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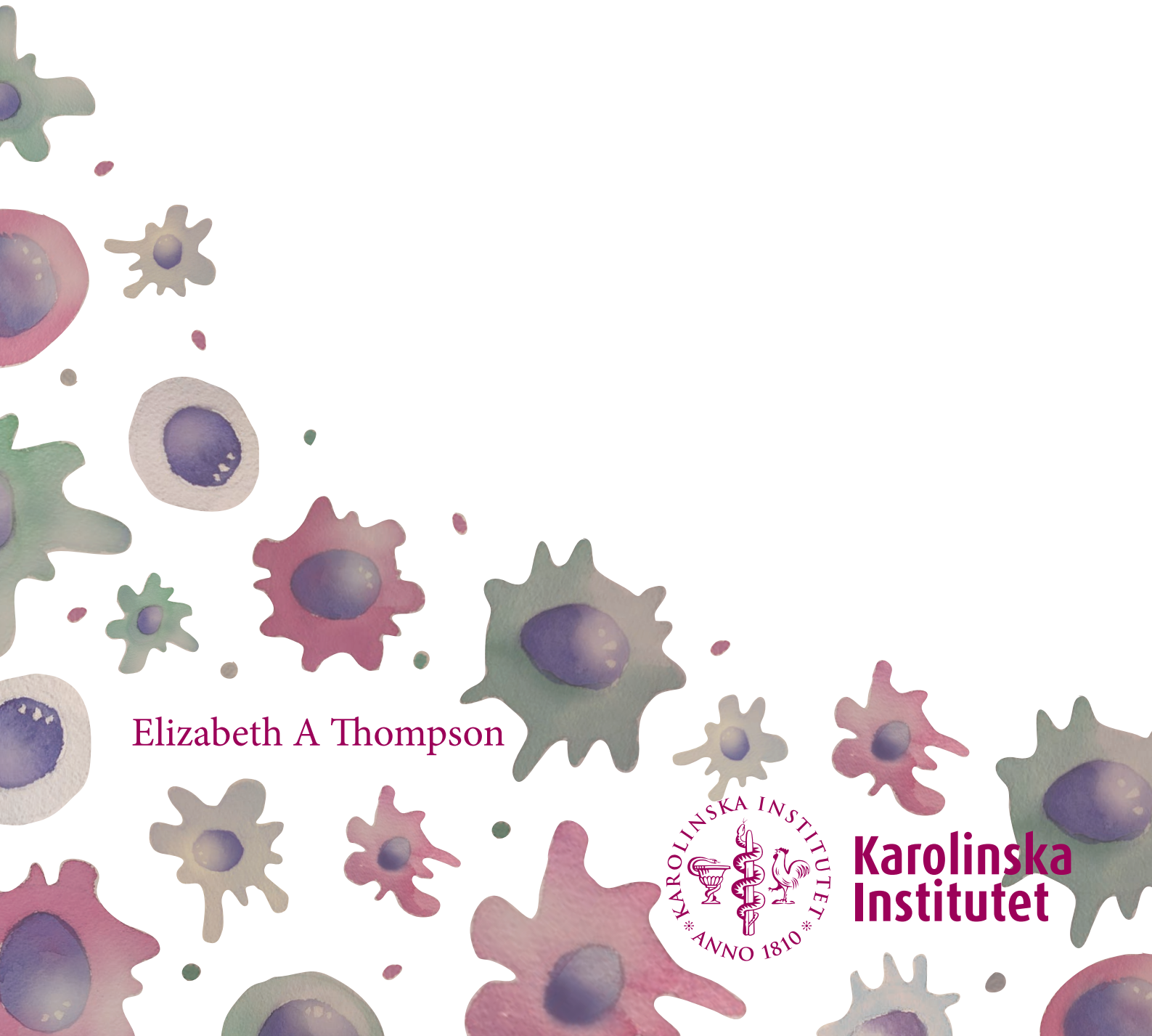
The Role of Adjuvant-Induced Innate Immune Activation in Shaping Vaccine Responses



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THE ROLE OF ADJUVANT-INDUCED INNATE IMMUNE ACTIVATION IN SHAPING VACCINE RESPONSES

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ABSTRACT

Adjuvants are components added to non-live vaccine formulations to enhance the effect of the vaccine by alerting the immune system to initiate a response against the vaccine. Powerful new adjuvants will be critical for the development of next generation vaccines to diseases such as tuberculosis, HIV-1/AIDS, malaria, and therapeutic cancer vaccines. My thesis work has focused on the responses induced by adjuvants targeting different immune-modulatory receptors in the innate immune system. The overall aim of the studies was to better understand the mechanisms by which adjuvants can alter innate immune activation and thereby influence the magnitude, polarization, and longevity of adaptive vaccine responses.

In **paper I**, I investigated an adjuvant combining the toll-like receptor (TLR)3-agonist, Poly IC:LC, and an agonistic monoclonal antibody targeting CD40 (anti-CD40Ab) for the potential to induce T cell responses. We found low T cell responses in the blood, but remarkable frequencies of vaccine-specific T cells restricted to the lung and bronchoalveolar lavage after vaccination. The majority of the vaccine-specific T cells in the lung expressed CD103, representing tissue-resident memory T cells (T_{RM}). However, we found that the anti-CD40Ab was widely disseminated after vaccination to all organs analyzed, and therefore lung-specific adjuvant activation alone could not explain the compartmentalized T_{RM}.

We consequently expanded the studies in **paper II** to compare the intravenous (IV) and subcutaneous (SQ) routes of administration. In contrast to IV, the CD40Ab stayed localized to the skin and the skin draining lymph nodes following SQ administration. While both groups induced equivalent vaccine-specific T cell homing to the lung, IV immunization induced a significantly higher proportion of CD103+ T_{RM}. IV immunization induced an innate profile skewed towards IL-10 production, which strongly correlated with the proportion of T_{RM}. By *in vitro* studies, we found that blood monocytes were the main producers of IL-10 and could mediate increased CD103 expression on T cells. IL-10 did not directly cause CD103 upregulation, but instead conditioned monocytes to release TGFβ which in turn induced the T_{RM} phenotype.

In **paper III**, I compared how adjuvants targeting either TLR4, TLR7/8, or TLR9 induced different innate immune responses to polarize the adaptive vaccine responses. In a large preclinical vaccine study, the TLR-adjuvants were added to polymer-based nanoparticles encapsulating the malaria transmission-blocking vaccine antigen Pfs25, to identify correlates of immunity leading to robust, long-lived, functional Ab titers. All groups induced high Ab titers and transmission reducing activity in mosquitoes at peak responses. However, the adjuvants targeting TLR7/8 or TLR9 induced higher levels of IFNα production and type I IFN associated gene signatures than the adjuvant targeting TLR4. The IFNα signature showed strong correlations with the increased Ab half-life observed in these groups. All adjuvants generated Pfs25-specific CD4 T cell responses when combined with the nanoparticle encapsulated antigen, which correlated with increased IgG Ab avidity.

In conclusion, the thesis provides increased understanding of the mechanisms by which adjuvants potentiate and regulate vaccine responses and will hopefully aid in refining future vaccine formulations.

LIST OF SCIENTIFIC PAPERS

I. **Elizabeth A Thompson**, Frank Liang, Gustaf Lindgren, Kerrie J Sandren, Kylie M Quinn, Patricia A Darrah, Richard A Koup, Robert A Seder, Ross M Kedl, Karin Loré

Human Anti-CD40 and Poly IC:LC Adjuvant Combination Induces Potent T Cell Responses in the Lung of Non-Human Primates

Journal of Immunology. 2015 Dec 30;496(2):371-81

II. **Elizabeth A Thompson**, Patricia A Darrah, Kathryn Foulds, Elena Hoffer, Sophie Norenstedt, Leif Perbeck, Ross M Kedl, Robert A Seder, Karin Loré

Monocytes Acquire the Ability to Prime Tissue-Resident T Cells via IL-10-Mediated TGFβ Release

Manuscript

III. **Elizabeth A Thompson**, Sebastian Ols, Kazutoyo Miura, Kelly Rausch, David L Narum, Mats Spångberg, Michal Juraska, Ulrike Wille-Reece, Amy Weiner, Randall F Howard, Carole A Long, Patrick E Duffy, Lloyd Johnston, Conlin P O'Neil, Karin Loré

TLR-Adjuvanted Nanoparticle Vaccines Differentially Influence the Quality and Longevity of Responses to Malaria Antigen Pfs25

JCI Insight. 2018 May 17;3(10)

PUBLICATIONS NOT INCLUDED IN THESIS

I. Lin A, Liang F, **Thompson EA**, Vono M, Ols S, Lindgren G, Hassett K, Salter H, Ciaramella G, Loré K. Rhesus Macaque Myeloid-Derived Suppressor Cells Demonstrate T Cell Inhibitory Functions and Are Transiently Increased after Vaccination. *Journal of Immunology*. 2018 Jan 1;200(1):286-294.

II. Liang F, Lindgren G, Lin A, **Thompson EA**, Ols S, Röhss J, John S, Hassett K, Yuzhakov O, Bahl K, Brito LA, Salter H, Ciaramella G, Loré K. Efficient Targeting and Activation of Antigen-Presenting Cells In Vivo after Modified mRNA Vaccine Administration in Rhesus Macaques. *Molecular Therapy*. 2017 Dec 6;25(12):2635-2647.

III. **Thompson EA**, Loré K. Non-human primates as a model for understanding the mechanism of action of toll-like receptor-based vaccine adjuvants. *Current Opinion in Immunology*. 2017 Aug;47:1-7.

IV. Liang F, Lindgren G, Sandgren KJ, **Thompson EA**, Francica JR, Seubert A, De Gregorio E, Barnett S, O'Hagan DT, Sullivan NJ, Koup RA, Seder RA, Loré K. Vaccine priming is restricted to draining lymph nodes and controlled by adjuvant-mediated antigen uptake. *Science Translational Medicine*. 2017 Jun 7;9(393)

V. Lindgren G, Ols S, Liang F, **Thompson EA**, Lin A, Hellgren F, Bahl K, John S, Yuzhakov O, Hassett KJ, Brito LA, Salter H, Ciaramella G, Loré K. Induction of Robust B Cell Responses after Influenza mRNA Vaccination Is Accompanied by Circulating Hemagglutinin-Specific ICOS+ PD-1+ CXCR3+ T Follicular Helper Cells. *Frontiers in Immunology*. 2017;8:1539.

VI. Salvador A, Sandgren KJ, Liang F, **Thompson EA**, Koup RA, Pedraz JL, Hernandez RM, Loré K, Igartua M. Design and evaluation of surface and adjuvant modified PLGA microspheres for uptake by dendritic cells to improve vaccine responses. *International Journal of Pharmaceutics*. 2015 Dec 30;496(2):371-81.

VII. Calantone N, Wu F, Klase Z, Deleage C, Perkins M, Matsuda K, **Thompson EA**, Ortiz AM, Vinton CL, Ourmanov I, Loré K, Douek DC, Estes JD, Hirsch VM, Brenchley JM. Tissue myeloid cells in SIV-infected primates acquire viral DNA through phagocytosis of infected T cells. *Immunity*. 2014 Sep 18;41(3):493-502.

CONTENTS

LIST OF ABBREVIATIONS

Ab	Antibody
Ad	Adenovirus
AIDS	Acquired immune deficiency syndrome
APCs	Antigen presenting cell
BCG	Bacillus Calmette-Guérin
BCR	B cell receptor
bNAbs	Broadly neutralizing antibodies
CAR	Chimeric antigen receptor
CD40L	CD40 ligand
cDC	Conventional dendritic cell
CMV	Cytomegalovirus
CSP	Circumsporozoite protein
DC	Dendritic cell
dDC	Dermal dendritic cells
EBV	Epstein-Barr virus
Env	Envelope
FcR	Fc receptor
FDC	Follicular dendritic cell
GLA	Glucopyranosyl lipid adjuvant
HBV	Hepatitis B virus
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HSV	Herpes simplex virus
IM	Intramuscular
IV	Intravenous
LCMV	Lymphocytic choriomeningitis virus
LLPC	Long-lived plasma cell
LPS	Lipopolysaccaride
mAb	Monoclonal antibody
MBC	Memory B cell
MDC	Myeloid dendritic cell

MHC	Major histocompatibility complex
MNPs	Mononuclear phagocytes
MPL	Monophosphoryl lipid A
NHP	Non-human primate
PAMPs	Pathogen associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PDC	Plasmacytoid dendritic cell
PRRs	Pattern recognition receptors
RSV	Respiratory syncytial virus
S1PR	Sphingosine-1-phosphate receptor
SHM	Somatic hypermutation
SQ	Subcutaneous
TB	Tuberculosis
TBV	Transmission blocking vaccine
T_{CM}	T central memory
TCR	T cell receptor
T_{EM}	T effector memory
T_{FH}	T follicular helper
T_H	T helper
TLR	Toll-like receptor
T_{PM}	T peripheral memory
T_{RM}	T resident memory
VLP	Virus like particle
VZV	Varicella zoster virus

1 INTRODUCTION

Few medical interventions have affected so many lives as vaccination. Vaccines have the ability to train the immune system to fight off external pathogens or even target infected or cancerous cells within the body. The immune responses generated from vaccination have brought several life-threatening infectious diseases under control, such as small pox, measles, mumps, etc. Previous vaccination efforts have been established largely by empirical methods, however infectious diseases that have so far eluded standard vaccination strategies offer significant challenges that require a more targeted and informed approach to vaccine design. Most currently available vaccines work by eliciting potent antibody (Ab) responses against a given pathogen, but current attempts indicate that to develop vaccines against diseases such as HIV-1, malaria, or therapeutic cancer vaccines, Ab responses alone may not be sufficient to mount a protective response. Instead, these diseases will likely need both humoral and cellular responses (Plotkin 2010). As of yet, non-live vaccines have not been able to induce strong cellular T cell-based responses in humans, or the broad Ab responses that will likely be necessary for rapidly diversifying infections such as HIV-1. Therefore, a better understanding of how vaccine responses are induced and maintained is critical for successful next generation vaccines.

As the overall goal of vaccination is to provide long-term immunological memory and protection, it is important to first have a deep understanding of the immune system tasked with providing this protection. The immune system is broadly divided into two arms, the innate and the adaptive. The innate arm provides rapid and so-called non-specific clearance or activation. These effects are mediated by a multitude of cells types, including phagocytic cells, eosinophils, basophils, mast cells, and NK cells. These cells jointly provide pathogen clearance through phagocytosis or cytolytic activity, activation of the complement system, and activating or educating the adaptive immune response. In contrast, the adaptive immune system has developed a highly diverse and specific repertoire, capable of recognizing specific foreign threats and keeping a history of encountered pathogens to provide a more rapid secondary response. The adaptive immune response utilizes two main components, the cellular and humoral, mediated by T cells and B cells, respectively.

In this thesis, I have aimed to enhance our collective knowledge of the immunology underlying the initiation of a vaccine elicited response. I will therefore provide a detailed introduction to the immune system, with a particular focus on the cell types evaluated throughout my work, namely, antigen presenting cells (APCs), T cells, and B cells. I will then describe some of the major obstacles currently facing the field of vaccinology, and discuss how different aspects of the vaccine, such as the choice of adjuvant, vaccine formulation, and route of delivery can all be used in combination to overcome these obstacles. A better understanding of the broad immunological response to these components of the vaccine can help tailor future efforts in vaccine design.

2 AIMS OF THESIS

The overall aim of this thesis was to better understand the mechanisms by which immune stimulatory adjuvants can alter the innate immune environment and thereby influence the ensuing adaptive responses following vaccination. We addressed this question by evaluating both innate immune activation and development of adaptive memory following immunization with several adjuvants that can specifically target the innate immune system. The specific aims were as follows:

Paper I: To study the requirements for induction of antigen-specific T cell responses with an adjuvant combining CD40 and toll-like receptor (TLR)3 targeting.

Paper II: To determine how the innate cytokine profile contributes to the priming of tissue-resident memory T cells (T_{RM}) with the CD40/TLR3 adjuvant.

Paper III: To compare how different TLR-ligand based adjuvants influence innate immune activation leading to differential long-lived Ab titers and T cell help.

3 IMMUNOLOGY

3.1 INNATE IMMUNE RESPONSES

When reacting to a natural infection, the innate immune system has the unique ability to respond rapidly and non-specifically by recognizing certain conserved patterns on pathogens known as pathogen associated molecular patterns (PAMPs). The innate immune system can be divided into several different cell types, which each possess distinct combinations of pattern recognition receptors (PRRs), making them especially equipped to recognize and respond to a variety of pathogens (O'Neill *et al.* 2013). Depending on which receptors are engaged, the cells of the innate immune system will respond accordingly in order to educate the ensuing adaptive responses. Vaccines can be improved by taking advantage of this early education, based on a better understanding of the underlying processes of early antigen recognition and cellular activation.

3.1.1 Monocyte and dendritic cell subsets

The cells in the innate immune system are a heterogeneous population that are present in circulation and resident in both lymphoid and non-lymphoid tissues. In my thesis, I focused primarily on APCs for their ability to elicit and instruct an adaptive response. The mononuclear phagocyte (MNP) system contains subsets of professional APCs and can be divided into monocytes, macrophages, and dendritic cells (DCs). The specific subsets have overlapping, but distinct functions, which will be discussed below. Although much research has focused on mouse MNPs in different compartments, less is known about the human counterparts. Despite the difficulties of studying human MNP biology, recent efforts have focused on finding homologies between mouse and human in terms of surface expression and immunological function to further understanding, and has led to a unified classification system for MNP subsets (Figure 1) (Guilliams *et al.* 2016; Haniffa *et al.* 2015).

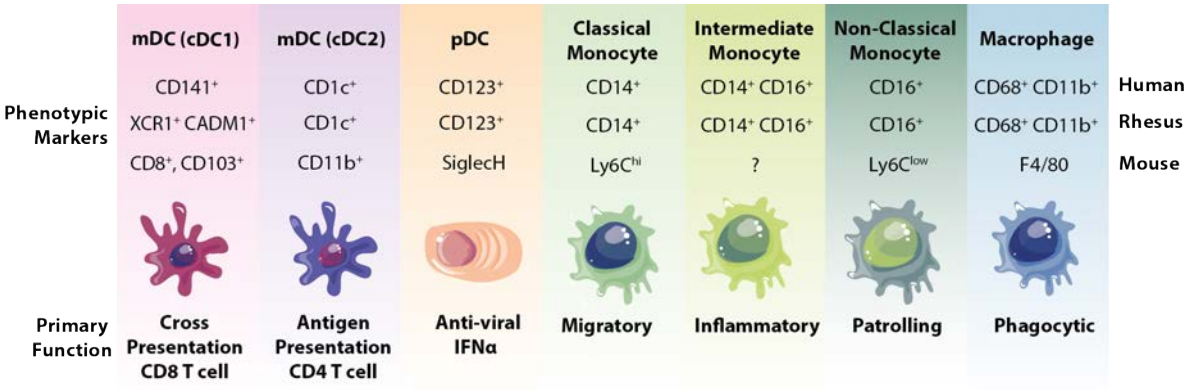

Figure 1: MNP subsets and function in human, rhesus macaque, and mouse.

Of the three main arms of the mononuclear phagocyte system, DCs are particularly significant because of their ability to initiate and modulate naïve T cell responses. They help efficiently eliminate pathogens and are often considered the bridge between the innate and adaptive arms of the immune system. These properties make DCs a prime target for vaccination efforts. DCs were first described as Langerhans cells (LCs) in the skin in 1868. However, in 1973 they were renamed and characterized by Ralph Steinman (Steinman & Cohn 1973, 1974). They were ultimately identified as the innate cells that are best at educating the

adaptive immune response (Steinman & Witmer 1978; Nussenzweig *et al.* 1980). DCs have now been extensively characterized, revealing several distinct cell populations. DCs are characterized as myeloid DCs (MDCs) or plasmacytoid DCs (PDCs). Myeloid DCs express CD11c and are also known as classic or conventional DCs (cDCs). Two main subsets exist, cDC1, which express CD141 and CADM1 in humans or CD8/CD103 in mice, and cDC2, which express CD1c in humans or CD11b in mice. While cDC1 are primarily associated with cross presentation of antigen to CD8 T cells, this distinction may be more pronounced in the mouse compared to human immune system (Segura *et al.* 2013). cDC2 are classic professional APCs and are especially equipped to present exogenous antigen via major histocompatibility complex (MHC)-II to CD4 T cells. PDCs on the other hand, are not as efficient at antigen presentation, but instead are highly effective in recognizing viral infections and in response produce high levels of type I IFNs, which can inhibit viral spread and help polarize T helper (T_H)1 responses.

Monocytes have classically been thought of as highly plastic cells that could differentiate into monocyte derived DCs or macrophages when cultured in specific cytokine milieu (Becker *et al.* 1987; Chomarat *et al.* 2000; Zhou & Tedder 1996) or upon entry into the tissue (Furth *et al.* 1973; Randolph *et al.* 1999). Therefore, tissue resident macrophage homeostasis was long considered dependent on continual recruitment and differentiation of monocytes. However, recent studies evaluating the ontogeny of MNP subsets have refined this view. Instead, it has become clear in mice that tissue macrophages are actually derived during early development and persist throughout adulthood, with a limited need for replacement by circulating monocytes. This concept was first demonstrated with microglia, the macrophages resident in the central nervous system. Microglial cells were found to in fact have a distinct ontogeny from circulating monocytes and originated from yolk-sac derived primitive myeloid progenitors (Ginhoux *et al.* 2010). This work has since been expanded to evaluate resident macrophages in multiple tissues to confirm the findings, showing that macrophages seed developing fetal tissues (Epelman *et al.* 2014; Hashimoto *et al.* 2013; Tamoutounour *et al.* 2013). In contrast, circulating monocytes are continually generated in the bone marrow from hematopoietic stem cells. Within different tissue microenvironments, macrophages have different phenotypes and functions specific for the environmental niche, but overall, they are particularly potent at phagocytosis.

Although macrophages have now been shown to have a distinct origin from monocytes, monocytes are in fact still a highly plastic population that have the capacity to differentiate into macrophages or DCs in the tissue when needed, in situations such as inflammation or infection. Even within circulation, monocytes are highly plastic, responding to danger signals and inflammation. In circulation, monocytes fall into different sub-populations, $Ly6C^{hi}$ and $Ly6C^{low}$ in mice, or classical (CD14+), non-classical (CD16+), and intermediate (CD14+CD16+) monocytes in humans. When using flow plots of CD14 vs CD16, human monocytes are often described as taking on a “waterfall” like shape, indicating a continuous differentiation between the subsets (Haniffa *et al.* 2015; Patel *et al.* 2017; Sugimoto *et al.* 2015). Upon inflammation, classical monocytes can readily differentiate, becoming intermediate CD14+CD16+ cells. To date, it is unclear whether there is a functional homolog to intermediate monocytes in mice. However, classical monocytes in both humans and mice readily migrate to sites of inflammation in tissue and can differentiate into peripheral mononuclear phagocytes. Interestingly, upon entering the tissue, monocyte derived

macrophages and DCs will take on many of the functional characteristics of resident populations. In particular, they often upregulate molecules associated with antigen uptake and presentation, a requirement for a successful immune response following infection and inflammation (Tamoutounour *et al.* 2013). Ly6C^{low} or CD16+ non-classical monocytes preferentially patrol the endothelium and are less likely to enter tissue and differentiate in response to inflammation (Cros *et al.* 2010). Recent reports have even highlighted the possibility of tissue monocytes, which traffic through lymphoid and non-lymphoid tissues at steady state without significant changes in their gene expression profiles (Jakubzick *et al.* 2013). These cells were implicated in antigen trafficking from tissue to lymph nodes during steady state, and therefore may be important for initiation of T cell responses.

3.1.2 Maturation and presentation

APCs are primed to be sentinels of the immune system, due to their distribution throughout the body, particularly at interfaces between the body and the environment. They are found in the blood, but are especially prevalent in airway epithelium, skin, and mucosal surfaces (Figure 2).

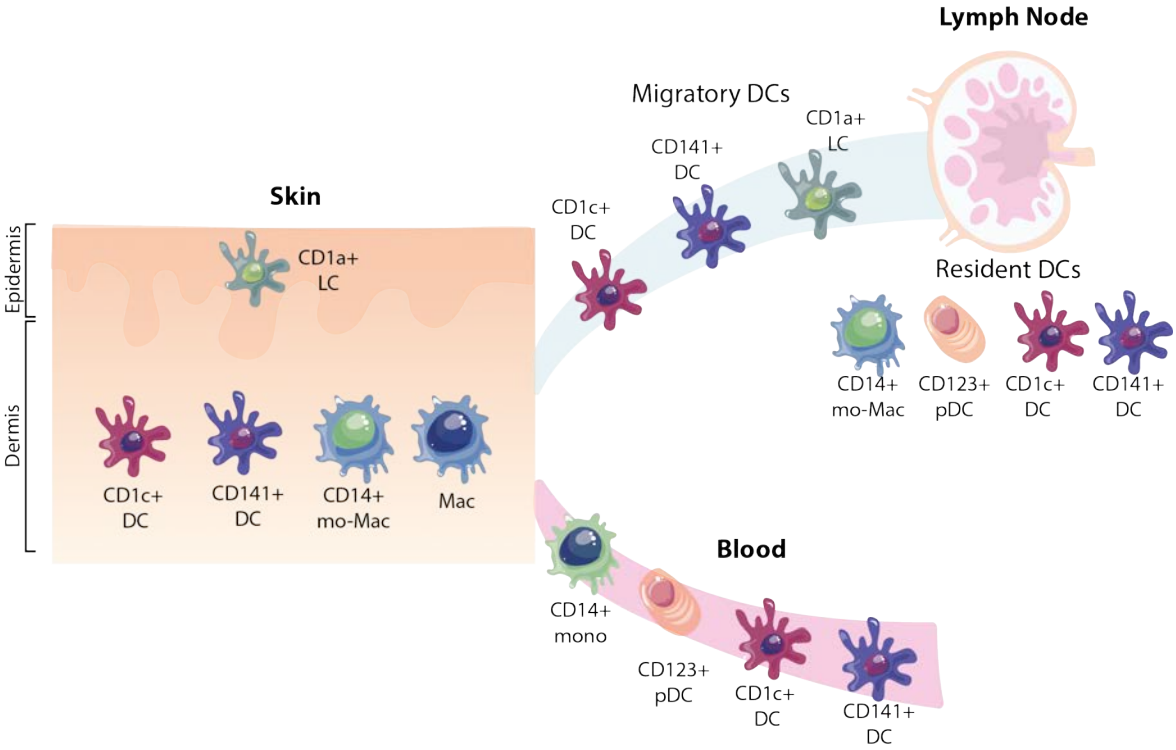

Figure 2: APC subset distribution in skin, blood, and lymph node.

In these vulnerable locations, the immature form of DCs can patrol for invading pathogens and capture antigens through several mechanisms. Like other cells of the innate immune system they are capable of phagocytosing microbes and particles. Additionally, they can sample extracellular fluid in a process called micropinocytosis (Sallusto *et al.* 1995) or utilize receptor mediated endocytosis through a variety of receptors such as C-type lectin receptors, DEC-205 (Jiang *et al.* 1995) or Fc receptors (FcR). These processes are highly efficient in DCs and therefore much lower concentrations of antigen are required for antigen presentation than compared to other APCs (Sallusto *et al.* 1995).

As a result of antigen uptake and external signals from PRR engagement, the DCs will undergo a process called maturation. During this process, the DC downregulates its capacity to acquire antigen, and instead shifts towards a phenotype better suited to initiate an adaptive immune response. Unlike in macrophages, where antigen is shuttled largely to lysosomes to be completely degraded, DCs traffic proteins to endosomal structures, which contain high levels of MHC-II. In these structures, DCs can degrade antigen protein into peptides and then assemble antigen-MHC-II complexes. The cell then re-distributes its MHC-II molecules from endosomes/lysosomes to the surface of the cell. Signals from TLR engagement, TNF-receptor family engagement (such as CD40) or cytokines from other innate cells further mature the DCs. DCs then upregulate co-stimulatory molecules, such as CD80/86 and CD70, critical for antigen presentation to T cells.

Upon maturation, DCs alter their chemokine receptor expression and migrate out of the tissue to lymphoid tissues, where they can interact with T cells and B cells. Under steady state conditions, DCs migrate from the periphery to lymph nodes for immune surveillance and to maintain peripheral T cell tolerance (Scheinecker *et al.* 2002). However, under inflamed conditions, DCs will downregulate chemokine receptors such as CCR1 and CCR5, while upregulating CCR7 to facilitate enhanced DC migration to the lymph node (Johnson & Jackson 2014). During homeostasis, low levels of the CCR7 ligand, CCL21, are expressed, but inflammation leads to new production and secretion of CCL21, allowing for DC adhesion and transmigration (Sallusto *et al.* 1998). This enhanced migration results in tissue DCs becoming the major subset in the lymph node (Jakubzick *et al.* 2008). Upon entry to the lymph node, tissue DCs carrying antigen from the periphery are now poised to find their cognate T cell and initiate an immune response.

Within the lymph node, professional APCs can present antigen to CD4 T cells via MHC-II, or by a process called cross presentation, present antigen to CD8 T cells via MHC-I. MHC-II is only expressed on professional APCs and presents antigens derived from the extracellular domain. In contrast, MHC-I is expressed on all nucleated cells and presents endogenous antigen to T cells. This provides a mechanism for CD8 T cells to recognize non-self antigen expressed on MHC-I and kill infected cells. However, cross presentation exists as a means to prime naïve CD8 T cells to pathogens that do not infect APCs. Cross presentation occurs through two primary pathways, the cytosolic and the vacuolar (Joffre *et al.* 2012). Through the cytosolic pathway, antigen is taken up extracellularly and escapes from the endosome into the cytosol where it is degraded by the proteasome (Kovacsovics-Bankowski & Rock 1995). In the vacuolar pathway, proteins are degraded in the phagosome and loaded onto MHC-I. Cross presentation is particularly important for protein-based vaccine development, where exogenous protein needs to be presented via MHC-I to prime a CD8 T cell response. Although this pathway seems to be largely restricted cDC1s in mice *in vivo* (Joffre *et al.* 2012), it is unclear if this distinction is as definitive in humans (Albert *et al.* 1998b, 1998a; Segura *et al.* 2013; Segura & Amigorena 2015; Tang-Huau *et al.* 2018). There is evidence that cDC1s are superior in humans, but information is limited and primarily derived from blood cDC1s (Bachem *et al.* 2010; Crozat *et al.* 2010; Jongbloed *et al.* 2010; Mittag *et al.* 2011). Further these studies showed that the cross-presentation capacity was increased following TLR stimulation. However, multiple DC subsets sorted from human tonsil showed similar capabilities of cross presentation at baseline (Segura *et al.* 2013). Therefore, it seems that the

pathways for cross presentation are conserved between species, but not dependent on a specific DC subset in humans.

3.2 T CELL RESPONSES

3.2.1 Generation of T cell responses

A strong understanding of the role of DCs in polarizing T_H cells can be used to enhance and adapt the immune response as necessary. Naïve T cells derived from precursors in the thymus move to the periphery where they can interact with DCs to become activated. DC priming of T cells in the lymph nodes has a large impact on the ultimate fate of the T cell, which is principally determined by three signals (Figure 3).

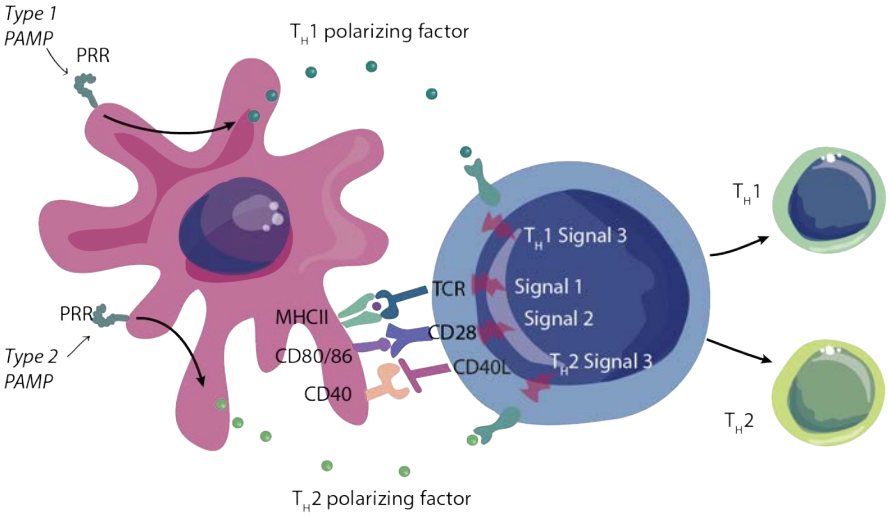

Figure 3: T cell activation requires three DC-derived signals.

The first signal is the initial contact between the DC and T cell, where the T cell receptor (TCR) binds to the peptide:MHC-II complex on the DC to determine the antigen specificity. A complementary TCR and antigen complex is not sufficient to stimulate a naïve immune response however. The second signal ensures that there is a threat associated with the antigen and arises from the binding of costimulatory molecules. As DC maturation leads to upregulation of costimulatory molecules such as CD80/86 and CD70, there is an increased likelihood of binding to their receptors, CD28 and CD27 respectively. With sufficient stimulation through signal 2, the T cell will become an effector cell, or will otherwise become anergic. Finally, signal 3 determines the polarization of the T cell to a T_H1 or T_H2 cell. The conditions under which a DC is primed will lead the DC to secrete certain factors that can influence the T cell polarization (Kapsenberg 2003). An intracellular infection requiring cellular immunity will condition DCs to make more T_H1 driving factors such as IL-12 (Macatonia *et al.* 1995; Trinchieri 2003), IL-23, IL-27, type I IFNs (Kadowaki *et al.* 2000; Wenner *et al.* 1996), and ICAM1. Instead, an extracellular pathogen, which would be more efficiently cleared by Ab responses, will stimulate DCs to produce more T_H2 factors such as the cytokines IL-4, IL-5, IL-9 or co-stimulatory factors such as MCP1 or Ox40L. Additionally, DCs that produce cytokines such as IL-10 and TGFβ have a regulatory effect and can induce T cells to take on a regulatory T cell phenotype. These factors are produced at low levels after DC maturation and will then increase with signal 2 engagement. However, these classifications are constantly evolving, and something that was evaluated throughout my thesis.

An additional pathway critical in the activation of naïve T cells is the CD40/CD40-ligand (CD40L) pathway. CD40 is broadly expressed on APCs, including DCs, B cells, and monocytes, as well as non-immune cells such as platelets and epithelial cells. CD40L is primarily expressed on T_H cells. During the DC-T cell interaction it is important the DC has strong cross talk with the T cell. T cells will upregulate CD40L after DC derived signals 1 and 2 (Grewal & Flavell 1998; Krug *et al.* 2001; Schulz *et al.* 2000) and bind to CD40 expressed on DCs to maintain contact during interaction. Further, CD40 ligation allows the T_H cells to license the DCs and leads to increased costimulatory and MHC molecule expression (van Kooten & Banchereau 2000) as well as proinflammatory cytokine production, such as IL-12 (Diehl *et al.* 2000). This DC licensing is critical for the generation of effective CD8 T cell responses and can lead to tolerance in the absence of CD40 (Buhlmann *et al.* 1995). In addition to DCs, B cells will also increase their antigen presentation capacity and proliferate in response to CD40 stimulation. However, the response to CD40 stimulation varies depending on the cell expressing CD40 and the microenvironment (van Kooten & Banchereau 2000).

3.2.2 Re-activation of memory T cells

Once activated, T cells can take on a variety of functions. Some will travel through the peripheral blood and continue to circulate, while other subsets will move to different tissue compartments, and even become resident there. The T cell's function is largely determined by imprinting from DCs during their initial education. A major goal of vaccination is to elicit memory T cells without an actual threat, to protect the host during future infections. Memory T cells can be recalled later during infection and have classically been categorized into two main subsets, effector memory (T_{EM}) and central memory (T_{CM}) (Sallusto *et al.* 1999). These cells can be distinguished based on their expression of the markers for lymph node homing and function, primarily CCR7 or CD62L and CD45RA or CD45RO (Figure 4A). T_{EM} are more cytotoxic and primarily located in blood, spleen, and non-lymphoid tissue. These cells are double negative for CD45RA and CCR7, with the lack of CCR7 expression explaining their inability to migrate to lymphoid tissue. Alternatively, long-lived T_{CM} are more proliferative and are CD45RA-/CCR7+. Through expression of lymph node homing markers such as CCR7, T_{CM} survey lymphoid tissue for their cognate antigen (Figure 4A) (Sallusto *et al.* 2004).

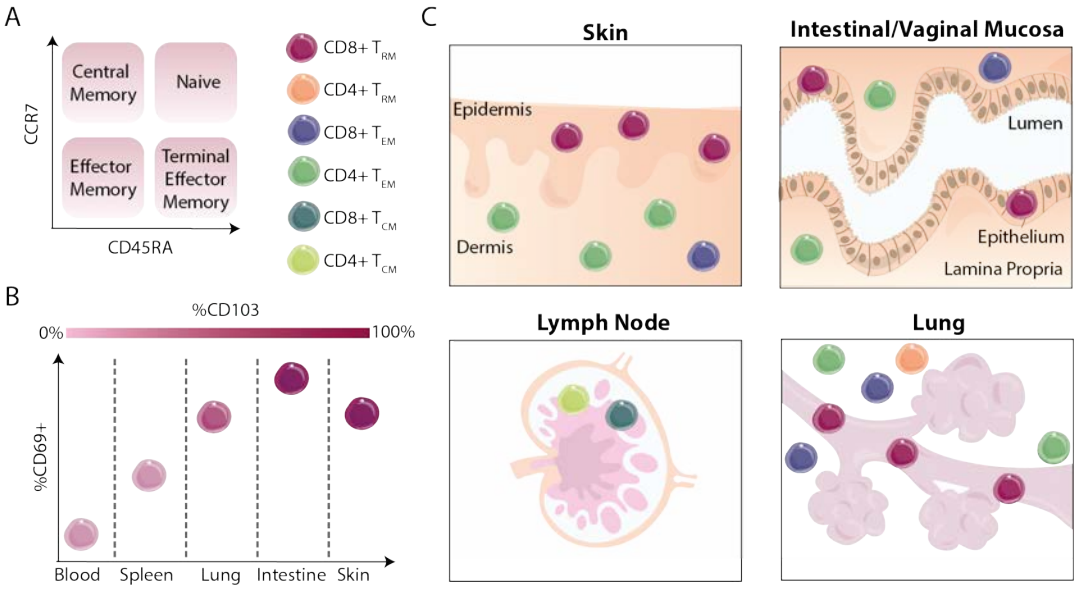

Figure 4: Memory T cell subsets in different anatomical compartments and phenotypic characterization.

However, recent studies evaluating the migration patterns, function, and development of memory T cells have challenged this dichotomous T cell lineage and revealed new subsets. It now seems that T cell subsets are more complex than cytotoxic T_{EM} in peripheral tissues and proliferative T_{CM} in lymphoid tissues. For example, cytotoxic T_{EM} are not typically found in the lymph node, however cells within the lymph node would need to provide cytotoxic effector function in case of infection by bacteria or virus within the lymphoid organ (Kastenmüller *et al.* 2012) and T cells that take up residence in the tissue have proliferative capacity for self-renewal unlike classical T_{EM} (Beura *et al.* 2018; Park *et al.* 2018). To clarify these distinctions, CX3CR1 can be used as a phenotypic marker that differentiates between memory cells with a cytotoxic profile (CX3CR1+) versus proliferative (CX3CR1-) (Böttcher *et al.* 2015; Gerlach *et al.* 2016). Additionally, CX3CR1 can distinguish three distinct subsets of memory T cells with high, intermediate, or low expression (Gerlach *et al.* 2016). Surprisingly, the $CX3CR1^{int}$ cells were the main subset in non-lymphoid tissues and the classical T_{EM} ($CX3CR1^{high}$) were found exclusively in the blood and spleen (Gerlach *et al.* 2016). This finding has dramatically altered our understanding of T cell migration patterns and development, and has even suggested the $CX3CR1^{int}$ cells be termed peripheral memory (T_{PM}). However, these findings need to be evaluated further in humans. Another revelation that argued against the role of T_{EM} in peripheral tissues was the discovery of tissue-resident memory T cells (T_{RM}). T_{RM} are memory T cells resident in a multitude of tissues to provide a front line of defense and do not recirculate. Recent quantitative data from mice shows that following infection the majority of memory T cells are actually tissue-resient and not recirculating memory T cells as previously believed (Steinert *et al.* 2015). T_{RM} can be distinguished by their expression of CD69 and/or CD103 (Figure 4B) and are the predominant subset in lungs, intestines, vaginal mucosa and skin (Figure 4C) but are also found in liver, brain and lymphoid tissues (Schenkel & Masopust 2014; Mueller *et al.* 2013). It seems that they have an even broader distribution in humans than mice (Thome *et al.* 2014), indicating a key role in protection and maintenance of immunological memory.

3.2.3 Tissue-resident memory T cells

Considering the high prevalence of T_{RM}, it is surprising that they were not discovered and characterized until 2008-2010. During this time, two groups demonstrated that T cells found in the skin and dorsal root-ganglia after herpes simplex virus (HSV) infections, or in the small intestine after lymphocytic choriomeningitis virus (LCMV) were in fact a new subset of tissue-resident T cells (Gebhardt *et al.* 2009; Masopust *et al.* 2010; Wakim *et al.* 2008). The reason T_{RM} went undetected for so long may be largely due to technical obstacles. Until recent technological advances, there were difficulties to differentiate circulating T_{EM} from bona fide resident T_{RM}. It has since been shown that many of the T_{EM} characterized in tissue were actually contaminating from the blood vasculature found in tissues (Anderson *et al.* 2012). Therefore, intravascular staining techniques were developed to specifically label cells found within the vasculature at time of tissue collection (Anderson *et al.* 2012). It has also been shown that standard methods for cell isolation from whole tissues result in a poor cell yield and are biased towards certain cell subsets (Steinert *et al.* 2015). When compared to quantitative microscopy, T_{RM} have been vastly underestimated using standard methods such as flow cytometry, and in fact far outnumber recirculating cells in the tissue (Steinert *et al.* 2015). These new methods, which include intravascular staining, tissue grafts, and parabiosis experiments, have now identified T_{RM} in a wide range of tissues (Mueller & Mackay 2016).

Aside from their wide distribution, T_{RM} have now been shown to be integral to secondary responses against infections. For example, they have been demonstrated to be critical in providing heterosubtypic immunity against influenza, protective T cell responses against respiratory syncytial virus (RSV), improved prognosis in cancer immunotherapy, and protection against several infections of the skin, most notably HSV (Muruganandah *et al.* 2018).

After identifying the phenotype and distribution of T_{RM}, much effort has focused on understanding their development. T_{RM} share a common clonality with T_{CM}, and appear to develop from a common precursor after entry into the tissue (Gaide *et al.* 2015; Mackay *et al.* 2013). Environmental signals such as cytokines or local antigen persistence have been shown to drive T_{RM} formation (Bergsbaken *et al.* 2017; Casey *et al.* 2012; Schenkel *et al.* 2016; Khan *et al.* 2016; Mackay *et al.* 2013, 2015; McMaster *et al.* 2018; Muschaweckh *et al.* 2016). For example, following HSV infection, T cells enter the skin via chemokine gradients, particularly via CXCL9/CXCL10 and CXCR3, and progressively acquire a transcriptional profile that is distinct from T_{CM}, which coincides with upregulation of CD69 and CD103 (Mackay *et al.* 2013). CD69 is an early activation marker, but can also be upregulated independent of antigen, and promotes tissue residence by inhibiting shingosine-1-P receptor (S1PR) and thereby inhibiting lymphocyte egress (Matloubian *et al.* 2004; Shiow *et al.* 2006). CD69 has been shown to be a key marker to distinguish T_{RM} from circulating cells in humans, and helped identify a core transcriptional profile across multiple donors and tissues (Kumar *et al.* 2017). CD103 is expressed on a subset of CD8 T_{RM} and is not expressed by CD4 T_{RM} in humans (Kumar *et al.* 2017). CD103 binds to E-cadherin, an adhesion molecule on epithelial cells, and therefore helps maintain residence in epithelial compartments. While CD69 is upregulated relatively quickly, CD103 may represent a late stage T_{RM} and could be dependent on antigen or specific tissue localization (Mackay *et al.* 2015). Although multiple cytokines have been implicated in driving T_{RM} differentiation and maintenance in the tissue, TGFβ has been most definitively characterized for CD103 upregulation (El-Asady *et al.* 2005; Mackay *et al.* 2013, 2015; Wang *et al.* 2004). TGFβ is constitutively expressed in many epithelial compartments (Kane *et al.* 1990; Koyama & Podolsky 1989) and has long been known to drive CD103 expression (Casey *et al.* 2012; El-Asady *et al.* 2005; Wang *et al.* 2004). T cell expression of TGFβ receptor 2 is required for upregulation of CD103 and induction of T_{RM} (Mackay *et al.* 2013). Following upregulation of the phenotypic markers CD69 and or CD103, T_{RM} are then maintained in the tissue long-term through local proliferation, particularly after secondary infection (Park *et al.* 2018).

Over time, T_{RM} begin to lose their cytotoxic capacity, indicating their function may be primarily to patrol and act as an alarm system, instead of providing direct effector function (Figure 5) (Beura *et al.* 2018; Schenkel *et al.* 2013; Mintern *et al.* 2007; Muruganandah *et al.* 2018; Park *et al.* 2018). In line with this function, T_{RM} have a dendritic-like quality and can travel randomly between keratinocytes to patrol the skin for infections (Ariotti *et al.* 2012). Although T_{RM} may have lower cytotoxic function, they are highly proliferative and multifunctional (Pizzolla *et al.* 2018). Therefore, after encounter with their cognate antigen, the T_{RM} slows its migration and loses its dendritic qualities (Ariotti *et al.* 2012; Gebhardt *et al.* 2011; Park *et al.* 2018). Here, the T_{RM} can proliferate and produce high levels of cytokines, such as IFNγ, which can act as an alarm and recruit other cells of the immune system. However, it was shown that skin T_{RM}

were still sufficient for protection against HSV in the absence of circulating memory T cells, indicating they are also capable of exerting function without recruiting circulating T cells (Mackay *et al.* 2015). The extensive recent work in mice has provided critical understandings of the developmental pathway and function of $T_{RM,}$ however it remains unclear how to best induce them via vaccination and how findings in mice will translate to humans.

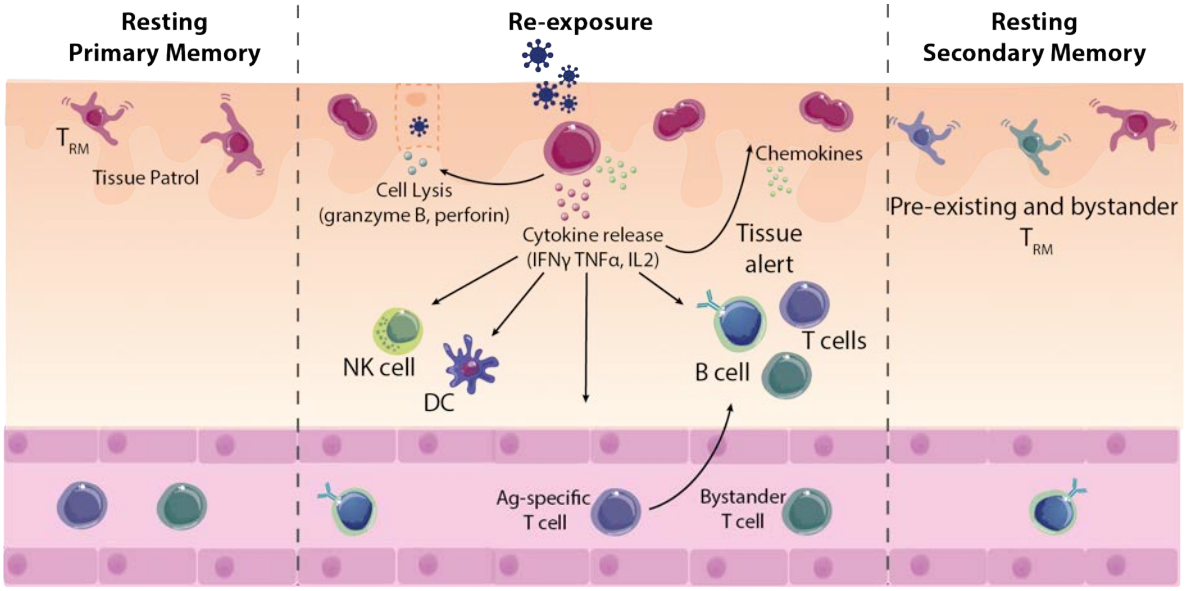

Figure 5: T_{RM} function during the event of antigen re-exposure.

3.3 B CELL RESPONSES

While much of the work performed in this thesis evaluates the induction and maintenance of T cell memory, most vaccines available today rely on Ab-mediated protection. Therefore, it is imperative to have a comprehensive understanding of the B cell biology required to generate protective Ab responses. Two primary cell types, memory B cells (MBCs) and long-lived plasma cells (LLPCs) mediate long-term Ab memory. MBCs provide rapid recognition to an incoming threat, and can respond by proliferation and enhanced Ab production. In contrast, LLPCs provide constant production of secreted Abs to provide a front line of defense.

3.3.1 Generation and re-activation of B cell responses

Naïve B cells express Abs of a single specificity on their cell membrane, which function as a B cell receptor (BCR). They migrate through the peripheral blood and can enter the lymphatic system to sample antigen within the lymph node either captured on follicular DCs (FDCs) or via subcapsular macrophages (Junt *et al.* 2007; Phan *et al.* 2009; Szakal *et al.* 1988). Upon BCR engagement, antigen is internalized and presented to CD4 T cells at the B cell-T cell border (Lanzavecchia 1990, Van Kooten and Banchereau 2000, Okada 2005). T cell help is critical for the induction of class switched and high affinity antibodies (Figure 6).

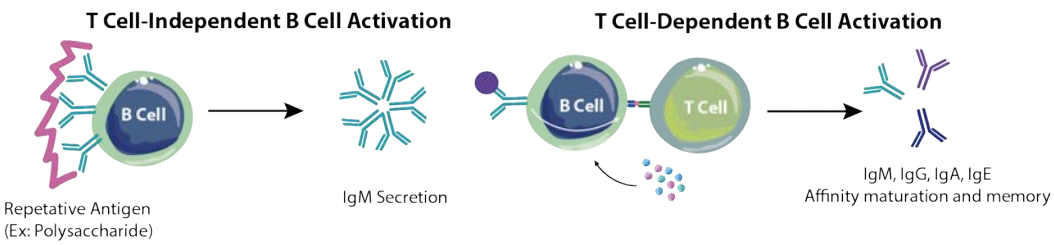

Figure 6: T cell-independent and dependent Ab production.

Through T cell help, B cells can follow two potential pathways, which may be dictated by the affinity of the Ab and the strength or duration of T cell contact (Allen *et al.* 2007; Schwickert *et al.* 2011). B cells will either receive signals to enter the germinal center or extrafollicular areas (Jacob *et al.* 1991; Liu *et al.* 1991). B cells that maintain longer contact with T cells are more likely to become a germinal center B cell, whereas short contacts are more likely to enter the extrafollicular pathway and become short lived Ab secreting cells (Schwickert *et al.* 2011).

The germinal center is a structure found within lymph nodes critical for development of mature B cells (Figure 7). It is within the germinal center structure that B cells can proliferate, differentiate, undergo class-switching and affinity maturation through a process called somatic hypermutation (SHM). Mutated B cells compete for signals to determine which cells have the highest affinity to be selected for differentiation into plasma cells or MBCs. As the germinal center matures, two compartments develop, the light zone and dark zone. Within the dark zone, B cells undergo proliferation and SHM. In the light zone, B cells interact with a specialized CD4 T cell, termed T follicular helper cell (T_{FH}), and sample BCR interactions with antigen sequestered on FDCs. T_{FH} secrete high levels of IL-21 to provide B cell help and act as a gatekeeper for B cells following SHM. Only B cells with the highest affinity for the antigen that can present to cognate T_{FH} will receive signals to stay alive. B cell migration between the two zones is an iterative process, where B cells undergo multiple rounds of mutation and competition for survival (Allen *et al.* 2007; Gitlin *et al.* 2014; Schwickert *et al.* 2007; Victora *et al.* 2010). Cells that do not have appropriately high affinity or have acquired autoreactivity die by apoptosis. Thus, out of the germinal center comes a diverse repertoire of B cells with high affinity antibodies. These B cells can be Ab secreting cells, either short-lived plasmablasts or long-lived plasma cells; or memory B cells. Upon secondary encounter, memory B cells can proliferate in response to BCR binding without the need for cognate CD4 T cell help. It is critical to induce both of these cell types for an effective vaccine, as plasma cells can constitutively produce protective Abs, but memory B cells can be rapidly reactivated, producing higher levels of Abs and restarting the germinal center reaction after booster vaccinations or natural infections.

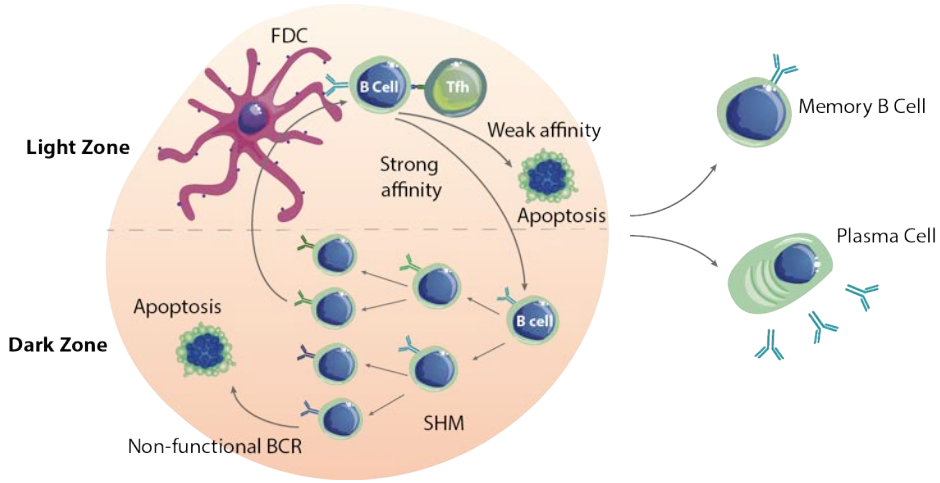

Figure 7: The germinal center reaction within a B cell follicle of a lymph node.

3.3.2 Antibody responses

Several models have been proposed for the maintenance of Ab titers over time (Amanna & Slifka 2010). However, more work evaluating the kinetics and maintenance of B cell

populations in combination with detailed measurements of Ab responses is required to fully delineate a working model. Currently, there are two primary categories of models, based on MBC replenishment or the lifespan of plasma cells (Amanna & Slifka 2010). Immunological memory based on MBCs could be maintained through several possible mechanisms, as illustrated below (Figure 8A). A seminal paper evaluating Ab titers and half-life against a variety of antigens in 45 volunteers over a period of almost three decades can be used to assess these models and to better understand maintenance of Ab titers (Amanna *et al.* 2007). Through chronic infection and repeated antigen exposure, Ab titers could stay elevated over the course of a lifetime, however this only represents a minority of infections (Figure 8A, black). Further, the half-life of two chronic infections, Epstein-Barr virus (EBV) and varicella zoster virus (VZV), differ dramatically (Amanna *et al.* 2007). While EBV generates very stable Ab titers, with a half-life of almost 12,000 years, titers against VZV decline over time and have a half-life of approximately 50 years. These responses are more short-lived than several acute infections, indicating that chronic infection or cross-reactivity of B cell clones should not be the major source of immunological memory. Additionally, if MBCs required persistent antigen, you would expect Ab titers to drop off after the clearance of antigen (Figure 8A, green). It has been demonstrated that small amounts of antigen can be retained on FDCs with a half-life of approximately 8 weeks (Tew & Mandel 1979). You would therefore expect Ab titers to follow a similar half-life, however both natural infection and vaccination have shown significantly longer half-lives. Polyclonal activation through means such as TLR stimulation or cytokine stimulation would generate random blips of Ab production overtime (Figure 8A, blue). *In vitro,* MBCs can respond to TLR and cytokine stimulation to differentiate into Ab secreting cells (Bernasconi *et al.* 2002), however Ab titers against irrelevant antigens would then increase in coordination with most immunizations or infection.

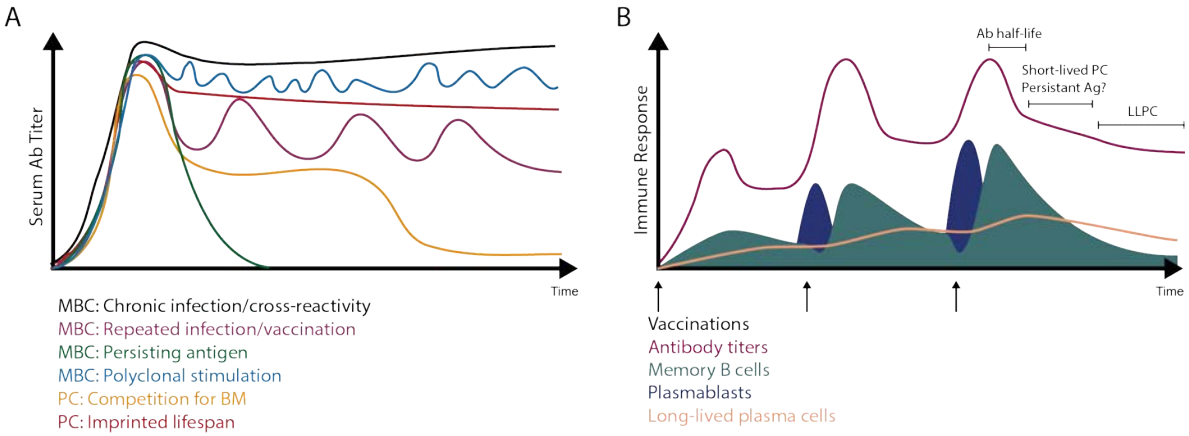

Figure 8: Models for Ab titer maintenance and B cell populations involved.

Finally, repeated infection or vaccination can lead to specific increases in Ab production that coincide with reinfection or immunization, that gradually decrease overtime until the next re-exposure (Figure 8A, purple) (Amanna *et al.* 2007; Genova *et al.* 2006). The peak in Ab titers appears after bursts of plasmablasts, or short-lived plasma cells that are derived from MBC restimulation and expansion (Figure 8B). While the MBC populations also expand and contract with repeated infections, the numbers of LLPCs appear to be established early after infection or vaccination and the numbers appear to remain relatively stable overtime (Lindgren *et al.* 2017; Sundling *et al.* 2013). While this pattern describes many vaccinations, immunizations against measles, mumps, and rubella have all generated long Ab half-lives,

ranging from 100-3000 years, and the titers can be maintained for decades without local outbreaks or exposure. Therefore, a fundamental question in vaccinology is how to imprint these types of long-lived responses using non-live vaccines without the need for continual boosting.

Two models have additionally been proposed for plasma cell-based Ab maintenance, which would not require replenishment by MBC. Although MBCs would still be critical for rapidly responding to reinfection, this model is theoretically possible since it has been demonstrated that LLPCs and Ab titers can be maintained overtime, even if MBCs have been depleted (Ahuja *et al.* 2008; DiLillo *et al.* 2008; Slifka *et al.* 1998). Differences in Ab half-lifes indicate that LLPCs also have different lifespans. One possible explanation is the plasma niche theory, which proposes that plasma cells compete for limited space in survival niches such as the bone marrow (Figure 8A, orange) (Radbruch *et al.* 2006). Once a LLPC is displaced from the survival niche it would die, making room for new LLPCs. However, this would predict that following each immunization there would be less space for previous LLPCs and there would consequently be a reduction of Ab titers against irrelevant antigens. Further, competition within the bone marrow would increase with age, indicating the Ab titers would decrease at a faster rate later in age. However, neither of these phenomena have been seen in an appreciable way (Amanna *et al.* 2007). Therefore, while there may be competition within the bone marrow, it is unlikely that this is the primary cause of differences in lifespan. Instead, it seems likely that when plasma cells are induced they are initially imprinted with a specific lifespan (Figure 8A, red). This model accounts for differences in T cell help during initial priming and the strength of BCR signaling or crosslinking, which could together imprint a specific lifespan of the LLPC. It would therefore be critical to initiate a strong germinal center response to initiate LLPCs capable of lasting decades after immunization. The proposed imprinted lifespan theory follows a biphasic model (Figure 8B). After an initial increase following immunization or infection, there is a rapid reduction in the Ab titers dictated by the Ab half-life, which is approximately 20 days. Following this rapid decline, the second phase is dictated first by short-lived plasma cells and possibly antigen retention, and finally Ab titers are maintained by LLPCs (Amanna & Slifka 2010). Together this model suggests that we need a much better understanding of the initiation of B cell responses within the germinal center response, to understand how to design vaccines to elicit LLPCs with an increased imprinted lifespan.

4 VACCINATION

Vaccines have been fundamental in efforts to alleviate human morbidity and mortality. Since the first recorded use of vaccination in 1796 by Edward Jenner to protect against smallpox, vaccines have been developed against an outstanding array of diseases and have been able to control or even eliminate many of them. However, today there are still significant challenges ahead for future vaccination efforts. Traditionally, vaccines have been developed using empirical methods and followed a standard protocol of isolate, inactivate, and inject (Figure 9) (Rappuoli 2000; Rappuoli *et al.* 2016). Today, a convergence of a variety of fields including genetics, immunology, structural biology, and computational biology have allowed for a new approach to vaccine development and design, termed "reverse vaccinology" (Rappuoli 2000; Rappuoli *et al.* 2016). This process does not rely on culturing the pathogen for inactivation, but instead starts with the genetic or structural sequences to identify potential vaccine candidates and antigens. Using this approach, a great deal of vaccine design is now required to elicit the optimal response. Multiple components of the vaccine can be altered and must now be considered when developing a new candidate, including antigen, adjuvant, formulation, and route of delivery (Figure 9). Together, a better understanding of all these components of a vaccine can help overcome the current challenges of vaccines, and will be discussed below.

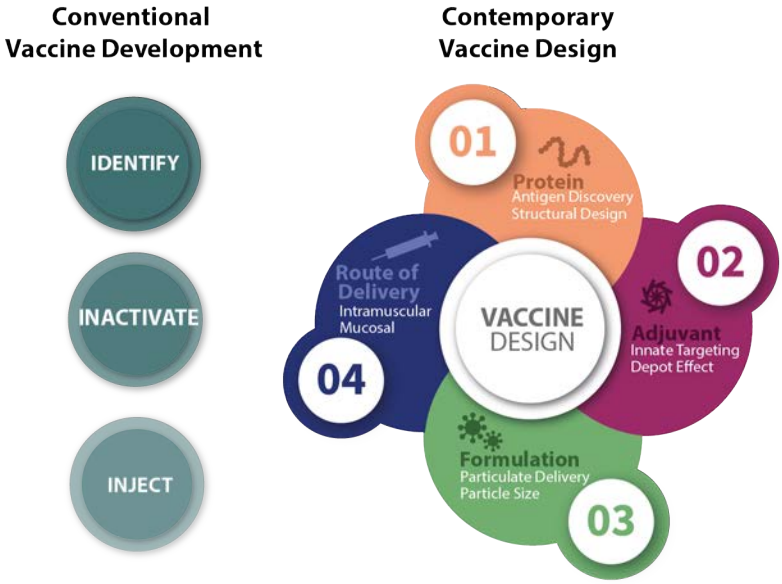

Figure 9: Requirements for conventional and contemporary vaccine design.

4.1 VACCINES FOR UNMET NEEDS

4.1.1 T cell-based vaccines

As discussed above, vaccines against HIV, tuberculosis (TB), malaria, and cancer immunotherapies will likely require induction of CD8 T cell responses. However, most vaccines to date rely on Ab mediated protection and it has been difficult to generate effective CD8 responses, in particular to sub-unit vaccines. Although viral vectored vaccines, such as those based on adenovirus (Ad) vectors have been able to generate strong CD8 T cell responses, setbacks such as failures of Ad5-based HIV-1 vaccine trials have slowed their clinical implementation. The STEP trial, one of the early HIV-1 large-scale efficacy trials based on Ad-5, was prematurely halted due to increased infection susceptibility in men with a high seroprevalence of Ad5 (Buchbinder *et al.* 2008). However, there is still motivation to generate an HIV vaccine that induces CD8 T cell responses, ideally in combination with potent Ab responses. The gold standard for an HIV vaccine has long been to establish broadly neutralizing antibodies (bNAbs), or antibodies that can neutralize multiple strains across different clades. However, bNAbs only develop in a small subset of HIV-infected individuals

and have proven to be exceedingly difficult to elicit via vaccination efforts. These Abs often take years of chronic infection to develop and have several unique properties, making them particularly difficult to develop (Geiß & Dietrich 2015). However, CD8 T cells, known to control HIV replication early after infection, may limit the viral reservoir and have been shown to be associated with disease non-progression (Boaz *et al.* 2002; Hess *et al.* 2004; Migueles *et al.* 2002; Streeck *et al.* 2008). Recent studies have identified a vaccine strategy that protects rhesus macaques from SIV infection mediated by CD8 T cells (Hansen *et al.* 2009, 2011, 2013). This strategy uses a cytomegalovirus (CMV) vector and elicits a T_{EM} response that is HLA-E restricted, and will soon be tested in clinical trials. The HIV/AIDS epidemic has also made clear the role for T cell responses in protection against TB. HIV makes patients highly susceptible to infection and reactivation of latent TB due to the decrease in CD4 T cells. It also seems that CD8 T cells are critical in protection against TB, although this has not been as thoroughly explored (Lin & Flynn 2015). CD8 T cells also likely play a role in heterosubtypic protection against influenza (Slütter *et al.* 2013, 2017; Zens *et al.* 2016), and could hold the key to avoiding yearly vaccination efforts presently needed. Finally, it is clear that T cell responses will be necessary for successful therapeutic cancer vaccines. The recent blockbuster success of checkpoint blockade therapies targeting PD-1 and CTLA-4 have demonstrated the potential to unlock T cell responses. Further, the advent of CAR-T cells and adoptive cell therapy have further emphasized the role of T cell mediated effect. However, work is still needed to design vaccines that can elicit *de novo* T cell responses to provide therapeutic effect.

4.1.2 Malaria vaccines

Malaria is another disease that causes a substantial global public health burden, but has so far eluded standard vaccination strategies. Several factors have complicated the development of a vaccine to malaria, including the complex life cycle of the *Plasmodium* parasite, large antigenic variation, and an overall poor understanding of the interactions between the parasite and human immune system. Candidate vaccines have been divided into three major categories based on the parasite life stage; pre-erythrocycic, blood stage, and transmission-blocking, each of which have distinct immunological requirements for efficacy (Figure 10). Much effort has focused on the development of pre-erythrocitic/liver stage candidates, which aim to block infection by targeting antigens expressed early in the life cycle. The most commonly studied antigen in this category is circumsporozoite protein (CSP), the predominant surface antigen on sporozoites which contains immunodominant B cell and T cell epitopes (Crompton *et al.* 2010). The RTS,S vaccine candidate, based on the pre-erythrocytic *Plasmodium falciparum* CSP protein, has shown around 40% efficacy immediately after vaccination for the four-dose regimen, but protection wanes over time. RTS,S efficacy has been correlated with anti-CSP Ab titers (Kazmin *et al.* 2017). However, an alternative pre-erythrocytic vaccine that has shown efficacy in controlled human infection models is based on whole irradiated sporozoites, which appears to function through hepatic CD8 T cell responses (Epstein *et al.* 2011; Ishizuka *et al.* 2016). Clinical symptoms of malaria are only seen after the parasite enters the bloodstream, and naturally acquired immunity typically develops against the blood stage of the parasite. Therefore, vaccines targeting the blood stage aim to mimic natural immunity, and instead of providing sterilizing protection they can reduce parasitemia and clinical symptoms, largely through antibodies and CD4 T cells (Crompton *et al.* 2010).

Finally, transmission-blocking vaccines (TBVs) are designed to target the parasite within the mosquito vector to interrupt the life cycle and thereby lead to malaria elimination (Hoffman *et al.* 2015). This is a unique approach that targets antigens in the mosquito midgut through vaccine-elicited antibodies taken up during the blood meal. This method takes advantage of the conserved antigens expressed within the mosquito midgut, which have not undergone centuries of immune pressure within the human host. Additionally, this is one of the few stages where the parasite exists extracellularly and in relatively small numbers, making it a prime target for antibodies. This would result in blockage of parasite replication in the mosquitos and thus limit the parasite burden overall. The caveat is that the vaccinated individuals themselves would not be protected, but with enough vaccinated individuals in a population malaria transmission could ultimately be eradicated. In my thesis we have evaluated a TBV targeting the most clinically advanced antigen, *P. falciparum* protein Pfs25. Although this antigen has progressed to clinical trials (Talaat *et al.* 2016; Wu *et al.* 2008), it has failed to induce robust and sustained Ab titers necessary for clinical implementation. While there are many possible targets for malaria vaccination, it is clear that all will require an in depth understanding of vaccine elicited immunity to guide rational vaccine design.

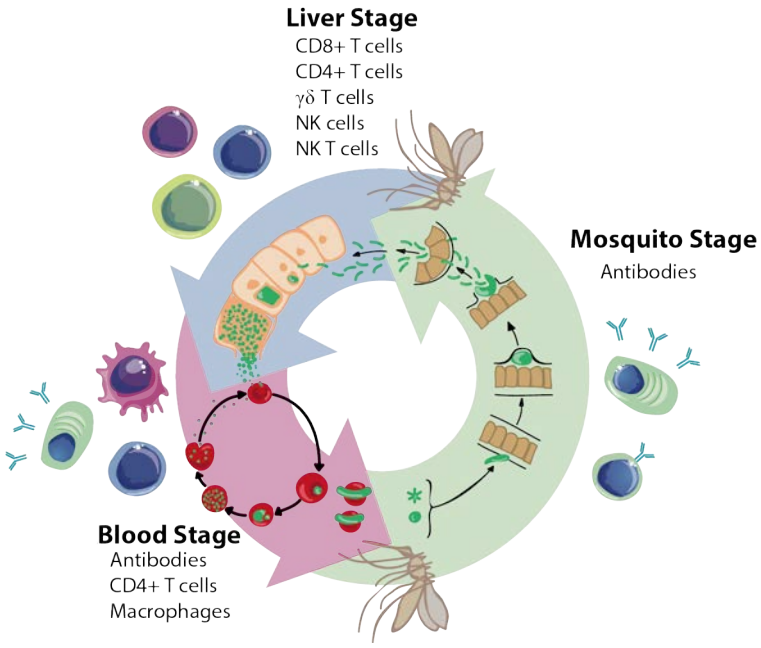

Figure 10: Different stages of plasmodium falciparum life cycle targeted by vaccination and proposed immunological mechanisms for protection.

There have been significant challenges for the field of vaccinology posed by both vaccines requiring CD8 T cell responses and the high levels of sustained Ab titers required for malaria vaccination. In my thesis, we have aimed to advance the understanding vaccine-elicited immunity to better inform rational vaccine design to help overcome these obstacles. Information of this kind can be used to tailor the appropriate vaccine adjuvant, formulation, and even route of delivery, to elicit the necessary adaptive immune responses.

4.2 ADJUVANTS

4.2.1 History of adjuvants

The increased understanding of basic immunological processes, as described above, has spurred the design of many novel vaccine adjuvants in recent years. Vaccine adjuvants are components added to non-live vaccine formulation in order to improve the immunogenicity and direct the resulting adaptive response. While adjuvants have been used for more than 90 years (Pasquale *et al.* 2015), they were largely developed empirically in the past. The most commonly used adjuvant, aluminum salts (alum), was the only licensed adjuvant in humans for 70 years, although the mechanism of action is still incompletely understood. Alum primarily increases Ab responses, and so far, no alum adjuvanted vaccine has been able to induce protective cellular responses. In general, most vaccines protect by eliciting protective Ab responses (Plotkin 2010), and there are still no fully effective vaccines for a variety of diseases that will likely require T_H1/CD8 T cell immunity, in addition to Ab responses.

Traditionally, adjuvants were primarily used to increase the magnitude of responses, but now it is becoming increasingly important to guide the specific type of adaptive response needed. In addition, as vaccines move towards using more purified antigens to increase safety, they become less efficacious and need stronger adjuvants to increase immunogenicity. Most of the purified antigens in use now typically lack PAMPS and therefore are incapable of initiating immune responses on their own (Coffman *et al.* 2010). Thus, many next generation adjuvants aim to exploit the power of the innate immune system to provide both an increased magnitude and qualitative alteration of the immune response. In fact, it seems almost all adjuvants enhance adaptive immunity by engaging the innate immune system, not the adaptive lymphocytes themselves (Coffman *et al.* 2010).

4.2.2 TLR-based adjuvants

Vaccine adjuvants have long been considered to function through two primary modalities, immunostimulatory agents or passive depots or vehicles. There is now increased evidence that even adjuvants long thought of as passive depots (e.g. alum) also stimulate innate immunity (Marrack *et al.* 2009; Mosca *et al.* 2008). In my thesis I focused on immunostimulatory compounds, including targeting PRRs and DC activating pathways, such as CD40. One of the most common ways to target PRRs is through natural or synthetic ligands to TLRs, of which a variety have been targeted. We have tested adjuvants targeting TLR3, TLR4, TLR7/8, and TLR9, all of which are in advanced stages of clinical or pre-clinical testing. These TLRs are located either on the cell surface or in endosomes (Figure 11), and are restricted to expression on distinct cell types (Figure 12) (Thompson & Loré 2017). Therefore, TLRs offer the ability to specifically target different cell types and compartments of the cell.

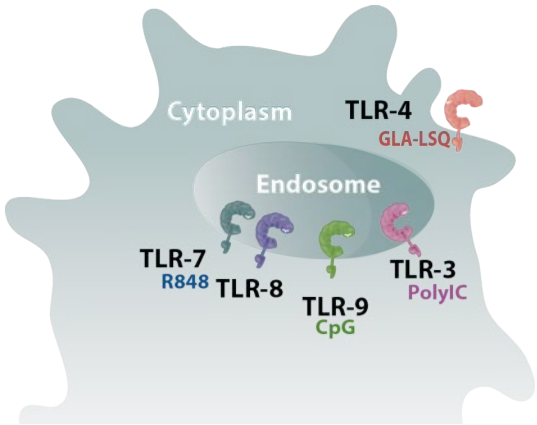

Figure 11: TLR distribution in the cells and examples of adjuvants targeting these TLRs.

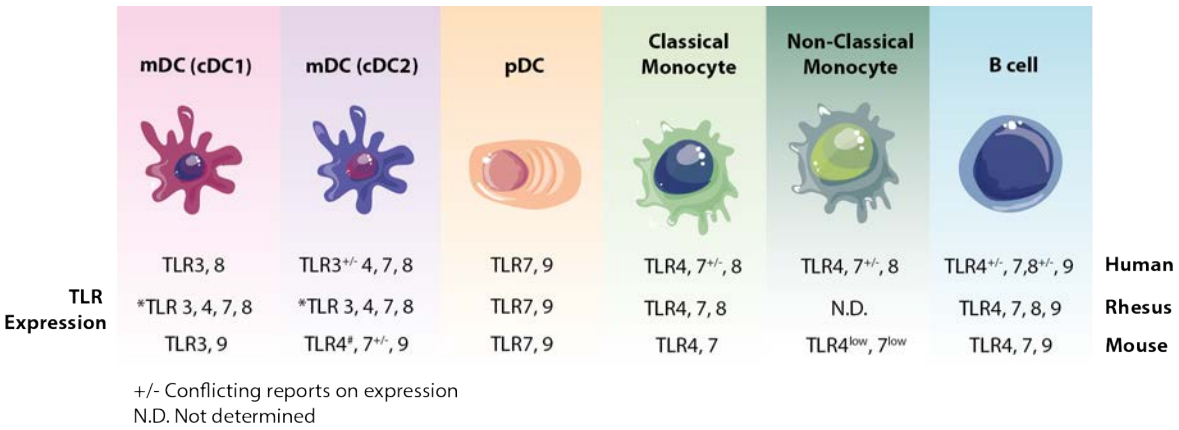

Figure 12: TLR distribution across APC subsets in human, rhesus macaque, and mouse.

Synthetic analogs of the TLR3 agonist, double stranded RNA, have been developed (PolyIC) and are able to potently activate innate immunity when used as an adjuvant (Longhi *et al.* 2009; Stahl-Hennig *et al.* 2009; Trumpfheller *et al.* 2008). PolyIC can act through two distinct pathways, activating TLR3 in endosomes or RIG-I and MDA5 in the cytosol. PolyIC activation of TLR3 in DCs induces IL-12 and type I IFN production as well as improving MHC-II expression and cross presentation (Grewal & Flavell 1998; Krug *et al.* 2001; Schulz *et al.* 2000). MDA5 activation occurs primarily in non-hematopoeitc cells (stromal cells) and induces strong production of type I IFNs, which may further enhance DC maturation and be critical for optimizing the generation of T_H1 and CD8 T cell immunity (Longhi *et al.* 2009). PolyIC and its derivatives (i.e. Poly IC:LC) have been tested in several clinical trials (Hartman *et al.* 2014; Kyi *et al.* 2018; Mehrotra *et al.* 2017; Okada *et al.* 2015; Pollack *et al.* 2014; Tsuji *et al.* 2013).

To date, the only licensed TLR-based adjuvants target TLR4. The natural ligand to TLR4 is bacterial lipopolysaccharide (LPS), but adjuvants primarily use a detoxified derivative, monophosphoryl lipid A (MPL), or a synthetic analog, glucopyranosyl lipid adjuvant (GLA). The hepatitis B virus (HBV) and human papilloma virus (HPV) vaccines are both adjuvanted with AS04, which combines alum and MPL (Garçon & Mechelen 2011). These two vaccines were licensed in 2005 and 2007, respectively (Garçon & Pasquale 2016). However, it has long been known that LPS was a potent stimulator of the immune system and could effectively function as an adjuvant, (Johnson & Jackson 2014), but the highly activating profile has been associated with a myriad of side effects (Beutler & Rietschel 2003). Since vaccine adjuvants are typically delivered to healthy individuals, it is critical to have a strong safety profile. New technology and formulations have been able to strike a balance between immune potency and unintended side effects. TLR4 can signal either via MyD88 or TRIF pathways, leading to proinflammatory cytokines or type I interferons, respectively (Lu *et al.* 2008). Additionally, targeting TLR4 on murine B cells leads to B cell proliferation and Ab secretion (Gururajan *et al.* 2007). However, human and non-human primates (NHPs) express low levels or non-functional levels of TLR4 and are therefore not responsive to stimulation (Bekeredjian-Ding *et al.* 2005). The success of TLR4-targeting vaccines in humans may consequently rely on innate activation of myeloid cells to provide proinflammatory cytokines.

TLR7 and 8 are expressed in the endosome and recognize single stranded RNA, as found in viruses such as HIV and influenza. Small molecule agonists have been discovered to target TLR7 and 8, with the most heavily studied being synthetic imidazoquinolines, such as

imiquimod and resiquimod (R848) (Adams *et al.* 2012; Dowling 2018; Maldonado *et al.* 2015; Sabado *et al.* 2015). Similar to the natural ligand, these synthetic small molecules can activate TLR7 and/or 8 within the endosome to initiate the activation of MyD88 and depending on the cell type will then drive a largely IRF7 dependent pathway initiating type I IFN genes or the NF-kB pathway to induce proinflammatory cytokines. While PDCs are prone to produce high levels of IFNα in response to TLR7 stimulation, monocytes and myeloid DCs primarily produce proinflammatory cytokines such as IL12 and are capable of skewing a T_H1 response. Despite the strong promise of TLR7/8 ligands such as R848 for vaccine adjuvants, they have to date failed to reach widespread clinical use due to the associated side effects. This is largely due to the small molecule size, which allows the adjuvant to diffuse away from the injection site and lead to systemic activation and reactogenicity. However, recent efforts at vaccine formulation have demonstrated that these effects can be overcome through multiple methods of formulation, such as encapsulation into nanoparticles (Ilyinskii *et al.* 2014; Kasturi *et al.* 2011, 2017), conjugation to polymer backbones (Lynn *et al.* 2015), and absorption to alum (Liang *et al.* 2017; Wu *et al.* 2014). These developments have ushered in a new generation of adjuvants, which can potently activate the immune system while mitigating the systemic effects of unformulated TLR7/8 agonists.

Finally, TLR9 agonists based on CpG have long been investigated for their potential as vaccine adjuvants. CpG-based adjuvants have been evaluated in a variety of phase I and II clinical tests for infectious diseases and cancer therapy (Bode *et al.* 2014). TLR9 recognizes bacterial DNA, which is rich in unmethylated CpG motifs, whereas eukaryotic DNA typically lacks these motifs. Multiple classes of synthetic analogs of CpG have been developed and while there are similarities between the classes, each preferentially activates TLR9 in different species or cell types. TLR9 is predominantly expressed by PDCs and B cells in humans (Hemmi *et al.* 2000; Takeshita *et al.* 2001). CpG class A (also known as D) principally target PDCs to produce IFNα through MyD88/IF-7 signaling, but have limited effect on B cells. IFNα can skew T_H1 responses and may therefore be useful for inducing CD8 T cell responses. Class B (or K) are highly efficient at stimulating B cell proliferation and Ab secretion. Class C have similarities with both Class A and B and can stimulate B cells to produce IL-6 and PDCs to produce IFNα. Therefore, the specific class of CpG can be tailored to the needed response for a given vaccine.

4.2.3 CD40 targeting adjuvants

An alternative innate pathway particularly important in generating T cell responses is the CD40/CD40L pathway as described previously. Targeting CD40 as a vaccine adjuvant has been tested in a variety of preclinical and clinical models. Mouse models have shown that DCs can receive CD40 stimulation independent of T cell help by administering either agonistic anti-CD40 Abs, soluble CD40L or an adenovirus vector expressing CD40L, all of which have shown enhanced CD8 T cell responses (Davis *et al.* 2006; Gladue *et al.* 2011; Hanyu *et al.* 2008). Preclinical data also suggests that combining a TLR-ligand with agonistic anti-CD40 Abs induces a several fold increase of CD8 T cell responses compared to either agonist alone (Ahonen *et al.* 2004; McWilliams *et al.* 2010; Sanchez *et al.* 2007).To further this notion, a mouse cancer model showed that delivering synthetic peptides together with anti-CD40 Ab and the TLR3 ligand, polyIC, (TriVax) induced high levels of antigen-specific T cell responses and tumor reduction (Assudani *et al.* 2008). Targeting CD40 may therefore be particularly

potent as a vaccine adjuvant with the synergy provided by another innate pathway. Recently, there is also increased interest in combining CD40 targeting with immune check point blockades, which is currently being tested in clinical trials (Vonderheide 2018).

Of all methods tested for targeting CD40, monoclonal Abs (mAbs) have achieved the most clinical attention (Figure 13). To date, all clinical trials involve testing the various CD40 Abs for their potency in cancer immune therapy. The range of CD40 mAbs have diverse activities, varying from agonism to antagonism, and further investigation is needed to fully explain the heterogeneity (Vonderheide & Glennie 2013). There are currently three major agonistic clones undergoing clinical investigation (Table 1) and several antagonistic clones. However, it has been difficult to directly compare data generated in mouse models to Abs in clinical use, as they use rodent and human isotypes, respectively. While mouse models showed that the Ab istoype and interaction with FcR may play a large role in determining the profile of CD40 Abs (Li & Ravetch 2011), recent reports evaluating the human IgG2 clone CP-870,893 (now known as RO70097890/Selicrelumab) instead propose that epitope specificity plays a dominant role (Richman & Vonderheide 2014). This clone is the most widely studied in clinical trials and also the most agonistic. Interestingly, this clone was chosen in part because the IgG2 isotype is known for having relatively low FcγR interaction. *In vitro* tests showed that the Ab maintained activity in the absence of cross-linking, unlike the mouse counterpart, and could still function with an inactive or removed Fc portion. Since it was found that clones commonly used in mouse studies bind to the CD40L binding site, whereas the human clone does not, it has been proposed that the differences lie in epitope specificity. However, this debate is far from over, as studies evaluating the human clone CP-870,893 in a mouse model expressing human CD40 and FcR again showed that *in vivo* activity was dependent on Fc signaling (Dahan *et al.* 2016). Therefore, it seems that even Abs designed to have low Fc engagement could be further optimized through Fc engineering.

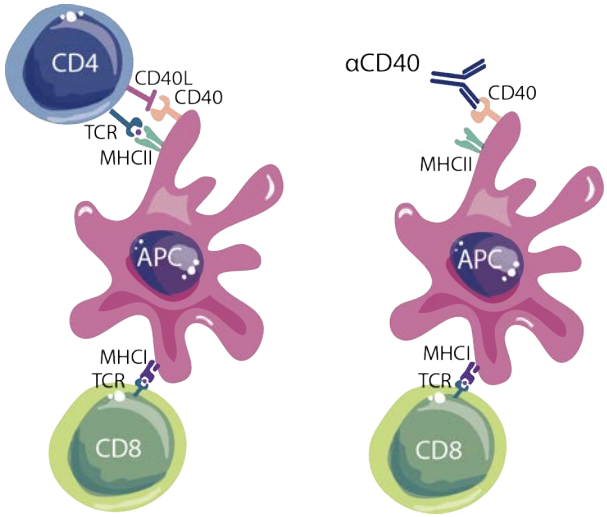

Figure 13: Proposed function of monoclonal anti-CD40 Ab targeting of APCs for enhanced antigen presentation.

Ab Name	*Ab Isotype*	*Company/ Institution*	*Clinical Trials Status*	*Conditions*	*NCT Numbers*
ADC-1013 (JNJ-64457107)	Fully human IgG1	Alligator Biosciences	1 Completed; 1 Recruiting	Neoplasms, Solid Tumors	NCT02379741, NCT02829099
Chi Lob 7/4	Chimeric IgG1	Cancer Research UK	1 Completed	Cancer, Neoplasms, Lymphoma, Non-Hodgkin, B-Cell	NCT01561911
CP 870,893	Fully human IgG2	Pfizer	1 Active, not recruiting; 6 Completed	Melanoma, Advanced Solid Tumors, Pancreatic Neoplasm, Adenocarcinoma Pancreas	NCT01103635, NCT02225002, NCT01008527, NCT00711191, NCT02157831, NCT00607048, NCT01456585
Selicrelumab (RO70097890)	Fully human IgG2	Roche	4 Recruiting	Pancreatic Cancer, Advanced/Metastatic Solid Tumors, Solid Tumors, Pancreatic Adenocarcionma	NCT02588443, NCT02665416, NCT02304393, NCT03193190

Table 1: Overview of agonistic CD40 mAb that have been tested in clinical trials.

While immunotherapies targeting CD40 have gained significant attention, there have also been safety concerns for such a highly immunostimulatory formulation. There is a potential for a myriad of side effects, including inducing cytokine release syndrome, autoimmune reactions, and cell death or tolerance due to overstimulation. However, it is important to note that none of these reactions have been seen in a significant way in the clinic (Vonderheide 2013). The strongest agonist, CP-870,893 (now Selicrelumab), has only led to mild cytokine release syndrome, which was transient and easily managed. There have also been no reported cases of autoimmunity following administration. However, it is still possible that local activation of DCs may overcome these potential hurdles. It has been shown that delivering the CD40 Ab SQ adjacent to the tumor restricts distribution to the tumor draining lymph node and lowers toxicity, while still maintaining the same effect (Fransen *et al.* 2011). Furthering this notion, CD40 targeting mAbs may benefit from alternative routes of administration, which allow for more localized and direct targeting of DCs.

4.3 THE ROLE OF VACCINE FORMULATION

Much work in recent years has focused on developing novel vaccine adjuvants, as described above. However, it has become increasingly clear that the simple combination of a correct antigen and adjuvant is not sufficient to generate protective responses (Moyer *et al.* 2016). Instead, it is necessary to properly formulate these sub-unit vaccines to achieve the proper biodistribution, pharmacodynamics, and ideally to mimic the molecular structure and appearance of the natural pathogen. A promising avenue to achieve all of these requirements is through nanoparticle formulation. Nanoparticulate formulation can improve antigen targeting to the lymph node, uptake and presentation to T cells, antigen retention within the lymph node, and B cell activation. Nanoparticles can be designed in a range of sizes, with multiple materials, and with different antigen densities and display. Therefore, they offer a prime technology to specifically enhance cellular and humoral responses.

Following immunization, antigen must be trafficked to lymph nodes to interact with B cells and T cells and initiate an immune response. Antigen can either traffic freely via lymphatic drainage or be transported by APCs. Typically, vaccines are delivered to the muscle, which has limited lymph drainage and therefore a large proportion of the antigen is transported via APCs (O'Hagan *et al.* 2012). In contrast, delivering vaccines via the SQ or intradermal route allows for more efficient lymphatic drainage, and higher levels of free antigen reaching the lymph node. However, the size of the particle is critical for efficient lymphatic drainage (Figure 14). The blood drains significantly higher amounts of fluid from the tissue than lymph, but is limited to small proteins due the tight junctions of the endothelial cells. It has been demonstrated that there is a direct relationship between particle size and lymphatic drainage, where particles above approximately 45kD traffic almost exclusively by the lymphatics (Miller *et al.* 2011; Supersaxo *et al.* 1990). This may explain the poor immunogenicity of peptide antigens and high cytotoxic events associated with low molecular weight adjuvants, due to increased systemic distribution via the blood stream. Therefore, formulating both antigens and adjuvants into nanoparticle structures, where the size and shape can be designed to avoid systemic exposure, offers possibilities to increase immunogenicity of relatively non-immunogenic antigens and increase the safety profile of highly activating adjuvants. The size of particle also plays a role in retention in the LN. Smaller particles (approximately 30nm) may flow through the lymph node and reenter circulation via the thoracic duct, reaching concentrations similar to intravenous administration (Kourtis *et al.* 2013). Therefore, synthetic nanoparticles, liposomes, and virus-like particles (VLPs) all offer opportunities to enhance vaccine drainage and retention in the lymph node based on size alone (Fifis *et al.* 2004; Kanekiyo *et al.* 2013; Link *et al.* 2012; Moon *et al.* 2011; Reddy *et al.* 2007). Nanoparticle size can also increase uptake by APCs and thereby more efficiently promote T cell responses (Manolova *et al.* 2008; Reddy *et al.* 2006).

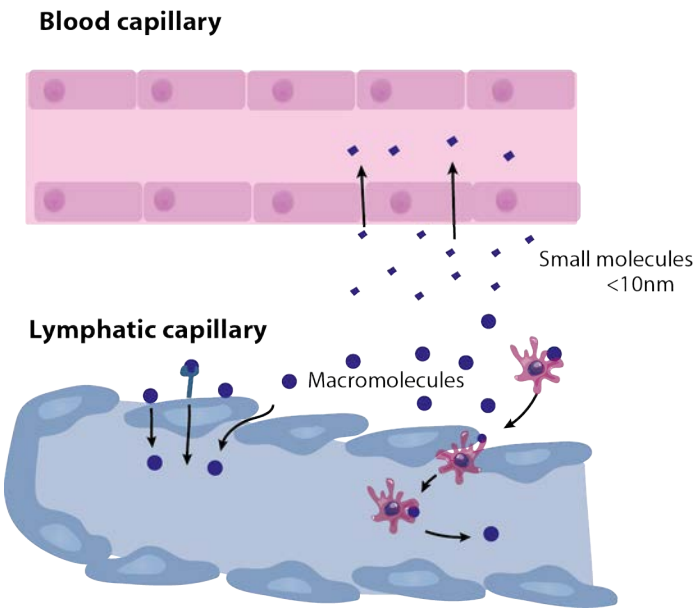

Figure 14: Size-dependent transport of antigen via blood and lymphatics.

Apart from increased lymphatic drainage, nanoparticles can provide a similar structure to pathogens. Bacteria and viruses often have a dense array of antigens expressed on the surface to facilitate infection. Therefore, increased antigen valency can better mimic natural structures, and has been shown to be superior in BCR cross-linking and activation of B cells, as well as antigen uptake by B cells and presentation to cognate T cells (Jegerlehner *et al.* 2002; Kim *et al.* 2006; Temchura *et al.* 2014). Prime examples of these repetitive structures are VLPs, which are utilized by HPV and HBV vaccines, and the malaria vaccine RTS,S. The highly successful nature of these new vaccines indicates this could be an approach to improve formulations where increased potency is required.

4.4 ROUTE OF DELIVERY

Another approach to influence vaccine-elicited responses is by using alternative routes of administration. The skin has gathered a lot of interest in the past decade after being recognized as an ideal anatomical target for vaccination due to its dense network of different subsets of DCs including epidermal LCs and populations of dermal DCs (dDCs), as well as increased lymphatic drainage as discussed above. DCs in the skin can easily be targeted and pick up vaccine antigen to be transported via lymphatics to the local draining lymph node (Teunissen *et al.* 2012). A more localized and contained response could lead to a better safety profile, dose-sparing, and an alternative homing pattern of vaccine-generated T cells (Teunissen *et al.* 2012). Understanding if there is differential T cell stimulation provided by phenotypically and functionally specialized DC subsets, with distinct plasticity in responding to extrinsic signals, represent essential information in the development of vaccines. The interest in targeting skin DCs for vaccination has motivated studies to compare skin vaccination to classical IM vaccination. Delivering the seasonal influenza vaccine in the skin compared to conventional IM injection resulted increased immune responses and dose sparing (Roukens *et al.* 2012). In fact, many of the recent clinical trials evaluating peptide-based cancer vaccines have been administered intradermally or SQ (Noguchi *et al.* 2013; Reuschenbach *et al.* 2016; Slingluff *et al.* 2013; Takahashi *et al.* 2013).

Additionally, delivery of vaccines via mucosal routes has been shown to be an effective method for induction of T cells that can home back to the site of immunization and site-specific T_{RM}, as well as mucosal IgA. Mucosal surfaces act as a barrier to the external world, and are therefore often a primary site of infection for a variety of diseases. Vaccine-elicited responses at the barriers may then be able to provide a front-line defense. There are already several clinically approved vaccines that are delivered via mucosal routes, most commonly via the oral route, including vaccines against polio, cholera, rotavirus, and typhoid (Shakya *et al.* 2016). Intranasal delivery offers easy access to the lung mucosa, and has been shown to induce protective T_{RM} following immunization for influenza, RSV, and immunotherapies for cancer (Kinnear *et al.* 2018; Nizard *et al.* 2015; Sandoval *et al.* 2013; Slütter *et al.* 2013, 2017; Zens *et al.* 2017). It is known that DCs from different mucosal tissues such as lung or gut can imprint T cell homing back to the site of infection (Iwata *et al.* 2004; Johansson-Lindbom *et al.* 2005; Mikhak *et al.* 2013; Mora *et al.* 2003, 2005). Targeting of local APCs via mucosal delivery may therefore explain the increased induction of T_{RM}. However, many of the barriers established to prevent invasion from foreign pathogens can also act as a barrier to vaccine uptake, thus special attention needs to be taken to vaccine formulation for successful mucosal delivery.

4.5 MODELS FOR VACCINATION

To date, most preclinical evaluations and mechanistic studies for vaccine formulations are performed in mice. However, these studies are not always translatable to humans as there are significant differences between the responses seen in mice and humans, likely due to differences in cellular expression patterns of PRRs. In addition, there are often very different conditions used in mouse studies when compared to clinical practice. Murine studies frequently use intraperitoneal or intravenous delivery routes, as opposed to the commonly used SQ or IM routes in humans. These different routes of administration can lead to different antigen presentation by specific DC subsets, as discussed above. In contrast, NHPs offer several distinct advantages over the traditional mouse model. NHPs have more similar DC

subsets and PRR expression to humans and more invasive sampling allow for more complete mechanistic studies and a bridge between mice and humans. Additionally, human reagents can be used in NHPs, allowing for more readily translatable data. *In vitro* studies using human peripheral blood cells can generate much data, but these *in vitro* studies have their limitations. *In vitro* cultures typically will not include cells that are not in circulation, which can also contribute to the immune response by adding a bystander effect. Further, these studies cannot easily replicate differences in anatomical distributions or persistence at an injection site. Therefore, it is important to have a clinically relevant model where you can follow up on *in vitro* findings and better evaluate vaccine formulations.

5 MATERIALS AND METHODS

5.1 SAMPLE MATERIAL

Approval for animal studies was granted by the Animal Care and Use Committees of the Vaccine Research Center, National Institutes of Health (NIH) (**I-II**) or the Stockholm Ethical Committee on Animal Experiments organized under the Swedish Board of Agriculture (**III).** Indian rhesus macaques were housed at Bioqual, USA (**I-II**), or Astrid Fagraeus Laboratory at Karolinska Institutet (**III**) and handled according to the standards of the Association for Assessment and Accreditation of Laboratory Animal Care. The work on human sample material was performed at both the NIH, USA and the Karolinska Institutet, Sweden and approved by the relevant Institutional Review Board (**I-II**). Signed informed consent was obtained in accordance with the Declaration of Helsinki.

5.2 IMMUNIZATIONS

In papers **I-II,** rhesus macaques were immunized with 1.5mg/kg anti-CD40Ab (clone 341G2 IgG2), 1mg poly IC:LC (Oncovir), and 1mg/kg HIV-1 Env peptides (Biomatik) either via the IV (**I-II**) or SQ (**II**) route of administration. The CD40Ab was tracked *in vivo* by conjugating it to Alexa680 according to standard protocols (Molecular Probes) prior to immunization (**I-II**).

In paper **III** rhesus macaques were immunized with 50µg of Pfs25 protein either formulated in a synthetic vaccine particle (SVP[Pfs25]) or as a conjugate (Pfs25-EPA). Group 1-3 received SVP[Pfs25] with 500µg CpG (Gr 1), 150µg R848 (Gr 2) or GLA-LSQ containing 12.5µg GLA and 25ug QS21. Immunizations were delivered in a volume of 1ml SQ. Rhesus macaques in group 4 were immunized IM with Pfs25-EPA containing 50µg of Pfs25 mixed with GLA-LSQ containing 25ug GLA and 50ug QS21 in a volume of 0.5ml.

5.3 RHESUS TISSUE AND BLOOD SAMPLING

Peripheral blood mononuclear cells (PBMCs) were purified by a density-gradient with Ficoll-Paque (GE Healthcare) according to standard protocols (**I-III**). For biodistribution studies (**I-II**), tissues were collected after termination and processed to a single cell suspension by manually disrupting the tissue and filtering through a 70um cell strainer. Liver samples were further purified with Ficoll-Paque. Lung and gut tissues were digested with collagenase treatment and mechanically disrupted using the gentleMACS Dissociator (Miltenyi).

5.4 CYTOKINE SECRETION ASSAY

Cytokine secretion was evaluated in plasma of rhesus macaques (**I-III**) or cell culture supernatants (**II**) using multiplex technology or commercially available ELISA kits according to manufactures protocols.

5.5 ANTIGEN RECALL ASSAY

For assessment of antigen-specific cytokine production, PBMCs or single cell suspensions from tissues were restimulated *in vitro*. $1.5x10^6$ cells were cultured in 200ul of complete medium per stimulation in a 96 well plate. Samples were stimulated as previously described with 2ug/ml of 9-13mer overlapping HIV-1 Env peptides (matched to immunization, **I-II**) or overlapping peptides (15mers overlapping by 11 amino acids) spanning the entire Pfs25

protein (**III**) in the presence of 10µg/ml brefeldin A (Sigma) overnight. Samples were stained the following morning to evaluate cytokine production.

5.6 MULTIPARAMETER FLOW CYTOMETRY

Samples were first stained with LIVE/DEAD Fixable Dead Cell kit (Invitrogen), then blocked with FcR-blocking reagent (Miltenyi). Samples were then surfaced stained with a panel of fluorescently labeled antibodies. For intracellular staining, cells were then fixed and permeabilized using Cytofix/Cytoperm solution (BD Biosciences) and followed by a panel of fluorescently labeled antibodies for intracellular staining. Samples were resuspended in 1% paraformaldehyde prior to acquisition on a Fortessa flow cytometer or modified Fortessa X-50 (BD Biosciences).

5.7 ISOLATION OF HUMAN APCS

Skin samples were obtained from patients undergoing breast reconstruction surgery (**II)** (Karolinska University Hospital, Stockholm, Sweden). To isolate skin APCs, skin pieces were cut using a skin graft mesher (Zimmer) then incubated in 2U/ml Dispase (Life Technologies) overnight at 4°C. After separating the epidermis from the dermis, both fractions were incubated in 26 WU/ml Liberase (Roche) and 20 U/ml DNase I (Roche) in a gently shaking incubator (80rmp) at 37°C for 1h. Cells were then filtered prior to use.

APCs were enriched in blood by using RosetteSep monocyte enrichment kit (StemCell Technologies) according to manufacturer's protocols (**II**). For cell sorting experiments, cells were further purified using positive selection with CD14 microbeads (Miltenyi) and isolated using an autoMACs (**II**) (Miltenyi).

5.8 B CELL ELISPOT

A B cell ELISpot was used to determine the frequency of antigen-specific B cell subsets (**III**). Briefly, ELISpot plates were coated with Pfs25 protein, PBMCs or bone marrow was added to wells directly for enumeration of plasmablasts and plasma cells, or after a four-day stimulation to recall memory B cells. Cells were then cultured overnight at 37°C followed by sequential incubation with biotinylated anti-human IgG (Jackson ImmunoResearch Laboratories), strepatividin-conjugated alkaline phosphatase (Mabtech) and developed using BCIP/NBT substrate (Mabtech). Non-specific spots were subtracted from antigen-specific wells.

5.9 MICROARRAY AND DATA ANALYSIS

Following RNA extraction and microarray, data was filtered to only include genes that were detected in all samples of at least one group and probes without associated GeneID were excluded. Microarray data has been submitted to National Center for Biotechnology Information GEO (accession no. GSE102909). Normalized data was analyzed to identify statistically significant genes using a paired two-tailed Students t-test. Probes were selected for further analysis if fold change > 2 and a raw p-value < 0.01. For functional pathway analysis, all genes were ranked by paired average fold change with respect to baseline for each vaccination group. Pre-ranked GSEA was performed with 1000 permutations using the Java interface (Broad Institute, software.broadinstitute.org/gsea/index/jsp).

6 RESULTS AND DISCUSSION

6.1 CD40AB INDUCES LUNG-RESIDENT T CELL RESPONSES (PAPER I)

As mentioned in the earlier sections, there is a growing need for vaccines that can elicit T cell-based responses. However, there is a considerable gap in knowledge of how to bridge studies performed in mice, which often readily develop CD8 T cell responses, to those in humans, which have been exceedingly difficult to generate robust T cell responses. In my studies, we therefore used the NHP model, whose immune system closely resembles that of humans. Data developed in NHPs should have the potential to be of much more physiological relevance and translational to humans. My first study focused on a vaccine adjuvant combining two components; Poly IC:LC to target TLR3 and an agonistic monoclonal CD40 Ab to target CD40. This adjuvant combination may ultimately be best suited for a therapeutic cancer vaccine with the aim to induce strong T cell responses. However, as a model antigen, we co-delivered the adjuvant with a pool of 6 HIV-1 envelope glycoprotein (Env) peptides, since they have been shown to be immunogenic in rhesus macaques. This model therefore allowed us to investigate each component of the adjuvant individually to determine the requirements for induction of CD8 T cell responses. The immunization was delivered IV to mimic the administration of many therapeutic cancer vaccine strategies.

Similar to what has been reported in mice (Ahonen *et al.* 2004; McWilliams *et al.* 2010; Sanchez *et al.* 2007), we found that both anti-CD40Ab and Poly IC:LC were required to induce potent T cell responses. Either adjuvant component alone only induced low levels of responses, but there was a synergistic effect when combining the two. However, in contrast to the previous studies in mice, we found that regardless of immunization group, there was limited detection of T cell responses in the blood of rhesus macaques. Instead, we found remarkably high frequencies (5-10%) of both CD4 and CD8 vaccine-specific T cells in bronchoalveolar lavage (BAL) of the animals (Figure 15A). Interestingly, CD40Ab and Poly IC:LC were required in the prime immunization to induce CD8 responses in the BAL, as boosting with either anti-CD40Ab or Poly IC:LC could not reprogram T cells to become resident after priming with either of the components alone. While we anticipated that this vaccine led to a general mucosal homing in the rhesus macaque model, we found that the responses were highly compartmentalized to the lung, and were found both in the lung tissue and the BAL (Figure 15B). Vaccine-specific T cells could not be detected at substantial levels in any other organ evaluated. The majority of the antigen-specific T cells in the lung expressed the classical markers CD69 and CD103 expressed by T_{RM} (Figure 15C).

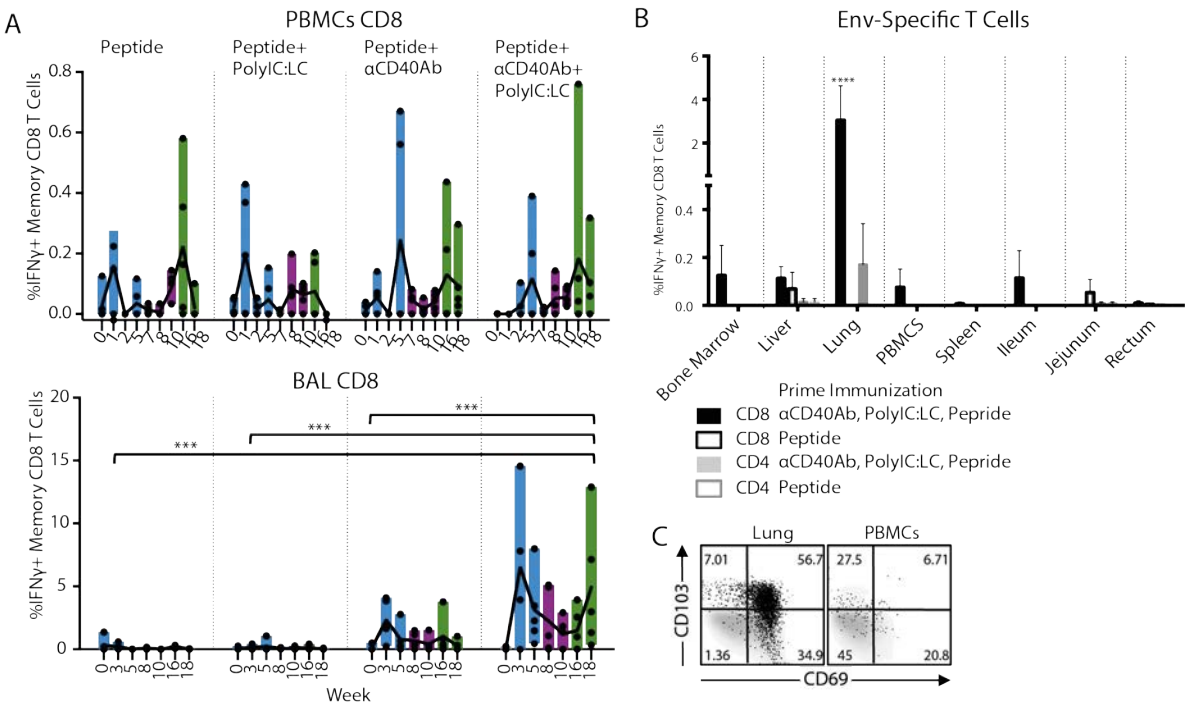

Figure 15: Vaccine-specific CD8 T cell responses in rhesus macaques following immunization with anti-CD40Ab, Poly IC:LC and HIV-1 Env peptides. (A) CD8 responses over time in PBMCs (top) and BAL (bottom) differ depending on prime immunization. (B) CD8 T cell responses in different tissues after boost immunizations. (C) Vaccine-specific T cells in the lung expressed CD103 and CD69.

To try to explain the compartmentalized T cell responses, we evaluated the biodistribution of the anti-CD40Ab, to determine if the adjuvant was also restricted to the lung. Since T cell priming by lung APCs has previously been shown to imprint lung homing (Mikhak *et al.* 2013), we reasoned that localized APC activation and T cell priming could instruct vaccine-specific T cells to home back to the lung. We labeled the Ab with alexa-680 to track the Ab *in vivo* and determine tissue distribution and cell subset targeting (Figure 16). At 24 hours after IV administration, the Ab was widely disseminated and was not preferentially targeting the lung. Therefore, we concluded that this did not explain the restricted T cell responses in the lung and BAL. However, it is possible that had we evaluated the biodistribution at an earlier time point, the lung may have been the first target organ. We also observed that the Ab primarily bound to monocytes and neutrophils, despite their medium-low level of basal CD40 expression. Both these cell populations have the ability to bind antibodies non-specifically via FcR, but can also upregulate CD40 upon immune activation. The immunization induced rapid cytokine secretion and systemic immune cell mobilization and therefore likely upregulated CD40 expression on these subsets.

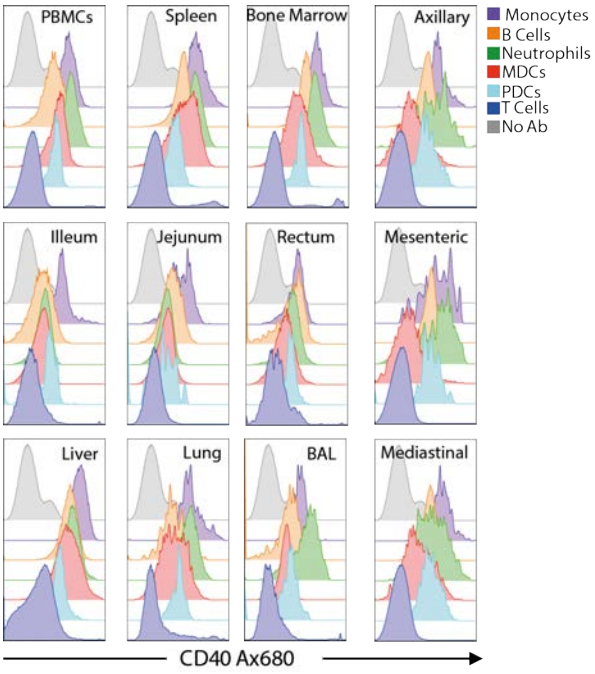

Figure 16: In vivo anti-CD40Ab binding and distribution 24 hours after IV administration in rhesus macaques.

The unexpected finding of T cell responses restricted to the lung following this immunization regimen, indicated that this adjuvant combination may be particularly powerful for tumors in the lung and pathogens targeting the lung. These findings also underscore the importance of evaluating immune responses in tissue in addition to peripheral blood, which is frequently the only sample available in human clinical trials. Without the BAL sampling allowed for in the NHP model, it may have appeared that the combined activation of anti-CD40Ab and Poly IC:LC could not induce robust T cell responses outside of the murine model. This realization initiated a general interest in mucosal biology and the ability to induce tissue homing and residence via vaccination. These questions then resulted in the design of study II.

6.2 IL-10 INDUCES TISSUE-RESIDENT MEMORY T CELLS (PAPER II)

From our findings from Paper I, it was unclear whether the route of administration or inherent signaling due to the anti-CD40Ab/Poly IC:LC adjuvant led to the establishment of highly restricted T_{RM} in the lungs of rhesus macaques. Therefore, this project was initially developed largely to evaluate the requirements for tissue homing. To evaluate the possibility of route of administration in inducing T cell homing to the lung, we expanded on the rhesus macaque model and used two distinct routes of administration to compare the contributions of systemic vs local APCs in T cell priming and education. By immunizing via the IV route as in paper I, or via the SQ route, we could evaluate the innate mechanisms leading to T cell homing to the lung and induction of a T_{RM} phenotype.

We again tracked the biodistribution of the anti-CD40Ab. Following SQ administration, we found that in contrast to IV administration, the Ab stayed highly localized in the skin and the skin draining lymph node, with macrophages and monocytes being the main APCs targeted. Immunizing the animals with the same adjuvant combination and Env peptides from paper I either via the IV or SQ route, we found that both regimens induced antigen-specific T cell homing to the lung (Figure 17A). This indicates that the lung homing ability of the T cells was not strictly a matter of route of administration, as both routes readily induced lung homing. In fact, many vaccine formulations have induced T cell responses in the lung, regardless of immunization route (Darrah *et al.* 2014; Liu *et al.* 2010; Park *et al.* 2013). As the lung is highly vascularized and regularly exposed to environmental threats, this may be an common result of a multitude of APC:T cell interactions and is not limited to education by lung APCs. However, while there were no significant differences in terms of proportion of antigen-specific T cells between the groups, there was a striking difference in the proportion of CD103+ T_{RM} within the antigen-specific population (Figure 17B). IV immunization induced a significantly higher proportion of CD103+ T_{RM} compared to SQ administration. Together this data suggests that multiple routes of administration may induce lung homing, however local signals or early education of the T cell response could alter the ability to take up residence. While the two routes of administration induced largely similar innate profile in terms of cell mobilization and cytokine secretion in the blood, there was a differential production of plasma IL-10 (Figure 17C), which strongly correlated with the proportion of T_{RM} (Figure 17D).

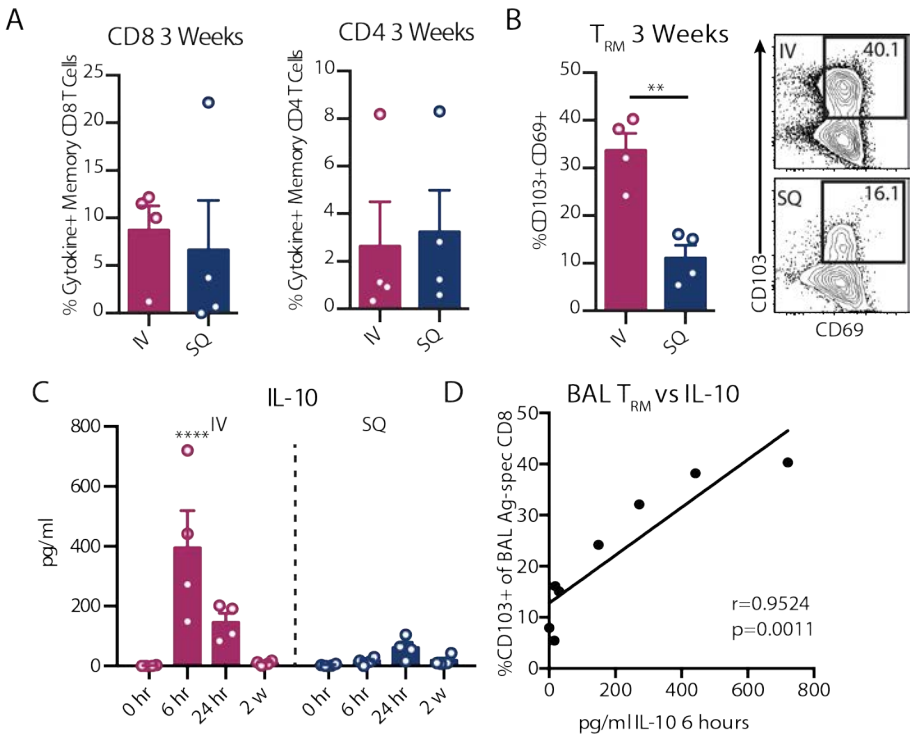

Figure 17: IV administration of anti-CD40Ab/Poly IC:LC/HIV-1 Env peptides induces lung-resident T_{RM} that correlates with systemic IL-10 6 hours after immunization

To be able to address mechanisms behind this finding and establish the connection between IL-10 and induction of T_{RM}, I developed an *in vitro* system using primary human cells. APCs purified from human skin or blood represented cells targeted by the SQ or IV routes of administration, respectively. Following stimulation with anti-CD40Ab and Poly I:C, APCs isolated from all compartments showed the ability to increase naïve CD8 T cell proliferation, however, only the blood APCs could increase CD103 expression on the T cells following stimulation (Figure 18A). Incidentally, blood APCs were also the only cells capable of producing high levels of IL-10 following stimulation, and the increase in CD103 expression could be inhibited with a neutralizing IL-10 Ab or increased with recombinant IL-10 (Figure 18B).

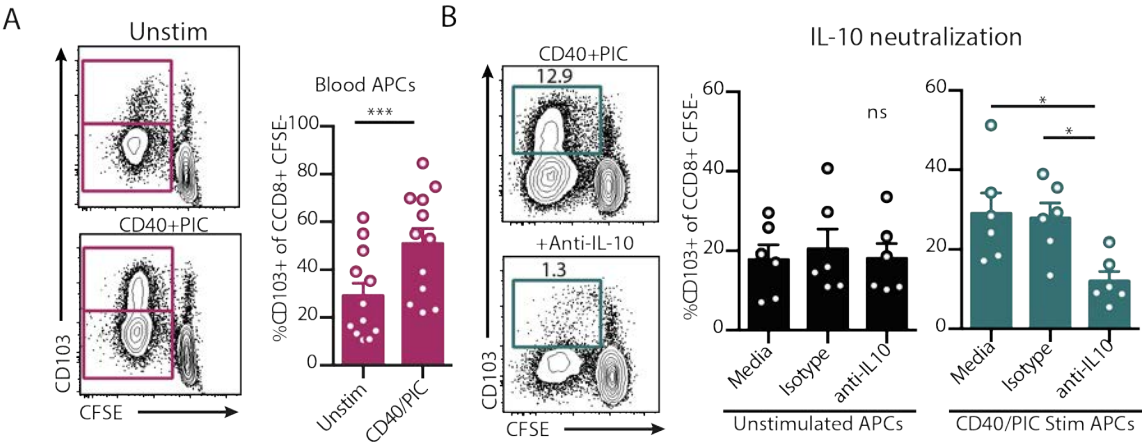

Figure 18: Blood APCs increase CD103 expression on naïve T cells following anti-CD40Ab/Poly IC stimulation in an IL-10-dependent manner.

However, we found that IL-10 did not directly signal through T cells to upregulate CD103, but instead conditioned monocytes to release TGFβ and thereby induce a T_{RM} phenotype. We found that the release of TGFβ from monocytes was increased if the cells were stimulated to produce IL-10. Blocking the IL-10 secretion with an anti-IL10 Ab also inhibited the release of TGFβ (Figure 19A) and adding recombinant IL-10 increased TGFβ secretion (Figure 19B). Active TGFβ constitutively expressed by epithelial cells has earlier been demonstrated to have role in the induction of T_{RM}, and has been implicated in the typical localization of T_{RM} to epithelial compartments. However, many details concerning the events leading to T_{RM} generation and a possible role for TGFβ prior to tissue entry or derived from activated immune cells within the tissue has not been well understood.

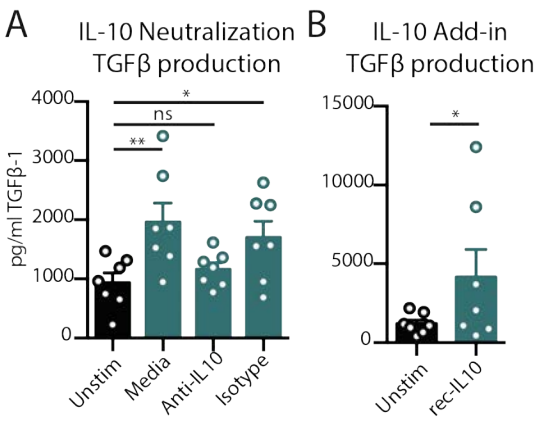

Figure 19: IL-10 mediates TGFβ release.

To begin to understand where and when the influence of IL-10 and TGFβ and interaction between monocytes and naïve T cells could occur, we established a timeline for the kinetics of CD103 upregulation. Naïve T cells exposed to TGFβ from the start of stimulation upregulated CD103 to the highest extent and could upregulate CD103 over several days. Further, naïve T cells primed in the presence of TGFβ were more susceptible to subsequent TGFβ signaling (Figure 20), indicating that priming in a lymph node in the presence of an IL-10 activated monocytes could already destine T cells to be more responsive to the tissue microenvironment (Figure 21).

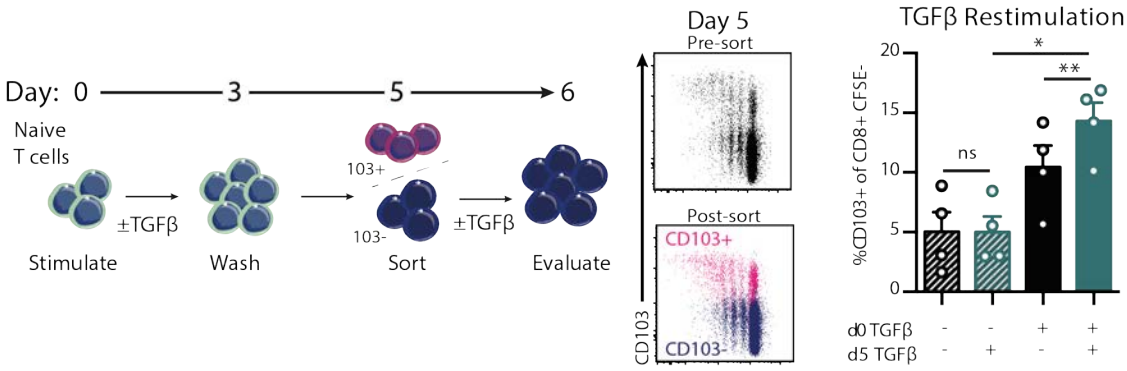

Figure 20: Naïve T cells primed in the presence of TGFβ are more susceptible to subsequent TGFβ signaling.

These findings indicate a possible mechanism for the recent success of recombinant IL-10 as an anti-tumor therapy and outline a new pathway to target with adjuvants for the induction of T_{RM}. Since multiple TLR ligands can induce IL-10, there are several possibilities for clinical implementation, including TLR-based adjuvants such as R848 or MPL and delivery of recombinant IL-10. These strategies could be employed with so-called "prime and pull" methods to target specific tissues as needed for immunotherapies or preventative vaccines.

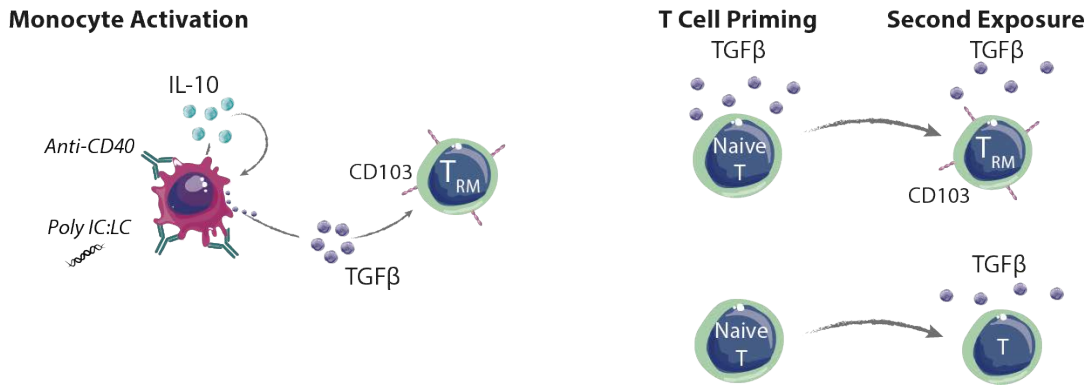

Figure 21: Summary of paper II. Activated monocytes produce IL-10 inducing the release of TGFβ, which in turn upregulates CD103 on naïve T cells and makes cells more responsive to late TGFβ signaling.

6.3 TLR-BASED ADJUVANT VACCINES DIFFERENTIALLY IMPRINT VACCINE RESPONSES (PAPER III)

In the third study in my thesis, the focus was again on how innate immune activation induced via different TLR-based adjuvants influences vaccine responses. Here, we took a broader approach to evaluate multiple arms of the immune response to find correlates of vaccine immunity. In a large preclinical vaccine study in rhesus macaques, I had the opportunity to side-by side compare how adjuvants targeting either TLR4, TLR7/8 or TLR9 influenced different aspects of innate immune activation and in turn polarized the humoral as well as cellular vaccine responses (Figure 22).

Group	# of Animals	Immunization	Route
1	6	SVP[Pfs25]+SVP[CpG]	SQ
2	6	SVP[Pfs25]+SVP[R848]	SQ
3	6	SVP[Pfs25]+GLA-LSQ	SQ
4	6	Pfs25-EPA+GLA-LSQ	IM

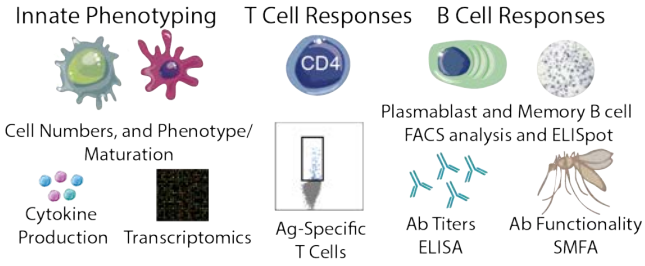

Figure 22: Immunization groups in paper III and immunological parameters evaluated.

An overall aim of the study was to evaluate the potency of a novel malaria vaccine that had been adjuvanted with the different TLR-agonists. As mentioned in the introduction, TBVs represent a key strategy to eradicate malaria. A major challenge in the development of such vaccines has been that the strong Ab titers induced in mice did not translate into successful clinical vaccine trials (Talaat *et al.* 2016; Wu *et al.* 2008). The leading antigen candidate, Pfs25, is poorly immunogenic on its own, although several studies in mice have demonstrated that multimerization of the protein greatly improves the vaccine responses. Despite this preclinical work, Pfs25-based vaccines have failed in clinical trials, indicating that more modern approaches to vaccine formulation are necessary. Further, mechanistic insights into how Pfs25 based immunity can be improved have been sorely lacking. We therefore utilized novel polymer-based nanoparticle vaccine formulations and multiple TLR-based adjuvants to overcome the poor immunogenicity of Pfs25. We tested these formulations in rhesus macaques and benchmarked against the most advanced formulation currently in clinical trials, Pfs25-EPA. Since transmission-blocking vaccines work via production of antibodies, the major focus was on the magnitude and longevity of the humoral response. While T cell responses and innate activation would have no direct protective effect, the study still offered an

opportunity to measure these parameters and correlate their levels with the magnitude and durability of the Ab response. These findings are important not only for transmission-blocking vaccines, but are also applicable to various non-live platforms. We therefore used data generated from the wide array of immunological assays performed in this study to perform multiparameter correlation followed by hierarchical clustering (Figure 23). This was a way to visualize how different arms of the immune response correlated with each other, and generated three primary clusters, that will be discussed in more detail below.

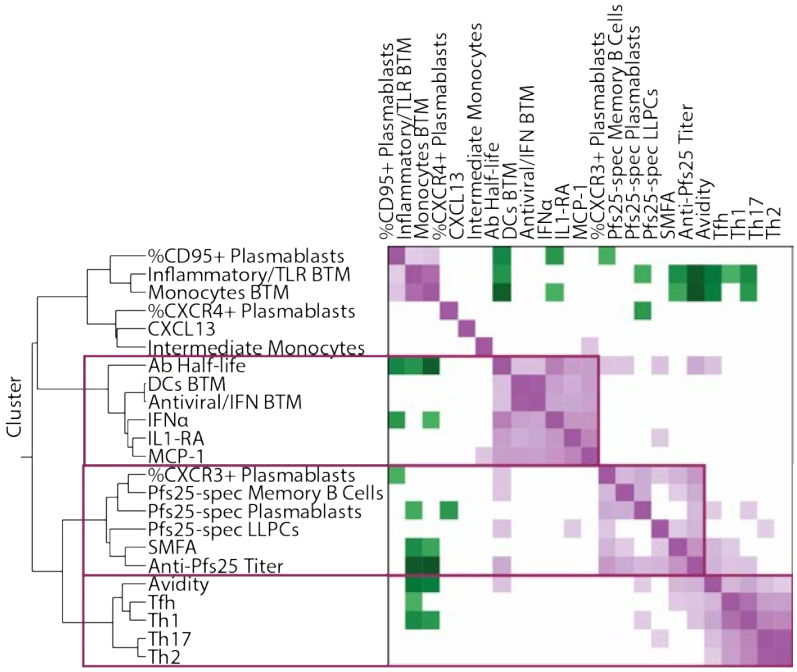

Figure 23: Parameters measured in the study at peak responses analyzed by multivariate nonparametric Spearman's test and hierarchical clustering. The three primary clusters identified are marked.

By following the animals long-term, we could demonstrate that the nanoparticle formulation increased Ab titers and transmission reducing activity in mosquitos (Figure 24A-B), which correlated with increased induction of vaccine-specific B cell subsets including plasmablasts and memory B cells in the blood, and plasma cells in the bone marrow (Figure 24C-E). Since the nanoparticles were not specifically engineered to express Pfs25 on the outside in a way that was optimal for BCR cross linking, we suspect the increased Ab titers and B cell responses were instead driven by increased trafficking to the lymph node and uptake by APCs. Interestingly we also found that the adjuvants had differing efficacies in the prime vs boost setting. While GLA-LSQ, the TLR4 ligand, showed a superior ability to prime responses, both CpG and R848 were better at boosting preexisting memory. This may a result of the TLR expression on B cells. Although B cells express TLR4 in mice, in humans and NHPS they express low levels or non-functional expression of TLR4. In contrast, B cells in humans and NHPs express both TLR7/8 and TLR9, and could therefore be boosted directly with R848 and CpG.

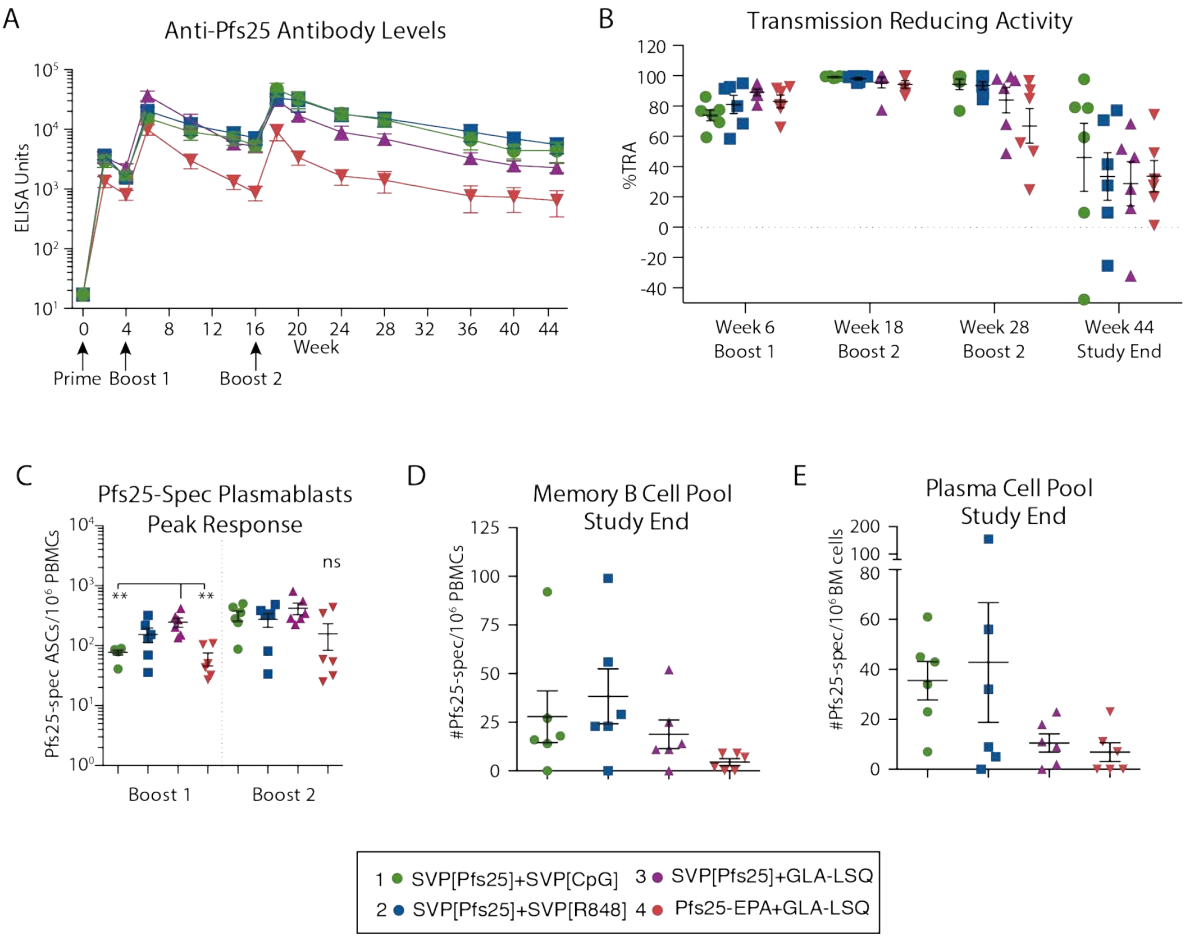

Figure 24: Ab titers and B cell responses following immunization. (A) IgG Ab titers against Pfs25 over course of the study. (B) Transmission reducing activity as assessed by percent reduction of oocyst count in mosquito midgut with the addition of plasma from immunized animals. (C-E) Pfs25-specific B cell subsets as determined by ELISpot.

Targeting distinct TLRs via the three different adjuvants induced differential innate immune profiles in terms of cytokine production and gene modulations (Figure 25A-B). We found that adjuvants containing R848 and CpG induced a more robust innate activation that GLA-LSQ. The increased innate activation was notably seen in the skewing of cytokine production, where R848 in particular induced high levels of IFNα production and gene modulation (Figure 25A-B). The IFNα signature at the protein and gene level showed strong correlations with a substantially increased Ab half-life (Figure 25C), indicating that early innate education may not necessarily influence the magnitude of the Ab response, but could potentially alter the longevity of the response or even alter the imprinted lifespan of the LLPC. IFNα has been shown to directly stimulate B cell proliferation and promote survival through resistance to Fas-mediated apoptosis. Therefore, by promoting B cell survival, IFNα could possibly increase the ability to take up residence as a LLPC and increase Ab half-life.

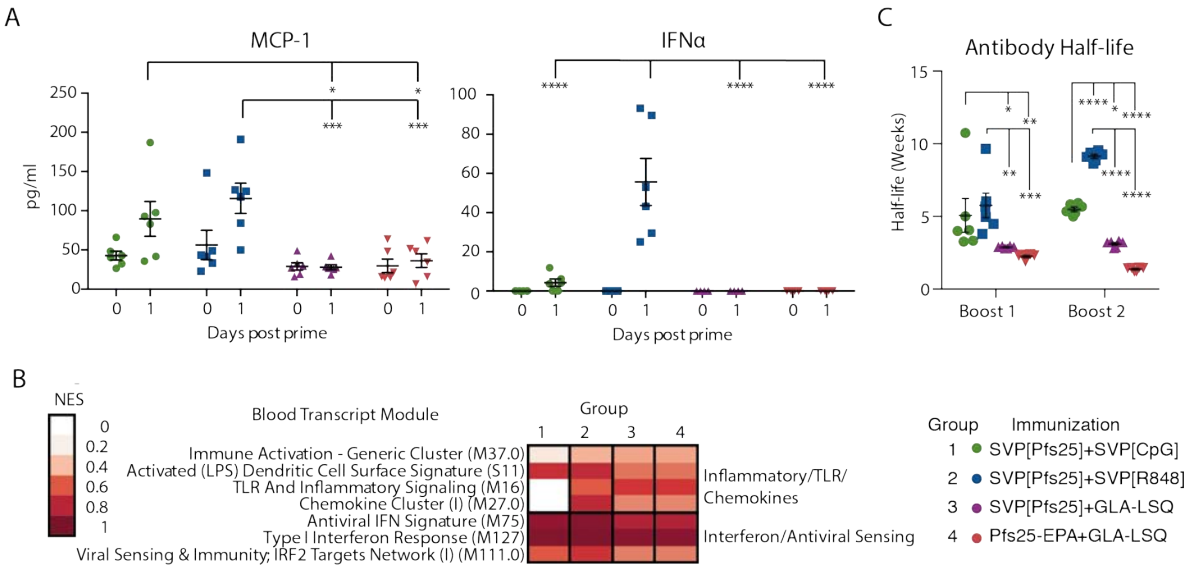

Figure 25: Innate immune profile 24 hours after immunization correlates with Ab half-life. (A) Plasma cytokine levels. (B) Innate gene modulation associated with inflammation/TLR signaling/chemokines and interferon/antiviral sensing as determined by Gene Set Enrichment Analysis using blood transcript modules. Shown is normalized enrichment score (NES). (C) Ab half-life after first and second boost, calculated using biphasic model.

Previous vaccination efforts against Pfs25 had not been able to generate detectible Pfs25-specific CD4 T cell responses, which could help explain the short-lived responses generated in clinical trials. We found that the nanoparticle formulation tested here could overcome the poor immunogenicity of Pfs25 and generated the first reported Pfs25-specific CD4 T cell responses (Figure 26A). The T cells were phenotyped based on their ability to produce different cytokines following antigen restimulation *in vitro*. The responding T cells predominantly produced IFNγ, indicating a T_H1 phenotype, or IL-21, indicating circulating T_{FH} cells. As discussed previously, T_{FH} are a highly specialized CD4 T cell that provide B cell help. T_{FH} found in the lymph node produce high levels of IL-21 and have been correlated with increased B cell activity and Ab levels following vaccination. However, removing lymph nodes during an ongoing immune response may negatively influence ensuing responses, therefore ways to evaluate T_{FH} and germinal center activity from peripheral samples have been established (Havenar-Daughton *et al.* 2016b, 2016a; Lindgren *et al.* 2017; Locci *et al.* 2013). Circulating T_{FH} have been evaluated based on a variety of cell surface markers or IL-21 production and have been shown to correlate with the level of T_{FH} in the lymph node and share a common clonality, making them a feasible readout for T_{FH} activity during vaccine trials. Both T_H subsets correlated with increased IgG Ab avidity (Figure 26B). This is likely due to increased T cell help and activation within the germinal center, leading to higher affinity antibodies. In line with this, we also saw higher levels of CXCL13 (Figure 26C), a chemokine involved in the organization of germinal center structures, which has been suggested as a plasma biomarker for germinal center activity, in groups receiving the nanoparticle formulation. Together, these data suggest that the PLGA-based nanoparticles increased Ab affinity and avidity by engaging T cell help. This is possibly mediated by enhanced antigen uptake by APCs typically seen with nanoparticles.

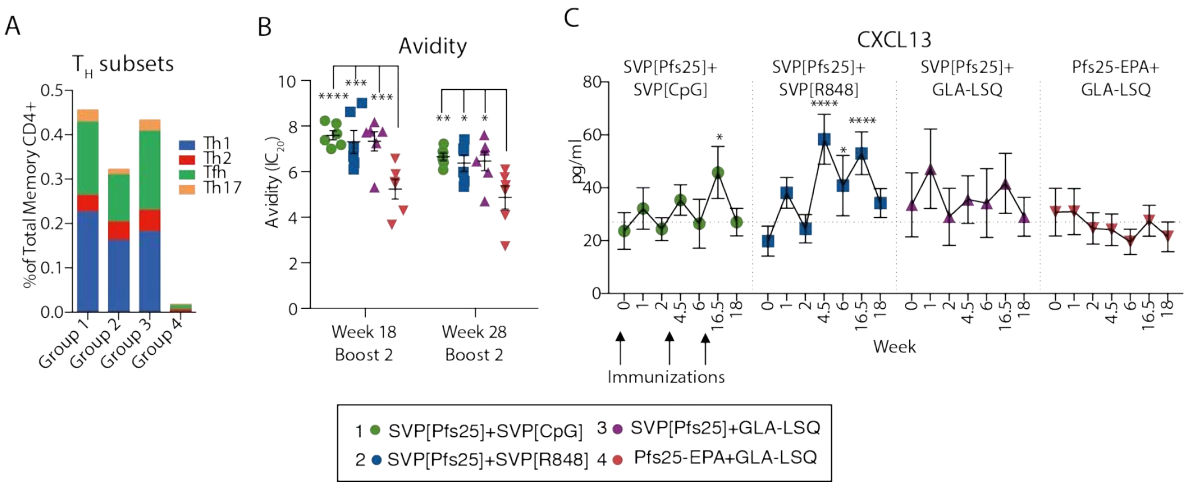

Figure 26: Induction of T_H subsets correlates with Ab avidity and germinal center activity. (A) Pfs25-specific T_H cells induced by nanoparticle formulation. (B) Ab avidity as determined by modified urea ELISA. (C) Germinal center activity as measured by plasma CXCL13.

Taking this type of broad approach to evaluate the generation of vaccine immunity allowed us to draw correlations between multiple arms of the immune system (Summarized in Figure 27). One of the more interesting findings was the potential relationship between IFNα produced early after immunization with the longevity of the Ab response. As this is a major goal of vaccination efforts, this finding can help guide future adjuvant design and help the field to refine future vaccine formulations. This may also help explain the highly successful nature of the yellow fever vaccine, which also generates a strongly IFNα skewed innate immune response. Future efforts can use the correlations described here to delve deeper into the specific mechanisms of enhanced immune responses and to evaluate the feasibility of clinical implementation.

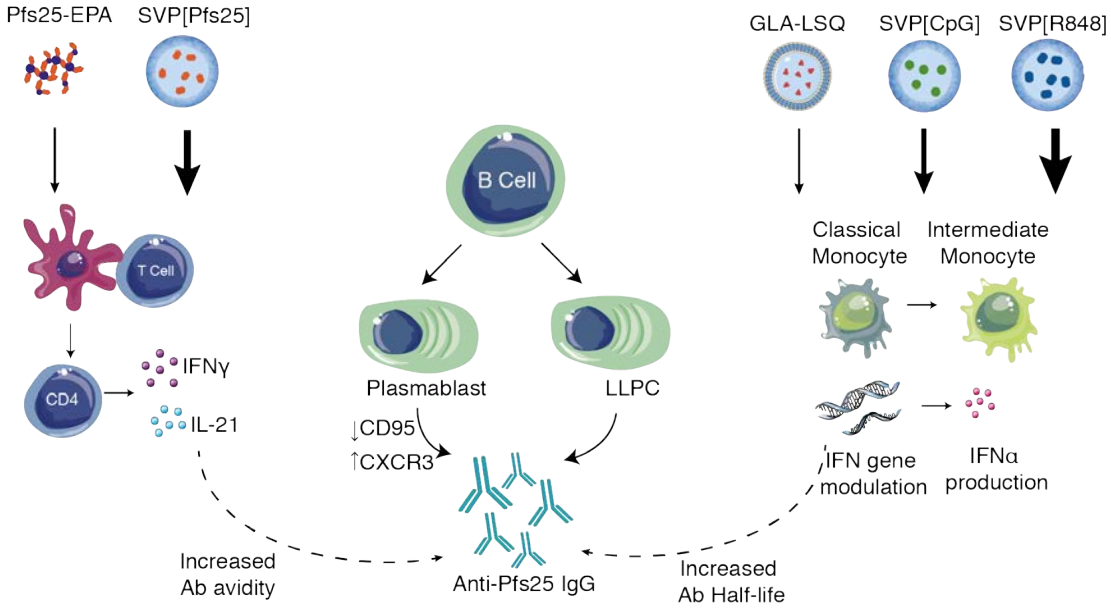

Figure 27: Overview of findings from study III. Formulation of Pfs25 in nanoparticles increased circulating T_H1 and T_{FH} Pfs25-specific CD4 T cells compared to Pfs25-EPA. Increased T cell responses correlated with increased titers and Ab avidity. TLR targeting adjuvants showed differing abilities to stimulate innate immune responses, R848 induced robust IFNα production, which correlated with increase Ab half-life. Plasmablast phenotype was also differentially regulated by vaccine formulation with CXCR3+ and CD95- cells correlating with the highest Ab titers.

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