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# IMMUNE MECHANISMS THAT DETERMINE THE QUALITY OF ANTIBODY RESPONSES TO VACCINES

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# IMMUNE MECHANISMS THAT DETERMINE THE QUALITY OF ANTIBODY RESPONSES TO VACCINES

## THESIS FOR DOCTORAL DEGREE (Ph.D.)

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*"Science is more than a body of knowledge. It is a way of thinking; a way of skeptically interrogating the universe with a fine understanding of human fallibility.*

*If we are not able to ask skeptical questions, to interrogate those who tell us that something is true, to be skeptical of those in authority, then, we are up for grabs…"*

Carl Sagan
*May 27th 1996*

# ABSTRACT

Effective vaccines for many dangerous infectious diseases are still lacking. An improved understanding of the mechanisms that determine the quality of vaccine responses is essential to guide future attempts to design effective vaccines. This thesis aimed to characterize the early and late immune responses of different clinically relevant vaccine platforms. All studies were performed in the non-human primate (NHP) model due to their physiological and immunological similarities to humans.

Protein subunit vaccines are commonly used when live attenuated vaccines are unsafe. Adjuvants are often needed in protein subunit vaccines to stimulate sufficiently potent immune responses. In **paper I** we developed an in vivo model to examine the initial immunological events after vaccine administration into the muscle. Specifically, we studied the mechanisms of the adjuvants Alum, MF59 and Alum-TLR7. We found that all adjuvants induced rapid infiltration of immune cells at the site of vaccine injection. Alum-TLR7 and MF59 showed stronger adaptive immune profiles compared to Alum, including higher antibody titers, better neutralization and a stronger germinal center (GC) response. However, their innate immune activation was very different. Alum-TLR7 exclusively induced a type I interferon response and excelled at dendritic cell maturation while MF59 promoted neutrophil recruitment to the vaccine draining lymph nodes (LNs).

In **paper II-III** we studied mRNA-based vaccines which have emerged as promising candidates against several diseases including Zika and influenza. In **paper II** we used fluorescently labeled lipid nanoparticles (LNP) encapsulating mRNA encoding for the fluorescent protein mCitrine, for tracking of vaccine uptake and mRNA translation separately. We found a rapid infiltration of immune cells to the site of injection as well as uptake and translation of the mRNA into antigen with both intradermal and intramuscular immunizations. A strong type I interferon response was induced at the site of injection and the draining LNs. Priming of T cells occurred in the vaccine-draining LNs. In **paper III** we investigated the adaptive immune profile of a mRNA vaccine encoding influenza hemagglutinin. Immunization induced a quick B cell response including vaccine-specific memory B cells in blood and plasma cells in the bone marrow. Formation of GCs was detected in the vaccine draining LNs. Circulating vaccine-specific $CXCR3^+$ T follicular helper cells, associated with a Th1 response, transiently appeared in the blood and correlated with antibody avidity in serum.

Finally, in **paper IV,** we focused on the methodological advancements of GC analysis. LNs were divided in two and each half was analyzed by either immunohistology or flow cytometry. We propose a method to analyze and present GC data to make a direct comparison possible between studies regardless of the technique. Altogether, the data presented in the thesis will add to the understanding of how vaccine responses are initiated and regulated. Ultimately this will help in the development of new or improved vaccines.

# LIST OF SCIENTIFIC PAPERS

I. Frank Liang, **Gustaf Lindgren**, Kerrie J. Sandgren, Elizabeth A. Thompson, Joseph R. Francica, Anja Seubert, Ennio De Gregorio, Susan Barnett, Derek T. O'Hagan, Nancy J. Sullivan, Richard A. Koup, Robert A. Seder, Karin Loré

Vaccine priming is restricted to draining lymph nodes and controlled by adjuvant-mediated antigen uptake

*Science Translational Medicine 2017 Jun 7;9(393)*

II. Frank Liang, **Gustaf Lindgren**, Ang Lin, Elizabeth A. Thompson, Sebastian Ols, Josefine Röhss, Shinu John, Kimberly Hassett, Olga Yuzhakov, Kapil Bahl, Luis A. Brito, Hugh Salter, Giuseppe Ciaramella, Karin Loré

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)*

III. **Gustaf Lindgren**, Sebastian Ols, Frank Liang, Elizabeth A. Thompson, Ang Lin, Fredrika Hellgren, Kapil Bahl, Shinu John, Olga Yuzhakov, Kimberly J. Hassett, Luis A. Brito, Hugh Salter, Giuseppe Ciaramella, Karin Loré

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 Nov 13;8(1539)*

IV. **Gustaf Lindgren**, Sebastian Ols, Elizabeth A. Thompson, Karin Loré

Comparative Analysis of the Germinal Center Response by Flow Cytometry and Immunohistology

*Manuscript*

# PUBLICATIONS NOT INCLUDED IN THESIS

I. Paola Martinez-Murillo*, Karen Tran*, Javier Guenaga, **Gustaf Lindgren**, Monika Àdori, Yu Feng, Ganesh E. Phad, Néstor Vázquez Bernat, Shridhar Bale, Jidnyasa Ingale, Viktoriya Dubrovskaya, Sijy O'Dell, Lotta Pramanik, Mats Spångberg, Martin Corcoran, Karin Loré, John R. Mascola, Richard T. Wyatt*, Gunilla B. Karlsson Hedestam*
* These authors contributed equally

Particulate array of well-ordered HIV clade C Env trimers elicits neutralizing antibodies that display a unique V2 cap approach

*Immunity 2017 May 16;46(5)*

II. Ang Lin, Frank Liang, Elizabeth A. Thompson, Maria Vono, Sebastian Ols, **Gustaf Lindgren**, Kimberly Hassett, Hugh Salter, Giuseppe Ciaramella, Karin Loré

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)*

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

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

*Journal of Immunology 2015 Aug 1;195(3)*

IV. Frank Liang, Aurélie Ploquin, José DelaO Hernández, Hugues Fausther-Bovendo, **Gustaf Lindgren**, Daphne Stanley, Aiala Salvador Martinez, Jason M. Brenchley, Richard A. Koup, Karin Loré, Nancy J. Sullivan

Dissociation of skeletal muscle for flow cytometric characterization of immune cells in macaques

*Journal of Immunological Methods 2015 Oct Vol 425 Pages 69-78*

# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| AID | Activation-induced deaminase |
| APC | Antigen presenting cell |
| BAFF | B cell-activating factor |
| BCR | B cell receptor |
| BM | Bone marrow |
| DC | Dendritic cell |
| cDC | Classical dendritic cell |
| Env | Envelope glycoprotein |
| FDC | Follicular dendritic cell |
| GC | Germinal center |
| GLA | Glucopyranosyl Lipid A |
| HA | Hemagglutinin |
| HIV | Human immunodeficiency virus |
| ICOS | Inducible T cell costimulator |
| ID | Intradermal |
| IFN | Interferon |
| IL | Interleukin |
| Ig | Immunoglobulin |
| IM | Intramuscular |
| LN | Lymph node |
| LNP | Lipid nanoparticle |
| MHC | Major histocompatibility complex |
| MPL | Monophosphoryl Lipid A |
| NHP | Non-human primate |
| PBMC | Peripheral blood mononuclear cell |
| PD1 | Programmed death-protein 1 |
| PDC | Plasmacytoid dendritic cell |
| SAP | SLAM associated-protein |
| SHM | Somatic hypermutation |
| TCR | T cell receptor |
| TLR | Toll-like receptor |
| $T_{FH}$ | T follicular helper cell |
| $cT_{FH}$ | Circulating T follicular helper cell |
| Th | T helper |

# 1 INTRODUCTION TO VACCINES

## 1.1 HISTORY OF VACCINES

Vaccines are among the greatest medical interventions in history to transform global health. Edward Jenner (1749-1823) revolutionized health care by learning that milk maidens infected by cowpox were resistant to smallpox (1). In one of the first documented vaccinations, Edward Jenner inoculated humans with cowpox to induce resistance to smallpox, a pivotal event for the field of immunology (1, 2). In the 19th century, another major discovery was made by Louis Pasteur (1822-1895) who introduced the "Germ Theory of Disease", replacing the previous theory of "Spontaneous generation". Within the new paradigm, where microorganisms caused disease instead of vice versa, strategies to prevent infection by microorganisms could be implemented. Emile Roux (1853-1933), Pasteur's assistant and successor, later discovered toxins from diphtheria as an agent of disease, an important finding for future toxoid vaccines. Two years later Emil Von Behring (1854-1917) and Shibasabura Kitasato (1853-1931) developed a treatment for diphtheria, based on serum from horses immunized with attenuated diphtheria bacteria (3, 4). Kitasato and Behring were the first to introduce the concept of antibodies and the latter was awarded the Nobel Prize in 1901 for the new treatment of diphtheria. Collectively, these discoveries, among others, laid the ground for the main strategy of vaccine development; isolate the microbe or its toxins, prepare vaccine formulations with antigens of said microbe or toxin, and administer them to induce protective antibodies. Early success with this strategy was tremendous with a substantial decline in many diseases, including the historical accomplishment of smallpox eradication in 1980 (5). Yet there are still many infectious diseases for which there are no effective vaccines, such as tuberculosis, malaria and human immunodeficiency virus (HIV). To combat these diseases, an improved understanding of the mechanisms of action by vaccines is needed to facilitate rational development of the next generation of vaccines.

## 1.2 TYPES OF VACCINES

There are currently many different types of vaccines that all work by activating and expanding cells with the antigen-specificity of interest (*3*). Although new promising vaccines that function by generating cytotoxic CD8 T cells are being developed, most vaccines work by eliciting protective antibodies (*6*). Differently formulated vaccines are grouped into vaccine "platforms" with their own distinct advantages, these are summarized in Figure 1. Live attenuated vaccines are whole pathogens that have been weakened to reduce proliferation and infectious potential in humans. The rubella and yellow fever vaccines are examples of live attenuated formulations. Such vaccines most closely resemble natural infection and have thus far proven to be the most effective means of vaccination, requiring few if any boost vaccinations, along with high protection (*7*). However, live attenuated vaccines pose the risk of reverting back to a pathogenic form and therefore cannot be used

against pathogens such as HIV and Ebola. Additionally, they cannot be used in immunocompromised individuals due to safety reasons. Another problem with vaccines containing live pathogens is the need for cold-chain storage, making the distribution of the vaccine exceedingly difficult (*8*).

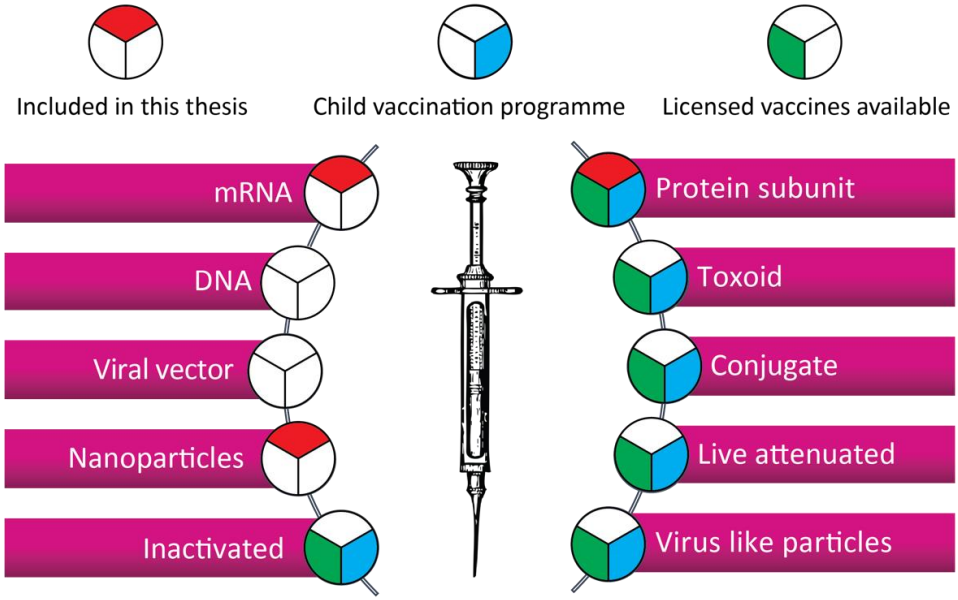


*Figure 1: Vaccine platforms and their current application in humans.*

Inactivated vaccines are produced by destroying the pathogen with radiation, heat or chemicals, rendering the pathogen harmless while keeping intact the antigens recognized by the immune system. Examples of such vaccines are influenza, Hepatitis A and poliovirus vaccines (*9*). Conjugate vaccines are designed from the bacterial coat and are co-administered with a carrier protein. A common bacteria, Streptococcus pneumoniae, is the target of such vaccines (*10*). Vaccination with weakened toxins prevents toxin-induced mortality and morbidity, one such being neurotoxin produced by Clostridium tetani (*10*). A recent vaccine platform licensed for human use is virus-like particle vaccines. They are made out of proteins that fold together into spheres that closely resemble the virus, but are without genomic material and are therefore incapable of proliferation. Virus-like particle vaccines were first licensed against Hepatitis B followed by human papillomavirus (*11*).

This thesis contains studies of vaccines against HIV and influenza. The main purpose of these studies was to gain a more detailed understanding of the immunological mechanisms of the different vaccine platforms used. In study I, protein subunit vaccines were used. Such vaccines contain only parts of the pathogen and therefore lack other microbial immunostimulators. Adjuvants, from Latin “to help”, are used as artificial immunostimulators to improve vaccine efficacy (*12*). Examples of protein subunit vaccines are annual influenza vaccines and HIV candidate vaccines. In study II and III mRNA vaccines were investigated. mRNA vaccines belong to a family of emerging vaccines that are not yet commonly used in a clinical setting but show great potential in scientific research and clinical trials (*13*).

# 2 AIMS OF THESIS

The general aim of this thesis was to better understand the innate and adaptive immune response induced by different vaccine platforms. Information on the type of immune response induced by vaccines is essential for their future improvement. The specific aims were:

**Paper I:** To investigate the innate and early adaptive immune response induced by three clinically relevant adjuvants; Alum, Alum-TLR7 and MF59

**Paper II:** To study the early immune response to mRNA vaccines

**Paper III:** To study the adaptive immune response to a mRNA vaccine encoding influenza hemagglutinin (HA)

**Paper IV:** To develop a method for immunohistological analysis of germinal centers that is comparable to flow cytometry

# 3 IMMUNOLOGY

## 3.1 INTRODUCTION

Our immune system has developed through the trial and error of evolution by natural selection to recognize harmless self from harmful non-self and self. The latter two include pathogens such as bacteria and viruses, as well as endogenous cells that are damaged or have turned cancerous. The capacity of the immune system to effectively recognize and neutralize a countless variety of such threats is a marvelous feat of nature. To achieve this, the immune system is equipped with two main pathways of pathogen recognition that can be broadly divided into innate and adaptive immunity (*14*). The innate immune system functions primarily by a set of receptors against common antigens of pathogens, an evolutionary memory against pathogens that have co-evolved with us (*15*). However, in an ever-changing environment, new pathogens can arise at any time, and current pathogens continuously develop new strategies to evade our fixed means of pathogen recognition. A prime example of this is the HIV, which hides its epitopes, i.e. the part of its antigens which is being recognized by the immune system, by covering them with non-immunogenic glycans. Adaptive immunity provides an alternative strategy by attempting to detect any structure that is not considered self and react to it if necessary. This process is slower since there can only be very few cells specific against any potential antigen. However, it is also more flexible since these cells can rapidly expand to large numbers of effector and memory cells with an incredible capacity to neutralize dangerous pathogens.

## 3.2 INNATE IMMUNITY

Innate immunity provides the first line of defense against invading pathogens at the sites where pathogens are usually encountered; mucosal tissues, skin and airways (*14*). Since the probability of encountering pathogens at these sites is high, more immune cells reside at these sites than in tissues that are less likely to encounter antigens, such as muscles (*16*). Additionally, innate immune cells are important for maintaining homeostasis within the body, contributing to both tissue repair and the control of commensal microbes (*15*).

The innate immune system detects and neutralizes pathogens via fixed systems consisting of cells, complement proteins and antimicrobial peptides (*17*). Pattern recognition receptors (PRRs) on innate immune cells recognize common features of pathogens. Activation of PRRs leads to the release of proinflammatory cytokines, including TNF and interleukin-6 (IL) (*18*). There are 4 main families of PRRs: Toll-like receptors (TLRs), RIG I-like receptors, nucleotide-binding oligomerization-like receptors and C-type lectin receptors (*19*). Depending on which PRR is activated, different innate immune responses are initiated to efficiently combat the invading pathogens. This also shapes adaptive immunity by the type of cytokines that are produced. Due to their ability to induce inflammation and shape adaptive immunity, the ligands for PRRs are utilized as vaccine adjuvants.

The initial recognition of pathogens by PRRs leads to the initiation of inflammation which is clinically characterized by rubor (dilation of blood vessels), tumor (swelling), dolor (pain), calor (temperature increase due to an influx of blood) and function laesa (loss of function). Dilation of blood vessels and the production of chemokines lead to extravasation of innate immune cells to the site of inflammation. The newly arrived innate immune cells produce more cytokines to amplify the immune response. Neutrophils and monocytes from circulation (or tissue resident macrophages) infiltrate first and use scavenger receptors to phagocytose microorganisms and degrade them (*20*). Neutrophils can also release their granules containing antimicrobial products and neutrophil extracellular traps consisting of DNA to neutralize invading pathogens (*21*). Of particular importance are antigen presenting cells (APCs) loaded with antigen, which is presented on the cell surface to the cells of the adaptive immune system. The cells most efficient at bridging the innate and adaptive immune system are professional APCs such as dendritic cells (DCs).

## 3.3 DENDRITIC CELLS

DCs are often called the sentinels of the immune system. They are specialized at taking up, processing and presenting antigens (*22*). There are two main categories of DCs; myeloid classic DCs (cDCs) efficient at antigen presentation and plasmacytoid DCs (PDCs) which excel at producing type I interferons (IFN) in response to viruses.

cDCs are a heterogeneous group of cells that reside in most tissues (*23*). Apart from the tissue-resident DCs such as Langerhans cells, cDCs and can be divided in two main subsets: cDC1s characterized by their expression of Batf3, XCR1 or CD141 and cDC2s characterized by their expression of IRF4, CD172 or CD1c (*23*). Traditionally cDC1 have been considered to excel at antigen cross-presentation over cDC2s, but these differences might not be as profound in humans as in mice (*24*). Generally, cDC1s are believed to participate mainly in responding to intracellular pathogens, while cDC2s respond to intra- and extracellular pathogens (*23*). cDCs express a large variety or PRRs to sense injury and pathogenic microbes. cDCs orchestrate the immune system by inducing tolerance to self-antigens and by the activation of naïve T cells. There are several factors contributing to their excellent ability at performing these tasks including: Their strategic localization in both lymphoid and non-lymphoid organs with a constant supply of self and foreign antigens, their high capacity for antigen processing and presentation (*25, 26*), their ability to migrate with antigen to draining LNs (*27*) and the fact that they excel at priming naïve T cells (*28*). Due to their unique ability to excel at activating T cells and tailoring them to combat specific kinds of threats, DCs are one of the main cell types of interest in the study of vaccines.

PDCs reside mainly in blood and LNs. They differ from cDCs in that they are not efficient at taking up antigen and they express low levels of major histocompatibility complex (MHC) class II and co-stimulatory molecules (*29*). Also unlike cDCs that express a wide range of

TLRs, PDCs mainly express TLR7/9. Although PDCs are very scarce compared to cDCs, they make up for their low numbers by their unique capability to produce vast amounts of type I IFNs. This provides protection against intracellular pathogens and skews the adaptive immune response towards an intracellular adapted T helper 1 (Th1) profile (*30*).

## 3.4 ADAPTIVE IMMUNITY

Pathogens that evade our innate immune mechanisms are often cleared by our adaptive immune response, which in contrast to the innate immune response is flexible rather than fixed (*14*). The primary cells of adaptive immunity, B cells and T cells, randomly generate unique receptors on their surfaces that specifically target specific epitopes of antigens (*14*). B cells and T cells specific for self-antigens are deleted through central and peripheral tolerance mechanisms. If these mechanisms fail, autoimmune diseases arise (*31*). Upon detection of their cognate antigen and proper stimulation, B cells or T cells are activated and proliferate. Once the infection is cleared some of the expanded cells will persist as immunological memory or effector cells to enhance protection from reinfection of the same pathogen. The process of clonally expanding B cells and T cells specific against vaccine antigens is the central mechanism of vaccination (*32*).

Adaptive immunity is broadly divided into humoral and cellular immunity. Cellular immunity is mediated by CD8+ T cells that kill infected cells and $CD4^+$ helper T cells that exhibit helper functions to support innate cells, CD8+ T cells and B cells. While CD8+ T cells are generally not the mediators of vaccine-induced protection, B cells are central (*32*). $CD4^+$ T cells are therefore crucial for vaccine responses due to their ability to help B cells. B cells are the effector cells of humoral immunity and produce antibodies, also called immunoglobulins (Ig) (*33*). Upon activation, B cells differentiate into memory B cells or plasma cells that are capable of producing and secreting large quantities of antibodies (*34*). Antibodies will bind to their specific antigen and exert one of several functions depending on the type of antibody, including opsonization by phagocytes, activation of the complement cascade or antigen-dependent cell-mediated cytotoxicity (*35*). Almost all vaccines function by eliciting durable antibody titers that protect from infection or severe disease. Sustainable antibodies produced by long-lived plasma cells residing in the bone marrow (BM) are thus critical for maintaining long-term protection after vaccination (*33*).

## 3.5 INTERACTION BETWEEN INNATE AND ADAPTIVE IMMUNITY

A core theme of this thesis is the co-operation between innate and adaptive immunity. Such co-operation is eloquently displayed by the activation of complement (innate immunity) by antibodies (adaptive immunity), to assemble the membrane attack complex (*35*, *36*). Innate immunity also functions to initiate the adaptive immune response by providing antigens and the right inflammatory signals. Naïve B cells and T cells originate in the BM and mature in the BM or thymus respectively before entering circulation (*14*). Guided by the chemokine receptors such as CCR7 and CXCR5 they migrate from the circulation to secondary lymphoid

organs in search of their cognate antigen (*37*, *38*). However, the cognate antigen must first arrive at secondary lymphoid organs to be given to B cells and presented to T cells by innate immune cells. Once DCs have taken up antigen in the periphery, they downregulate chemokine receptors such as CCR1 and CCR5 and upregulate CCR7 to migrate towards draining LNs (*39*). DCs present processed antigens on MHC class II to $CD4^+$ T cells which will be described in detail later. Antigens also arrive at the LNs through lymphatic drainage with innate immune cells or freely diffused in lymphatic fluid. A group of macrophages lining the sub-capsular sinus efficiently take up antigen and shuttle them on to B cells that transport them to B cell follicles. Once T cells and B cells are activated within the LN some of them enter the germinal center (GC) reaction, which as will be discussed later is crucial for the generation of high affinity antibodies. The GC reaction is also dependent on innate immune cells such as follicular dendritic cells (FDCs) which are stromal cells located in GC (*29*). Due to this close connection between innate and adaptive immunity, there is a great effort in understanding and targeting innate immune cells to enhance adaptive immunity. DCs have received particular attention since they are important APCs (*40, 41*).

## 3.6 B CELL DIFFERENTIATION AND ACTIVATION

Naïve B cells are generated from the BM. Before exiting the BM, developing B cells must create a B cell receptor (BCR) with random specificity by V(D)J recombination. Within the BM, strong binding of the BCR indicates an autoimmune BCR, since only self-antigen are presumed to be in the BM. This leads to the negative selection of that B cell clone (*42*). Conversely, no binding or activation of the BCR gives no proof that the BCR works, which also results in the deletion of this B cell clone (*42*). Hence B cells need a functioning BCR with no or low self-reactivity in order to enter circulation. Once in circulation B cells migrate back and forth between the lymphatic and vascular systems in search of its cognate antigen. The organs of the body are all draining to LNs where the antigens are concentrated by an intricate network of stromal and innate immune cells. This greatly increases the probability that a B cell will encounter its cognate antigen by probing the LNs of the body. The random generation of B cell specificity ensures that a wide variety of specificities are represented, however very few cells for each specificity are generated. B cells are APCs and present peptides from their cognate antigen on MHC class II after internalization of the antigen by BCR binding (*43*). $CD4^+$ helper T cells that are specific for an epitope on the same antigen will then provide help to the B cells by activating CD40 with CD40L and by the production of cytokines. The activation of naïve B cells by $CD4^+$ helper T cells also leads to switching from IgM to IgG, IgE or IgA, depending on which cytokine is provided, in a process termed class switch recombination. B cells can also be activated without T cell help, in a process called T independent activation. This is usually achieved by activation of the BCR in combination with TLR activation, or activation by antigens with highly repetitive structures that cause cross-linking of the BCR (*44*). This activation, however, is generally considered not to cause antibody class switch and usually results in low-affinity IgM antibodies being secreted. Overall the B cell is destined for one of three general pathways after T cell-

dependent activation; 1. Become a short or long-lived plasma cell 2. Become a memory B cell or 3. Enter the GC reaction. The end goal of most vaccination strategies is the induction of long-lived plasma cells and memory B cells with the right quality of antibodies. Hence a fundamental understanding of the mechanisms that determine B cell fate after an encounter with its cognate antigen is important to our future attempts to design new better vaccines.

## 3.7 T CELL DIFFERENTIATION AND ACTIVATION

T cells undergo their initial selection in the thymus. T cells are generated with a T cell receptor (TCR) with a random specificity. For CD4 T cells to develop, the TCR needs to recognize MHC class II, failure to do this results in apoptosis (*45*). The TCR of newly generated T cells is tested within the thymus (*45*). To prevent autoimmunity, T cells that bind antigen too strongly within the thymus will undergo apoptosis or differentiate into regulatory T cells (*45, 46*). This central tolerance mechanism ensures that T cells entering the circulation have a viable TCR that has a low probability of causing autoimmunity.

Once outside the thymus, T cells circulate the body in search for their cognate antigen. Unlike B cells that recognize antigens in their native form, T cells only recognize their antigen when it is degraded into peptides and presented on MHC class II molecules. Although several cell-types express MHC class II and can present antigen to CD4 T cells, only professional APCs are able to efficiently prime naïve T cells (Figure 2) (*47*). Priming is achieved by three signals. Signal one: the binding of TCR to MHC class II molecule and peptide complex. Signal two: the activation of the CD28 receptor by CD80/86 on APCs. And signal three: cytokines secreted by the APC.

Signal three, in particular, has the additional advantage of being able to shape the T cell into different subtypes, most classically termed Th1, Th2 and Th17 (*48*). Additionally, Th9 and Th22 subtypes have also been characterized and are thought to protect against cancer and bacteria respectively. However, these subtypes are less defined and will not be further discussed in this thesis (*48*). Since detailed differentiation studies are more suited for mouse studies, where knockout models are available, a lot of our current knowledge of Th cell differentiation comes from mice. However, it is important to note that while Th1 and Th2 differentiation are very similar between mouse and humans, Th17 differentiation seems to differ between the species (*48*). Here the differentiation mechanisms for human T cells will be discussed since it is most applicable to the studies of the thesis.

Th1 cells are mainly thought to enhance protection against intracellular pathogens. In the 1990s it was discovered that IL12 was instrumental to Th1 differentiation, and the transcription factor T-bet was revealed to be crucial for the phenotype in the year 2000 (*49*). Additionally, IFNγ promotes the expression of itself and activates STAT1 which increases T-bet expression (*49*).

Th2 cells are associated with parasite infections and allergy (*48*). They secrete IL-4, IL-5 and IL-13. The differentiation of Th2 cells is less clearly understood than that of Th1 and Th17 cells. The main cytokine for Th2 differentiation, IL4, which turns on the Th2 transcription factor GATA3 is not produced in large amount by DCs (*50*). Hence it is possible that other innate immune cells play a crucial role in the differentiation of Th2 cells, and some studies suggest that full differentiation is only achieved after leaving the LN to sites of inflammation, where innate immune cells can provide the necessary IL4. In addition, Th2 cells have traditionally been thought to excel at B cell help although this is now more attributed to T follicular helper cells ($T_{FH}$) (*51*, *52*).

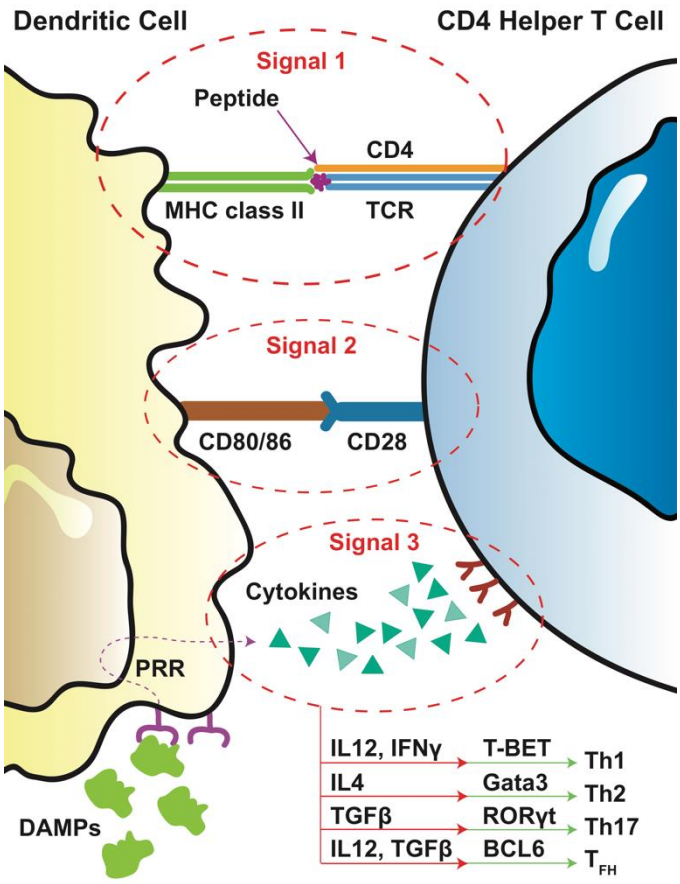


*Figure 2: T cell activation by an antigen presenting dendritic cell.*

Th17 cells are involved in several autoimmune disorders and provide defense against extracellular bacteria and fungi. Their characteristic cytokine IL17 induces the production of chemokines and other cytokines that leads to the recruitment of neutrophils and monocytes (*53*). Th17 cells express the chemokine receptor CCR6 (*54*). Th17 differentiation is controlled by the transcription factor RORγt which is upregulated upon stimulation with TGFβ, other cytokines that increase RORγt expression but are not essential for its induction are IL6, IL21, IL23 and IL1β (*55*, *56*).

# 4 GERMINAL CENTERS

## 4.1 THE GERMINAL CENTER RESPONSE

GCs were described more than 125 years ago by Walther Flemming and were first believed to be the birthplace of lymphocytes (*57*). Today we know they are rather the cradle of differentiated high-affinity B cells. It has long been known that repeated immunizations increase the affinity of antibodies against administered antigens in a process termed affinity

maturation (*58, 59*). A better understanding of this phenomenon was reached since it was discovered that affinity maturation was not achieved by better selection of B cells with appropriate V(D)J rearrangement, but rather by mutations into V(D)J sequences of lower affinity (*60-63*). The mutations to the BCR are driven by the activation-induced deaminase (AID) enzyme, which specifically mutates the BCR upon cell division in a process termed somatic hypermutation (SHM) (*35, 64, 65*). The AID enzyme sometimes induces off-target mutations, occasionally in oncogenes, which increase the risk for B cell lymphomas (*66*). The GC arises in a B cell follicle with naïve B cells. When a B cell follicle is occupied by rapidly dividing GC B cells, naïve B cells that reside in the follicle are pushed away to the edges of the GC, forming the B cell mantle (*57*). GCs develop around 4-6 days after antigen administration and take a few days more to fully mature (*67*). In the actual GC, two main areas are found, called the dark zone and the light zone. B cell proliferation and SHM occurs in the dark zone, whilst $T_{FH}$ dependent selection of high-affinity B cells occurs in the light zone. High-affinity B cell clones are selected in a Darwinian evolution by natural selection like process, in the micro-cosmos of the secondary lymphoid organs. If the GC B cell fails to find its cognate antigen or performs poorly at antigen presentation it undergoes apoptosis. However, after successful presentation of its antigen to a $T_{FH}$ cell the GC B cell is sent back to the dark zone for additional rounds of proliferation. This process is then repeated so that newly generated GC B cells continuously cycle back and forth between the dark zone and the light zone, with higher affinity clones being selected for survival. This is how the GC achieves its main goal of affinity maturation. GC B cells that receive proper stimulation in the light zone are then selected to become either memory B cells or short or long lived plasma cells (*57*). To me, the GC is one of the most fascinating structures in the human body. It is at the center of vaccine immunology due to its ability to produce affinity-matured long-lived memory B cells and plasma cells. An overview of the GC reaction is presented in figure 3.

## 4.2 FOLLICULAR DENDRITIC CELLS

FDCs were discovered in 1965 and are one of many stromal cells that participate in the underlying architecture of the LN (*68*). In this thesis, FDCs will be exclusively discussed due to their central role in the GC reaction. FDCs are generated from perivascular precursor cells and migrate to LNs to enable and support the GC reaction (*69*). FDCs are essential to the formation and maintenance of GCs, one major function being the recruitment of B cells and T cells via the production of CXCL13, the chemokine for CXCR5 (*70, 71*). Additionally, FDCs support GC B cell survival by the production of cytokines, such as B cell-activating factor (BAFF) or IL6 (*72*). One of the most interesting functions of FDCs is their ability to retain and cycle intact antigen on their cell surface (*73*). This provides B cells with intact antigens that they can use in their competition for $T_{FH}$ cell help (*74*). Additionally, since antigens can be bound to the surface of the FDC by complement receptors, the affinity of BCRs could perhaps be further assessed, since it has been shown that while there is no difference in antigen uptake between low and high-affinity BCRs for soluble antigen, high-affinity BCRs have an advantage in uptake of antigens that are attached to a surface (*75*).

Antigens can reach FDCs in different ways depending on the size of the antigen and its previous interactions with the immune system. Antigens with a size less than 70 kDa can be transported directly via a conduit network to the FDCs (*76*). None of the vaccine formulations in this thesis are below this threshold. Larger antigens that have bound complement attached to them can be picked up by sub-capsular sinus macrophages by complement receptor recognition (*70*). The antigens are then transferred by the macrophage from the sub-capsular sinus to the opposite side of the macrophage facing B cell follicles. These antigens are picked up by naïve B cells in a non-cognate way by complement receptor binding and transport the antigen to FDCs (*74, 77, 78*). Since delivery of antigen to the FDCs is crucial for a strong GC reaction, the size of antigens and the way they interact with the complement system is thus of great interest in vaccine design. Mannose-binding lectins that recognize carbohydrates on microbes can bind complement (*79*). It was recently shown that sugars and valency of antigens on vaccine nanoparticles determined binding to mannose binding lectins, which influenced antigen transport to FDCs in a complement-dependent manner (*80*). This is a prime example of how a detailed understanding of the fundamental biology of stromal cells can have significant implications on vaccine design. It also underscores the need for continued research into the mechanisms of vaccination from the delivery of the vaccine to the binding of the antibody to its target epitope.

## 4.3 GERMINAL CENTER B CELLS

Since GC B cells are the precursor to affinity matured memory B cells and plasma cells, they are extensively investigated by immunologists. Before a GC is started naïve B cells are activated by the antigen and T cells at the B: T cell border. In the next phase, activated B cells migrate towards the B cell follicles in a CXCR5 dependent manner (*57*). After intense rounds of proliferation, some B cells enter the core of the B cell follicle to form an early GC (*81*). The GC B cells stay inside the GC due to changes in the expression of G protein-coupled receptors while their naïve B cell counterparts are pushed aside (*82*). Dark zone B cells have traditionally been called centroblasts and express high levels of CXCR4 (*83*). The chemokine for CXCR4, CXCL12 is believed to be provided by a network of reticular cells residing in the dark zone (*84*). Dark zone B cells express high levels of AID, which as previously mentioned induces mutations into the BCR upon cell division. Mutation rates are quick since GC B cells are among the fastest diving cells in the body with divisions every 6-12 hours (*85, 86*). Any such mutation could either improve or worsen the affinity against the antigen. Subsequently, B cells enter the light zone by the down-regulation of chemokine receptors such as CXCR4 (*82*). Light zone GC B cells are called centrocytes and highly express activation markers such as CD86 (*82*). Light zone B cells compete for uptake of antigen from FDCs in an affinity-dependent manner (*75*). The B cells that manage to bind the most antigens and present it to $T_{FH}$ cells are given stimulation with CD40L, BAFF, IL4, IL21 (*57, 82*). The B cells that do not receive enough stimulation instead undergo apoptosis and are engulfed by GC macrophages. The B cells that are given T cell help however, either re-enter the dark zone for additional rounds of proliferation or differentiate into either plasma cells or

memory B cells (*82*). Short-lived plasma cells function as immediate protection against on-going infection and their role in vaccination is not known. Memory B cells patrol the body in search for their cognate antigen and upon renewed exposure they restart the GC reaction or proliferate and differentiate into plasma cells. There are three reasons why the memory B cell response is more effective than a naïve B cell response. 1) The number of memory B cells are at any given point higher than the number of naïve B cells specific for a certain antigen. 2) Memory B cells are more easily activated compared to naïve B cells. 3) Most memory B cells have already undergone affinity maturation in the GC and are thus likely already of high affinity at first antigen encounter. While memory B cells provide protection only after activation, long-lived plasma cells migrate to the BM where they continuously produce antibodies, independent of circulating antigen. Together, memory B cells and long-lived BM plasma cells constitute the main end goals of vaccination (*32*).

## 4.4 T FOLLICULAR HELPER CELLS

$T_{FH}$ cells are crucial for an efficient GC reaction (*51, 87*). Several groups discovered that $CXCR5^+$ T cells, now termed $T_{FH}$ cells, are efficient at activating B cells (*38, 88*). As previously mentioned, CXCR5 is the chemokine receptor for CXCL13 which promotes migration to B cell follicles in secondary lymphoid organs. $T_{FH}$ cells were shown to be different from previously discovered T cell subsets since they express little of their traditional transcription factors. $T_{FH}$ cells express high levels of BCL6, IL21, programmed death protein 1 (PD1) and inducible T cell costimulator (ICOS) (*89, 90*). BCL6 is the transcription factor driving the $T_{FH}$ phenotype (*91-93*). Today CXCR5 and PD1 surface markers are used to reliably detect $T_{FH}$ cells (*94, 95*). $T_{FH}$ cells are differentiated from CD4 T cells early on after antigen exposure (*51*). This requires antigen presentation by APCs that express ICOSL and OX40L (*96*). Interestingly immune complexes containing RNA, which activates TLR7 on APCs, upregulates OX40L which benefits $T_{FH}$ cell development (*97*). In humans, the cytokines that promote $T_{FH}$ cell differentiation are TGF-β, IL-12, and IL-23 (*98*). Powerful evidence to support the role of IL-12 and IL23 are the observations that humans lacking IL12R1β have decreased numbers of circulating $T_{FH}$ cells, less memory B cells and lower avidity antibodies (*99*). IL2 and IL10 have been described as inhibitors of $T_{FH}$ development (*100-102*). The increasing production of IL2 in LNs after antigen exposure might therefore provide an obstacle for $T_{FH}$ cell differentiation. $T_{FH}$ cells might therefore be prone to develop early after antigen exposure, making sure that the humoral response is directed against the initial invading pathogen and not antigens from commensal bacteria that enter after protective barriers have been breached, that might not be as dangerous (*51*). Conversely, this might constitute a problem with dangerous secondary infections such as bacterial infections after influenza-induced pneumonia. To finalize and maintain their phenotype $T_{FH}$ cells need stimulation from B cells via CD80, CD86 and ICOSL (*96*). Without cognate B cells or proteins that enable long $T_{FH}$ cell B cell synapses such as SLAM-associated protein (SAP), GC $T_{FH}$ cell development is severely impaired (*103*).

The purpose of $T_{FH}$ cells is to provide stimulatory signals to B cells that have the highest affinity for the antigen within the GC (*52*). This is achieved by indirectly sensing affinity through the number of loaded MHC class II molecules on the B cells surface. The higher the affinity of the BCR, the more antigen it can acquire which means it can present more antigen on MHC class II and receive more $T_{FH}$ cell help. Help is provided by $T_{FH}$ cells with CD40L stimulation of CD40 on B cells and by cytokines such as IL21. Additionally, depending on the pathogen B cell antibody types can be tailored by the secretion of certain cytokines, IFNγ (Th1) and IL4 (Th2) (*104*). $T_{FH}$ cells have recently emerged as key players in developing protective vaccine responses and are currently under intense pre-clinical and clinical investigation.

## 4.5 CIRCULATING T FOLLICULAR HELPER CELLS

It has been difficult to study $T_{FH}$ cells in humans since the collection of secondary lymphoid organs where they reside is an invasive procedure. Recently a subset of $CXCR5^+PD1^+$ cells in circulation have been termed circulating $T_{FH}$ cells ($cT_{FH}$). These $T_{FH}$ like cells could help the study of $T_{FH}$ cells in human health and disease since blood is easily accessible. Although BCL6 is required for the generation of $cT_{FH}$ cells they do not express BCL6 once in circulation (*105-107*). However, they do express the transcription factor c-Maf, which can upregulate CXCR5 and induce IL-21 production (*107*). $cT_{FH}$ cells can be further divided based on CXCR3 and CCR6 expression; $CXCR3^+CCR6^-$ $cT_{FH}1$, $CXCR3^-CCR6^-$ $cT_{FH}2$ and $CXCR3^-CCR6^+$ $cT_{FH}17$ cells (*108*). Since mice and humans that lack the SAP protein needed for final $T_{FH}$ cell differentiation in the GC still have unchanged numbers of $cT_{FH}$ cells, it is believed that $cT_{FH}$ cells are generated before entry to GCs (*109*). It is believed that their primary function is to differentiate into GC $T_{FH}$ cells upon antigen re-exposure to enhance antibody responses (*109*). $cT_{FH}1$ are found in circulation after influenza vaccination and correlate with high avidity antibodies (*110, 111*). $cT_{FH}2$ cells are found after HPV vaccination (*112*) and Malaria infection (*113*). $cT_{FH}17$ cells were induced by vaccination with a recombinant vesicular stomatitis virus expressing Ebola virus antigens, capable of replication (*114*). $CXCR3^-$ $cT_{FH}2/17$ cells were found to correlate with broadly neutralizing antibodies in $HIV^+$ individuals (*107*). Much remains to be elucidated on the origin and function of $cT_{FH}$ cells and their role as biomarkers for diseases and vaccine-efficacy.

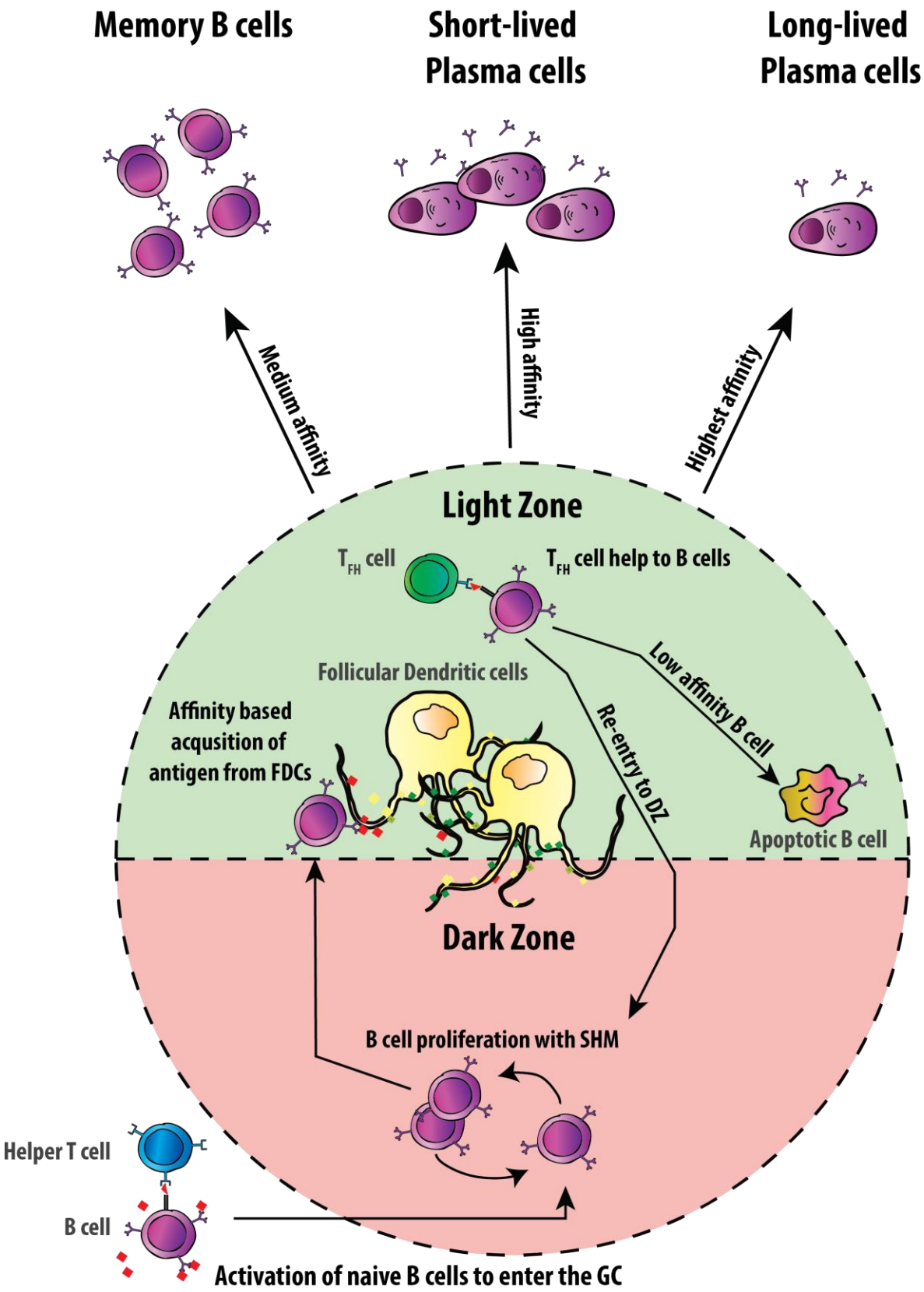


*Figure 3: Overview of the germinal center reaction.*

# 5 VACCINE ADJUVANTS

## 5.1 HISTORY AND DISCOVERY OF ADJUVANTS

Adjuvants are included in vaccines to enhance the immune response against antigens included in the vaccine. As previously mentioned this is of particular importance for protein subunit vaccines that lack other immunostimulatory molecules from the pathogen. Already in 1921, it was shown that the immune response against a diphtheria toxin vaccine could be enhanced by the addition of insoluble aluminum salts (this adjuvant group will hereafter be called Alum for simplicity) (*115*). In the 1950s a new adjuvant reached clinical use, a water-in-oil emulsion called Freund's incomplete adjuvant (*116*). Although it improved the efficacy of influenza vaccines, its use was discontinued due to side effects such as sterile abscesses. It would take until 1997 until a new adjuvant besides Alum was licensed for human use (*117*). This was an oil-in-water emulsion adjuvant called MF59. As for Alum, MF59's adjuvant effects were discovered by chance rather than by design. This trend of "luck" as the driver of adjuvant discovery was broken by the design of TLR agonists as adjuvants. A proof of concept that TLR agonists work was shown by the cream imiquimod (TLR7/8 agonist), which is licensed for topical use against actinic keratosis (*118*), basal cell carcinoma (*119*) and genital warts caused by HPV 1, 2, 4, and 7 (*120*). Shortly after the implementation of imiquimod, AS04 was released as an adjuvant in vaccines for human papillomavirus and hepatitis B virus (*121, 122*). AS04 consists of Alum and Monophosphoryl Lipid A, a TLR4 agonist which interacts with TLR4 similarly to lipopolysaccharides of bacteria (*123*). A synthetic version of Monophosphoryl Lipid A has also been made called Glucopyranosyl Lipid A (GLA), which was used in paper II-III of the thesis. Since our understanding of the signaling pathways of the immune system has dramatically increased, we are now in an unprecedented position to develop and test new adjuvants. This has caused some to say that we have entered a new golden age of vaccine adjuvant development.

Although the antigen used in paper I of this thesis was the HIV-1 Env glycoprotein, the primary research focus was the immune events that determine the quality of a vaccine. In the case of protein subunit vaccines this is likely primarily achieved by the adjuvants. As such, comparing the different immune responses induced by the adjuvants in vivo was the main focus of study I. An introduction to some of the known mechanisms of action of each adjuvant included in the thesis will be discussed below.

## 5.2 ALUMINUM ADJUVANTS (ALUM)

As mentioned, Alum is the oldest and most commonly used adjuvant. Pope and Glenny discovered that Alum could pull down toxins from solutions and then discovered that it also enhanced the immune response to toxins in guinea pigs (*124*). Hence its implementation was

used long before the mechanisms of action where discovered. Due to its long and widespread use, it has an extensive safety record. It is used in the national vaccination programs for many clinically approved vaccines.

It has been known since long that Alum and similar adjuvants induce infiltration of immune cells to the site of injection (*125-127*). At the site of injection, fibrinogen is converted to fibrin, which along with neutrophil extracellular traps creates a meshwork where Alum is trapped (*21, 128-130*). It has been proposed that Alums adjuvanticity is therefore conferred through a "depot effect" or the DNA from the neutrophil extracellular traps. None of these theories seem to provide a satisfactory explanation of the adjuvant effect of Alum in subsequent studies, especially the depot effect since the removal of the vaccine injected site shortly after immunization and thus any antigen depot did not affect the vaccine response (*131-133*). The more likely theory is that Alum simply induces inflammation, for instance by lysing lysosomes, leading to release of lysosomal enzymes into the cytoplasm (*133-135*). Inflammation induced by Alum leads to the production of cytokines and chemokines that recruit and activate more innate immune cells (*136*). Alum has also been shown in several studies to activate the NALP3 inflammasome, which leads to the production of IL-1β that stimulates T cell responses (*137-139*). Although some have modeled this as Alums main mechanism of adjuvanticity, it could not be confirmed by others (*139, 140*). Different mouse models, routes of vaccine delivery, the antigen of choice and more makes it difficult to pinpoint the exact mechanism of action for Alum or any other adjuvant. The differences in the mouse and human immune system also make it hard to know how translatable these results are. More information is warranted on the mechanisms of action of our most widely used adjuvant.

### 5.3 MF59

The mechanisms of action of MF59 has been extensively studied since its implementation in 1997, although a lot of details remain to be elucidated. The adjuvant has now been given hundreds of millions of times and has proven to be safe and efficient. One particular advantage has been the improvement of influenza vaccination for the elderly, who respond poorly to un-adjuvanted influenza vaccines (*117*). Similar to the elderly, children also respond poorly to un-adjuvanted influenza vaccines and MF59 has been shown to increase the vaccine response in young individuals (*141, 142*). By increasing the efficacy of the vaccine, MF59 adjuvanted vaccines can use a lower antigen dose and require fewer vaccinations while remaining effective (*143, 144*). Additionally, MF59 has been shown to increase the breadth of the immune response, which is important in influenza vaccine contexts to protect against heterologous strains (*145*).

In several studies, MF59 has been superior to Alum at generating vaccine responses (*146, 147*). The depot effect hypothesis was falsified early on by investigating the clearance of

radiolabeled MF59 at the site of vaccination, which showed rapid clearance of both MF59 and the antigen (*148*). Early studies also showed that MF59 could be found in LNs draining the vaccination site (*149, 150*). It seemed that most of this transport was done intracellularly and that APCs were the main cell type to transport the adjuvant (*151*). Upon direct stimulation with the adjuvant in vitro, the cells that respond the most are monocytes, macrophages and granulocytes, and they all respond similarly by the production of chemokines (*117*). The precise mechanism of how MF59 activates the cells is still unknown, although TLRs and the NALP3 inflammasome do not seem to play a part (*152, 153*). It appears that as with Alum, although by different means, MF59 induces inflammation at the site of injection that is crucial for its adjuvanticity. Consistently, the genetic profiles of MF59, Alum and CpG have been compared with the general finding that MF59 induces a broader range of inflammatory genes, many encoding for cytokines and chemokines (*136*). This response is local as the injection of adjuvant into non-vaccinated sites does not confer adjuvanticity (*148*). Preliminary studies have suggested MyD88 and apoptosis-associated speck-like protein containing CARD as crucial for MF59 adjuvanticity (*152, 153*). Much remains to be elucidated on the mechanisms of action of MF59 and on how much of the data from murine studies is translatable to humans.

## 5.4 ALUM-TLR7

In this thesis, we tested a new promising adjuvant formulation which combines Alum with a TLR7 agonist. This had previously been shown to be superior to Alum in generating antibody responses (*146*). A potential synergistic effect might be found from these adjuvants since Alum excels at inflammation and cell recruitment while TLR7 can directly activate cells such as B cells and DCs. Alum-TLR7 is not yet in clinical use and more information is needed on the innate and adaptive immune profiles induced by this adjuvant. In Paper I, we compared it side by side to Alum alone and to MF59.

## 5.5 GLA

GLA activates TLR4 in a similar fashion to LPS (*154, 155*). It has been shown to induce a Th1 like profile in mice and has been safe to administer in higher species (*156-158*). In study II-III of this thesis the GLA was used to evaluate if there was a need for an adjuvant with the administration of mRNA vaccines.

# 6 NUCLEIC ACID VACCINES

## 6.1 DNA VACCINES

Nucleic acid vaccines consist of either DNA or mRNA encoding specific antigens (*41*). Of the nucleic acid vaccines, technology for producing viable DNA vaccines has been available the longest, and there is an extensive literature on them (*159*). DNA vaccines function by being transcribed to mRNA which is then translated to a protein that the immune system recognizes. DNA therapeutics has shown potential for a long time, with a major breakthrough in 1992, when it was shown that a DNA vaccine could induce antigen-specific antibody responses. Still, there is no licensed human DNA vaccine available today (*160*). However, there is a DNA vaccine licensed for West Nile-virus in horses (*161*). One of the major advantages of DNA vaccines over conventional vaccines is that they can be produced synthetically, at a lower price than most vaccines, and are easier to transport (*159*). In addition DNA vaccines have been repeatedly shown to induce both humoral and cellular immune responses, making the platform promising for both infectious diseases and cancer vaccine therapeutics (*162*). However, there are several pitfalls with the DNA vaccination strategy that has likely slowed down its clinical application. Since DNA needs to enter the cell nucleus, DNA vaccines require either a viral vector or advanced delivery techniques (*159*). Also, pre-existing immunity to the viral vector and logistical problems with advanced delivery methods makes DNA vaccines less ideal for large scale vaccination. There is also a concern of the incorporation into the genome causing unknown consequences or the development of antinuclear antibodies, inducing autoimmunity (*159, 162*). It remains to be seen what future DNA vaccines has for veterinary and human use. Will it soon show its true potential or will it be mRNA vaccines that live up to the promises of nucleic acid vaccination?

## 6.2 mRNA VACCINES

The literature of evidence showing the efficacy of mRNA vaccines is steadily growing (*13*). mRNA was first discovered as a carrier of genetic information in 1961 (*163*). In 1990 it was shown that mRNA injected into mice produced translated proteins (*164*). There were early problems with translation however since administered mRNA is recognized by the innate immune system via TLR 3, 7 and 9, leading to its degradation (*165-167*). Since administered mRNA molecules were quickly degraded and therefore did not yield high protein output, the potential of mRNA vaccines could not be unlocked. A major breakthrough was achieved in 2005 when it was discovered that modification of synthetic mRNA, for instance by methylation mimicking that of natural mRNA, could greatly reduce TLR activation (*168*). Other improvements to the synthetic mRNAs were the additions of 5’ and 3’ untranslatable regions which increased stability and translatability of the mRNA, and the addition of the 5’ cap region protecting the mRNA from exonucleases (*169-171*). These discoveries, among others, unlocked the potential of mRNA vaccines and several immunogenicity studies follow

suit, showing induction of protective titers in animal models (*172*). Additionally, it was shown that by delivering the mRNA vaccines in lipid nanoparticles (LNPs) the immune response could be further enhanced (*173*).

There are two main categories of mRNA vaccines, "normal" and self-amplifying. Self-amplifying mRNAs translate RNA-dependent RNA polymerases, which in turn makes negative copies of the self-amplifying mRNA that is used to produce more mRNA. This in vivo amplification has the advantage of lowering the dose needed to achieve sufficient protein translation. Also, the replication of the mRNA can trigger innate immune pathways which can have an adjuvant effect (*174*). This thesis contains mRNA vaccines that are "normal" i.e. not self-amplifying. mRNA vaccines can be given intramuscularly (IM) or intradermally (ID) similarly to live or subunit vaccines. There are three main delivery formulations for mRNA therapeutics; naked RNA-, conjugate- or nanoparticle-delivery (*175*). Since entry to the cytoplasm is sufficient for translation of mRNA, the proportion of translation is higher than for DNA vaccines, which need entry into the nucleus of the cell.

A particularly useful area where mRNA vaccines could be applied is influenza vaccination. The WHO tries to estimate what strains of influenza will be circulating each year and gives recommendations on which strands of influenza should be included in the yearly vaccine. mRNA vaccines can be produced within weeks, which offers an advantage over the more slowly produced sub-unit vaccines, such as the trivalent influenza vaccines (*175*). Subunit vaccines are not only slow to produce, but errors in the purification of the proteins can result in whole batches being discarded, leaving society without a vaccine until the next batch can be produced. Also, influenza still poses a pandemic threat and more effective means of dealing with pandemic influenza are desperately needed. For instance, it is estimated that up to 100 million people died by "the Spanish flu" in 1918. As comparison 37 million people died as a consequence the first world war (*176*). Due to the reasons mentioned above, quick scalable production of influenza vaccines is key to global health, which makes mRNA vaccines suitable candidates for vaccination against this pathogen. The mRNA vaccine platform developed by Moderna Therapeutics was shown to induce HA inhibition titers above the level of protection against influenza HA in both mice, ferrets, NHPs and humans (*177*). In project II-III we got the chance to further analyze their mRNA vaccine platform for influenza by focusing on characteristics of the innate and adaptive immune response induced. Basic research on the immune events occurring in vivo after mRNA vaccine administration is needed to improve future development and implementation of mRNA vaccines.

# 7 NHPs AS A MODEL FOR VACCINATION

To improve human and animal health and to prevent future catastrophes caused by pandemics, continued vaccine research is needed. For every experiment, careful ethical consideration should be applied to make sure that the benefits are believed to outweigh potential harm caused by experimentation. Since biology is extremely complicated, in vivo

models are crucial for improving our understanding of vaccines. It is our obligation to make sure that any harm to animals is minimized which is summarized by the three R's. Reduce, which implies that the minimum number of animals needed to provide satisfactory data should be used. Refine, which implies that researchers should strive for proper planning before starting animal experiments, removing unnecessary repeat experiments. Refine also implies to ensure proper animal care from the animal's birth until death. And replace, which implies that whenever a non-animal model or an animal model which is believed to cause less suffering for the animal, can be used to gather the same data, that model should be used instead. Most vaccine studies are done in mice. Mice are ideal for hypothesis testing, knockout experiments and allow for precise control of the environment and other factors. Mice, however differ from humans in many of their cell types, frequencies and expression of PRRs and other molecules. Their anatomy is also vastly different from that of humans. Vaccines are often given intraperitoneally in mice experiments rather than IM as in humans since the muscles of mice are very small. This makes results in mice hard to translate to humans. In this thesis, all vaccines are already in clinical use or likely to be used in the near future. They have already been tested in other preclinical studies. To gain insight that is truly translational we used the NHP model to test new hypotheses and to confirm those that have been tested in mice. NHPs have similar anatomy to that of humans. Their immune system is very comparable to that of humans with similar PRRs. Another advantage of the NHP model is that most markers used are cross-reactive to human, which means the same reagents can be used for both NHP and human studies.

# 8 MATERIALS AND METHODS

The main methods of the thesis will be outlined below. Methods are described in greater detail within the individual papers.

## 8.1 IMMUNIZATIONS

Approval for study I was granted by the Animal Care and Use Committees of the Vaccine Research Center, National Institutes of Health (NIH). Study II-IV was approved by the Local Ethical Committee on Animal Experiments (Stockholm, Sweden). Animals were housed at BIOQUAL or the Division of Veterinary Resources, NIH in USA for study I. Animals were housed at Astrid Fagraeus Laboratory at Karolinska Institutet, Sweden, for study II-IV. All above according to guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care.

In study I, animals were given 1 ml IM vaccinations at four different sites. Vaccines contained either 100 µg of fluorescently labeled Env alone or mixed with fluorescently labeled MF59 at a 1:1 ratio, or adsorbed to 1 mg of alum or alum-TLR7. Animals also received adjuvant alone or PBS as controls at separate sites. Detailed vaccination schedules are outlined in results and discussion and in the papers.

In study II-III animals received a LNP/H10 mRNA vaccine (50 µg). This was administered either ID or IM with or without the inclusion of the GLA adjuvant (5 µg). For innate immune tracking animals were also vaccinated with an Atto-655 labeled LNP/mCitrine mRNA vaccine (50 µg). Detailed vaccination schedules are outlined in results and discussion and in the papers.

## 8.2 PROCESSING OF TISSUES

Peripheral blood mononuclear cells (PBMCs) were collected via a ficoll density gradient. Muscles were processed and digested using Liberase as previously described (*178*). Liberase TH (0.26 WU/ml, Roche, Indianapolis, IN, USA) together with DNase (0.1 mg/ml, Sigma) was used to digest skin, for 1 hour in 37 °C under agitation. Complete media was used to quench the Liberase activity. Skins were filtered using 70 mm cell strainers (BD, Stockholm, Sweden) and were subsequently washed with PBS before being stained for flow cytometry. Scissors were used to mince LNs before mechanically pushing them through 70 mm cell strainers with a plunger. LNs cells were subsequently washed and stained for flow cytometry.

## 8.3 FLOW CYTOMETRY STAININGS

Cells were first stained with LIVE/DEAD Fixable Dead Cell kit (Invitrogen). Depending on the experiment, cells were then stained with different combinations of fluorescently labeled surface markers. When intracellular staining was performed, cells were permeabilized with

either Cytofix/Cytoperm™ (BD) (Study I-III) or transcription factor buffer set (BD) (Study IV) according to manufacturer's instructions.

## 8.4 IMMUNOHISTOLOGY STAININGS

Tissues embedded in optimal cutting temperature embedding media were thawed from -80 °C to -20 °C. Approximately 7 µm thick sections were obtained using a CryoStar$^{TM}$ cryostat. Slides were allowed to dry for 15 minutes before being fixed in 2 % formaldehyde solution diluted in PBS. Slides were blocked with fetal calf serum and permeablized using a triss-buffer saline and saponin (2 %) based permeablilization buffer. Slides were subsequently stained with unconjugated primary antibodies, followed by biotinylated secondary antibodies. The secondary antibodies were visualized by the addition of streptavidin conjugate fluorophores. Images were acquired using a Nikon Eclipse Ti-E motorized confocal microscope.

## 8.5 ANTIGEN RECALL ASSAY

To test for antigen specific cells, PBMCs were re-stimulated in vitro. For study II-III H10 overlapping peptides (matching protein from mRNA of previously administered vaccines) at 2 µg/ml concentration and Brefeldin A were used for over-night stimulation and cytokine retention respectively. Cells were then stained for surface markers and cytokines to assess antigen specificity.

## 8.6 ELISA

For detection of antigen specific IgG, clear flat-bottom immune 96-well plates (Thermofisher) were coated with 100 ng of H10 protein for 3 h in 37 °C. Coated wells were washed and blocked for 1 hour with 2 % milk powder diluted in PBS (blocking buffer). Plasma samples diluted in blocking buffer were incubated in wells at 37 °C for 1 h. For avidity ELISAs an additional step was added with sodium thiocyanate (NaSCN) at 1.5-4.5M. Antibodies were then detected with an anti-monkey IgG HRP antibody (Nordic Labs) diluted in washing buffer (0.05 % Tween 20 in PBS). TMB solution (BioLegend) was used to detect the HRP antibody. Plates were read at 450 nm using an ELISA reader (PerkinElmer).

## 8.7 B CELL ELISPOT

B cell ELISpot was used to enumerate antigen-specific memory B cells and plasma cells as previously described (*179*). Briefly, plates were coated with anti-human IgG for detection of antibodies from stimulated memory B cells or unstimulated plasma cells. Antibodies were then detected via the addition of HA (antigen specific IgG) or anti human IgG (total IgG).

## 8.8 HEMAGGLUTININ INHIBITION ASSAY

Turkey red blood cells diluted in PBS to 0.5 % were used to evaluate HA inhibition by serum antibodies. Non-specific blocking of agglutination was prevented by incubating serum with receptor destroying enzymes over night at 37 °C. Serial dilutions of the serum was used to evaluate its inhibition efficacy against agglutination of the red blood cells by HA from H10N8 influenza A virus.

# 9 RESULTS AND DISCUSSION

## 9.1 IMMUNE MECHANISMS OF VACCINE ADJUVANTS (PAPER I)

As mentioned above, little is known of the mechanisms of action of vaccine adjuvants. An improved understanding of how adjuvants work and the type of responses they induce would facilitate the development of new, improved adjuvants and better match adjuvants with specific diseases. In the first study of my thesis, which I entered as a master's student and early Ph.D. student, we aimed to compare the first immunological events occurring in vivo after administration of three distinctly different adjuvants. We chose to study Alum combined with a TLR7-agonist (Alum-TLR7) and the oil-in-water emulsion adjuvant MF59. They had both been shown to induce high antibody titers and T cell responses compared to many other adjuvants tested side-by-side in NHPs in a prior study led by our collaborators (*180*). We also included the conventional adjuvant Alum as a benchmark. The adjuvants were administered together with the HIV vaccine candidate envelope glycoprotein gp120 (Env) since this antigen is well characterized and tested in both preclinical and clinical vaccine trials and is relevant together with these adjuvants. The adjuvants and Env were provided by Novartis (now Glaxo Smith Kline; GSK) which means that they had been quality control tested and formulated according to their standards.

Studies on the mechanisms by which adjuvants work have previously mostly been performed in mice, however, such results are not always translatable to humans (*181-183*) especially since mice have a different expression repertoire of TLRs compared to humans and NHPs. To build on the knowledge gained from the murine studies, we therefore used rhesus macaques since they have an immune system and anatomy more similar to humans.

The overall purpose of Paper I was to evaluate how the distinctly different adjuvants influenced the initial immunological steps after vaccine administration such as mobilization of cells, cell activation, vaccine antigen uptake and presentation. Additionally, we aimed to characterize early adaptive events initiated by innate immunity, such as the formation of GCs which was my primary focus in the study. Therefore, a lot of the analyses were on the immune responses at the site of injection and in the vaccine draining LNs. Animals were divided into four groups receiving either Alum, Alum-TLR7, MF59 or no adjuvant together with Env. Each animal received four injections, i.e. in the left and right deltoid or quadriceps muscle with

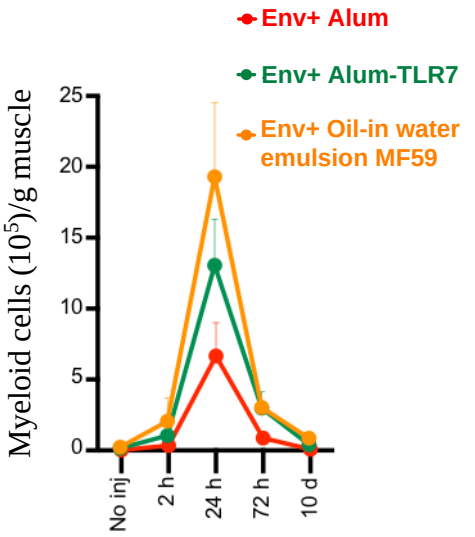


*Figure 4: Infiltration of myeloid cells (monocytes, neutrophils and cDCs) to the site of injection.*

either adjuvant together with Env, adjuvant alone or PBS. This experimental set up allowed for that each animal had their own PBS control and that we could restrict the number of animals used for experimentation. Sample collection was performed at termination at 2 hours, 24 hours, 72 hours or 10 days after immunization.

The recruitment of immune cells to the site of vaccine administration is a prerequisite to stimulate efficient vaccine responses (*184*). This is especially important for administration sites such as the muscle, which contains very few immune cells at steady state (*16, 178*). As mentioned earlier, protein subunit vaccines need adjuvants since they often lack immunostimulatory properties and are poorly immunogenic themselves. In line with this, we found that infiltration of immune cells to the site of injection or vaccine-draining LNs was only induced in the presence of adjuvants, as it was very limited at sites injected with PBS or Env alone. Neutrophils and monocytes were the most abundant cells to infiltrate the injection sites. Similar to data from murine studies (*150*), we found that the infiltration of immune cells in both muscle and LNs was rapid and transient with the highest numbers detected at 24 hours with a decline at 72 hours (Figure 4). Overall, MF59 and Alum-TLR7 generated stronger infiltration of immune cells e.g. myeloid cells such as neutrophils, monocytes and myeloid DCs to the site of injection and to vaccine-draining LNs compared to Alum.

In order to track vaccine uptake and its distribution, Env was labeled with the fluorescent dye Alexa680. $Env^+$ cells were readily detected at the site of injection and exclusively in the LNs draining the site of injection, indicating that the dissemination of vaccine antigen was very local (Figure 5). Neutrophils and monocytes again represented the most frequent cell populations that took up the antigen ($Env^+$), likely due to their high phagocytic abilities and their abundance. While there was no statistical difference in antigen uptake between the adjuvant groups, the animals receiving MF59 showed more $Env^+$ neutrophils in the vaccine-draining LNs as well as more neutrophil migration in general to these sites. While both MF59 and Alum-TLR7 induced recruitment of PDCs to the vaccine-draining LNs, only the Alum-TLR7 group showed antigen uptake by PDCs at a higher level.

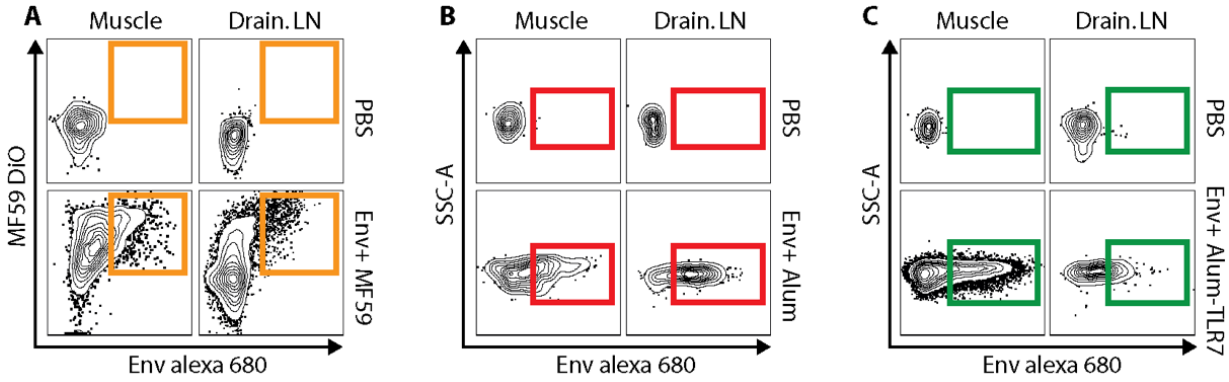


*Figure 5: Uptake of antigen (Env) in vivo in the presence of MF59, Alum or Alum-TLR7 adjuvant by MDCs at the site of injection and draining LNs.*

The TLR7 receptor detects single-stranded RNA and its activation is known to result in the induction of high levels of type I IFN and skewing towards Th1 responses (*185*, *186*). Hence, we expected to find evidence of type I IFN production in the Alum-TLR7 group. We used detection of the MxA protein as a surrogate marker for type 1 IFN production since MxA expression is tightly regulated by type I IFNs (*187*). MxA was only detected at the site of injection and in vaccine-draining LNs in the animals in the Alum-TLR7 group (Figure 6A). Additionally, only Alum-TLR7 induced PDCs to produce type 1 IFN in vitro (Figure 6B).

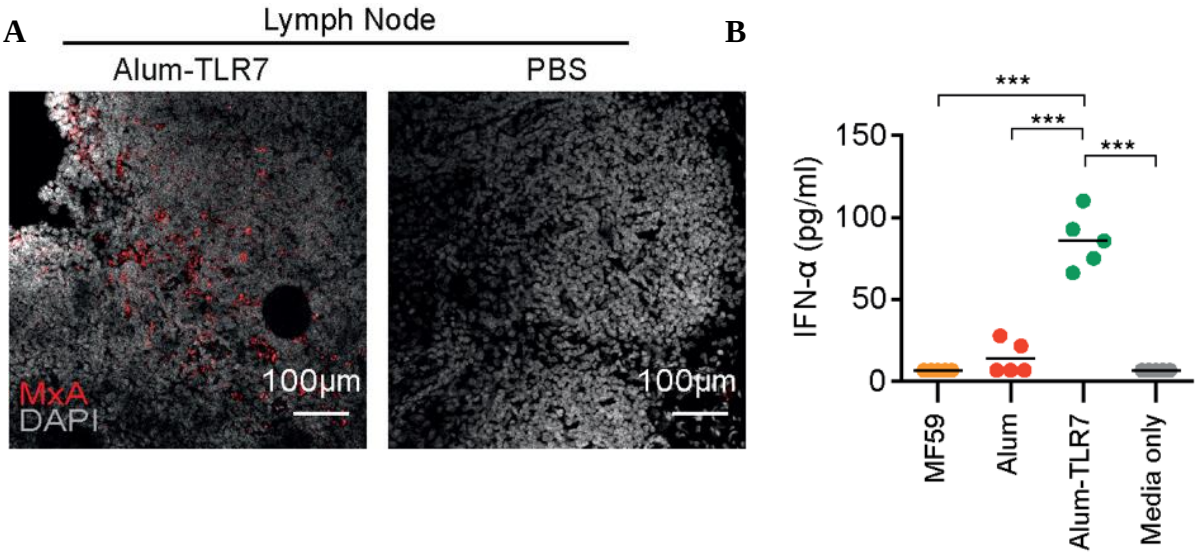


*Figure 6: MxA expression is only induced by Alum-TLR7 at the site of injection and in draining LNs and only Alum-TLR7 induces type 1 IFN production by PDCs in vitro.*

In line with the type I IFN milieu induced by Alum-TLR7 we also detected a predominant Th1 type CD4+ T cell response characterized by IFNγ in this group (Figure 7). In contrast, MF59 induced a mix of IFNγ (Th1) and IL4 (Th2) producing CD4 T cells.

Ultimately the strength of a vaccine is determined by the quantity, quality, longevity and breadth of the antibodies induced. All of these are the product of a strong GC response. To analyze the GC response in vaccine-draining LNs we developed a staining protocol using CD3, PD1 and Ki67. GCs were defined as clusters of GC B cells ($CD3^{-}Ki67^{+}$) and $T_{FH}$ cells ($CD3^{+}PD1^{+}$). We confirmed that $CD3^{-}Ki67^{+}$ cells were GC B cells by their expression of CD20. The software CellProfiler™ was used together with a customized algorithm to enumerate cells within GCs and to measure the area of the GCs. We found that the animals receiving Alum-TLR7 and MF59 showed

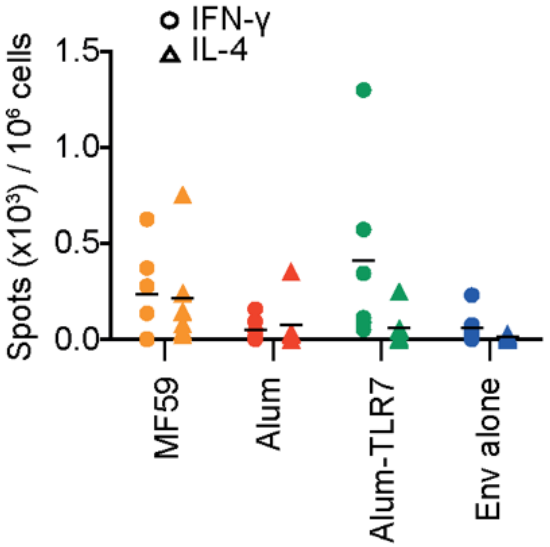


*Figure 7: Cytokine ELISpot on PBMCs after vaccination with different adjuvants.*

equally strong induction of GCs and were both superior over Alum (Figure 8). This pattern was found for GC area and $T_{FH}$ cells for both MF59 and Alum-TLR7, while only MF59 had statistically higher numbers of GC B cells over Alum.

Therefore, although MF59 and Alum-TLR7 have distinctly different innate immune profiles; MF59 inducing a mixed Th1/Th2 response with enhanced neutrophil recruitment and Alum-TLR7 a strict Th1 profile with type I IFN production, they both demonstrated enhanced GC activity. The requirements for the initiation of a GC reaction basically involves four essential processes (which could be simultaneous events); 1. the transport of antigen to FDCs in B cell follicles, 2. retention of antigen within these follicles, 3. activation of innate immune cells e.g. monocytes or DCs with production of cytokines and antigen presentation to T cells, and finally, 4. the activation of T cells and B cells to enter the GC. These processes can be influenced by different factors in the LN milieu. In our study, we speculated that the strong neutrophil recruitment by the MF59 adjuvant or type I IFN production and activation of immune cells induced by Alum-TLR7 affected these processes. However, the depletion of neutrophils has been shown to not reduce the adjuvant effect of MF59 in mice (*150*). This may mean that other immune cells e.g. monocytes could compensate for the loss of neutrophils. In any event, there are several potential mechanisms by which neutrophils could enhance the immune response to vaccines, such as the production of chemokines that recruit other immune cells to the site and cytokines, including BAFF for support of B cell survival. Neutrophils can also contribute with transportation of antigens and even antigen presentation (*47, 188*).

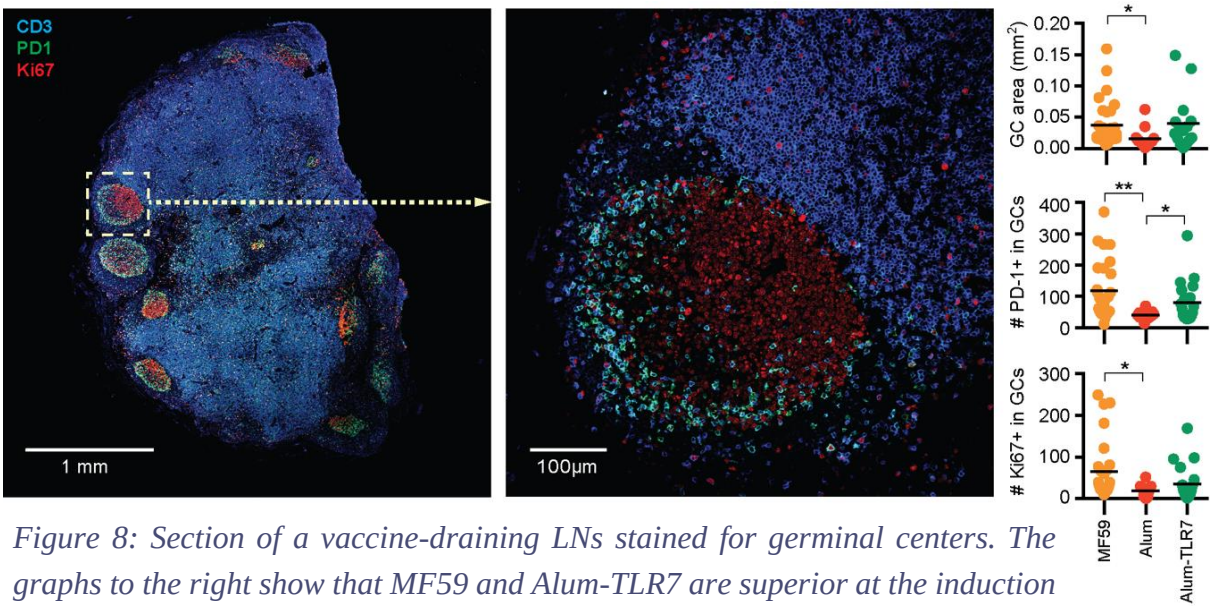


*Figure 8: Section of a vaccine-draining LNs stained for germinal centers. The graphs to the right show that MF59 and Alum-TLR7 are superior at the induction of GC responses over Alum.*

Similarly, it remains to be elucidated to what degree Alum-TLR7 adjuvanticity is achieved by direct TLR activation or by the indirect effect of type I IFN production (*189, 190*). Naïve B cells have low or no expression of TLRs, however BCR activation and differentiation into memory B cells induce expression of TLR 2, TLR6, TLR7, TLR9 and TLR10 (*191*). Hence,

although TLR stimulation can lead to polyclonal activation of memory B cells, with the production of Igs, and even provide a mechanism for sustaining the memory B cell pool, it is not enough for the activation of naïve B cells (*192*). T cell-dependent activation of naïve B cells requires three essential steps: 1. Binding of antigen to the BCR, 2. Activation by CD40L from cognate CD4 helper T cells, 3. Additional stimuli as provided by TLRs (*191*). Previous studies in mice have shown that TLR7 stimulation of B cells was sufficient to induce proliferation and IgG production, while type I IFN was required for fine tuning the isotype switching of antibodies (*193*). In our study and in most other contexts, TLR7 stimulation and type I IFN stimulation of B cells are processes that likely occur simultaneously. Either way, the differential immunological milieus identified with the different adjuvants in our study likely played a critical role in polarization of the Th1 vs Th2 responses with the different adjuvants. This could, in turn, result in different antibody isotypes produced, and ultimately decide whether a vaccine response is protective or not (*194*).

A prior study investigating how potently these different adjuvants induce adaptive vaccine responses to Env had shown surprisingly small differences between Alum, MF59 and Alum-TLR7 in terms of the level of SHM (*180*). This is in contrast to our findings where the larger GCs we detected in the Alum-TLR7 and MF59 groups presumably would generate more SHM. In agreement with this presumption, a separate study where we investigated a liposome-based Env vaccine found that the animals with superior neutralizing antibodies after vaccination also had the largest GCs (*195*).

Collectively, the data from Paper I demonstrate that adjuvants generating stronger antibody responses, such as MF59 and Alum-TLR7 over Alum, have stronger innate immune responses leading to superior GC formation. We show that while MF59 and Alum-TLR7 both reach strong end goals, such as high T cell and B cell responses including the formation of GCs, the innate immune profile leading up to this is very different. Alum-TLR7 induces type I IFN production, while MF59 induces the recruitment of neutrophils to vaccine draining LNs. It is plausible that the Alum-TLR7 and MF59 would be best suited to stimulate responses to different pathogens requiring different types of responses. Insights from this study may therefore help the future implementation of these adjuvants.

## 9.2 INNATE AND ADAPTIVE RESPONSES TO mRNA VACCINES (PAPER II-III)

The recognition of Paper I resulted in the opportunity to further develop our rhesus macaque model to study the early immune mechanisms induced by mRNA vaccines. Since the field of mRNA vaccines is in its infancy there is limited knowledge of the innate and adaptive immune responses they induce. The aim of Paper II-III was therefore to characterize in detail the immune responses by mRNA vaccines. In these studies, we collaborated with Moderna Therapeutics that designed and provided the mRNA vaccines.

In this project, the animals were divided into three groups receiving modified mRNA encoding the HA of H10N8 influenza A virus packaged in LNPs. One group received the vaccine ID one IM and the final group received the vaccine IM together with the TLR4-based adjuvant GLA. We found that all animals induced neutralizing antibody titers against HA above the reported level required for protection against influenza after vaccination (Figure 9). The addition of a GLA adjuvant showed no superior responses over the un-adjuvanted vaccines. This indicated that the mRNA/LNP vaccine formulation was immunogenic in itself and did not need an adjuvant. It is possible that other adjuvants may have been able to enhance the vaccine responses, but we conjectured that the mRNA/LNP vaccine formulation induced sufficient innate immune activation to generate a potent immune response. We therefore set out to characterize the innate immune response induced after administration. For these experiments, we used an mRNA construct encoding for the fluorescent protein mCitrine (Paper II), enabling detection of the cells that had translated the mRNA. The mRNA was delivered in fluorescently labeled LNPs to enable identification of

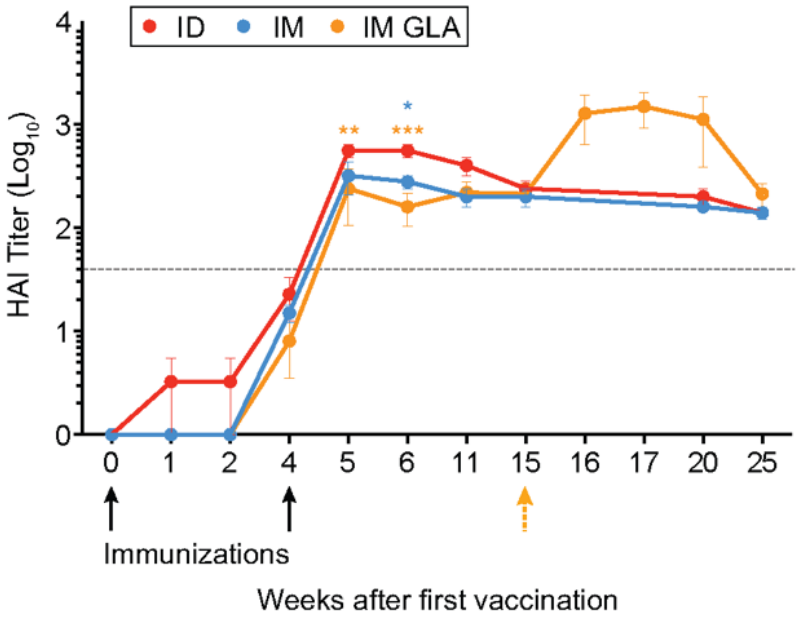


*Figure 9: HA mRNA vaccine delivered in LNPs induces high HAI titers, the dotted line indicates the threshold of protection.*

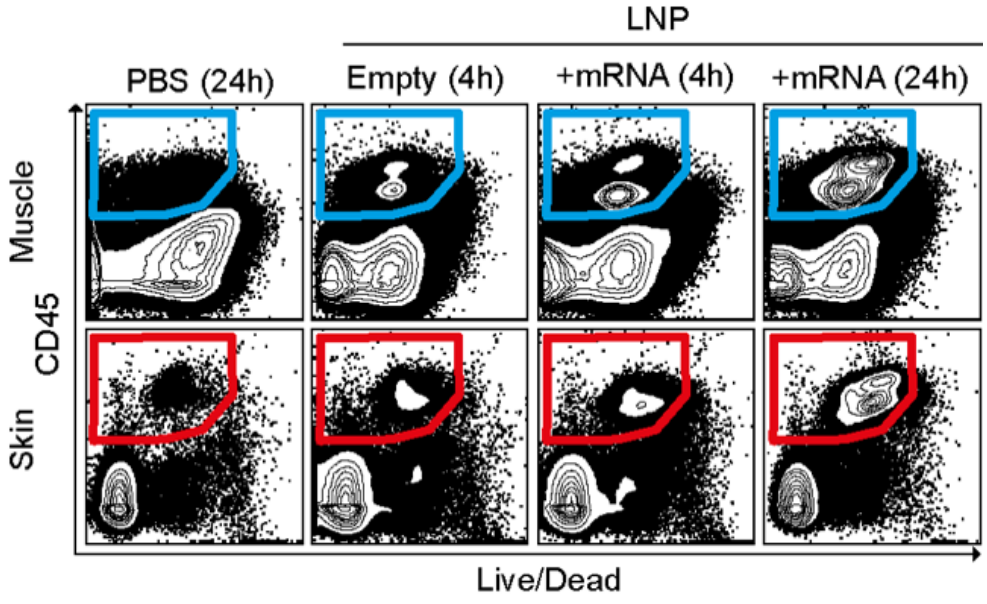


*Figure 10: Infiltration of immune cells to the site of injection after mRNA LNP vaccine administration.*

cells that had taken up the vaccine and cells that had translated the mRNA. Similar to Paper I, samples were collected at different time points (4 hours, 24 hours and 9 days) after administration of the mRNA vaccine or PBS as a control.

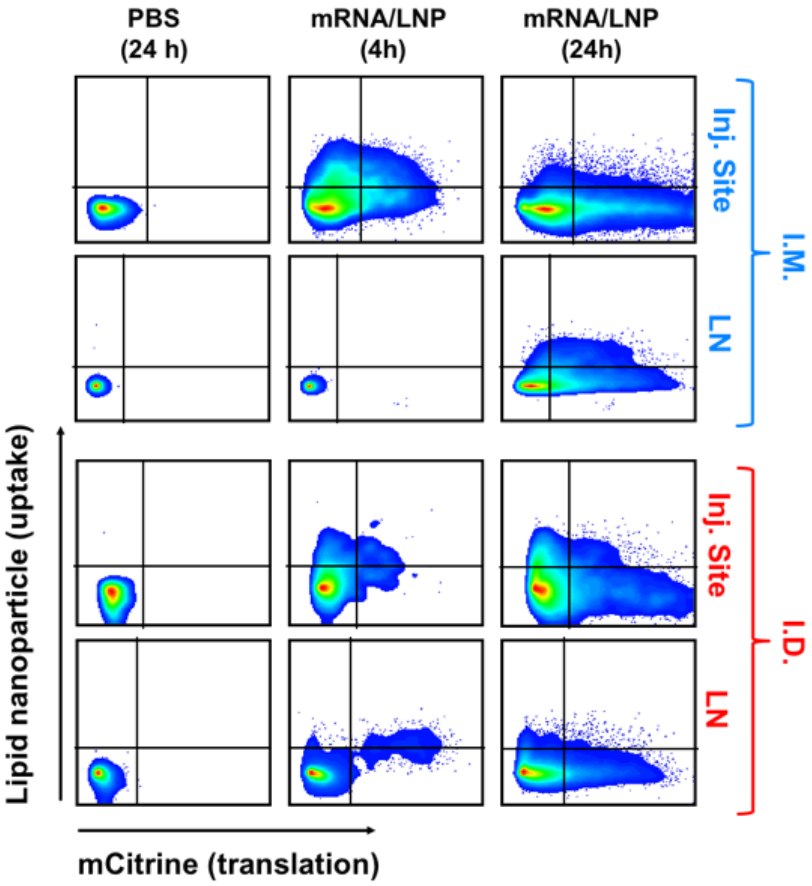


*Figure 11: Uptake and translation of the LNP mRNA vaccine by immune cells at the site of injection and in draining LNs.*

As found for protein/adjuvant administration in Paper I, the mRNA vaccine also induced rapid recruitment of immune cells to the site of injection, with the highest recruitment measured at 24 hours (Figure 10). Infiltration of immune cells was induced by LNPs regardless if it contained mRNA. As for the adjuvants tested in Paper I, the LNP/mRNA vaccine was mostly taken up by neutrophils and monocytes. However, for mRNA vaccines to function translation is required in addition to uptake. The majority of the cells translating the mRNA were found to be monocytes, followed by various DC subsets (Figure 11). In contrast translation by neutrophils was almost

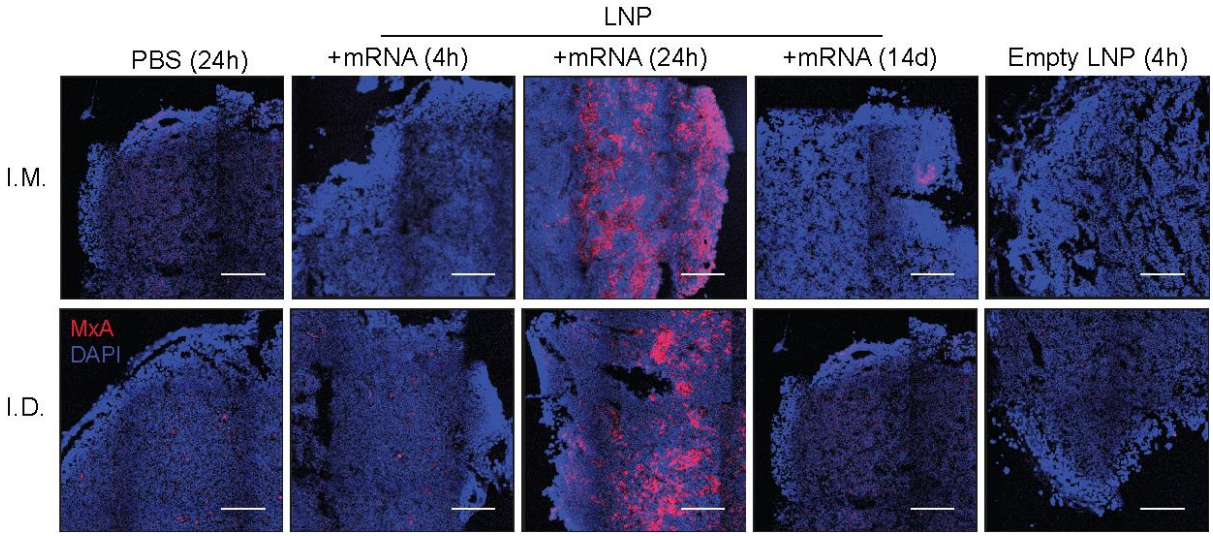


*Figure 12: mRNA vaccine administration leads to type I IFN production in vaccine draining LNs as shown by the production of the type I IFN inducible protein MxA. Scale bars are 200 µm.*

negligible. As for the protein/adjuvant administrations in Paper I, the mRNA vaccine was also detected exclusively at the site of injection and the LNs draining the site of injection.

Although the mRNA constructs used for vaccination has been modified to induce less innate immune activation in order not to interfere with translation efficiency, the recognition of the constructs by TLRs or other PRRs is not completely abolished (*13, 168, 174*). We used the expression of MxA as a surrogate marker for type I IFN production again. A clear upregulation of MxA at 24 hours after vaccination was found in the vaccine-draining LNs (Figure 12). No MxA was found at 4 hours or 14 days after vaccination, indicating that the production of type I IFN in response to mRNA vaccination has a limited time frame. This fits well with the translation data showing peak translation at 24 hours after vaccination in vaccine-draining LNs. Collectively paper II shows that LNP mRNA vaccines are immunogenic, induce rapid infiltration of immune cells with monocytes being the main cell type translating the vaccine. The vaccine response is restricted to the site of injection and vaccine-draining LNs where the induction of type I IFN is promoted, presumably by TLR7 activation by the mRNA.

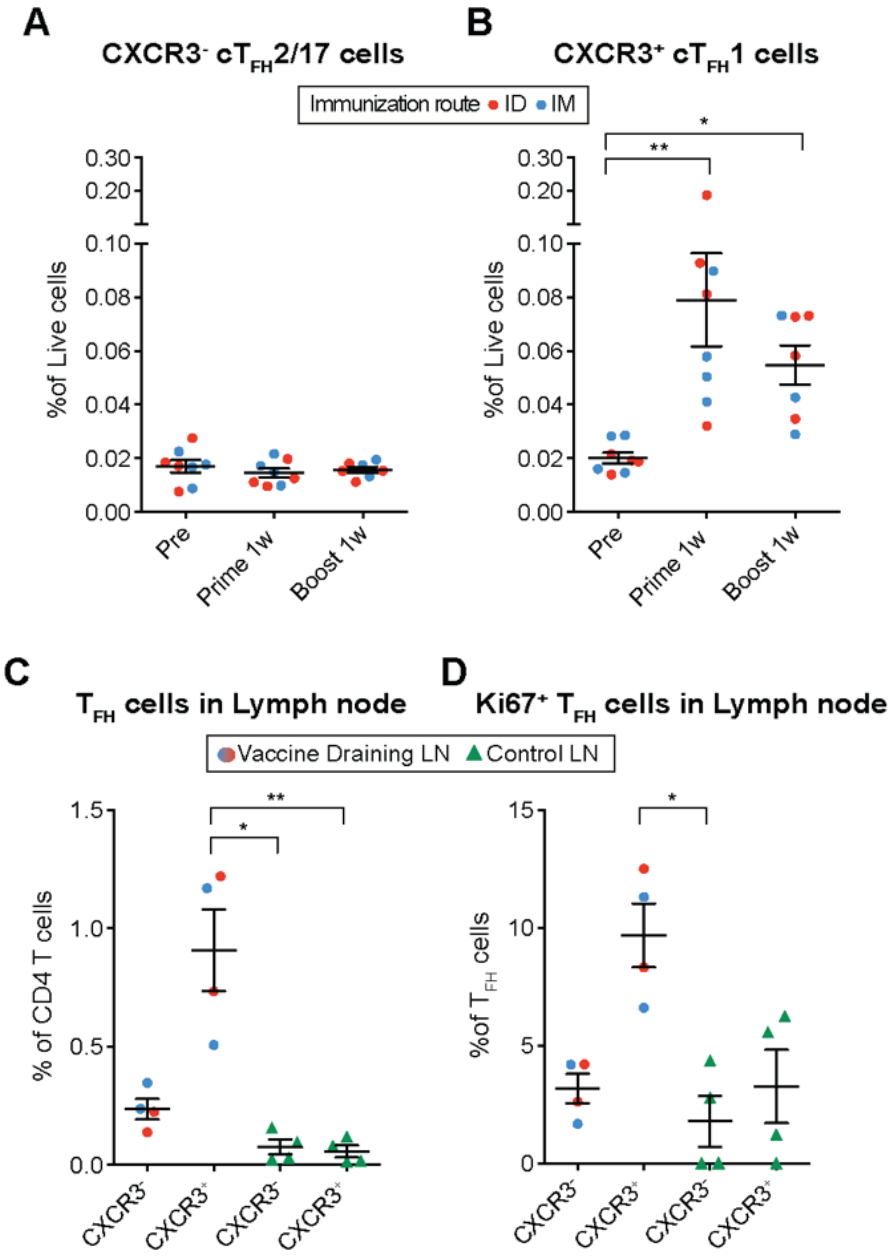


*Figure 13: CXCR3⁺ cT$_{FH}$ cells are induced 7 days after vaccination and CXCR3⁺ T$_{FH}$ cells are proliferating in vaccine-draining LNs.*

While type I IFN production is indicative of a Th1 response, the Th1/2/17 classification is ultimately centered around T helper cells with different effector functions, including the

skewing of B cells to produce antibodies of different isotypes and qualities. In paper III we therefore focused on details of the adaptive immune response induced by mRNA vaccines, such as the phenotype and kinetics of the T cell and B cell response and the induction of GCs in vaccine draining LNs.

In the animals receiving the influenza mRNA vaccine we therefore investigated whether detectable levels of circulating $T_{FH}$ cells were induced and whether they showed a polarized phenotype based on the chemokine receptor CXCR3$^{+}$(Th1) or CXCR3$^{-}$ (Th2/17). Strikingly there was only evidence of CXCR3$^{+}$ c$T_{FH}$1 cells and no increase of the Th2/17 populations (Figure 13 A-B). There was also a higher percentage of CXCR3$^{+}$ $T_{FH}$ in the vaccine-draining LNs compared to control LNs, and those $T_{FH}$ were to a higher degree Ki67$^{+}$, indicating that they were proliferating (Figure 13 C-D). Altogether, the data suggest that the mRNA vaccine induces a strict Th1 type phenotype, likely shaped by the strong type I IFN response.

Biomarkers early after vaccination that predict long term protection are key to making vaccine research more efficient and to quickly assess the quality of the immune response in vaccinated individuals. It was recently shown that circulating $T_{FH}$1 cells correlate strongly with long term avidity of influenza antibodies in humans (*110*). We thus investigated whether there was a similar pattern in our study. First, we analyzed antigen-specific IgG titers and antibody avidity and found that while IgG titers increased rapidly with each vaccination, antibody avidity increased slowly over time (Figure 14). We analyzed multiple time points and the latest time point in the study was around 38 weeks. We had samples available from

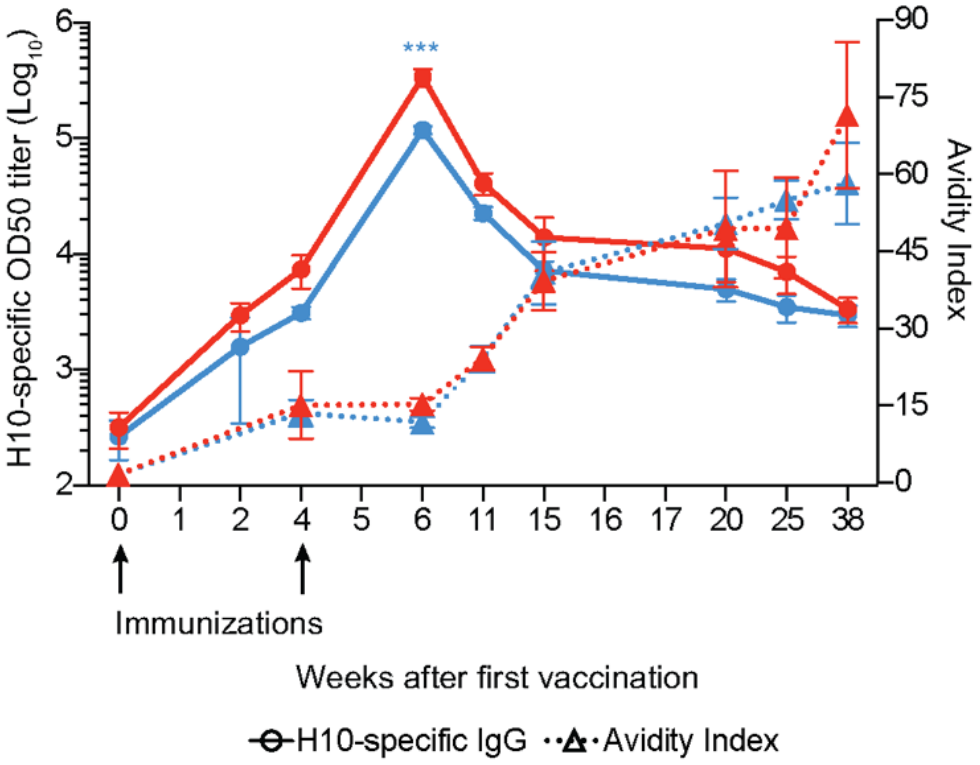


*Figure 14: H10 specific IgG titers and H10 specific IgG avidity index after mRNA vaccine administration.*

this point from 6 animals and found that there was a strong correlation between the cTFH 1 cells and antibody avidity (Figure 15). The mechanisms behind this correlation remain to be elucidated, but it is plausible that circulating TFH reflect the overall strength of conventional TFH activation within GCs.

The induction of GCs was also evaluated by immunohistology in tissue sections (Figure 16). Each GC was evaluated separately and there was a clear increase in size and cell numbers of individual GCs 14 days after vaccination. Thus, we concluded that the mRNA vaccine was able to induce GC formation, enabling the formation of affinity matured memory B cells and long-lived plasma cells. Since GCs take approximately 7 days to mature and it is in these that affinity maturation of antibodies occur, the delayed increase in antibody avidity index seen in Figure 14 can be naturally explained by GC kinetics (*57*). The avidity index will also increase as less competitive clones of antibodies are being depleted, while affinity matured antibodies remain.

To further dissect the immune response, we investigated the kinetics of peripheral memory B cells and BM PCs by ELISPOT analysis, after vaccine administration (Figure 17). The memory B cell pool expanded and contracted with each immunization and seemed to plateau around week 15. To our surprise, plasma cells were found to be seeded already at two weeks after prime immunization. This indicates that GC formation occurs quickly after immunization since plasma cells homing to the BM are induced late in the GC reaction (*196*). After initial seeding, the number of plasma cells in the BM seemed stable, as the numbers did not fluctuate with the boost immunization, similarly to what has been reported by others (*179*). Much remains to be elucidated on how the maintenance of long-lived plasma cells in the BM is controlled. Do plasma cells with higher affinity against influenza HA that arrive in the BM after the boost immunization outcompete any lower affinity plasma cell clones? If that is the case, how is this selection process achieved? Plasma cells display little or no BCRs on their cell surface and can therefore not compete for antigen uptake like the process in a GC (*197*). Even if they could, the BM is not thought to harbor antigens to any great extent, as this would likely interfere with de novo generation of B cells since strong BCR activation in the BM of newly generated B cells leads to apoptosis, as protection against autoimmunity. Another scenario where new plasma cells would simply replace old plasma cells in the BM at random makes little sense. Since we know that some vaccines such as smallpox and yellow fever

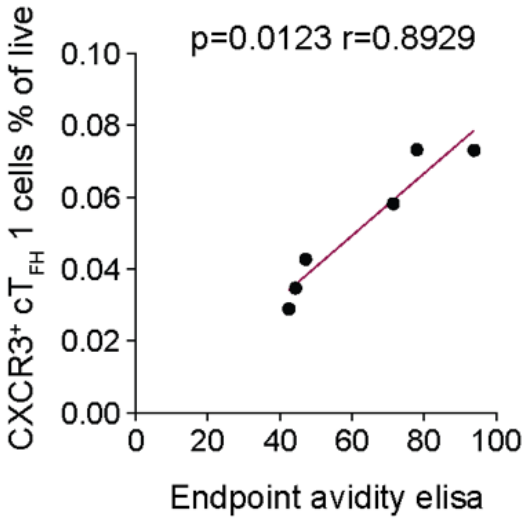


*Figure 15: The frequency of cTFH1 cells is a vaccine biomarker for long term antibody avidity.*

induce long-lived plasma cells that can survive over a lifetime and are not replaced by the continuous repopulation of new long-lived plasma cells created during an individual's lifetime (*198*). This conundrum of plasma cell niches within the BM is an area that needs more research to improve future vaccine design strategies.

Collectively Paper II and III shows that mRNA vaccines are immunogenic in a NHP model. The vaccine is mainly taken up by monocytes and neutrophils while most mRNA translation occurs in monocytes. The vaccine is immunogenic on its own and did not benefit from the inclusion of the GLA adjuvant. As mentioned earlier the use of mRNA for vaccination has become feasible due to modifications to make the mRNA more stable and less potent at stimulation of innate immunity. However, some innate immune activation may be necessary to induce vaccine responses, or otherwise, an adjuvant may be needed.

Finally, the vaccine induces a Th1 skewed response, strong GC activation and strong antibody avidity that correlates strongly with circulating $T_{FH}1$ cells. The skewing towards a Th1 profile might be beneficial for influenza vaccines in terms of antibody isotype produced since it has been shown in mice that Th1 skewed antibodies and not Th2/17 skewed antibodies protect against influenza infection, although they have the same affinity to bind antigen (*194*).

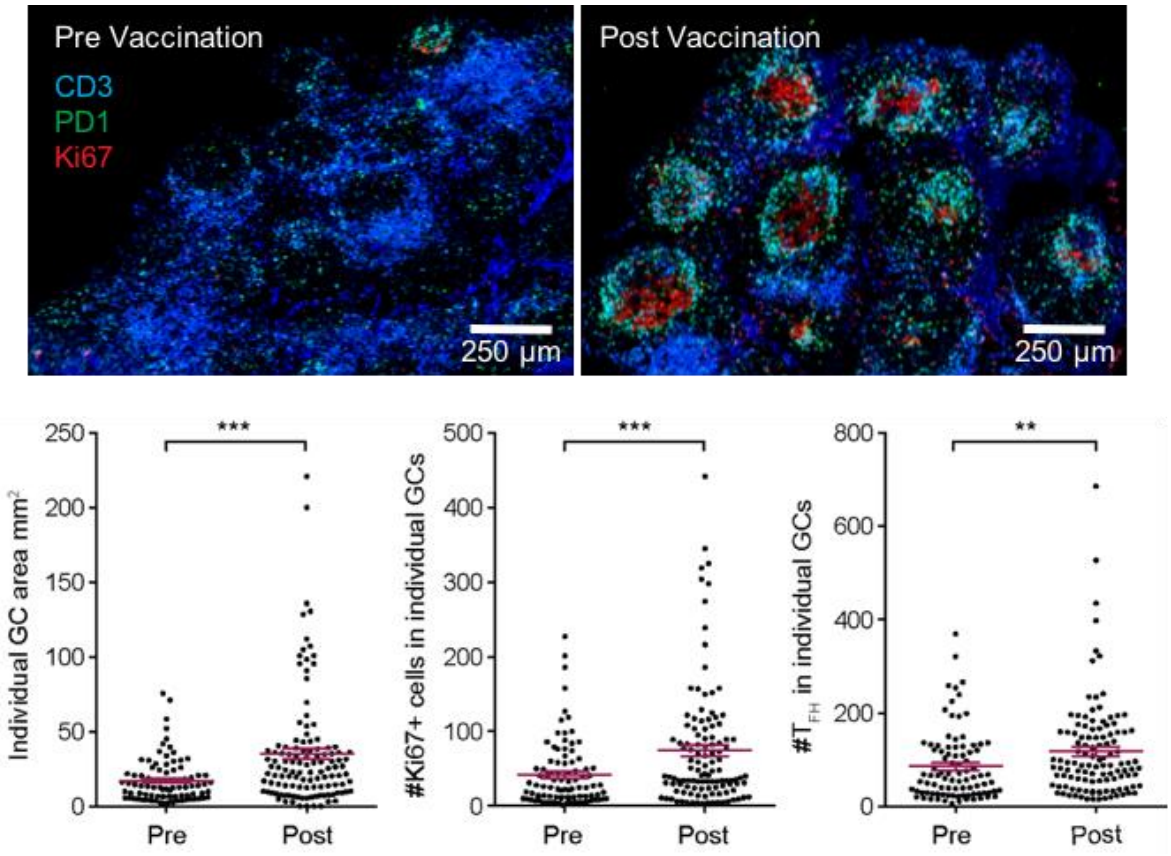


*Figure 16: mRNA vaccines induce strong GC reactions two weeks after boost vaccination.*

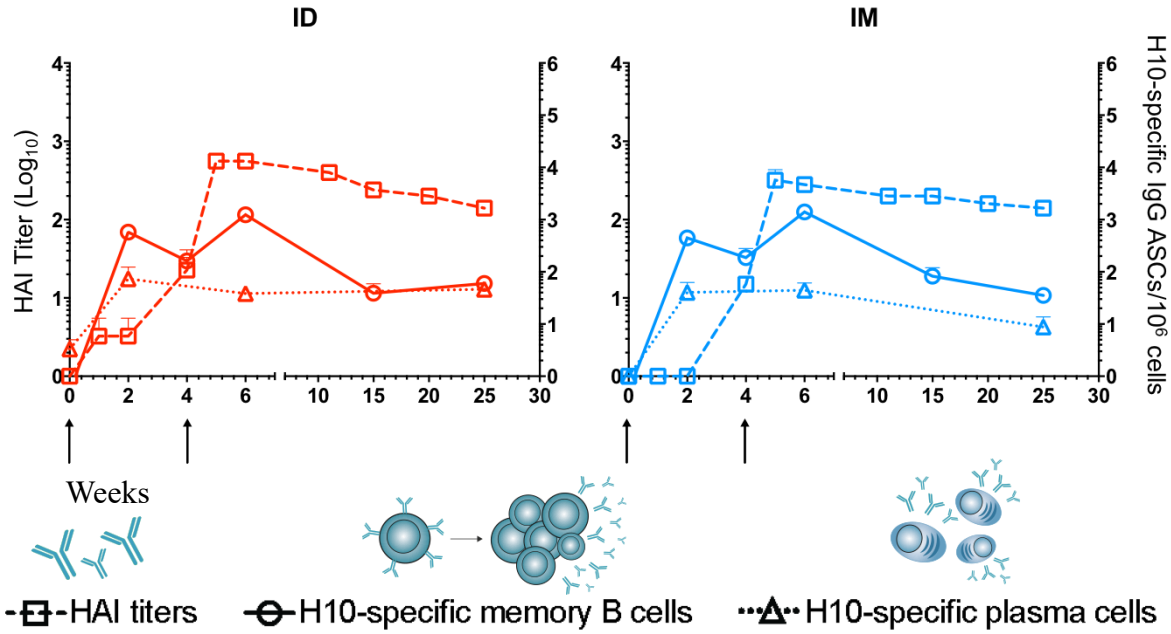


*Figure 17: mRNA vaccine induces a potent humoral immune response with early induction of antibody secreting cells (ASCs) including BM plasma cells and circulating memory B cells.*

## 9.3 A NOVEL METHOD FOR GC ANALYSIS BY IMMUNOHISTOLOGY THAT COMPARES TO FLOW CYTOMETRY

The analysis of GCs is often applied in the study of vaccine immunology, autoimmunity and cancer. In Paper I and III of the thesis, the analysis of GCs was one of my main interests, since GCs are essential to the generation of long-lived vaccine responses and affinity maturation of antibodies. Since we were interested in tracking fluorescently labelled vaccines and analyze various effector molecules which are not always easily detected by flow cytometry, we opted to analyze vaccine draining LNs by immunohistology in some situations. We therefore developed a staining protocol to stain for GCs in NHP LNs. This protocol was used for paper I and III of this thesis, and a paper investigating a liposome-based Env vaccine, not included in this thesis (*195*). We discovered that while there are established methods for the analysis of GCs by flow cytometry, there was no consensus on how to analyze GCs by immunohistology. Also, there was no data on how the two methods compare, which made comparisons between studies using the different methods difficult.

The aim of Paper IV was therefore to address the lack of comparison between the methods and also the lack of consensus on how to analyze GCs by immunohistology. We compared the two methods within the same LNs. Eighteen mesenteric LNs were cut in half and each half was analyzed by either flow cytometry or immunohistology (Figure 18).

GCs were detected by immunohistology by staining for T cells ($CD3^+$), $T_{FH}$ ($CD3^+PD1^+$) and GC B cells ($CD3^-Ki67^+$). We confirmed that $CD3^-Ki67^+$ cells were B cells by their expression

of CD20 in a separate staining. Two consecutive sections from each LN were obtained and were stained for either CD3, PD1 and Ki67 or CD3 and CD20. The consecutive staining with CD20 was included since the number of B cells was needed to normalize GC B cell data and since all antibodies did not fit into one protocol. To analyze the images, we developed a custom-made algorithm for the analysis of GCs using the freely available CellProfiler™ software. Data was collected after manually confirming that cells were accurately counted in a few control LNs.

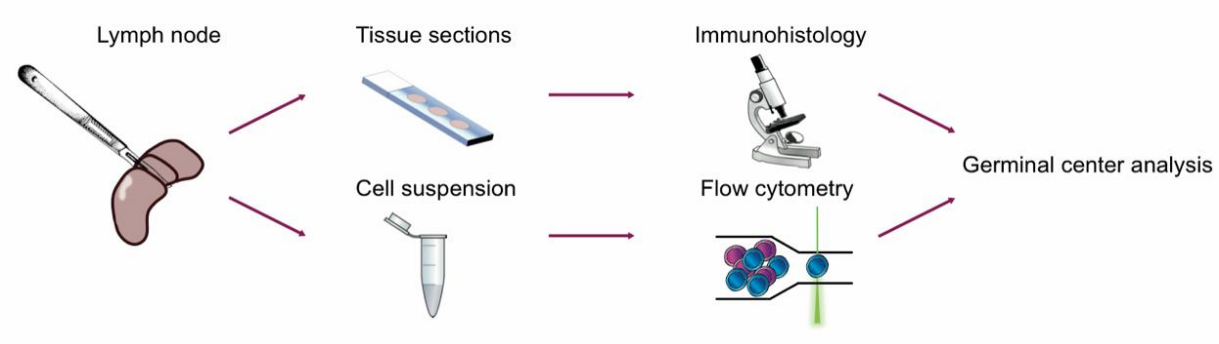


*Figure 18: Study layout for analysis of GCs with immunohistology and flow cytometry in the same LN.*

The flow cytometry panel was designed in accordance with previous studies, using the minimum number of antibodies required for accurate detection of GC B cells and $T_{FH}$ cells (*199*, *200*). Gating from live singlet cells, GC B cells were defined as $CD3^-CD20^+Ki67^+BCL6^+$ and $T_{FH}$ cells as $CD20^-CD3^+CD4^+CXCR5^+PD1^{++}$.

The number of GCs in secondary lymphoid organs is often used as a readout in experimental setups (*182*, *183*, *201*). However, defining the precise number of GCs can be difficult, since GCs in close proximity are hard to discriminate and the minimum number of cells required to qualify as a GC is arbitrary (Figure 19). Also, if the number of GCs is considered, the data can easily be distorted and nonsensical. For instance, a LN with ten small GCs will score higher than one with eight large GCs. Instead, the total number of cells in GCs or total GC area can be used to measure GC activation, which more closely resembles data collected by flow cytometry.

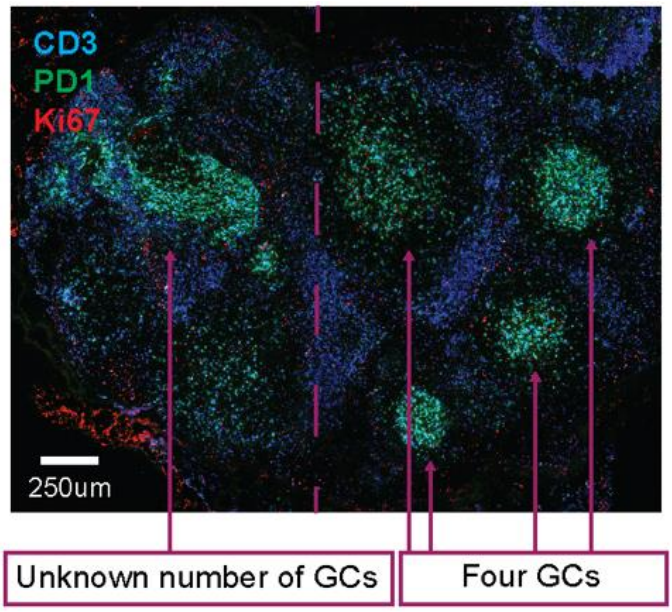


*Figure 19: GC staining illustrating the difficulty in enumerating the precise number of GCs.*

The major aim of this study was to investigate what means of analysis by immunohistology related most closely to flow cytometry. In flow cytometry, cell numbers are often presented as % of live or % of parent. Similarly, data from immunohistology needs to be normalized by an appropriate factor. For immunohistology, we normalized GC cell numbers by the number of GCs or their parent cells, i.e. T cells or B cells, and GC area was normalized by LN area. This data was then compared to similar data acquired by flow cytometry. We found that using GC numbers as a means of normalization did not relate well to flow cytometry data, which could be due to the pitfalls with accurately counting GCs discussed above (Figure 20A). Next, we compared GC area normalized by LN area to $T_{FH}$ cells or GC B cells normalized by total live cells by flow cytometry (Figure 20B). While GC area did not correlate with GC B cells by flow cytometry, it correlated well with $T_{FH}$ cells by flow cytometry. We previously established that $T_{FH}$ cells within individual GCs better correlated with the area of these GCs compared to GC B cells. This means that GC area better represents the number of $T_{FH}$ cells within them. This could explain why GC area correlates better with $T_{FH}$ by flow cytometry compared to GC B cells. Finally, we compared $T_{FH}$ cells or GC B cells normalized by parent cells to the same parameter by flow cytometry and found good correlations for both GC B cells and $T_{FH}$ cells (Figure 20C).

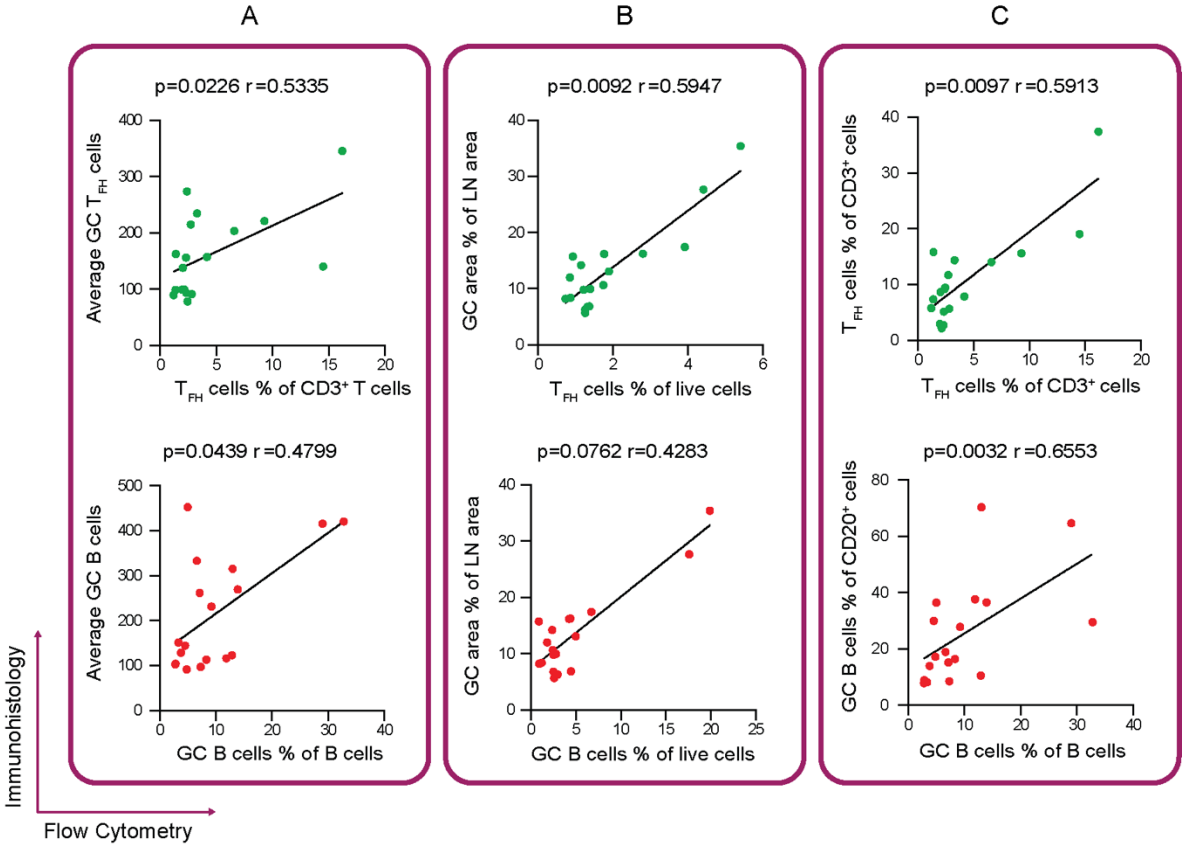


*Figure 20: Correlation of immunohistology and flow cytometry data of GCs within matched LNs.*

Based on our findings, we propose to analyze GCs by immunohistology as; GC area % of LN area or total $T_{FH}$ cells or GC B cells % of parent, to enable comparable data to that of flow cytometry.

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