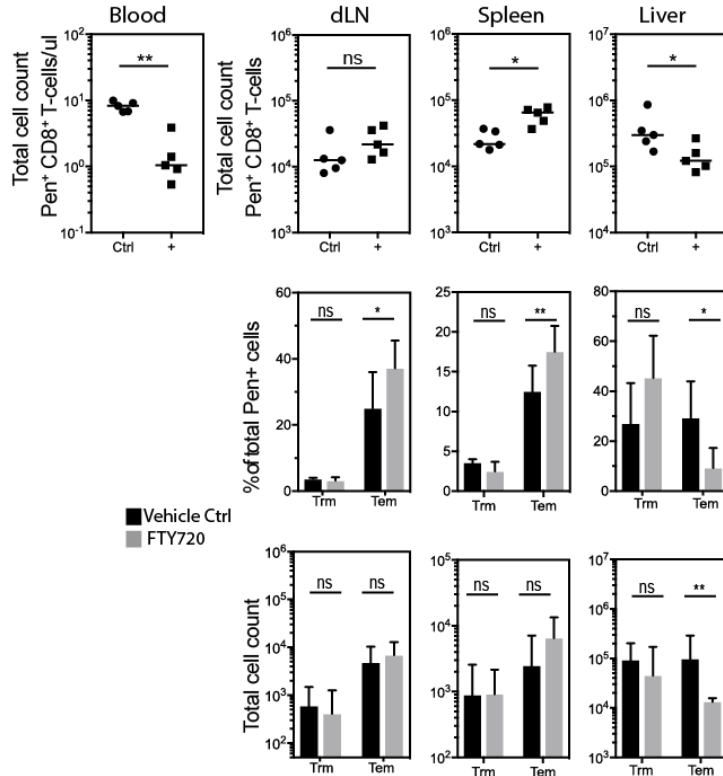


Fig. 5.8: Liver Pen⁺ CD8⁺ T-cells home back to liver tissue at higher frequency than to spleen in recipient mice.

(A) Illustration of experimental design: CD45.1⁺ mice received Ad.OVA-im/Np.OVA-iv vaccination. Spleen and livers were harvested 3 weeks (wks) later, CD8⁺ T-cells were enriched and labeled with intracellular cell trackers (CFSE and APC-Cell Tracker respectively). Pen⁺ cells from liver and spleen were then transferred into a naïve CD45.2 hosts in a 1:1 ratio between organs and organs harvested after 1 wk. **(B)** Frequency of CFSE⁺ or APC⁺ Pen⁺ T-cells of total transferred lymphocytes one week post transfer. **(C)** Liver:Spleen ratio of the CFSE⁺ or APC⁺ cells, or CFSE⁺:APC⁺ ratio in respective organs. p<0.001(***); p<0.005(**); p<0.05(*); not significant (ns).

Figure 5.9

A



B

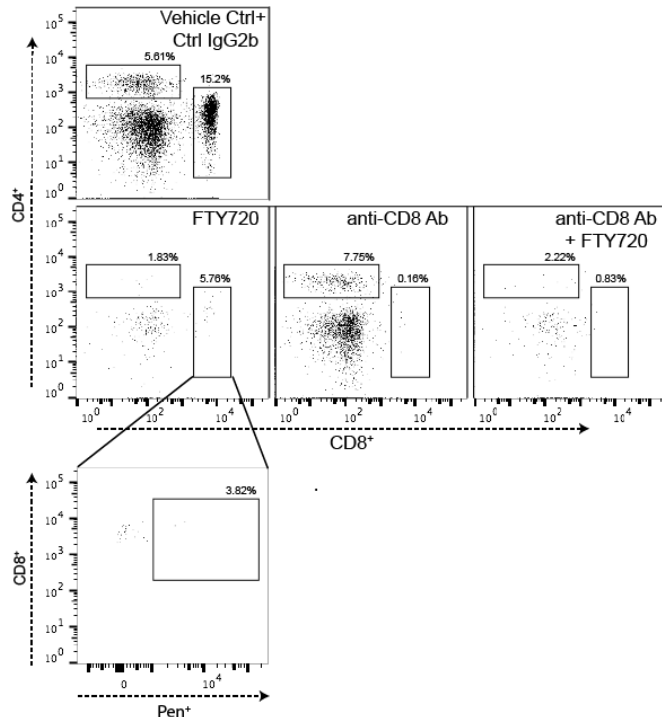


Fig. 5.9: Flow plots showing representative lymphocyte depletion and inhibition of egress from lymphatics following administration of depleting antibody and FTY720. (A) C57BL/6 mice (n=5) were vaccinated with Ad.OVA-im/Np.OVA-iv. Three weeks after, mice were treated with ip injections of FTY720 or with vehicle control (Vehicle Ctrl). Total cell counts of Pen⁺ CD8⁺ T-cells in blood (cell number/μl), in liver draining lymph node (dLN), Spleen, and Liver (top). Frequency and total cell count of Pen⁺ CD8⁺ T-cells Trm and Tem in respective organs (bottom). Kruskal Wallis test corrected with Dunn's multiple comparison test, p<0.01(**), p<0.05 (*), not significant (ns). (B) C57BL/6 mice (n=5) were vaccinated with Ad.OVA-im/Np.OVA-iv. Three weeks after, mice were treated with ip injections of anti-CD8 depleting antibody, FTY720 and anti-CD8 and FTY720, or with isotype and vehicle control (Vehicle Ctrl + Ctrl IgG2b). Peripheral blood was obtained prior to spz challenge, and lymphocytes were analyzed with flow cytometry. Representative flow plots showing respective lymphocyte depletion, and frequency of Pen⁺ CD8⁺ T-cells following FTY720 administration.

V.d – Discussion

Prime-target vaccination regimen utilizing a combination of viral vectors and PLGA-Np, is able to induce a high frequency of Ag-specific CD8⁺ T-cells in liver tissue, sufficient for protection against spz challenge. As mentioned in Chapter IV, this is a substantial improvement over current vaccination strategies, and has the potential for valuable clinical translation. Next, some of the critical questions regarding the mechanisms of vaccine protection were addressed, determining the phenotype, location and durability of the cellular immune response generated. Hence, multi-parameter histocytometry and flow-cytometry techniques were used to address two fundamental questions: firstly, what was the *in-situ* effector mechanism that resulted in immune protection and secondly, what is the phenotype and location of the cellular immune response mediating protection. Furthermore, longevity of the immune response generated following prime-target vaccination was investigated, and hence long-term studies to determine vaccine efficacy over time were performed.

To determine the cellular immune response at the site of hepatocyte infection, multi-parameter confocal imaging was used, to be able to screen multiple effector functions simultaneously *in situ* under various conditions. From the images obtained, polyclonal CD8⁺ T-cell were found to cluster in large numbers around infected hepatocytes, producing IFN γ and Granzyme B, as had previously been observed [73, 85]. Although other effector molecules such as TNF α , Perforin and FasL have all been implicated in cytotoxic CD8⁺ T-cell effector killing, staining for these markers was not successful. Probably, these effectors molecules are also being produced locally, as has been reported by flow-cytometry ex-vivo analysis by others [85, 287] . Interestingly,

Ki67 positive CD8⁺ T-cells were detected in clusters formed around OVA-spz. Although lymphocytes are predominantly thought to undergo cell division inside lymph nodes following priming phases, cell proliferation upon-recall response of memory CD8⁺ T-cells might also occur in the tissue. Such local cell proliferation might be interesting, and further work will be required to confirm that Ki67⁺ T-cells are indeed undergoing full cell-division in peripheral tissue (Ki67 is a marker denoting permission to undergo cell division, not necessary active cell division [288]) and that such cues were provided directly in the peripheral organ and not in secondary lymphoid organs.

The large cellular clusters observed were found to require cognate antigen recognition to recruit activated T-cells and clear infected hepatocytes, suggesting that TCR engagement is crucial, with by-standard activation insufficient in mediating protection [73, 289]. This data is in fact supported by work done by Cockburn et al., showing that CD8⁺ T-cells killing requires contact-mediated processes following direct recognition of the infected hepatocyte and that killing of bystander parasites at a distance via the secretion of inflammatory mediators is unable to provide sterile protection [289]. By vaccinating animals with an irrelevant antigen and subsequently challenging them, it was further confirmed that Ag recognition was crucial to obtain sterile protection. Although high levels of hepatic inflammation have been shown to potentially reduce spz infectivity when administered one day post-challenge [287], it was shown here that any inflammation potentially induced by iv-injection of viral vectors was not able to mediate protection. Furthermore, in accordance to seminal literature in the field, even in the prime-target vaccination regime, CD8⁺ T-cells were confirmed to play a crucial role in

mediating protection, as following cell depletion of this subset, vaccine protection was completely ablated.

It was hypothesized that sterile protection was dependent on the induction of high numbers of Ag-specific CD8⁺ T-cells. In fact, sterile protection induced by prime and target vaccination was found to scale in a dose-dependent manner, with higher levels of Ag-specific CD8⁺ T-cells in the liver correlating with increased protection. In fact, experiments in the field using adoptive transfers of Ag-specific CD8⁺ T-cells have been shown to be protective when sufficiently high numbers of cells are transferred [82]. This is also in accordance with work done by Harty and others which describe a critical CD8⁺ T-cell threshold to mediate protection during the liver stages of the disease, showing that particularly high numbers of Ag-specific CD8⁺ T-cells are important to find all of the few infected hepatocytes [81, 290]. In summary, in support of what is known in the field, prime-target vaccination was shown to mediate protection via cytotoxic CD8⁺ T-cells, producing effector functions locally following cell-cell contact with infected hepatocytes presenting cognate-antigen.

Following careful *in situ* phenotypic characterization using histo-cytometry, of CD8⁺ T-cells during the spz infection, the phenotype of these cells within the hepatic tissue following prime-target vaccination was investigated. Ag-specific CD8⁺ T-cells were found to express markers of Trm (notably, CXCR6 and CD69). Ag-specific Trm were found to be predominantly in the vasculature, speculated to be patrolling the liver sinusoids looking for cognate antigen (as has been shown by intra-vital microscopy performed both on Trm and T-effector cells [85, 257]). What is the actual mechanism of retention within the hepatic tissue is yet unclear. CXCL16 has been shown to be crucial,

but its direct role in T-cell retention is an area of continuing investigation. Furthermore, it was observed that small clusters of Kupffer cells, CD4⁺ and CD8⁺ T-cells form at steady state post prime-target vaccination. Whether such interactions are important for Trm survival or maintenance is still unclear, potentially tissue-resident macrophages are providing a chemokine network (or alternatively a source of deposited antigen) allowing for Trm retention. Understanding the mechanism of Trm retention in the liver might an important future direction, with further work needed to address such crucial questions.

In agreement with a previous study [83, 85], the number of liver Trm CD8⁺ T-cells was found to correlate with survival in the context of liver-stage malaria. However, it had remained unanswered whether Trm alone were sufficient in mediating protection to spz challenge or if they acted as sentinels: recruiting CD8⁺ effector and effector-memory cells from the circulation or liver dLN. To address this, a combination of FTY720 and splenectomy operations to inhibit or remove T-effector CD8⁺ lymphocytes from the circulation (hence allowing to isolate only the liver Trm) were performed. Notably, following challenge, Trm were found to be sufficient in mediating protection against liver-stage malaria suggesting that a resident antigen-specific Trm population was indeed effective at finding all infected hepatocytes. During a natural infection, it is most probable that Tem/Tcm are able to contribute directly to help clear all infected hepatocytes; however, they probably lag behind Trm in their ability to enter the tissue and become activated. Hence, Trm probably provides a critical advantage in clearing effectively all spz-infected hepatocytes.

Taken together, it was shown that this prime-target approach substantially improves current antigen-based liver-stage malaria vaccines, providing a potent and

flexible immunisation platform by generating high levels of liver Trm able to clear the hepatic spz infection in the short time frame provided.

Chapter VI

Discussion and Conclusion

Chapter VI

Discussion and Conclusion

VI.a – Thesis overview

Malaria, caused by *Plasmodium* species, is one of the most critical infectious diseases, causing about 500,000 deaths annually, with particularly high morbidity and mortality in sub-Saharan Africa [2]. With growing anti-malarial drug and insecticide resistance, an effective vaccination regimen with high efficacy is needed urgently. A variety of different vaccine strategies have been investigated, with candidates in the pre-erythrocytic, erythrocytic and mosquito stages of the *Plasmodium* life-cycle showing varying degrees of efficacy. The most advanced candidate in the field, a RTS,S/AS01 formulation, completed Phase III field trials with partial efficacy, but is still years from WHO pre-qualification and licensure due to relatively low efficacy [41]. An alternative pre-erythrocytic approach, not based on Ab-responses to CSP, has been to generate cellular immunity directed against infected hepatocytes. Early studies showed sterile protection after RAS vaccination in mice [60] and humans [59], with later work demonstrating that CD8⁺ T-cells have a leading role in protection against spz challenge [64-66]. To this end, substantial effort has been invested in optimizing a viral vector platform, with a leading approach comprising of a recombinant simian Adenovirus vector prime, subsequently boosted with Modified Vaccinia Ankara expressing the same antigen. Ideally, a combination of optimal pre-erythrocytic and erythrocytic sub-unit vaccines will provide an effective anti-malaria vaccine approach.

A greater understanding of CD8⁺ T-cells memory formation and maintenance (both in a quantitative and qualitative fashion) would be of great importance for the field as a whole. Additionally, understanding what rules govern CD8⁺ T-cell spatial dynamics will greatly inform future CD8⁺ T-cell inducing vaccine design. In fact, despite the substantial progress that has been made, vaccines aimed at inducing cell-mediated immunity against malaria have been hindered by a number of challenging spatio-temporal factors pertaining to parasite biology and liver microanatomy. Firstly, using only peripheral vaccination regimens, it is hard to generate and maintain the high numbers of *Plasmodium*-specific circulating T-cells necessary to confer liver-stage protection. Secondly, it has been difficult to localize a robust and long-lived cell-mediated memory immune response to the target organ, able to clear the parasitic infection in the short time window of liver stage malaria (5-7 days for *P. falciparum* infections in humans, 2 days in the context of *P. berghei* mouse infections). In this Thesis, initial work investigated the dynamics of CD8⁺ T-cell generation following viral vector vaccination, with preliminary evidence that antigen may be important in regulating CD8⁺ T-cell anatomical localization (Chapter III). Following on from this work, a prime and target vaccination approach was developed, specifically aimed at inducing high numbers of liver Trm cells, shown to be important for liver-stage malaria protection.

The approach here focused on generating a highly translatable and deployable vaccine strategy, which can induce high numbers of liver Trm cells against full-length antigens in the human population. Targeting CD8⁺ T-cells to the liver was achieved using either PLGA-protein loaded nanoparticles or a recombinant viral vector, such as human or chimpanzee Ad or MVA. It was shown (Chapter IV) that this approach generates

durable Ag-specific CD8⁺ T-cells resident in the liver and dramatically increases the efficacy of subunit immunization against liver-stage malaria using clinically relevant antigens and clinically tested malaria vaccine candidates. Furthermore, the phenotype of the protective immune response in prime-target vaccination was subsequently investigated, showing that liver Trm cells were sufficient to mediate protection in this malaria challenge model (Chapter V).

After demonstrating that prime and target was highly efficacious and following the completion of toxicology testing in mice and rabbits, a small phase I clinical trial has started in Oxford, UK to address safety and tolerability of iv administration of ChAd63.ME-TRAP in human healthy volunteers. This dose escalation trial involved 3 subjects per dose group (Groups 1-3), with ChAd63.ME-TRAP doses of 5×10^8 vp, 5×10^9 vp and 5×10^{10} vp administered intravenously. No severe adverse events were found at the injection site, and some mild-moderate systemic events were noted (such as fever, headaches and malaise) (*manuscript under review*). Close monitoring of laboratory parameters was followed to assess for any derangement of liver function tests and Disseminated Intravascular Coagulation (DIC), however no transaminitis was observed. Overall, iv ChAd63.ME-TRAP was well tolerated. Following vaccination, vaccine immunogenicity was assessed via *ex-vivo* IFN γ ELISPOT using peripheral blood mononuclear cells (PBMCs), and at the highest vaccine dose, a remarkably high response was observed (at least comparable to a previous clinical trial where the same dose (5×10^{10} vp) of ChAd63.ME-TRAP was administered via the im route [98]). Although future trials will need to assess liver CD8⁺ T-cell Trm induction post vaccination, the initial positive safety and immunogenicity data

will support exciting future clinical testing of the safety, immunogenicity and efficacy of prime-target regimens in humans.

VI.b – Chapter conclusions and inferences

VI.b.i – Chapter III: Memory localization following Adenovirus

The immune system plays a crucial role in protecting our bodies from pathogen invasion. One of the cardinal features of the adaptive immune system is the formation of memory T- and B-cell populations allowing for enhanced protection upon secondary encounters. Memory lymphocytes present two cardinal quantitative and qualitative features. In the context of T-cells, memory populations result in higher numbers of clonally identical, antigen experienced cells than a naïve individual. Furthermore, memory T-cells show enhanced functionality, with increased abilities to produce effector molecules and cytokines, proliferative potential and unique localization within the host (as discussed in greater detail in Chapter I). The experiments in Chapter III described the kinetics of Ad.OVA, MVA.OVA and Ad.OVA/MVA.OVA T-cell expansion following intramuscular administration, showing that Ad.OVA/MVA-im results in high numbers of Ag-specific CD8⁺ T-cell upon expansion, which correlates with high-frequencies of memory CD8⁺ T-cells. Through the use of adoptive transfers of memory CD8⁺ T-cells, it was demonstrated that high numbers of Ag-specific CD8⁺ T-cells generated from the priming phases are crucial, resulting in higher numbers of memory T-cell maintained post-boost. Following on from this, the properties of Adenoviral vectors were further investigated and through the use of adoptive transfers of OTI and OTII T-cells, the

presence of persistent antigen presentation through T-cell upregulation of CD69 and early proliferation in dLN and spleen was demonstrated. Lastly, some preliminary data showed that this antigen persistence is able to shape memory CD8⁺ T-cell location and maintain a Tem phenotype. Understanding what determines memory T-cells localization post-vaccination is still an area of ongoing research with two, not mutually exclusive hypothesis proposed in the field: the first is related to antigen persistence and TCR engagement; the second is the induction of homing molecules during the priming phases of the response (a mechanism also known as imprinting).

Antigen persistence and immunological memory

The hypothesis of antigen persistence in maintaining and influencing CD8⁺ T-cell memory has been investigated since the early 90s [291]; with evidence showing that successful CD8⁺ T-cell induction from vaccinations can be influenced by long-term antigen persistence in the host [292]. In fact, it is commonly thought that vaccination with live or live-attenuated viral vaccines are much more efficient than killed or non-replicating viral vectors [293]. Two main interpretations can be made that would have implications in memory CD8⁺ T-cell generation, which could be characterized as quantitative vaccine factors (higher antigen loads can be reached with live-attenuated, replicating, viral vaccines) or qualitative vaccine properties (ie unique induction of a priming microenvironment, maturation and Ag-deposition in different APCs, production of cytokine and survival factors). The persistence of Ag following vaccination might have unique implications for memory T-cell survival (with constant TCR engagement providing increased levels of homeostatic proliferation and survival signals) as well as

shape long-term T-cell migration dynamics between tissue and lymphatic tissue (with TCR engagement resulting in arrest and local retention).

Within the LN environment, unique lymphoid tissues structures adapted for long-term maintenance of antigen have been described. In fact, the importance of antigen persistence has been well determined in the context of B-cell formation, affinity-maturation and antigen deposition in germinal centers. Seminal work has revealed that the magnitude of the Tfh cell response is dictated by the amount of antigen (which directly correlated with the magnitude of the germinal center B-cell response) [294]. Maintenance of the Tfh cell phenotype also required sustained antigenic stimulation by germinal center B-cells. Aside from B-cells, antigen can be retained long-term in germinal centers by Follicular Dendritic Cells (FDC), described to be “dynamic antigen libraries”. The mechanism of Ag deposition by FDC has yet to be fully resolved, with the belief that long-term antigen-retention involves the recycling of intact immune-complexes within non-degradative vesicles [295, 296]. Although these pertain to the humoral branch of the immune system, one could predict that a parallel system involving long-term antigen presentation by DC, FRCs, and CD169⁺ sub-capsular sinus macrophages might function in a similar manner, with more stable p-MHC complexes retained long-term on the surface of these cells.

Antigen persistence has been shown to be required for effective DC licensing and CD8⁺ T-cell cross-priming, allowing for effective CD8⁺ T-cell memory formation [297]. In fact, early work by Zinkernagel and colleagues showed that the duration of T-cell memory depended on the antigen dose (and clearance) used in immunisation [239]. Similarly, viral persistence has been shown to influence the differentiation of CD4⁺ T-

cells [298], hence impacting the T-lymphocyte “flavor” of the immune response generated. Indeed, many viral infections remain in the host tissues for years (even when assumed to be eliminated, such as in the case of LCMV) [293, 299]. In the context of non-replicating Adenoviral vectors, they have been shown to persist in multiple organs following intramuscular administration [222, 223], with involvement of both hematopoietic and non-hematopoietic antigen presenting cells (although not precisely characterized) [231]. Future quantitative work is required to further investigate the relationship between persistent antigen-presentation and memory phenotype, location and number in the context of prime-boost vaccination strategies. Altogether, these data seem to suggest there is an intimate interaction between Ag and lymphocytes, in multiple aspects of the immune response.

Imprinting of Memory T-cells

Aside from Ag-recognition, a large body of literature suggests that the location of T-cell priming can influence the expression of homing molecules during Teff and memory CD8⁺ T-cell formation stages [300]. For example, T-cells primed in mesenteric lymph nodes and Peyer’s patches upregulate the integrins $\alpha 4\beta 7$ and the chemokine receptor CCR9 following interactions with DCs in a retinoic acid dependent manner [301, 302]. On the other hand, T-cell activation in skin dLN results in increased expression of E and P-selectins, CCR4, and CCR10 induced in part by the metabolism of vitamin D3 by skin DCs [302-304]. However, the molecular controls behind migrational imprinting are still unknown, with epigenetic regulation speculated to play a key role in determining the expression of specific integrins and chemokine receptors [300]. Although

the ability of DCs to mediate imprinting to various organs via the upregulation of certain markers has been shown, imprinting of a migrational phenotype does not necessarily require priming in local LN specific environments. In fact, after systemic infections, it has been shown that CD8⁺ T-cells can form memory cells in the gut [305]. Similarly, a respiratory infection can prime T-cells that migrate to mucosal tissue [306], or skin infections can result in lung memory CD8⁺ T-cell pools [307]. Such T-cell imprinting appears to be transient: with most T-cell activation resulting in the upregulation of gut-homing, skin-homing or lung-homing CD8⁺ T-cells [300]. From the data presented in this thesis, one may speculate that although early imprinting probably occurs during the priming phases, throughout the course of the immune response persistent antigen presentation might play a crucial role in shaping memory T-cell migration. Future work using histo-cytometric analysis following Ad.OVA-im vaccination and subsequent adoptive transfers of OT-I T-cells as surrogate markers for antigen-presentation, will be able to determine whether DCs or stromal cells are able to persistently present antigen in the context of MHC class I. It is likely that a combination of TCR engagement as well as imprinting of T-cells during the priming phases of the response are both involved in determining memory T-cell localization and migration dynamics. Furthermore, the tissue microenvironment will most likely further shape memory T-cell phenotype (discussed in greater detail in the subsequent sections) [189].

The work in this chapter, although somewhat preliminary, suggested that a potential model to generate hepatic CD8⁺ T-cell memory would be to attract T-cells to the hepatic tissue via antigen presentation within the sinusoidal vasculature. It was speculated that if T_H1 were promiscuously traveling throughout the body in search of cognate

antigen, that by providing large quantities of SIINFEKL loaded MHC class I molecules, Teff might arrest upon antigen encounter, interact with their surrounding tissue thus acquiring the needed chemokine receptors/transcription-factors to survey the local environment and potentially survive long-term in it. These observations led to the development of a subunit liver-stage malaria vaccine approach, later called prime and target.

VI.b.ii – Chapter IV: Prime and Target Vaccine Approach

A prime and target vaccine strategy was developed to address some of the limitations of heterologous prime-boost viral vector vaccinations. Specifically, it was hoped that the new vaccine strategy would address four goals: firstly, generating large numbers of Ag-specific CD8⁺ T-cells in the liver microenvironment without inducing T-cell exhaustion or tolerance; secondly, provide a vaccine strategy that was safe for clinical use; thirdly, utilize vaccine types that were deployable on a large scale and with ease of manufacture; and lastly, provide vaccine efficacy that resulted in long-lasting sterile immunity to *P. berghei* spz-challenges. Importantly, preliminary work showed that prime and target regimen could achieve most of the outlined aims. Following a peripheral prime, administering a high dose of antigen to the liver (either via iv injection of viral vectors or PLGA protein loaded nanoparticles), resulted in sterile protection in the *P. berghei* mouse model of liver-stage malaria. Although promising, further improvements are necessary for a highly-efficacious human vaccine. Specifically, certain aspects outlined could be the focus of important future research: choice of antigens, delivery platform, vaccine safety in the target infant populations, and longevity of protection.

Optimizing choice of antigen used

Efficacy of sub-unit vaccines will in part depend on antigen choice, with extensive recent work attempting to determine novel protective liver-stage malaria antigen [101, 102]. Hence, efficacy of the prime-target vaccination was assessed with a variety of different transgenic *P. berghei* parasites expressing either a model antigen (OVA), or clinically relevant liver-stage malaria antigens (LSA-1, LSAP-2 and TRAP) [101, 103], and subsequently found to mediate sterile protection at least 3-weeks post vaccination. Though encouraging, for effective translational deployment of prime and target vaccination, a combination of protein antigen(s) with particularly immunodominant epitopes as well as abundant peptide-MHC I presentation on infected hepatocytes will be preferred. The exact mechanism of antigen presentation following hepatocyte infection by spz is still unclear. It has been shown that effective presentation of antigen in two different transgenic models requires cytosolic protein degradation and MHC class I loading via Transporter Associated with Antigen Processing protein (TAP), an ATP-binding-cassette transporter family member [70, 74]. However, whether proteins are taken up *in vivo* from endocytosis/micropinocytosis and presented via cross-presentation or if direct spz-invasion is required is unknown. Shedding of surface proteins such as TRAP and CSP via proteolytic cleavage is a central feature of invasion by apicomplexans [308-310], making these fragments potentially more easily available for antigen processing by hepatocytes, Kupffer cells, Liver Sinusoidal Endothelial cells (LSEC), and Hepatic Stellate cells. Furthermore, the cell biological mechanisms of how the proteasomes access proteins from the PVM or from inside the parasitophorous

vacuole are still not identified. Although some *in vitro* culture work has been done using cell lines, detailed *in vivo* work will need to be pursued in the future. With respect to OVA Tg-spz models used in this thesis, at three weeks post-vaccination no difference was found when OVA protein was driven under a Hep17 (PVM only) promoter or under Hsp70 (cytosolic) promoter [208]. This was a little surprising as it was predicted that protein location within the intracellular hepatic stages of the parasite might influence MHC-class I peptide loading and presentation. Whether a discrepancy between the two transgenic lines could be observed at later time points, more than 3-weeks post-vaccination, or when administering sub-optimal vaccine doses, is an important future research avenue as it may provide insight into the choice of future translational antigens. In essence, further work is needed to improve our understanding of which antigens are abundantly presented on human MHC-class I following spz challenge (with the added complication that HLA genes show extreme polymorphism within the population), allowing for high levels of Ag-specific CD8⁺ T-cells induction.

Several techniques can be used to identify protective T-cell epitopes using either direct or reverse immunology. Indeed T-cell epitopes can be determined via mass-spectrometry experiments [311]. Alternatively, several computer algorithms have been developed, able to predict proteasome cleavage patterns and TAP translocation and have been shown to identify CD8⁺ T-cell epitopes with high predictability (in contrast to CD4⁺ T-cell epitope predictions which are much lower due to the structural properties of the MHC class II binding pocket) [312]. Algorithms such as SYFPEITHI [313], BIMAS [314], and NetChop [315], have been used successfully. In fact, in cancer immunotherapy the field has advanced to such a level that the ability to identify cancer exome-based

neoantigens has been widely applied [316], suggesting that similar techniques could be applied successfully in the context of liver-stage malaria. Additionally, with the advance of CRISPR technology and generation of GAP-parasites, antigen screens could be refined to include only the liver-stages required to mediate protection.

Optimization of antigen delivery platform to hepatic tissue

Aside from antigen choice, further developments on delivery platform and route of administration will be important, with future developments with modification of Np and viral vectors as promising starting points. Data in this thesis showed that PLGA protein-loaded nanoparticles were particularly efficacious in inducing high frequencies of Trm resulting in liver-stage malaria protection. Potentially more complex nanoparticle formulations [269], with increased depot effects from slow release PLGA compounds, unique charge and/or cell targeting, might have important consequences in the longevity of the response. In these experiments, 25µg of Np.OVA intravenously was the routine dose administered; however, what the total protein load in each target cell remains unknown. For example, it is unclear if the targeting of only a few cells with high-antigen loads (with formulations of high antigen load per Np.OVA particulate) or many cells with low-antigen load (obtained from low antigen load per Np.OVA particulate) would result in differences in Trm distribution, and/or number, and subsequent vaccine efficacy. Furthermore, future work utilizing nanoparticles in combination with adjuvants and/or chemokines might be of future interest. No significant improvement in the number of SIINFEKL-Pen⁺ CD8⁺ T-cells recruited to the liver when MPL-R848 adjuvants were combined to Np.OVA was observed. However, other adjuvants or chemokines couplings

(such as CXCL16), might be interesting to investigate, not only for their impact on the longevity of the Trm response, but also for potential effects on the phenotype of Trm CD8⁺ T-cells generated (for example, previous work had shown that the poly-functionality of memory CD8⁺ T-cells might be important for liver stage malaria vaccine efficacy [98]).

In addition to protein-loaded nanoparticles, recombinant viral vectors were administered intravenously and assessed in their ability to generate high numbers of Ag-specific liver resident CD8⁺ T-cells. Both Adenovirus and MVA viral vectors, despite having highly different viral tropisms and expression kinetics, were able to induce similarly high numbers of Ag-specific CD8⁺ T-cells, with Adenovirus viral vectors resulting in an increased frequency of CXCR6⁺ CD69⁺ tissue-resident CD8⁺ T-cells. Indeed, a recent report showed that recombinant AAV type 8 viral vector might also be an effective targeting agent [85]. AAV viral vectors are known to have high liver-tropism, with the ability to express high-levels of antigen for extended period of time [317, 318]. Work at the Jenner Institute has compared MVA.OVA-iv, Ad.OVA-iv or AAV.OVA-iv, and showed significantly lower Trm generation following AAV administration, suggesting AAV may not be a particularly effective targeting agent in a prime-target regimen (*data not shown, experiments performed by Alex Spencer*). Regarding the priming phases of prime and target, further investigation into optimal viral vector intramuscular priming might result in higher numbers of Trm (as suggested partially by trends observed in Fig. 4.7B; albeit non-statistically significant). Of particular interest would be to understand if higher CXCR6⁺ expression upon priming, known to be up-regulated upon CD8⁺ T-cell activation, would result in increased Trm induction and

maintainance [319, 320]. This could be directly investigated by comparing the upregulation of CXCR6 on Ag-specific CD8⁺ T-cells between different viral vectors such as Ad and MVA, as well as utilizing adoptive transfers of CXCR6⁺ Teff and following Trm formulation.

Lastly, an interesting experimental approach would be to develop a prime-target vaccination strategy that avoids intravenous injections (improving both safety and ease of introduction). The oral route of administration is particularly appealing, especially as certain naturally occurring viral infections have complex life-cycles involving gastro-intestinal to liver transitions. For example, the development of non-replicative Hepatitis A viral vectors is particularly interesting (with considerable caveats regarding safety, pre-existing immunity and a small single-stranded 7.5kb RNA small genome) [321]. An alternative “antigen-carrier” could be an attenuated form of *Listeria monocytogenes*, able to undergo translocation across the intestinal mucosa and then disseminate into the liver via the bloodstream [322]. Potentially even more promising would be the use of AAV5 viral vector, which appears to be stable following oral administration and has been reported to be found in the hepatic tissue [323]. Although preliminary work administering AAV5 orally did not appear to result in much Ag-specific Trm formation following administration (*data not shown*), potentially higher doses might result in a better outcome.

Altogether, the choice of viral vector to maintain high numbers of CD8⁺ Ag-specific Trm in the liver will be of crucial importance. Due to the biological complexities of viral vectors which cannot be easily manipulated (regarding antigen delivery, viral tropism, induction of local inflammation, and viral vector clearance) utilizing PLGA

nanoparticles might be a valuable experimental alternative to tease apart the requirements for Trm induction, phenotype alteration and long-term Trm maintenance requirements.

Safety and longevity in the target infant population

On a last note, there are a number of remaining questions regarding vaccine long-term safety and efficacy in the target infant population to be resolved. Regarding safety, as Teff CD8⁺ T-cells are recruited to the hepatic tissue with antigen, it is possible that transient hepatic inflammation and/or hepatic damage could occur. A small phase I clinical trial involving human healthy volunteers was recently completed at the Jenner Institute with a positive safety and immunogenicity profile, warranting optimism. Future work will need to expand these findings, combining both the prime and subsequent target injections, while carefully monitoring safety in a larger cohort.

Lastly, regarding long term efficacy in the target population, it is worth remembering that infant immune systems vary substantially from the adult population both in the innate and adaptive compartments. Indeed, infants tend to have an immune system slightly biased towards tolerance (believed to be a consequence of life *in utero*), resulting in greater numbers of immature DC and monocyte populations, as well as weaker CD8⁺ T-cell responses (hypothesized to be caused by impaired priming). Aside from numerical differences between adult and infant populations, there are also qualitative differences. For example, neutrophils and monocytes show weak bactericidal functions, poor responses to inflammatory stimuli and reduced ability to undergo inflammatory activation [324, 325]. Intracellular signaling following PRRs activation is also greatly diminished resulting in impaired cytokine output, such as secretion of IFN-

α/β upon viral exposure [326]. Although much of this work has been performed using infant cord-blood, or studying pre-term infants, clear differences between adult and child immune physiology exist [327]. Recent data in the field on the use of ChAd-MVA regimens in infants are encouraging [328], with further work in the field investigating the differences between adult and infant immune systems important to improve vaccine responses in the target population.

VI.b.iii – Chapter V: Understanding the tissue specific response

Having determined the protective ability of prime-target vaccination using intravenous administration of Np.OVA or viral vectors, it was important to gain a greater understanding of the tissue-specific response that was mediating the sterile protection.

Characterizing the early immune response following spz challenge

Detailed histo-cytometry was performed in animals that had been vaccinated with or without prime-target, and subsequently challenged with either transgenic spz-OVA or just spz. Hepatic tissue was then harvested 24hrs into the liver-stage response. From this, it was observed that around infected hepatocytes, large cluster of lymphocytes were recruited in an Ag-dependent manner only when the animals were vaccinated with prime-target regimens. Furthermore, high-frequencies of CD8⁺ T-cells producing IFN γ and Granzyme B were observed. Although the duration of CD8⁺ T-cell and cognate target cell interactions were not assessed, it is known from intravital two-photon microscopy that effective degranulation of CD8⁺ T-cells requires several hours to kill one target cell

[329]. It was furthermore interesting to note that several cells within the clusters were Ki67⁺, suggesting that liver Trm are able to proliferate within the hepatic tissue.

Of interest, was the large number of CD4⁺ T-cells at the infectious site. Although this was not conclusively investigated, it is likely that a large fraction of these cells would be NKT cells, which are present at high-frequencies in the liver sinusoids [173]. The crucial role of CD8⁺ T-cells in protection from liver-stage malaria was confirmed by antibody-depletion, revealing that CD8⁺ T-cells are necessary to clear all infected hepatocytes. Due to the multitude of cells observed around infected hepatocytes (as well as other hepatic resident cells not stained: NKT, NK, $\gamma\delta$ T-cells, neutrophils and monocytes) future work will focus on characterizing all involved players. Furthermore, a time-course will be performed to understand the kinetics of CD8⁺ T-cells recruitment and subsequent activation. In fact, the identity of the “sentinel” cell upon spz-infection is still not known, and therefore a careful longitudinal study utilizing histo-cytometric analysis could provide some fundamental insight into the protective liver-stage immune response.

Lastly, one could speculate that a prime and target vaccination would result in quantitative and qualitative changes in the local hepatic microenvironment involving local hepatic immune populations such as Kupffer cells, sinusoidal LSECs, NKT cells, and hepatic stellate cells. Although CD8⁺ T-cells are essential to mediate protection, local changes in the hepatic environment such as ability to present antigen, cytokine secretion and kinetics, number and positioning of surveying immune cells might be important in protection against liver-stage spz challenges.

Determining the phenotype of CD8⁺ T-cells

Having demonstrated that prime-target vaccination induces high levels of liver CD8⁺ T-cells which cluster around infected hepatocytes producing effector functions, and knowing from the literature that they are necessary to mediate spz-protection, a detailed characterization of their location and phenotype was performed. Indeed, CD8⁺ T-cells were confirmed to be in the liver sinusoids, and found to be expressing liver Trm markers such as CXCR6 and CD69 both via histo-cytometric and flow-cytometric analyses. Furthermore, through the combination of splenectomy and administering FTY720, it was shown that Trm T-cells were sufficient to mediate protection in the context of spz-challenge. This is an important finding: together with previous work [90], it appears that Trm are not only necessary, but also sufficient to mediate protection in the context of liver-stage malaria mouse spz challenges. Together these data suggest that a detailed understanding of Trm biology would be very useful to design future liver-stage malaria vaccines.

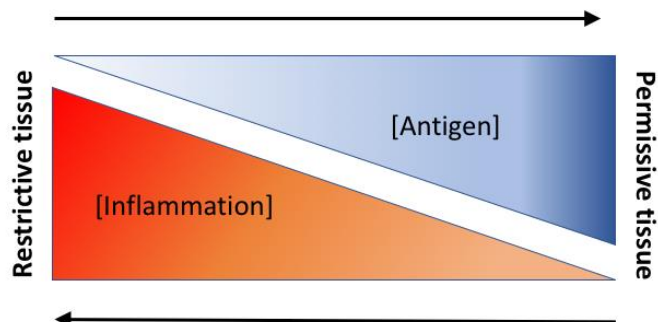
The requirements for the induction of liver-resident Trm was subsequently investigated. As mentioned in the prior section, Trm liver induction required antigen such as in the form of Np.OVA, with Np.adj particulates showing no increase in number of liver SIINFEKL-Pen⁺ CD8⁺ T-cells. This is also in accordance with recent work by Fernandez-Ruiz et al., showing that AAV8 is able to generate liver Trm [85]. However, the presence of adjuvants (in this case a combination of MPL and R848) could have a dose sparing effect when only 10µg of OVA were administered (Fig. 4.7).

Speculating on liver-stage Trm biology

Looking at the broader picture of Trm mechanisms, evidence suggests that there is a tight interaction between unique tissue physiology and microanatomy and Trm phenotype, with the tissue probably constraining and shaping Trm functions and properties. In fact, as mentioned in Chapter I, tissues can be described as being permissive or restrictive depending on whether CD8⁺ T-cells effector and memory cells can circulate through the tissue in the absence of inflammation [178]. Indeed, one may speculate that there is a correlation between what is required for Trm induction and tissue “accessibility”, whereby Trm induction may be dependent on antigen recognition in permissive tissues that constitutively express high-level of tissue-homing chemokines, while non-specific inflammation might be sufficient in restrictive tissue (Fig. 6.1).

For example, in the context of skin Trm, local administration of OVA is not able to induce Trm formation, while local injections of OVA+LPS are able to result in CD8⁺ OTI Trm cells at the site (*personal data not shown*). For the generation of other Trm-based vaccines, understanding the requirements for antigen and/or inflammatory cues for Trm induction in the tissue of interest will be critical.

Fig. 6.1: Potential model for Trm induction requirement in permissive or restrictive tissue. Trm induction in permissive tissues requires cognate Ag-specific interactions for long term retention; conversely, in restrictive tissues non-specific inflammation allows for Trm induction, with specificity tightly controlled upon entry into the tissue rather than engagement with.



Following careful multi-parameter, confocal imaging, it was observed that although the majority of liver Trm were found within the sinusoidal vasculature (as previously described [85, 172]), small clusters of Trm were also found including Tem, CD4⁺ T-cells and Kupffer cells in them, in part resembling some of the previously described MLCs [188]. This opens an interesting question: what are the requirements for liver Trm retention: is chemokine/survival factor sensing sufficient or is there a requirement for constant TCR engagement?

With respect to chemokine Trm retention, in the genito-urinary tract, such MLCs clusters were found to create a “chemokine network” with tissue-resident macrophages producing CCL5 and CXCL9 to retain Trm [188]. More recent reports in the skin revealed the formation of similar clusters, with CD11b⁺ and CD8⁺ T-cells producing CCL5, in part required for CD4⁺ skin Trm retention [330]. However, neither report investigated whether TCR signaling was also occurring in the clusters, with blocking antibodies to CCL5 and/or CXCL9 reducing Trm numbers but not completely ablating them. In the context of hepatic Trm, knock-out studies have shown the importance of CXCR6 and CXCL16 interactions as being crucial for Trm retention [86]. However, what is the source of CXCL16 is still under debate, the vasculature liver endothelium (LSEC) has been shown to constitutively express CXCL16 [173], as well as Kupffer cells (Fig. 6.2). In fact, it has been shown to be upregulated in the presence of pro-inflammatory cytokines such as IFN γ and TNF α on vascular macrophage populations [331]. Although the majority of Trm are in close association with the endothelium, transient interactions with Kupffer cells might be involved to retain liver Trm. Furthermore, CXCL16 is a unique chemokine (similar only to CX3CL1), having a

transmembrane domain and a cleavable mucin-rich extracellular stalk [332]. In fact, CXCL16 (also known as scavenger receptor for phosphatidylserine and oxidized low-density lipoproteins, SR-PSOX), is both a scavenger receptor for oxidized low-density lipoprotein [333] and bacteria [334], as well as a chemokine. Cleavage of CXCL16 has been shown to require ADAM protease 17 and 10, both expressed by Kupffer cells [335, 336]. Interestingly, microarray data has shown ADAM19 to be upregulated in liver Trm when compared to spleen memory CD8⁺ T-cells in two independent studies; however, whether ADAM19 is also able to cleave CXCL16 has not been investigated (with little to no knowledge available regarding ADAM19 function and substrates) [83, 85]. Hence, the interactions between CXCR6⁺ Trm and CXCL16 are still unknown: does Trm retention require the cleaved form of CXCL16, creating an active migration gradient? Which cell produces the ADAM-proteases? Can CXCR6 bind to the cleaved and un-cleaved forms of the chemokine and with what affinity? Interestingly, CXCL16 was shown to induce a strong chemotactic response in activated CD8⁺ T-cells in *in vitro* migration assays, and was effective at inducing calcium mobilization inside these cells [337]. An alternative hypothesis, potentially complementary, would involve IL-15 and TGFβ as being crucial for Trm survival, as has been reported in skin, gut and lung Trm populations [170]. Indeed, high levels of TGFβ are present in hepatic tissue. Hepatic TGFβ expression is known to be a key regulator of liver development, steady-state physiology and hepatic-pathology (ie often found to be dysregulated during inflammation and fibrosis, cirrhosis and hepatocellular carcinoma) [338, 339]. Utilizing receptor knock-outs and/or inhibiting receptor function with mono-clonal antibodies might determine if IL-15 and TGFβ also play a role in liver Trm long-term survival.

In addition to local chemokine/cytokine signaling, TCR engagement by Trm, could also play a significant or partial role in Trm retention. Preliminary evidence from Chapter III would support the hypothesis that memory T-cells (either Tem or Tcm) are recruited and retained in tissues where antigen is present, with TCR engagement known to result in T-cell migration decline and tissue retention. Through the use of both confocal microscopy and flow-cytometry the data presented in this thesis has shown that Trm express CXCR6 at high frequencies; however, it is unknown what maintains CXCR6 expression in CD8⁺ T-cells in the liver. Possibly TCR-engagement might be crucial for Trm maintenance in the hepatic environment, with TCR signaling allowing for CXCR6 and CD69 upregulation as has been shown for CD8⁺ T-cells activation [337, 340, 341]. Further evidence also comes from studies that have shown that prolonged antigen presentation is required for optimal CD8⁺ T cell responses to clear infected hepatocytes, with decreased antigen resulting in lower numbers of liver-CD8⁺ T-cells memory formation [72]. Future experiments using anti-SIINFEKL-MHC blocking antibodies will determine if TCR engagement is required for Trm long-term retention.

On a last note, it is interesting to mention that irrespective of the type of target vaccination regimen used (including repeated viral vector intravenous administrations and increased PLGA-protein dosage), it was not possible to obtain higher numbers of Ag-specific CD8⁺ T-cells in the liver than approximately one million. Although further investigation is warranted, it is possible that a liver Trm niche might act as a limiting factor in determining the total number of Trm within the tissue. The properties of the hepatic Trm niche are yet to be investigated with key questions such as, is there a maximum number of Trm cells that the hepatic tissue can accommodate and if so, what

constrains it? How do secondary liver-infections alter the Trm population (ie: is the niche competitive? What are the “rules” for established Trm and new entry lymphocytes)?

These are some crucial questions that will be important for the development of improved liver-stage malaria vaccines.

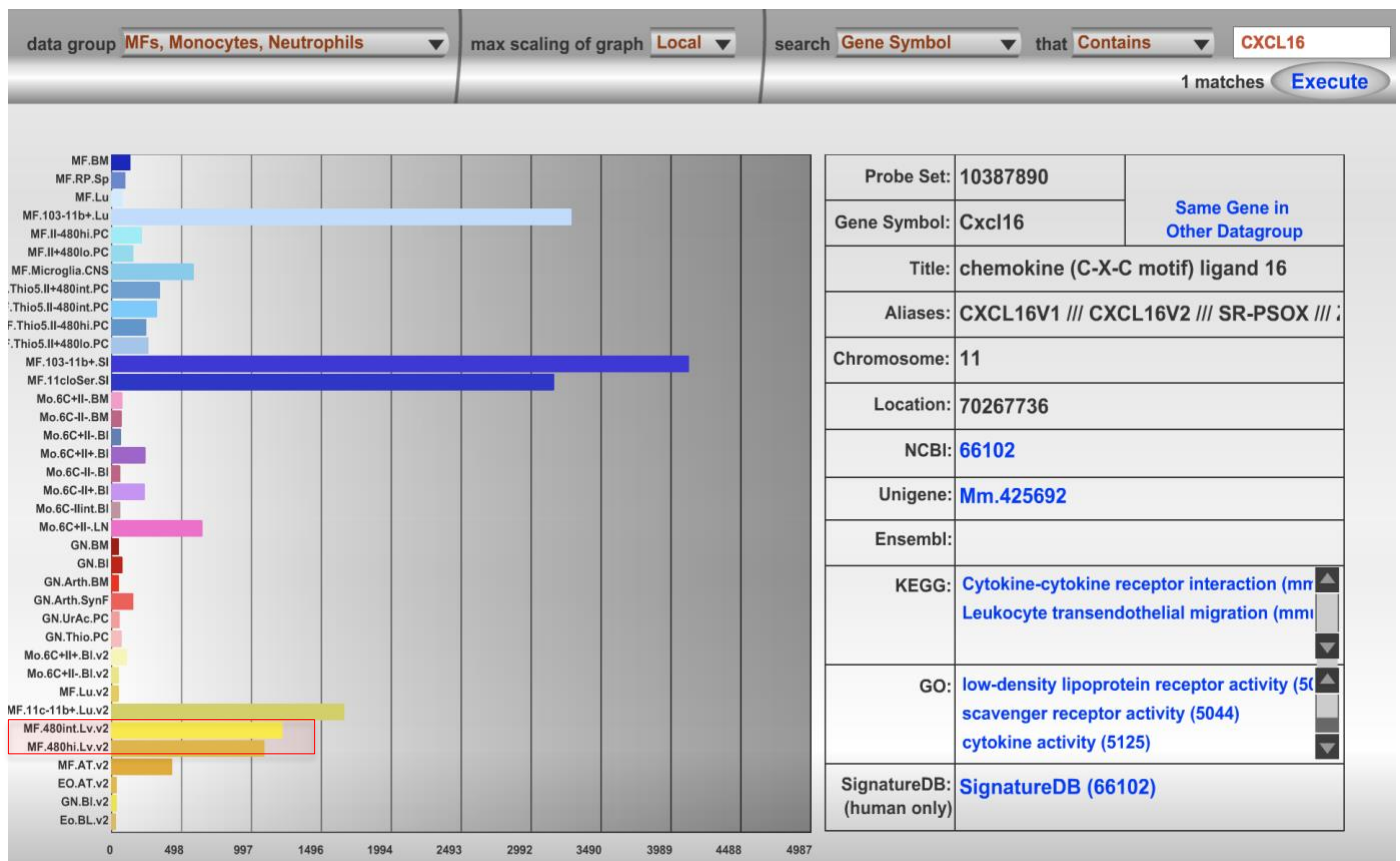


Fig. 6.2: CXCL16 expression in macrophage, monocyte and neutrophil populations. Highlighted in red are liver F4/80^{+/int} macrophages showing high expression levels of CXCL16 at steady state (second only to spleen and lung macrophages). (Figure taken from the public dataset, ImmGen)

VI.c –The use of prime and target vaccination outside the context of liver stage malaria

Most pathogenic encounters arise from specific routes of infection: with pathogen tropism and infectious life-cycle carefully selected to allow pathogen replication and dissemination. Local Trm cell induction at barrier sites results in an important immunological protective parameter for a multitude of infectious diseases, as discussed in Chapter I. Prime and target vaccination is able to induce high numbers of liver Trm, which was shown to be protective in the context of liver-stage malaria. As this is a subunit vaccine, the same strategy could be used for other diseases confined to specific anatomical locations by altering the antigen of choice. Specifically, applications in other infectious diseases such as viral hepatitis, influenza, pneumonic plague and solid tumors could be of particular interest.

In the context of hepatic infectious, CD8⁺ T-cells have been shown to be crucial at clearing both Hepatitis B and Hepatitis C infected cells [342-344]. As proof of principle, using viral vectors expressing Hepatitis B Surface Antigens (HBsAg) in a prime and target vaccination regimen, a ten-fold increase of Ag-specific CD8⁺ T-cells in the liver was observed (Fig. 4.12). Although highly speculative, it would be interesting to see if intravenous administration of VLP (formulated with a HBsAg core), might also be used in combination with viral vectors to induce high levels of Ag-specific CD8⁺ T-cells within the liver tissue. Though VLPs are significantly smaller than PLGA-Np used (with HBsAg VLPs averaging about 22.8nm in diameter [345]), ordered VLP aggregates might result in larger particles resembling PLGA-nanoparticles. In the possible positive outcome that VLPs result in functioning “targeting agents”, an intriguing vaccine regimen would be to combine a liver-stage malaria vaccine with a Hepatitis B vaccine.

This would involve an Adenoviral vector prime expressing the malaria antigen of choice (M_A) fused with HBsAg. Subsequently, an intravenous administration of a VLP-M_A would be administered, resulting in high numbers of CD8⁺ liver Trm against both antigens (with potentially interesting induction of high antibody titers). Aside from hepatic infections, Trm might result in protection in other localized viral infections. For example, CD8⁺ T-cells have been shown to release protective cytokines in the context of lung infections such as influenza and pneumonic plague [346-348]. In fact, a recent report has shown that CD8⁺ Trm in the upper respiratory tract and nasal epithelial tissue can block the transmission of influenza virus to the lung, preventing the development of severe pulmonary disease [349]. Recruitment of Teff CD8⁺ T-cells to lung tissue could be easily achievable with intranasal or aerosol administration of Adenovirus viral vectors post intramuscular prime, as shown by Lambe et al., [350].

Aside from infectious agents, prime and target vaccines could potentially be harnessed in the context of cancer immunotherapy. Indeed immunotherapies that boost the ability of the endogenous immune system to remove cancerous cells have involved a multitude of strategies such as antibodies against T-cell check-point proteins (namely anti-CTLA-4 and anti-PD-1 immunotherapies [351, 352]) and adoptive T-cell therapy [353]. Indeed, there is good evidence to suggest that cytotoxic CD8⁺ T-cells play a central role in cancer immunotherapy, in multiple diverse cancers such as melanoma, cancers of the bladder, lung, stomach, renal, head and neck and Hodgkin's lymphoma [316]. To target cancerous cells via CD8⁺ T-cell recognition of altered MHC-I molecules, antigens could come from two different proteins. One option is the presentation of non-mutated proteins to which central T-cell tolerance is incomplete (ie: due to restricted tissue

expression in adults or temporal restrictions such as proteins only found in developmental stages). Another option is CD8⁺ T-cell recognition of an abnormal protein, not encoded in the genome, such as a neo-antigen. Although these are promising candidates, especially if formed by viral oncogenes, a large fraction of the mutations in human cancers are patient-specific, requiring deep-sequences on a per-tumour basis to predict potential neoantigens [354]. A potential use for prime and target would be to develop personalized Ad.NeoAntigen viral vectors that activate CD8⁺ T-cells in healthy LN (away from the tumour tolerogenic microenvironment and/or tumours dLN which are at times invaded by malignant tissue) and target them to the cancerous tissue with local intra-tumoural injections, increasing antigen presence at the tumour site.

Antigen could be delivered either in the form of protein-adjuvant nanoparticles and/or viral vectors ideally trophic for cancerous cells. It would be particularly interesting not only to attempt to increase antigen load (hence peptide-MHC I presentation) on the cancer cells, but also to stimulate specifically the innate branch of the immune system (ie: APCs in tumour site) by utilizing *ad-hoc* adjuvant combinations. In an elegant paper by the Krummel lab, it was shown that only a few DCs are required to activate T-cells at tumour sites, with CD103⁺ DC cells being particularly potent, albeit found sparsely in proximity to the tumour [355]. They show that GM-CSF and FLT3L cytokines are specifically potent inducers of these antigen-presenting cell subset. Injections of PLGA.NeoAntigen protein particles in combination with PLGA.FLT3L/GM-CSF loaded particles might be an intriguing research possibility. The potential advantage of prime-target vaccination is the ability to formulate multiple adjuvant and antigen combinations,

generating a powerful experimental platform for targeting immune cells at specific localized sites and dissecting the induced immune response.

VI.d –Final Remarks

In this Thesis, I have tried to combine basic science knowledge to tackle a challenging translational problem, improving a liver-stage malaria vaccine strategy. With the birth of a new field in the context of memory T-cell subsets, tissue-resident T-cells have showed incredible capacity to mediate protection in many mouse infectious diseases. Harnessing our understanding from experimental models into translational medicine will be paramount for developing next-generation vaccines. It will be exciting to be at the forefront in the field, adopting prime and target vaccine regimens in human clinical trials against liver-stage malaria. I am very hopeful that by endeavoring in novel and robust bench to the bedside research with an emphasis on challenging known dogmas, an effective liver-stage malaria vaccine will be developed.

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