From: Department of Medicine, Huddinge
Center of Infectious Medicine
Karolinska Institutet, Stockholm, Sweden

INNATE IMMUNE RESPONSES IN VIVO AFTER ANTIGEN ADMINISTRATION

– IMPLICATIONS FOR VACCINE DEVELOPMENT–

Frank Liang

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Innate immune responses in vivo after antigen administration – Implications for vaccine development –

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Frank Liang
Center for Infectious Medicine
Department of Medicine, Huddinge
Karolinska Institutet

Principal Supervisor:
Karin Loré

Karolinska Institutet
Department of Medicine, Solna
Clinical Immunology and Allergy Unit

Co-supervisor(s):
Jan Andersson

Karolinska Institutet
Department of Medicine, Huddinge
Center for Infectious Medicine

Richard A Koup

National Institutes of Health
Vaccine Research Center
Immunology Laboratory

Opponent:
Andrea Cerutti

Affiliation 1:
Icahn School of Medicine at Mount Sinai
Division of Clinical Immunology
Department of Medicine

Affiliation 2:
Catalan Institute for Research and Advanced Studies (ICREA)
Hospital del Mar d'Investigacions Mèdicques

Examination Board:

Francesca Chiodi

Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology

Sören Andersson

Örebro University Hospital
Department of Laboratory Medicine

Else Marie Agger

Statens Serum Institut
Infectious Disease Immunology
Vaccine Research and Development

ABSTRACT

Inducing high magnitude of antibodies with epitope breadth over prolonged periods of time is likely a prerequisite to prevent several of the world's most serious infectious diseases such as HIV-1, malaria and tuberculosis for which there are no vaccines yet. A much better understanding of the innate immune mechanisms that are critical for inducing strong responses to vaccination is therefore essential. The overall aim of this thesis was to characterize early innate immune responses in vivo after administration of antigens. This includes studies of the recruitment of immune cell subsets to the site of antigen injection (e.g. skin or muscle), local cell activation and presence of inflammatory mediators, antigen uptake and transport and finally initiation of adaptive immunity in lymph nodes (LNs). To be able to approach this in humans in vivo, we first utilized skin punch biopsies collected from sites injected with purified protein derivate (PPD), which is a mixture of mycobacterial antigens used in the tuberculin skin test (TST). By performing tissue staining of cryosections we show in paper I that several subsets of dendritic cells (DCs), including the plasmacytoid DCs (PDCs) normally not residing in skin, infiltrated the dermis in the positive TST indurations, which was in contrast to donor-matched saline-injected skin. The positive TST indurations were associated with cell death and high expression of the antimicrobial peptide LL37, which together can provide means for PDC activation and IFNα production. In line with this, IFN-inducible MxA was highly expressed in the positive TST sites. We expanded the studies in paper II and DC accumulation was also found in skin biopsies taken after skin tests using antigens from either mumps virus or *Candida albicans*. Further, TST indurations of HIV-1^+ individuals also showed DC infiltration but to a lower degree, which likely reflect on the reduced integrity of their immune system. To this end, the level of DCs in the positive TST reactions correlated with the level of infiltrating T cells.

The skin antigen tests represent recall of immunological memory responses locally. To enable studies of local innate immune activation after vaccine administration and priming of naïve responses, we developed a nonhuman primate (NHP) model in the second part of the thesis. After establishing and validating protocols for sample collection and tissue processing in paper III, we examined in paper IV how the distinct vaccine adjuvants; alum (benchmark), MF59 (emulsion) or alum with TLR7 agonist, influence the innate responses leading to adaptive immunity. HIV-1 envelope glycoprotein (Env) was administered as the vaccine antigen together with the adjuvants. We found a rapid infiltration of neutrophils, monocytes and DCs to the vaccine-injected muscle with all adjuvants. Env^+ cells were readily detected in the muscle and draining LNs. In line with the finding of alum-TLR7 and MF59 being superior over alum in terms of inducing both antibody- and T cell responses, alum consistently showed lower innate activation. While alum-TLR7 consistently induced robust DC maturation and type IFN I responses, MF59 induced neutrophil homing to LNs. Comparison of antigen presentation capacity of Env^+ cells in the draining lymph nodes showed that myeloid DCs exceled at stimulating Env-specific $CD4^+$ T cell responses. However, neutrophils were also capable of antigen presentation. Despite inducing different innate activation, both MF59 and alum-TLR7 enhanced the priming of Env-specific T cells in vaccine-draining LNs as well as increased the differentiation of T follicular helper cells and germinal center formations compared to alum. In summary, our findings demonstrate the initial immune events at the sites of antigen delivery, including vaccination in vivo. These early immunological responses shape the quantity and quality of adaptive immunity. Understanding the mechanisms by which distinct adjuvants influence vaccine response will help in the selection of the best-suited adjuvant to improve vaccine efficacy to a given pathogen.

LIST OF SCIENTIFIC PAPERS

I. Emily Bond***, Frank Liang***, Kerrie J Sandgren, Anna Smed-Sörensen, Peter Bergman, Susanna Brighenti, William C Adams, Senait A Betemariam, Molebogeng X Rangaka, Christopher Lange, Robert J Wilkinson, Jan Andersson and Karin Loré. Plasmacytoid denritic cells infiltrate the skin of positive tuberculin skin test indurations.
*shared first authorship.
Journal of Investigative Dermatology 2012;132(1):114-23

II. **Frank Liang**, Emily Bond, Kerrie J Sandgren, Anna Smed-Sörensen, Molebogeng X Rangaka, Christoph Lange, Richard A Koup, Grace A McComsey, Michael M Lederman, Robert J Wilkinson, Jan Andersson and Karin Loré. Dendritic cell recruitment in response to skin antigen tests in HIV-1-infected individuals correlates with the level of T-cell infiltration.
AIDS 2013, 27:1071-1080

III. **Frank Liang***, Aurélie Ploquin*, José DelaO Hernández, Hugues Fausther-Bovendo, Gustaf Lindgren, Daphne Stanley, Aiala Salvador Maritinez, Jason M Brenchley, Richard A Koup, Karin Loré** and Nancy J Sullivan**. Dissociation of skeletal muscle for flow cytometric characterization of immune cells in macaques.
*shared first authorship, **shared last authorship
Journal of Immunological Methods, 2015, S0022-1759(15)30013-2.
DOI:10.1016/j.jim.2015.06.011.

IV. **Frank Liang**, Kerrie J Sandgren, Gustaf Lindgren, Elizabeth A Thompson, Joseph R Francica, Anja Seubert, Ennio De Gregorio, Susan Barnett, Derek O'Hagan, Srinivas Rao, Nancy J Sullivan, Richard A Koup, Robert A Seder and Karin Loré. The adjuvants MF59 and Alum combined with TLR7 agonist differentially stimulate neutrophil and dendritic cell functions leading to enhanced vaccine responses.
Manuscript

Publications not included in this thesis:

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 non-human primates.
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Chuanfeng Wu, Brian Li, Rong Lu, Samson J Koelle, Yanqin Yang, Alexander Jares, Alan E Krouse, Mark Metzger, **Frank Liang**, Karin Loré, Colin O Wu, Robert E Donahue, Irvin SY Chen, Irving Weissman, Cynthia E Dunbar. Clonal tracking of rhesus macaque hematopoiesis highlights a distinct lineage origin for natural killer cells.
Cell Stem Cell 2014, 3;14(4):486-99

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Book chapter in the Encyclopedia of AIDS, Springer New York 2014 Sep 20: 1-14. DOI 10.1007/978-1-4614-9610-6_133-1. ISBN 978-1-4614-9610-6.

Kerrie J Sandgren, Anna Smed-Sörensen, Mattias N Forsell, Maritna Soldemo, Willian C Adams, **Frank Liang**, Leif Perbeck, Richard A Koup, Richard T Wyatt, Gunilla B Karlsson Hedestam, Karin Loré. Human plasmacytoid dendritic cells efficiently capture the HIV-1 envelope glycoprotein via CD4 for antigen presentation.
Journal of Immunology 2013, 191(1):60-9.

CONTENTS

PREFACE

Vaccines are one of the most important discoveries in human medicine and have saved millions of people worldwide from death related to infectious diseases. Understanding the mechanisms of how vaccines work is vital for developing vaccines against severe diseases, to which no preventive vaccines currently exist. Vaccines stimulate the immune system, which consists of a remarkable variety of immune cells with unique features and functions. I am intrigued by how vaccines stimulate these immune cells and the sequential immunological events leading to protective immunity.

In analogy with a light switch, vaccines "turn on" the immune system.

So who is flipping on the light switch in the first place?

Vaccines interact with the immune system for the first time at the site of delivery. The immune responses occurring directly after administration impact the generation of immunity. The main focus of my thesis has been the early immune responses following antigen delivery.

The outline of the thesis starts with a brief historical introduction on vaccines followed by basic immunology of responses to non-self antigens including components of vaccines. A general overview on immune responses (especially those mediated by dendritic cells) is described, starting with the delivery or exposure of foreign antigens and ending with the generation of immunity. The introduction ends with brief description on HIV-1 infection and its vaccine development, plus the role of nonhuman primates as models for vaccination. Thereafter, the aims of the thesis and method description are presented. The main findings in the papers included in my thesis are presented and discussed in the results section. Finally, this thesis will end with concluding remarks, acknowledgements, references and reprints of the original papers.

Stockholm, 2015-09-08 *Frank Liang*

LIST OF ABBREVIATIONS

AIDS	acquired immunodeficiency syndrome
Alum	aluminum salt based adjuvant
AMP	anti-microbial peptide
APC	antigen presenting cells
APRIL	a proliferation-inducing ligand
AS	adjuvant system
BAFF	B cell activating factor
BCG	Bacille Calmette Guérin
Bcl	B cell lymphoma transcription factor
BCR	B cell receptor
BDCA	blood dendritic cell antigen
CCR	CC chemokine receptor
CXCR	CXC chemokine receptor
CD	cluster of differentiation
CFA	Freund's complete adjuvant
CFSE	carboxyfluorescein succinimidyl ester
CLR	C-type lectin receptor
CMV	cytomegalovirus
CpG	cysteine-phosphate-guanine motifs on microbial DNA
CTL	cytotoxic T lymphocyte
DAMP	danger associated molecular pattern
DC	dendritic cell
DC-SIGN	DC-specific intracellular adhesion molecule-3 grabbing non-integrin
DDC	dermal dendritic cell
DiO	3,3'-Dioctadecyloxacarbocyanine Perchlorate
DNA	deoxyribonucleic acid
DTH	delayed type hypersensitivity
Env	HIV-1 envelope glycoprotein
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
FDC	follicular dendritic cell
GC	germinal center
HA	hemagglutinin
HAART	highly active anti-retroviral therapy
HIV-1	human immunodeficiency virus type 1
HLA	human leukocyte antigen
HMGB-1	high motility group protein box 1
ICOS	Inducible T cell co-stimulator
IFN	Interferon
Ig	immunoglobulin
IL	interleukin
LC	Langerhans cell
LN	lymph node
LPS	lipopolysaccharide

MDC	myeloid dendritic cell
MHC	major histocompatibility complex
MoDC	monocyte-derived dendritic cell
MPL	monophosphoryl lipid A
MTB	Mycobacterium tuberculosis
MxA	myxovirus resistace protein A
NHP	nonhuman primate
NK cell	natural killer cell
NLR	nucleotide-binding oligomerization domain-like receptor
PAMP	pathogen associated molecular pattern
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PD-1	programmed death receptor 1
PDC	plasmacytoid dendritic cell
PFA	paraformaldehyde
Poly I:C	polyinosinic-polycytidylic acid
PPD	purified protein derivate
PRR	pattern recognition receptor
rAd5	recombinant adenovirus serotype 5
RLR	retinoic acid-inducible gene 1-like receptor
RNA	ribonucleic acid
RPMI	Roswell park memorial institute medium
SHM	somatic hypermutation
SIV	simian immunodeficiency virus
STING	stimulator of interferon genes
ssRNA	single stranded ribonucleic acid
TB	tuberculosis
TCR	T cell receptor
Tfh cell	T follicular helper cell
Th cell	T helper cells
TLR	toll-like receptor
TNF	tumor necrosis factor
TST	tuberculin skin test

1 INTRODUCTION

1.1 HISTORY OF VACCINATION

Vaccination is one of the most successful medical interventions implemented to prevent mortality. So far, vaccines have saved countless lives and remain essential for prevention of death due to infectious diseases worldwide (1, 2). One of the most important milestones in public health is the eradication of smallpox disease by a vaccine that was developed almost two centuries ago (3). Reports of vaccination go back to ancient China, where inoculation of dried pus, fluid or scabs from smallpox lesions into the skin or nasal cavity of healthy people resulted in protection against this fatal disease. However, while most of the inoculated individuals established protective smallpox immunity, some of them developed the disease instead (1, 2). By using substance from lesions caused by the milder cowpox disease for inoculation, Edward Jenner demonstrated in 1796 that protection against the much more severe smallpox disease was feasible without disease transmission. The beneficial effects of these inoculations were observed empirically and Jenner was likely unaware of the specific agent causing smallpox disease and the immune responses underlying smallpox immunity. A century later, Louis Pasteur and Robert Koch discovered that infectious diseases were caused by microbial pathogens. Subsequently, Pasteur used different strategies known as attenuation, to transform pathogens to vaccines by reducing their capacity to cause disease and yet retain the ability to generate immunity. Using the attenuation strategy, Pasteur and Roux generated the first human vaccine, which was a rabies vaccine (1). Based on Jenner and Pasteur's empirical methods, i.e. using a pathogen strain closely related to the strain that causes severe disease in humans followed by the attenuation process, the vaccine against tuberculosis (Bacille Calmette Guérin, BCG) was subsequently generated. In fact, BCG is currently still the only clinical tuberculosis vaccine available, despite its suboptimal efficiency to protect from pulmonary disease.

1.2 THE IMMUNOLOGY OF VACCINATION

Vaccines are either preventive or therapeutic. While preventive vaccines inhibit initial infection, therapeutic vaccines induce immunity against existing disease. Either way, vaccination is aimed to stimulate the immune system to mount a response that protects and/or controls a disease. As during natural infection when pathogens breach the protective skin or mucosal barrier, vaccination also involve introduction of foreign substances. This triggers immune responses that lead to short-lived or lasting immunological memory (4). The immune system is divided into innate and adaptive immunity and has evolved to respond to pathogens by different defense mechanisms. While innate immune cells are quick to respond to pathogen invasion by a series of wide-range targeting molecules, adaptive immune cells confront the specific pathogen more precisely (5). The outcome of pathogenic insults is the generation of immunological memory, which is broadly defined as cell-mediated immunity (T cell responses) and humoral immunity (B cell responses). These series of events most likely recapitulates the fundamentals of early vaccine responses (4, 6). The type of adaptive

immune response as well as its quality and durability after infection or vaccination are dictated by the stimulation provided by the innate immune cells (2, 7). A more detailed understanding of the immune events leading to protective immunity would help the design of better vaccines.

1.2.1 Sentinel cells of the innate immune system

The immune system has physical, chemical, and cellular defense mechanisms against invasion by pathogens and foreign substances. The skin and mucosal tissues face the external environment and are constantly exposed to pathogens or foreign molecules. Thus, these tissues are armed with a large and complex network of innate immune cells that function as sentinels of innate immunity. This network consist of many different cells, including the resident phagocytic cells such as dendritic cells (DCs) and macrophages, which are professional antigen presenting cells (APCs) that scan the tissues for antigens of either self or non-self origin. Among the APCs, the DCs have the superior capacity to initiate and regulate adaptive immune responses (7-9). Vaccines such as the BCG vaccine and some influenza vaccines are delivered to the skin and the nasal cavity respectively and are likely to interact with these sentinel cells. Encounter of pathogens or foreign antigens like vaccine components activate innate cells, which subsequently initiate an array of functions to ultimately destroy pathogens as well as limit their infectivity and dissemination.

1.2.2 Sensing of foreign antigens by innate immune cells

The initiation of immune responses depends on the ability to sense the presence of foreign antigens. Pattern recognition receptors (PRRs) sense pathogen structures and products known as pathogen associated molecular patterns (PAMPs). The PRRs comprise the C-type lectin receptors (CLRs), NOD-like receptors (NLRs), stimulator of interferon genes (STING), RIG-I-like receptors (RLRs) and toll-like receptors (TLRs) (9, 10).

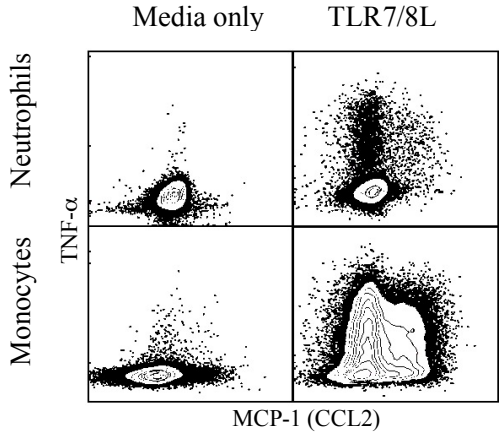

Figure 1. Flow cytometer sorted human monocytes and neutrophils produce pro-inflammatory cytokine (TNF-α) and chemokine (MCP-1) after stimulation with TLR7/8 ligand.

The TLRs bind to specific PAMPs such as pathogen-derived nuclei acids (TLR3, TLR7, TLR8, TLR9) or bacterial and fungal products (TLR1, TLR2, TLR4, TLR5, TLR6), which activates the immune cells. As discussed below, each type of innate immune cell expresses a distinct repertoire of TLRs (9-13).

The activation mediated by TLRs stimulates several immune functions for efficient pathogen elimination. These functions include production of different mediators like cytokines (molecules that activate and/or regulate cellular functions) and chemokines (molecules attracting cells towards a specific site) (Fig. 1). Capacity to present antigens and stimulate adaptive immune cells such as T cells and B cells are also enhanced in activated APCs (5). However, activation of immune cells can also occur in the absence of microbial PAMPs, e.g. during tissue injury or autoimmune disorders. Such "sterile inflammation" is mediated by danger associated molecular patterns (DAMPs), which include self-derived antigens such as antimicrobial peptides and nucleic acids (14). To this end, the innate immune cells are attractive targets in vaccine development since their activation through TLRs may potentially influence the quality of vaccine-induced immunity (6).

1.2.3 Connecting innate immunity with adaptive immunity

In general, efficient vaccines are defined by their ability to induce durable and protective adaptive immunity. The adaptive immune system recognizes a given pathogen by sophisticated recognition systems consisting of T cell receptors (TCRs) and B cell receptor (BCRs). Initially, T cells and B cells are less differentiated and referred to as naïve. In order for naïve T cells to fully carry out their effector functions against novel pathogens or antigens, they need to receive proper stimulation by innate immune cells (15). As described below, DCs are critical activators of naïve T cells through their high expression of co-stimulatory molecules and major histocompatibility complex (MHC) loaded with processed antigen fragments (i.e. peptides), which are recognized by cognate TCRs. Extracellular proteins that are taken up by APCs are processed into peptides that are complexed with MHC class II molecules such as human leukocyte antigen (HLA)-DR. In contrast, intracellular proteins and those derived from intracellular pathogens are presented on MHC I.

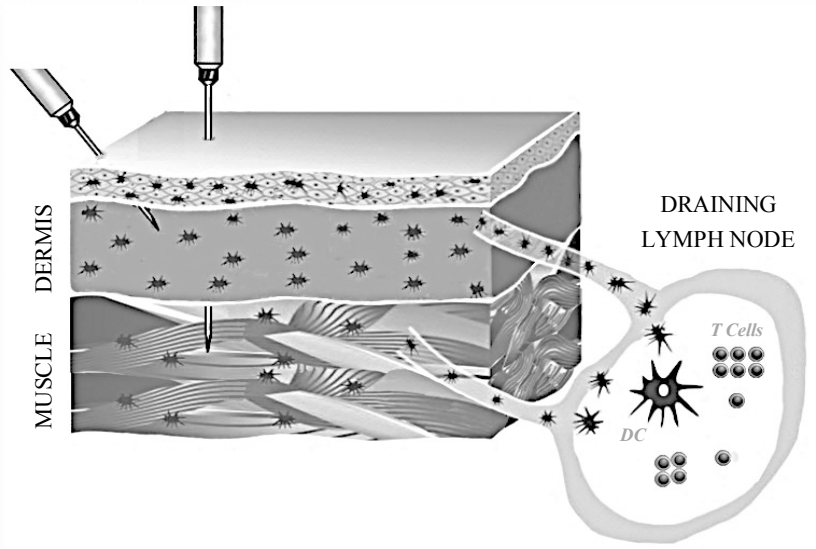

Figure 2. Administration of intradermal versus intramuscular vaccines

Most vaccines are injected into the muscle but some are delivered to the dermis of the skin and the immunological events occurring after vaccine administration are likely similar to those induced during pathogen exposure (Fig. 2). It is believed that subsequent to vaccine

delivery, DCs infiltrate the site of vaccine administration to capture antigens and become activated. Subsequently, activated DCs migrate to the draining lymph nodes (LNs) to prime naïve T cells via antigen presentation and co-stimulation, which leads to expansion of antigen-specific T cell clones and differentiation to different T helper (Th) cells (16, 17). However, vaccine antigens from the delivery site may also passively reach the LNs and be presented by LN-resident DCs (18). In simple terms, naïve $CD4^+$ T cells primed via MHC II pathway yield Th1 or Th2 effectors cells. Several additional $CD4^+$ Th cell subsets have been described, including Th17 cells involved in anti-bacterial and anti-fungal responses, T follicular helper (Tfh) cells mediating the augmentation of antibody responses (19) and T regulatory (Treg) cells with immune suppressive functions (17). In contrast, MHC class I presents antigens to $CD8^+$ T cells, which differentiate into CTLs that kill infected cells or tumor cells. In this regard, vaccines that promote differentiation of Th1 helper cells and CTLs (cell-mediated response) are likely important for control of chronic infections and tumors (10, 13). In contrast, the Th2 helper cells support antibody production (humoral response) by providing B cell stimulation. Later on, some of the Th cells will become a part of the memory lymphocyte pool (20, 21).

1.2.4 Considerations in vaccine development

Most current vaccines work by inducing production of antibodies, which are the main effector molecules of the humoral immunity that target extracellular pathogens. Diseases such as diphtheria, tetanus, and measles are brought under control by vaccine-induced antibody responses. Intracellular pathogens such as human immunodeficiency virus (HIV)-1 would probably require both humoral and cell-mediated immunity to target free virus and infected cells respectively. Some of the challenges to establish preventive vaccines relate to the complexity of the pathogens in terms of their route of infection, life cycle and ability to escape immune responses (2). Desirable features of a vaccine include rapid generation of long lasting protection and the ability to induce the type of immune response that best eliminates the pathogen. An ideal vaccine should also induce protective immunity in immune-compromised individuals who are especially susceptible to infections. Elderly and young children may also need more efficient vaccines to compensate for reduced ability to mount robust immune responses. Importantly, vaccines need to have good safety and tolerability profiles while remaining potent to stimulate the immune system. The several challenges in vaccine development stress the need for a better understanding of how vaccines interact with the human immune system. Characterization of host-pathogen interactions and production of better recombinant vaccine antigens would also greatly contribute to improved design of vaccines against diseases to which no preventive vaccines currently exist, such as HIV-1, malaria and Ebola. In addition, understanding of how vaccine adjuvants stimulate immune responses that result in cell-mediated or humoral immunity or both, would help formulate vaccines that best target a given disease.

1.3 VACCINE ADJUVANTS

1.3.1 The role of vaccine adjuvants

Many of the existing vaccines consist of live attenuated or killed whole pathogens and thereby retain a high degree of similarity to the real pathogen in terms of microbial structures and contents. These vaccines have therefore inherent abilities to induce strong immune responses on their own (6). However, the inactivated polio vaccine and attenuated BCG vaccine are relatively inefficient despite containing intrinsic PAMPs. Further, attenuated vaccines that are in most cases efficient and safe can be precarious due to the risk of live attenuated vaccine strain to regain original virulence (2, 4). Furthermore, live attenuated pathogens are also not suitable for vaccinating immune-compromised individuals (4). To improve safety profiles, many of the licensed vaccines are non-live subunit vaccines that consist of specific proteins or capsular polysaccharides (3), which correspond to those found on the pathogens. These antigens are selected based on the efficiency and versatility of the adaptive immunity they are able to induce. Recombinant technologies in vaccine development have enabled production of scalable amounts of highly purified proteins for subunit vaccines. These vaccines lack PAMPs such as nucleic acids and other pathogenic components with intrinsic capacity to induce strong immune responses (22). Since activation of innate immune responses is a prerequisite for generating immunological memory, protein subunit vaccines are formulated together with an adjuvant to enhance their immune-stimulatory properties. In fact, the word adjuvant comes from the Latin verb *adjuvare* meaning “to help”. Since adjuvants amplify immune responses, their immune stimulatory properties can also result in dose sparing which means that less amount of the vaccine antigen per vaccination and/or fewer vaccinations can be used without compromising the quantity and quality of subsequent immune memory (13).

Adjuvants can be broadly divided into delivery systems, immune potentiators and adjuvant systems i.e. combination of adjuvants (2, 23-25). Many novel adjuvants are in pre-clinical studies or clinical trials and include; oil-in-water emulsions, cytokines, bacterial toxins, polymeric microparticles, liposome-based adjuvants, polysaccharides, TLR agonists, saponin-based molecules and combinations thereof. Currently, there are only a few adjuvants approved for human vaccines. Alum, emulsions and virosomes (liposome-based adjuvant) are referred to as delivery systems since co-delivery of protein antigens with these adjuvants greatly enhance cellular antigen uptake compared to when protein antigens are administered alone (13, 26). Increased antigen uptake likely leads to better antigen presentation. However, delivery systems can also induce immune activation although they are not believed to directly target and activate specific immune cells by specific PRRs. In contrast, it is well established that monophosphoryl lipid A (MPL) derived from bacterial cell wall component called lipopolysaccharide (LPS) targets and activates cells via TLR4. Combination of the immune potentiator MPL and alum (AS04) is currently licensed for vaccines against hepatitis B virus as well as human papilloma viruses that cause cervical cancer.

The quantity, quality and durability of the generated immune responses set the criteria for vaccine efficacy, and how adjuvants enhance these criteria is of great interest in vaccine development. For example, adjuvants that induce high antibody levels may enable quicker pathogen clearance and mutation-prone pathogens could be more efficiently targeted if the adjuvants induced antibodies with broad specificity. However, the efficacy of a vaccine may also depend on adjuvants capable of inducing both humoral and cell-mediated immunity. How adjuvants work is not fully understood but some are thought to activate immune responses by mimicking the effects of PAMPs (2, 6), and/or enhance the functionality of both innate or adaptive immune cells, or prolong the persistence of the administrated vaccine in vivo (depot effect) and thereby extending the time for vaccines to interact with immune-competent cells (27). Recently, considerable efforts have been focused on understanding the mechanisms of action of adjuvants. Indeed, strategies in vaccine development include formulating vaccine antigens with suitable adjuvant(s), which induces immune responses that best target the specific disease with improved efficiency, while remaining safe and tolerable.

1.3.2 Aluminum adjuvants

The most commonly used clinical adjuvant is based on different insoluble aluminum salts (aluminum hydroxide or aluminum phosphate), commonly referred to as alum. Alum has been used for almost 70 years in licensed vaccines. The vaccine antigens are adsorbed to alum by electrostatic forces, where negatively charged antigens adhere to the aluminum hydroxide while positively charged antigens are better attached to aluminum phosphate (27). Alexander T Glenny discovered alum already in 1926. Alum mainly enhances antibody-based vaccine responses and thereby suitable for targeting extracellular pathogens, which are vulnerable for antibody-mediated immune responses. Despite being extensively used, the mechanisms behind alum's adjuvant effect remains incompletely understood. It has been proposed that alum causes a depot effect as described above. It has also been suggested that adsorbing vaccine protein antigens to alum facilitates antigen uptake since the antigen become particulate. Recently, several mouse studies suggested that alum induces inflammation at the delivery site by engaging different pathways dependent on TLR signaling (28). However, there are also reports showing that TLRs are not involved (29) and that alum causes release of metabolites due to cell damage that activates other cells (30). Despite the unclear mechanism of action, alum is by far the most clinically used adjuvant and it induces sufficient responses for many vaccines and has an exceptional safety profile. However, alum mainly induces antibody responses, which may not be sufficient to clear e.g. intracellular pathogens. Intense research is currently pursued to develop new adjuvants that are able to induce better antibody- as well as cell-mediated immune responses.

1.3.3 Emulsion adjuvants

Distinctly different from alum are the emulsion adjuvants based on water and oil mixtures. Emulsion adjuvants exist as a "water-in-oil" emulsion when aqueous droplets are dispersed within an oily media and when the opposite is formulated, it is called "oil-in-water" emulsion. The earliest report on an emulsion used as an adjuvant was reported by Jules T

Freund in 1937 and this empirically discovered adjuvant is commonly known as Freund's adjuvant (31). Freund's adjuvant consists of paraffin oil plus surfactant and when killed mycobacteria are included, it is referred to as complete Freund's adjuvant (CFA) (10). Although CFA has been shown to be efficient in stimulating both humoral and cell-mediated immunity, this emulsion adjuvant is not approved for human vaccines since it causes painful and harmful reactions at the injection site (10, 23, 31). In contrast, highly refined and well-characterized oil-in-water emulsion adjuvants such as MF59 and AS03 are well tolerable and are licensed for pandemic influenza vaccines (10, 32, 33). Both of these emulsions are based on squalene, a precursor molecule to natural cholesterol that is more readily metabolized than paraffin oil in CFA (10, 33), which may explain why these adjuvants have better safety profiles. The other components in MF59 consist of surfactants that enable dispersion of approximately 160 nm wide squalene droplets within the aqueous citrate buffer. In addition to surfactants, the AS03 emulsion also contains α-tocopherol (vitamin E), which has immune enhancing properties (34).

Nearly 100 million doses of MF59 have been commercially distributed and its safety profile has been extensively assessed (33). Influenza vaccine given together with MF59 has proven to be effective and safe among the elderly and young children, which are especially at risk for influenza-related fatality and do not respond well to traditional influenza vaccines due to their insufficient capacity to mount strong immune responses. In fact, several studies based on large cohorts of elderly participants, infants and young children show that MF59 significantly increased the efficacy of influenza vaccines and the generated antibodies had broad specificity (33). Since influenza virus mutates frequently, the broadened specificity of antibodies induced by the vaccine is particularly important. Especially since the vaccine is based on strains predicted to cause disease, which mean that the degree of protection is likely related to the cross-reactivity of the vaccine-induced antibodies. Moreover, dose sparing was observed in MF59-adjuvanted pandemic influenza vaccine since it induced protective antibody levels with lower antigen doses and fewer immunizations (33). How MF59 stimulates immune responses is largely unclear and is thought to induce a so-called "immunocompetent environment" that facilitates innate immune events such as infiltration of immune cells and expression of molecules involved in cellular activation, migration and antigen presentation (33, 35).

1.3.4 TLR agonist-based adjuvants

In contrast to alum and MF59, which mainly enhance antibody responses, immune potentiators have the potential to activate cell-mediated immune responses. While the mechanisms of action of alum and emulsion adjuvants are largely unclear, the actions of immune potentiators are directly related to activation of specific PRRs such as the TLRs (10, 13). Most immune potentiators are TLR agonists but so far, only MPL is approved. MPL is chemically removed of the toxic properties of LPS and activates immune cells like DCs by binding to TLR4 (Table 1). Other adjuvants in clinical trial that involve TLR stimulation are CpG DNA and Imidazoquinolines, which are agonists for TLR9 and TLR7/8 respectively

and these adjuvants enhance both cell-mediated and humoral immunity as well as promote sustained immune responses (10, 23, 24, 32). Importantly, the TLR7 agonist Imiquimod that is licensed as topical therapy for genital warts was shown to induce higher antibody responses in elderly participants of an influenza vaccine trial where intradermal immunization was given immediately after applying Imiquimod on the skin of the injection area (36). Since TLR7 agonist is already implemented as a topical treatment in the clinic, their approval for use in human vaccines may be facilitated.

	TLR1	TLR2	TLR3	TLR4	TLR5	TLR6	TLR7	TLR8	TLR9
$CD1c^+$ MDCs									
$CD141^+$ MDCs									
$CD16^+$ MDCs									
PDCs									

Table1. TLR expressed by blood DCs, where the level of expression is indicated accordingly: Black (+ + +), dark grey (+ +), medium grey (+) light grey (+/–) and white (–)

However, TLR agonists are usually co-formulated with delivery systems such as alum since immune potentiators are small molecules that diffuses rapidly from the site of delivery (24). Using combinations of adjuvants may enable cellular activation via multiple innate receptors, which could be more effective than activating a single pathway (10). Nevertheless, immune responses initiated by a single pathway may in turn activate other pathways and/or activate adjacent immune cells in a bystander manner. Adjuvant systems have been shown to alleviate several challenges in vaccine development by eliciting effective immunity against complex pathogens. As reported in malaria vaccine trials, MPL plus a saponin-derived molecule (QS21) together with either an oil-in-water emulsion or liposomes induced increased protection via enhanced antibody and cell-mediated responses compared to alum alone as adjuvant (10, 23). Recently, a small molecule TLR7 agonist adsorbed to alum has shown potent adjuvanticity, which demonstrates the benefits of combination adjuvants (24, 37).

1.4 THE EARLY IMMUNE RESPONSES AFTER ANTIGEN DELIVERY

1.4.1 Recruitment of antigen presenting cells to antigen delivery sites

Recruitment of DCs to the site of antigen exposure and internalization of antigen by these cells are thought to be essential in the generation of adaptive immune responses and control of immunity (7, 8, 38, 39). This is most likely the case for development of responses to vaccines too. However, recruitment of adjacent tissue-resident immune cells or from the circulation (e.g. neutrophils, monocytes and blood DCs) require inflammation and one of the earliest innate responses in tissues exposed to foreign material like whole pathogens, non-self antigens or vaccines, is the secretion of cytokines and chemokines. DCs are found in blood and at body surfaces such as the skin and mucosal tissues as well as lymphoid tissues. Thus, the dense population of DCs residing in skin is optimally located for exposure to intradermal vaccines (40), which could mean that they can readily take up vaccine antigens and rapidly

become activated by the vaccine itself or the vaccine-induced inflammatory milieu. Chemokines and cytokines secreted by recruited cells may in turn recruit and activate other immune cells. In contrast to the skin, muscle tissue contains relatively few resident APCs (41) and infiltrating immune cells from the circulation such as DCs are likely those that mainly interact with intramuscular vaccines (6, 42). In this regard, upregulation of chemokines and cytokines in the adjuvant-injected muscle has been reported in mice (35).

1.4.2 Non-classical and atypical antigen presenting cells

Although DCs are critical for priming of naïve immune responses, mouse studies show that non-classical innate immune cells and atypical non-hematopoietic cells are also able to present antigens (43). The atypical APCs include non-hematopoietic cells such as epithelial, endothelial, stromal and skeletal muscle cells and are likely the initial producers of cytokines and chemokines for recruiting bone marrow-derived immune cells. However, non-hematopoietic cells cannot migrate to draining LNs to prime naïve T cells and probably mediate in local memory responses. Skeletal muscle cells, which are especially exposed to intramuscular vaccines and bone marrow-derived non-classical APCs like neutrophils, do not constitutively express MHC II required for $CD4^+$ T cell dependent adaptive responses. However, MHC II expression can be induced in skeletal muscle (44) during inflammatory conditions and neutrophils (45) express both MHC II and T cell co-stimulatory molecules under these conditions. The expression of MHC I expression for $CD8^+$ T cell responses is detectable on neutrophils and nearly absent in normal skeletal muscle but can be upregulated in presence of cytokines (46-48). In addition, TLR4 is expressed by human neutrophils (49) and TLR7 can be detected on skeletal muscle of patients with inflammatory muscle disorders (50), which suggest that these cells have the necessary PRRs to respond to licensed adjuvants targeting these TLRs. Moreover, mouse neutrophils have reported to be able to capture antigens in the periphery and shuttle vaccine antigens to LNs and express chemokine receptors for homing to LNs (51, 52). Interestingly, human neutrophils in spleen have reported to support antibody responses via cytokines (53) and mouse neutrophils seem capable of inducing T cell responses to protein antigen (54). Although these reports suggest that neutrophils positively support adaptive responses, the opposite effect has also been observed in mice (55). In general mouse and human neutrophils differ in expression of several markers involved in both innate and adaptive immunity (56). For example, mouse and not human neutrophils produce the immunosuppressive cytokine IL-10 (57) and the co-stimulatory molecule B7-H3 activate T cells in humans but inhibits T cell activation in mice (56, 58). The proportions of neutrophils and lymphocytes are also different between mice and humans (56). However, human neutrophils have also been shown to suppress $CD4^+$ T cell responses, while supporting B cell responses (53). Thus, the role of neutrophils in vaccine-induced adaptive responses needs further elucidation.

1.4.3 Human dendritic cells in blood

Human DCs comprise multiple subsets with distinct functions and represents approximately 0.5 % of peripheral blood leukocytes (59). In general, human blood DCs can be divided into

two major subsets: myeloid DCs (MDCs) and plasmacytoid DCs (PDCs). While PDCs are normally absent in skin, MDCs are found residing in normal skin. However, the MDCs in both blood and skin are further subdivided into several subsets (Fig. 3). The existence of multiple DC subsets with distinct functions suggest a division of labor regarding the stimulation and regulation of adaptive immune responses (39). In humans, PDCs express the interleukin (IL) -3 receptor (CD123), CD303 (BDCA-2) and CD304 (BDCA-4) (60, 61). PDCs are especially competent in antiviral immunity due to their capacity to rapidly produce high amounts of type I IFNs in response to viruses, which can also activate other APCs in a bystander manner (62). However, they may also play role in tolerance in mucosal tissue (63). PDCs sense viral nucleic acids via TLR7 and TLR9, and respond readily to synthetic agonists to these receptors (64), which indicate their susceptibility to activation by adjuvants targeting these TLRs. Interestingly, reports have also suggested that DAMPS such as host cell-derived self-nucleic acids can activate PDCs (65, 66). Type I IFNs have also been shown to support antibody responses in humans (67, 68) and mice (69). In addition, PDCs have also been reported to support Th1 responses in presence viral antigens (70, 71). Thus, an adjuvant that targets e.g. TLR7 could lead to strong PDC activation and bystander activation of other cells, which subsequently could support both humoral and cell-mediated immunity.

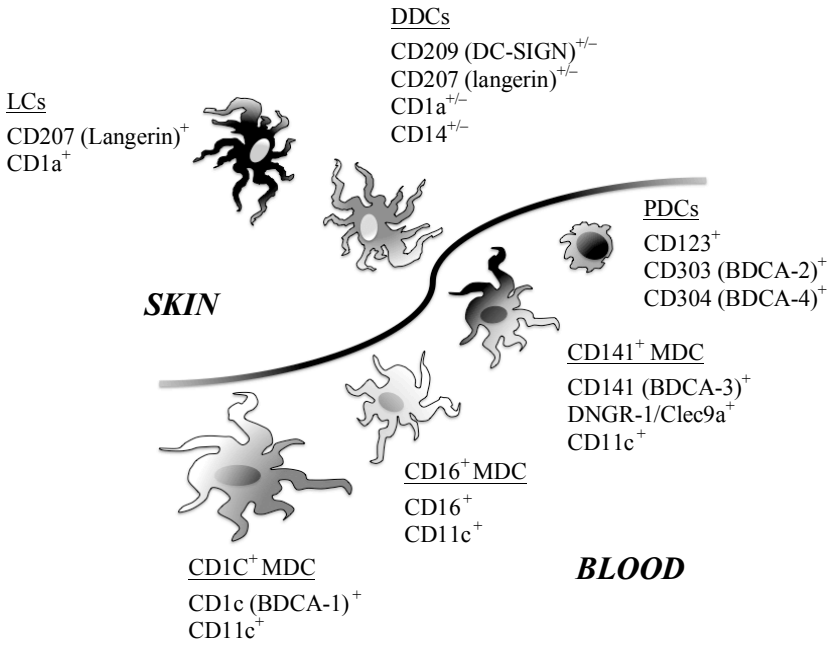

Figure 3. The phenotype of human DC subsets in skin and blood.
LC (Langerhans cells), DDC (Dermal DCs, MDC (Myeloid DC), PDC (Plasmacytoid DC)

While two subsets of PDCs in human blood have been reported (72), circulating MDCs are subdivided into distinct subsets defined by their respective expression of CD1c (BDCA-1)$^+$ MDCs, CD141 (BDCA-3)$^+$ MDCs and CD16 (61, 73). PDCs, CD1c$^+$ MDCs and CD141$^+$ MDCs are also found residing in lymphoid tissues (74) where antigen presentation occurs. All DC subsets are capable of presenting antigens, although the MDC subsets and in particular CD1c$^+$ MDCs, have shown to be more efficient in stimulating CD4$^+$ T cell responses compared to PDCs (75-78). CD1c$^+$ MDCs express TLR4, TLR7 and TLR8 (73, 75)

and when they infiltrate vaccine delivery sites, TLR-targeting adjuvants may activate them to induce specific adaptive immune responses. For example, activated MDCs can produce IL-12, which supports cell-mediated responses by inducing CTL response. Cross-presentation enables extracellular antigens to be presented on MHC I (for $CD8^+$ T cell responses) instead of MHC II (for $CD4^+$ T cell responses). $CD141^+$ MDCs have been reported to be superior in cross-presentation (61, 79, 80), which may induce CTLs to control of intracellular infections. However, the contribution of antigen presentation by $CD141^+$ MDCs may be small since they comprise only 0.04% of PBMCs and recently human $CD1c^+$ MDCs as well as PDCs have shown to cross-present antigens as well (81, 82). In contrast, the $CD16^+$ MDCs are the most abundant DC subset in the blood and have lower capacity to present antigens compared to other MDC subsets (73, 75). The $CD16^+$ MDCs have been suggested to have a more inflammatory phenotype due to their rapid production of pro-inflammatory cytokines such as TNF-α. Compared to other MDCs, TLR4 is highly expressed by $CD16^+$ MDCs (73, 75), and they might contribute to the adjuvant effect of MPL in licensed vaccines.

1.4.4 Human dendritic cells in skin

While MDCs and PDCs are found in blood and lymphoid tissues, only DCs of the myeloid origin are found in the skin during normal conditions, which make these MDCs readily available target cells for intradermal vaccines or skin test antigens. The human skin is divided into the outermost layer called epidermis, which faces the external environment, and the underlying dermis. While the epidermis is cell dense, the dermis consists mainly of collagen matrix with scattered cell populations such as macrophages and DCs. Skin-residing DCs are broadly defined as epidermal Langerhans cells (LCs) and dermal DCs (DDCs) (83). The LCs were the first identified skin-DC subset by Langerhans in 1868 but Steinman and Cohn first demonstrated their function in 1973. LCs highly express langerin (CD207), CD1a and Birbeck granules, which are unique in LCs (83). However, langerin expression has also been observed in some DDCs, but it is unclear if they represent epidermal LCs migrating to draining LNs or a distinct DDC subset. The DDCs are heterogeneous and their definition varies due to lack of specific markers. Nevertheless, DDCs can be divided into two major subsets; DC-$SIGN^+$ (CD209), $CD14^+$, $CD1a^{low}$ and $langerin^-$ DDCs and DC-$SIGN^-$, $CD14^-$, $CD1a^+$, $langerin^-$ DDCs (83) and referred to as $CD14^+$ DDCs and $CD1a^+$ DDCs. Regarding TLR expression, $CD14^+$ DDCs and LCs seem to express TLR4, which suggest that they might respond to TLR4 agonist adjuvanted vaccines delivered to the skin (84, 85). Live attenuated BCG vaccine and yellow fever vaccine are safe for administration to dermis and subcutis respectively. These vaccines do not contain adjuvants but they have intrinsic PAMPs themselves that likely target TLRs. Whether subunit protein vaccines formulated with TLR agonists can be delivered to the human skin needs to be further addressed.

1.4.5 Phenotypic maturation of dendritic cells

The quality of vaccine-induced immunity may depend on how an adjuvant influences DC activation, which involves upregulation of proteins necessary for antigen presentation, T cell stimulation and migration to lymphoid tissues. The functional properties of DCs depend on

whether they are in a resting or activated state, which is referred to as "immature" and "mature" respectively. DC maturation involves morphological and functional changes subsequent to activation via e.g. TLRs (5, 86). In normal conditions, DCs in peripheral tissues are described "immature" with an exceptional capacity to take up antigens and express relatively low levels of MHC II, T cell co-stimulatory molecules e.g. CD80, CD86, CD40 (86). Upon activation, mature DCs reduce their antigen uptake capacity and instead upregulate MHC and co-stimulatory molecules as well as the chemokine receptor, CCR7 for migration to draining LNs. The amount of T helper cells and their qualitative properties are likely dependent on antigen presentation in secondary lymphoid organs, where rare antigen-specific naïve T cells interact with mature DCs.

1.4.6 Shaping of T cell immunity by dendritic cells

Generally, three types of stimuli are involved to prime T cells leading to proliferation (i.e. clonal expansion) and differentiation (15, 87, 88). The first stimulus involves engagement of MHC-peptide complex and TCR. Simultaneously, T cells receive the second stimulus when co-stimulatory molecule on DCs, e.g. CD80 binds to CD28 on T cells. The third stimulus consists of cytokines, where IL-12 (IL-12p70) and IL-10 derived from DCs induce differentiation of Th1 and Th2 effector cells respectively with distinct cytokine profiles. While Th1 effector cells secrete primarily IL-2 and IFN-γ, Th2 effectors secrete IL-4, IL-5, IL-10 and IL-13 (20) and the type of Th effector cells determine the outcome of infections. For example, at the site of pathogenic insult, Th1 effector cell secrete IFN-γ to enhance clearance of infected cells by macrophages and release of IL-5 by Th2 effector cells facilitate killing of parasitic worms. Priming of $CD8^+$ T cells leads to expansion and differentiation into CTLs secreting e.g. IFN-γ, TNF-α, and IL-2 plus the cytolytic effector molecules; granzyme A/B and perforin. As mentioned above, cross-presentation by $CD141^+$ MDCs plus IL-12 derived from $CD1c^+$ MDCs has been reported to induce antigen-specific $CD8^+$ T cells responses (79, 89). The CD40-CD40L interaction between $CD4^+$ T cells and DCs is also believed to help DCs induce $CD8^+$ T cell responses (21, 90) and this interaction may enable protein-based vaccines to induce CTLs. However, DC and T cell interaction could also lead to tolerogenic responses (63, 87), which may contribute to the tolerability aspect of adjuvants. Regarding skin-DCs, epidermal LCs were found to be more efficient in inducing CTLs compared to $CD14^+$ DDCs in mouse studies (84). LCs were also better than $CD14^+$ DDCs at cross-presentation and generated CTLs displaying higher TCR avidity and more abundant cytotoxic effector molecules (84).

1.4.7 Antibody responses mediated by dendritic cells

Since most vaccines typically induce antibody responses (91), understanding the events that determine the quality and quantity of antibodies is highly relevant in vaccinology. One of the goals for preventive vaccines is to elicit neutralizing antibodies that can block infection by binding to epitopes on pathogen molecules to hinder successful infection. Therefore, neutralizing antibodies with broad specificity are needed for pathogens that frequently alter their epitopes by mutation (92). The quality of an antibody can also be defined by its affinity

i.e. the strength it binds to the epitope, which increases the probability for the antibody to remain bound to its epitope and sustain neutralization. Generating neutralizing antibodies with high affinity requires affinity maturation associated with somatic hypermutation (SHM), where gene segments affecting the variable regions are recombined to generate mutated antibodies with augmented functions (93, 94). More often, antibodies bind antigens in order to make them susceptible for phagocytosis. Gene recombination is also involved in switching of the invariant part of the antibody called Fc-region. The Fc-region determines the isotype (or class) of the antibody and enables interaction with cells through isotype-specific Fc-receptors during e.g. uptake of antibody-coated antigens. Thus, how efficient vaccine-induced antibodies perform these functions may reflect on the capacity of the vaccine to provide protection. Several events such as isotype-switch, SHM and generation of long-lived plasma cells secreting high affinity antibodies occur in the germinal centers (GCs) where Tfh cells provide of T cell help to antigen-specific B cells (19, 95, 96).

In regards to DCs supporting GC reactions, human $CD14^+$ DDCs but not LCs or $CD1a^+$ DDCs have been shown to polarize naïve $CD4^+$ T cells to Tfh-like cells (97). In contrast, LCs seem better than $CD14^+$ DDCs in inducing naive $CD4^+$ T cells into Th2 effector cells that secrete IL-4, IL-5 and IL-13, which are known to support antibody responses (97). Further, IL-12 produced by human monocyte derived DCs (MoDCs) has been shown to induce Tfh-like cells that secrete IL-21 for regulation of antibody responses in vitro (98). Both T cell dependent and independent B cell responses leading to isotype switch are also supported by PDCs (68) and the DC- and neutrophil-derived cytokine, B cell-activating factor (BAFF) promote B cell survival and plasma cell differentiation (68, 99, 100). To this end, antibody responses induced by protein-based vaccines are likely dependent on T cell help and TLR7 and TLR9 expressed by PDCs and B cells may enhance these responses. In addition, mouse studies have reported that DCs can facilitate antibody responses by providing antigens to B cells (101).

1.4.8 T follicular helper cells and germinal center reaction

In general, antibody production is T cell independent or dependent. Responses to proteins typically requires $CD4^+$ T cell help (102). While naïve B cells can acquire large particulate antigens attached on macrophages, follicular dendritic cells (FDCs) or DCs, small antigens might directly pass into the B cell zone from afferent lymph vessels (103). After encountering protein antigens, B cells migrate to the T cell-B cell border to receive T cell help. Antigen presentation and co-stimulation between the antigen-specific lymphocytes lead to a short B cell proliferation and the B cell progenies then differentiate along two pathways (96). While the follicular pathway give rise to germinal centers (GCs), the extrafollicular pathway result short-lived plasma cells. In the follicular pathway, some of the expanded progenies migrate to B cell zone to proliferate intensely and thereby give rise to the so-called dark zone of the GC. Proliferating B cells in the dark zone are called centroblasts, which undergo SHM and isotype-switch. Subsequently, centroblasts exit the cell cycle to become centrocytes and acquire antigen supplied by FDCs, which they present to Tfh cells in the so-called light zone.

Interaction with Tfh result in; a) survival and selection of high-affinity centrocytes, b) recycling of centrocytes back to the dark zone for another round of SHM and isotype-switch, or c) differentiation into long-lived plasma cells and memory B cells. The positively selected B cells with high affinity BCR leave the GC either as plasmablast (precursor to the antibody secreting plasma cells) or as memory B cells, which rapidly differentiate into plasma cells upon antigen re-exposure (104). The GC reaction may therefore correlate with the quality of vaccine-induced humoral response since it gives rise to long-lived plasma cells secreting antibodies with improved functionality (94). Therefore, adjuvants that induce appropriate stimulation of APCs at the site of administration may reflect on the subsequent Tfh cell differentiation and GC formations (Fig. 4).

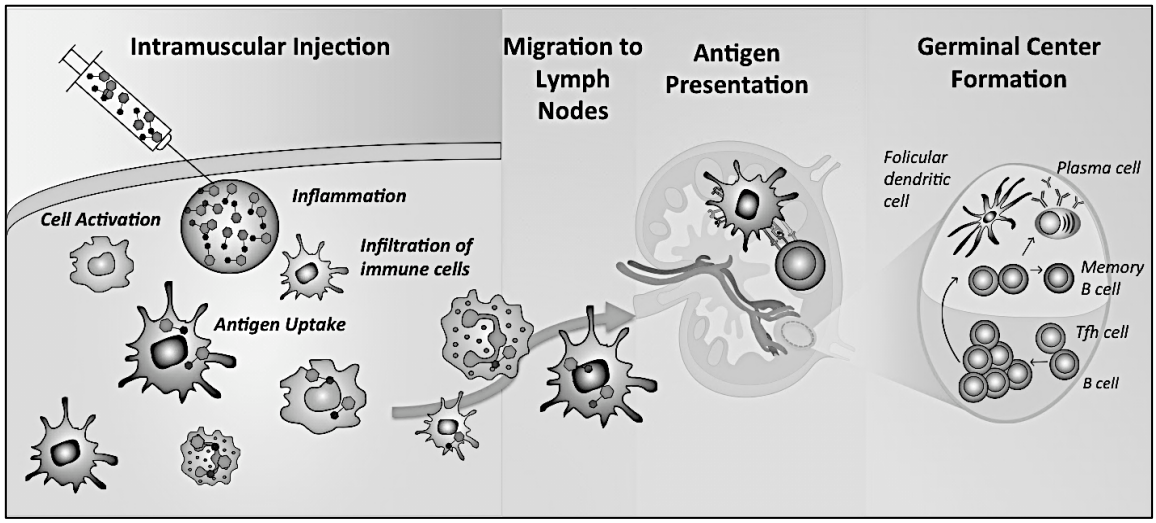

Figure 4. General view on the immune events occurring from vaccine administration to the generation of adaptive immunity.

1.4.9 Establishment and stimulation of memory responses

Upon re-encounter with the same pathogen, the immunological memory enables quicker and more robust responses. As mentioned, this concept represents the basis for vaccination. In fact, repetitive immunization (i.e. boosting) is needed for many vaccines to maintain antigen specific immunity at protective levels. Protection is mediated by antibodies secreted by long-lived plasma cells residing in the bone marrow in an antigen-independent manner to maintain constant antibody levels in blood and body fluids (105). During antigen re-encounter, memory B cells proliferate rapidly and differentiate to plasma cells, which can be indicated by transient elevation of antibodies in the blood (16). In the cellular immune system, tissue-resident memory T cells are able to provide immediate effector function upon antigen re-exposure. Instead, recall responses are mediated by central memory T cells in T cell areas of secondary lymphoid organs where they quickly proliferate after antigen presentation by DCs (16).

1.4.10 Skin antigen tests for cell-mediated immunological memory

While local memory responses in skeletal muscle remain elusive, one of the earliest characterized memory responses is the delayed type hypersensitivity (DTH) reaction in skin.

In the clinic, measuring DTH responses enable assessment of the ability to mount cell-mediated memory responses to various antigens including antigens derived from mumps virus or *Candida albicans* commensal yeast or purified protein derivate (PPD) from Mycobacterium tuberculosis (MTB). The skin tests are frequently used on immune-compromised individuals to evaluate the integrity of their cell-mediated immunity. These skin antigen tests are normally delivered intradermally. The most common skin test is using PPD as antigen for evaluation of pre-existing immunity to MTB or its vaccine (BCG). This test is called Mantoux test or tuberculin skin test (TST). At 48-72 hrs after injection with PPD, the inflammatory DTH reaction causes an induration (rigid protruding swelling) at the injection site that can be visualized and palpated (106, 107). The diameter of the induration represents the estimate for the TST and considered positive at ≥10 mm according to WHO standards, and sometimes at ≥ 5 mm where tuberculosis exposure are low. The DTH reaction is likely initiated by DCs taking up the skin test antigens and presenting them to skin-residing memory T cells, which then secrete cytokines that activate DCs or other cells locally in a direct or bystander manner. Mature DCs then migrate to draining LNs in order to present antigens to antigen-specific memory T cells, which respond by proliferation and home to the antigen injection site to maintain inflammatory responses such as recruitment of immune cells until the antigen is cleared (106).

1.5 VACCINES AGAINST HUMAN IMMUNODEFICIENCY VIRUS

1.5.1 Human immunodeficiency virus

HIV is an enveloped RNA virus that belongs to the retrovirus family and that causes acquired immunodeficiency syndrome (AIDS) (108). Like other retroviruses HIV carries the viral enzyme reverse transcriptase, which transcribes its RNA to DNA that can be incorporated to the host genome and cause chronic infection. Two types of HIV infect humans. While HIV-2 is less transmissible, cause slower disease progression and mainly prevalent in the Africa, the more virulent HIV-1 is the predominant type spreading throughout the world (109). HIV-1 is divided into three major groups (M, N & O) and group M, which can be further subdivided several clades (i.e. subtypes), is responsible for the majority of HIV-1 infections worldwide. While clade B is most prevalent in the Americas and Western Europe, clade C is the major subtype in e.g. South Africa (110). HIV-1 infects its main target cell, the $CD4^+$ T cells, by binding to the CD4 receptor and chemokine receptor CXCR4 or CCR5. However, HIV-1 can also infect other cells such as DCs (111-113), although the main hallmark of HIV disease is the severe depletion of $CD4^+$ T cells. According to WHO staging systems, AIDS diagnosis usually follows CD4 T cell counts that are less than 200 /μL blood. AIDS patients are susceptible to several opportunistic infections including invasive Candida infection, Pneumocystis carinii pneumonia, and Kaposi sarcoma, which usually cause AIDS-related deaths. Highly active anti-retroviral therapy (HAART) is generally efficient at controlling the viral load and thereby prevents the decline of $CD4^+$ T cells and progression to AIDS.

1.5.2 Dendritic cells and HIV-infection

The numbers of both MDCs and PDCs are declined in blood early in the infection (114, 115). However, while some MDC subsets are replenished after HAART, recovery of PDCs seems less efficient (116, 117) and treated patients show lower levels of both DC subsets compared to uninfected individuals (116). The mechanism behind depletion of blood DCs is unknown although pro-apoptotic markers are increased in MDCs of HIV-1^+ individuals (118) and infection, although very low, of both DC subsets has also been reported (119). However, DCs have also shown to mobilize to lymphoid tissues (120-122), which may cause the decline of DCs in blood. Whether DC functions remain intact in HIV patients are not clear. Both MDCs and PDCs from HIV-1-infected patients showed impaired antigen presentation capacity in vitro (119). In addition, influenza and herpes simplex viruses stimulated lower type I IFN levels in PBMCs (116) and isolated PDCs (123) from HIV-1^+ individuals, but can be restored after HAART (123). However, there are also reports showing MDCs and PDCs in HIV-1^+ individuals more readily secrete cytokines compared to healthy subjects and may thereby contribute to the chronic immune activation during HIV-1 infection (124, 125). Although these results seem contradictory, they imply that DC functions likely are affected during HIV-1 infection.

1.5.3 HIV vaccines

According to WHO, almost 37 million people live with HIV worldwide and 2 million HIV-related deaths have been reported during 2014. When HIV was established as the cause of AIDS in the early 1980's, there was great optimism that preventive HIV vaccines could be developed relatively quickly (126). After 30 years of intense HIV research, there is still no protective vaccine. Multiple HIV vaccine approaches have been developed, but as mentioned below only few have advanced to human efficacy trials (126, 127).

Since current vaccines mostly protect via antibodies, the first HIV-1 vaccine clinical trials (VaxGen) tested recombinant envelope protein of HIV (Env, gp120) administrated together with alum as the adjuvant. The vaccine induced strain-specific gp120 antibodies but they did not prevent infection of field strains of HIV-1 in high-risk populations. Subsequent trials (STEP) explored a recombinant adenovirus type 5-based vaccine encoding different HIV-1 proteins aimed to stimulate cell-mediated immunity. This trial was halted before completion due to lack of prevention of HIV-1 infection. In 2009, the RV144 trial used a vaccination strategy comprised of canarypox viral vaccine vector followed by Env gp120 in alum boost, which provided a 31.2% efficacy. Lastly, an efficacy trial was launched the same year (HVTN 505), using DNA vaccine encoding HIV-1 proteins from clades A, B and C followed by adenoviral vector encoding e.g. Env from the same clades. Unfortunately, this latest efficacy trial also failed to prevent HIV-1 infection or reduce virus levels and was stopped two years later (126).

There are clearly significant challenges in HIV-1 vaccine development. Although no clear correlation between protection and neutralizing antibodies was found in the RV144 trial, this

so far most successful efficacy trial resulted in an optimistic view of the effect of non-neutralizing antibodies which in combination with innate cells such as NK cells may contribute to protective responses (126, 128). Given that Env is the only viral protein expressed on HIV's surface, understanding its detailed structure is likely critical for inducing HIV-1-specific antibodies, including those able to neutralize. In fact, one of the major obstacles in HIV-1 vaccine development is constructing structurally optimal Env that can induce broadly neutralizing antibodies (128). There is still no proven consensus whether antibody or T cell responses are the most feasible type of immunity that can be simulated by a vaccine to prevent and/or control HIV-1 infection. Vaccines that induce both cell-mediated and humoral immunity may be most efficient. Nevertheless, HIV-1 protein antigens that are structurally accurate would need adjuvants to elicit sufficiently potent and durable vaccine responses. Understanding how such responses can be induced i.e. the influence of innate cells such as DCs, capture, present HIV-1 antigens, stimulate adaptive responses and how adjuvants affect these processes is receiving increasing attention (128).

After the modest success of the RV144 HIV-1 vaccine trial where alum-adjuvanted HIV-1 Env was used as a boost, MF59 has been suggested to replace alum in future clinical trials to increase efficacy using this vaccine regimen (129). MF59 has been shown to be a potent and safe adjuvant for HIV candidate vaccine trials in humans (130) and NHPs (131). In fact, this prompted some of the studies in this thesis where the effect of MF59 versus alum was investigated in terms of their capacity to generate immune responses to HIV-1 Env.

1.6 NONHUMAN PRIMATE MODELS FOR VACCINE RESPONSES

Nonhuman primates (NHPs) play a crucial role for modeling vaccine responses in humans, including new vaccines designed for malaria and HIV-1 (132). Although inbred rodent strains are frequently used and suitable for addressing specific research questions, they differ substantially in their distribution of immune cell populations, phenotype and PRRs compared to humans (56). In contrast, humans and rhesus macaques share high degree of similarities as shown by the comparable distribution of lymphocyte and APC populations, which can be identified using same phenotypic markers (Fig. 5) (64, 133-137). In adjuvant research where TLR agonists provide means to target specific immune cells rhesus macaques provide a powerful model since their DCs express similar sets of TLRs as humans, which is in contrast to mice (137, 138). Rhesus DCs also respond to TLR stimulation by upregulating maturation markers and produce cytokines in a similar manner (64, 138, 139). Thus, the similar expression and functionality of TLRs on both rhesus macaque and human DCs indicate that rhesus macaques model in vivo adjuvant responses more accurately compared to rodents (137). The memory lymphocyte pool in rhesus macaques, including Tfh cells and plasma cells, has also been characterized (136, 137, 140, 141), which enables both enumeration and functional analysis of these effector cells before and after vaccination. Importantly, antigen doses used in rodents may not be proportionally representative for clinical use (10, 33). Moreover, as in human vaccination, most antigens in NHP studies are delivered in the muscle

instead of the intraperitoneal route commonly used in mouse studies. Intradermal delivery of adjuvant alone (142) or together with antigen (143, 144) in rhesus macaques has also been employed.

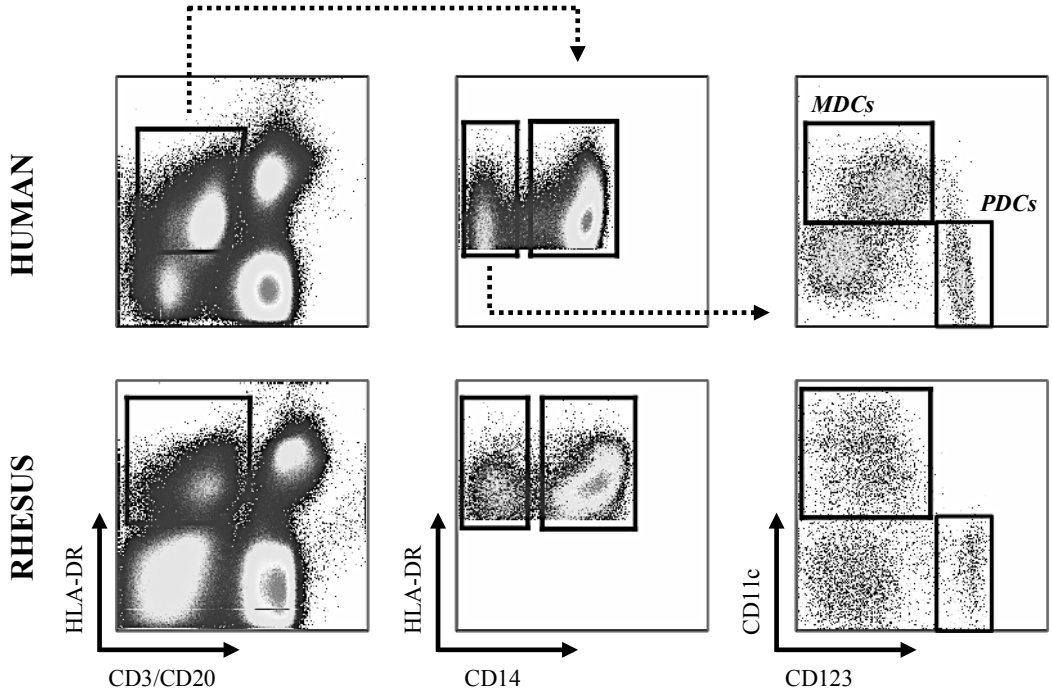

Figure 5. Flow cytometric identification of human and rhesus DC subsets in PBMCs with antibodies against human markers. DCs are lineage$^-$, HLA-DR$^+$, CD14$^-$. Myeloid DCs are CD11c$^+$ and plasmacytoid DCs are CD123$^+$.

Rhesus macaques are frequently used as in vivo models to evaluate efficacy of candidate vaccines against HIV-1 (145, 146). Although rhesus macaques more rapidly progress to AIDS, the signs of disease progression in terms of viral load and CD4$^+$ T cell counts are similar in SIV and HIV-1 infection (145, 147-149). The depletion of blood DC subsets in humans is mirrored in SIV$^+$ rhesus macaques (150). Numerous vaccine immunogenicity studies using NHPs report potency and efficacy by measuring antibody levels and/or T cell responses in blood. However, the immune events initiated locally at the immunization site and those giving rise to measurable adaptive immune responses week(s) later remain elusive. Based on the above, rhesus macaques represent an excellent in vivo model for studying local immune responses at the site of vaccine delivery as well as initiation of adaptive immunity.

2 AIMS OF THIS THESIS

The overall aim of this thesis was to investigate the early innate immune responses in vivo after administration of antigens. We addressed this both during recall of immunological memory responses locally (clinical skin tests) as well as priming of naïve responses in the presence of different adjuvants (vaccination). The specific aims for the individual studies are:

Paper I: To study the recruitment and function of human dendritic cell subsets in tuberculin skin tests.

Paper II: To compare recruitment of dendritic cells and T cells in response to skin antigen tests in healthy versus HIV-1 infected individuals.

Paper III: To establish a nonhuman primate in vivo model to study early local innate immune responses at the site of intramuscular immunization.

Paper IV: To model how distinct adjuvants induce innate stimulation to enhance vaccine responses in nonhuman primates.

3 MATERIALS AND METHODS

The methods used in the papers for the thesis papers are briefly outlined below. For more detailed description, see "materials and methods" of each paper. The bold roman numerals indicate the paper(s), where the specific method or treatment was applied.

3.1 ANTIGEN DELIVERY

3.1.1 Skin test antigens

The volunteers in studies of local immune responses after intradermal delivery of skin test antigens were recruited in Cape Town, South Africa (**I-II**) or Cleveland, USA (**II**). The South African cohort is from an area with frequent exposure to MTB (151). Thus, subjects with previous tuberculosis diagnosis and/or symptoms were excluded. Intradermal injection of PPD (**I-II**) was done according to international standards and TST reaction ≥10 mm was considered positive. Same subjects also received intradermal saline-injection on the other arm as control. The Cleveland cohort received antigens from *C. albicans* and mumps virus (**II**), but considered positive at ≥5 mm and non-injected skin served as control.

3.1.2 Antigens for intramuscular injections

Antigens modeling vaccines for studies of intramuscular immunization (**IV**) consisted of HIV-1 Env gp120 monomer derived from the Tv1 or SF162 strains. Env was fluorescently labeled with AlexaFluor 680 (Molecular Probes) and administrated alone or mixed 1:1 with oil-in-water adjuvant MF59 labeled with DiO. Alternatively, labeled Env was adsorbed to aluminum hydroxide adjuvant (alum) or to alum pre-adsorbed with a TLR7 agonist. Animals were generally injected with Env alone or Env together with one of the adjuvants at two different sites. The third and fourth site respectively was injected with PBS and adjuvant alone. In a separate group of animals, each site received Env plus adjuvant at a different time point. To model immune responses to viral vector vaccine (**III**), replication-incompetent recombinant adenovirus serotype 5 (rAd5) encoding Ebola glycoprotein was used. During general anesthesia, animals were placed in recovery position and injections were administered in a 90° angle at the midpoint of the encircled injection site.

3.2 COLLECTION OF BIOPSIES FOR IN SITU ANALYSIS

For the in situ studies of human skin, local anesthetic was administered prior collection of punch biopsies from non-injected skin (**II**) and skin intradermally injected with skin test antigen or saline (**I-II**) at 48 hrs post-injection as described (152). For the studies of rhesus macaque skeletal muscle (**III-IV**), biopsies were collected during necropsy. Animals were placed in same position as during immunization and a biopsy punch was pressed through skin, adipose and connective tissue at the center of the encircled skin area (i.e. needle entry point) to create a puncture mark on the underlying muscle. The muscle tissue surrounding this puncture mark indicated antigen-exposed muscle from which the punch biopsies were collected. LNs draining the injected muscles were also harvested. All biopsies were snap-frozen in liquid nitrogen.

3.3 TISSUE STAINING AND IN SITU ANALYSIS

3.3.1 Immunohistochemistry

To analyze and quantify DC subsets and markers of inflammation in situ at the intradermal delivery site of skin test antigens or saline (**I-II)** as well as non-injected skin (**II**), fixed sections of snap-frozen human skin biopsies were permeabilized with saponin (Sigma). Unspecific staining due to endogenous peroxidase, avidin and biotin, were blocked with hydrogen peroxide and avidin-biotin blocking kit (Vector). Incubation with primary antibodies was done overnight. Non-specific binding of the secondary antibodies was blocked with sera (Dako) from the species the secondary Abs were derived from. Sections were incubated with biotinylated secondary antibodies (Dako) and staining was visualized by Vectastain Elite ABC kit (Vector) containing streptavidin-conjugated peroxidase followed by 3,3'-diaminobenzidine (DAB) peroxidase substrate kit (Vector). Cell nuclei were counterstained with haematoxylin. All incubations were done at RT. The DAB staining was analyzed with bright field light microscope coupled to computerized image analysis system as previously described (153). Quantification of skin sections was performed in the dermis except for Langerin, which was done in the epidermis only. Melanin-rich cells between epidermis and dermis were excluded from the analysis.

3.3.2 Immunofluorescent staining

For in situ analysis of human skin (**II-III**) and rhesus muscle and draining LNs (**IV**), cryosections were stained as above, but visualized by streptavidin-conjugated fluorophores (Molecular Probes). To investigate the distribution of labeled antigen and adjuvant in the muscle (**IV**), sections were incubated with wheat germ agglutinin to visualize muscle fiber bundles. All incubations were done at RT and in dark. Sections were mounted with SlowFade Gold antifade regent (Molecular Probes) with or without DAPI for nuclear counterstaining. Imaging and analysis was done with confocal microscopy.

3.3.3 Analysis of germinal centers

To assess the number and size of germinal centers in LNs (**IV**), tiled images of entire LN sections were acquired. The GC formations in B cell areas were defined via $Ki67^+$ $CD3^-$ cells (proliferating B cells in the dark zone) and $PD\text{-}1^+$ $CD3^+$ cells (Tfh cells in the light zone). $Ki67^+$ $PD\text{-}1^+$ cells in T cell areas were excluded since these are most likely activated T cells. Enumeration of GCs and measurement of GC area plus the whole LN section were performed in a blinded fashion on tiled images using Fiji software.

3.4 PREPARATION OF SINGLE CELL SUSPENSION OF SOLID TISSUES

3.4.1 Skeletal muscle tissue

Multiparametric flow cytometry was used to simultaneously enumerate and phenotype the infiltrating immune cells after intramuscular antigen delivery (**III-IV**), as well as assess their activation status and antigen uptake capacity in vivo (**IV**). A cubical piece of approximately 15 cm^3 was dissected from the antigen injection site. Each dissected muscle tissue was weighed and normalized to 4 g by removal of adipose and connective tissues as well as excess muscle tissue. Muscle were then cut into small pieces and digested with RPMI

containing Liberase (Roche), which is a collagenase mixture, and DNase (Sigma) without agitation for 2 hrs at +37C°. Liberase activity was inactivated by RPMI supplemented with FCS. Inactivated digestion was filtered through 70 μm cell strainers (BD), washed and single cell suspension was resuspended to approximately 1 g tissue/ml.

3.4.2 Processing of lymph nodes

For flow cytometric evaluation of immune cell accumulation in LNs draining injected muscle and presence of labeled antigens and adjuvants (**IV**), LNs were cut into smaller pieces, passed through 70 μm cell strainer with a plunger, washed and resuspended to 5 x10^6 cells/ml.

3.5 FLOW CYTOMETRY

Multiparameter flow cytometric analysis was applied to both human (**I**) and rhesus macaques (**III-IV**) samples. Cells were washed with PBS, incubated with Aqua dye (Molecular Probes) for live/dead staining and blocked with FcR-blocking reagent (Miltenyi) before adding a cocktail of fluorescently labeled monoclonal antibodies. Cells were washed after incubation, fixed and on average 2 x10^6 events per sample were acquired using an LSR II flow cytometer (BD). Analysis was done with FlowJo software (Tree Star). Number of cells was obtained by normalizing to 1 x 10^5 viable cells (% Aqua$^{dim/-}$ cell subset divided by10^2 and then multiplied with 10^5). In some samples, a known number of AccuCount beads (Spherotech) were added to samples before acquisition and numbers of cell subsets were calculated (cell subset count / bead count) x (bead number / sample volume), as described (154).

3.6 ISOLATION OF ANTIGEN PRESENTING CELLS

3.6.1 Isolation of primary dendritic cells for in vitro assays

DCs were isolated from buffy coats, aphaeresed blood and elutriated monocytes for in vitro stimulations (**I**). Enrichment of DCs was done prior to DC isolation. RosetteSep monocyte enrichment cocktail (Stemcell Technologies) was added to buffy coat to deplete lymphocytes, granulocytes and erythrocytes during ficoll separation. MDCs and PDCs were magnetically isolated using anti-CD1c and anti-CD304 (BDCA-4) microbeads (Miltenyi) and AutoMACS (Miltenyi).

3.6.2 Isolation of immune cells for ex vivo antigen presentation assay

To compare antigen presentation capacity of Env$^+$ APCs in the draining LNs from rhesus macaques, flow cytometry sorting was applied to ensure high cell purity (**IV**). Cell suspensions of LNs draining muscle injected with AlexaFluor 680-labeled Env plus adjuvant or PBS, 24 hr earlier, were stained to identify CD14$^+$ monocytes, CD11c$^+$ MDCs and CD66 abce$^+$ neutrophils. Using FACSAria (BD), Env$^+$ cells were sorted and placed in co-cultures with sorted autologous CD4$^+$ T cells from stored PBMC samples from peak Env-specific immunity.

3.7 FUNCTIONAL ASSAYS

3.7.1 Antigen uptake by human primary dendritic cells in vitro

Uptake of PPD (Statens Serum Institut) (**I**) was assessed by labeling it with AlexaFluor 488 (Molecular Probes) and adding it to enriched APCs or isolated MDCs and PDCs for 2 hrs at $^{+}$37C° or $^{+}$4C°. PPD-uptake was analyzed by flow cytometry or confocal microscopy.

3.7.2 In vitro stimulation of human primary dendritic cells

For DC activation in vitro (**I**), magnetically isolated PDCs were stimulated with either PPD, LL37 (Innovagen), CpG ODN (class B 10103, Coley), human DNA (Biochain) or LL37 complexed with DNA or CpG ODN for 16 h at +37°C. In some experiments, supernatants of stimulated PDC were added to MDCs to assess bystander stimulation, PPD uptake and proliferation of $CD4^+$ T cells. For in vitro stimulation with adjuvants, flow cytometer sorted monocytes, MDCs, PDCs and neutrophils were incubated with MF59, alum or alum-TLR7 overnight. While expression of co-stimulatory molecules CD80, CD86 and CD40 was assessed by flow cytometry, cytokine secretion was measured by ELISA (PBL) or Luminex assay (Invitrogen) according to the manufacturers instructions.

3.7.3 Ex vivo antigen presentation assay

For comparison of antigen presentation capacity, Env^+ APCs isolated from rhesus LNs and autologous $CD4^+$ T cells as described above (**IV**) were co-cultured for 5 days and responding Env-specific $CD4^+$ T cells were analyzed via CFSE dilution using flow cytometry. To investigate the presence of Env-specific $CD4^+$ T cell responses in LNs after prime or boost (**IV**), LNs draining vaccination sites were collected at different time points as indicated and processed into cell suspensions. The suspensions were CFSE-labeled and cultured for 5 days in absence or presence of either Env protein (HIV-1 Tv1 strain) or Env peptides. $CD4^+$ T cells were gated during FlowJo analysis to measure Env-specific responses by CFSE dilution.

4 RESULTS AND DISCUSSION

This thesis has evolved around the early innate immune responses such as immune cell recruitment and local immune activation after administration of antigen and onset of immune responses in vivo. The studies were performed by first studying the local immune responses after clinical skin tests in humans (**paper I-II)** and second by studying vaccine administration in a rhesus macaque model (**paper III-IV)**.

4.1 INFLAMMATORY RESPONSES AFTER SKIN ANTIGEN TEST DELIVERY

In **paper I-II** we utilized human skin punch biopsies sampled after clinical skin tests. This was a valuable material to assess innate immune activation in vivo 48 hrs after antigen delivery in humans. As described in section 1.4.10, the DTH reaction induced by pre-existing immunity to skin test antigens including PPD used for TST, evaluates the presence and integrity of cell-mediated memory responses. Positive DTH reaction indicated by the induration is caused by inflammation, which may be initiated only a few hours after the skin test antigen delivery due to detectable erythema (155, 156). Neutrophils and monocytes are known to arrive first to inflammatory sites (157-159). In line with this, the majority of infiltrating cells during very early time points (4–6 hrs) in TST reaction have been shown to be $CD11b^+$ cells, which represent neutrophils and monocytes (155). T cells are detected around 12 hrs later (156). HLA-DR^+ APCs have also been shown to accumulate in this reaction (156, 160, 161). Since DTH reactions are based on reactivation of memory responses, they unlikely model the events occurring during primary intradermal vaccinations. In addition, the antigen mixes used for the skin tests do not contain immune stimulatory substances unlike unadjuvanted clinical intradermal vaccines against e.g. influenza, BCG or rabies, which contain intrinsic PAMPs. In **paper I**, the inertness of PPD was demonstrated by the lack of immune cell recruitment or activation in negative TST reactions found in individuals with no pre-existing immunity. In contrast, local immune activation in the positive indurations appears as a consequence of inflammation and cytokines such as IFN-γ secreted from recruited antigen-specific memory T cells in the TST reaction (106, 161, 162). Indeed, IFN-γ was detected in our skin biopsies material from the TST site in a previous study (152). Inflammatory cytokines such as IFN-γ, TNF-α and IL-1β have been reported in human skin biopsies already at 6-24 hrs after PPD-injection, which is considerably ahead of the peak DTH response occurring at 48-72 hrs (161, 162). Intradermal delivery of recombinant IFN-γ into the skin of healthy volunteers was shown to induce local inflammatory responses such as upregulation of chemokine genes and promote influx of immune cells (163).

4.1.1 Dendritic cells are recruited to the site of skin antigen test injection

In **paper I**, we performed an in situ characterization and quantification of multiple DC subsets in the skin of healthy donors receiving intradermal injection of PPD and saline on separate sites. Inflammation was detected by the robust immune cell infiltration in the

positive TST induration as indicated by the increased cellularity in the dermis. There was a higher percentage of HLA-DR$^+$ cells in the TST reaction compared to the saline-injected control skin. As shown in **paper II**, the presence of DC infiltration was accompanied by significantly higher levels of CD3$^+$ T cells in the positive TST reaction as compared to TST negative test sites. This suggests that there is a recruitment of DCs following PPD-injection as long as there is concurrent infiltration of pre-existing antigen-specific memory T cells to the site. Several markers defining CD123$^+$, CD303$^+$ PDCs and subsets of MDCs such as Langerin, DC-SIGN, CD11c, CD68 and CD141 were used to identify the infiltrating DC subsets. Both total CD11c$^+$ DCs and CD123$^+$ PDCs were significantly increased in the TST site compared to control skin (Fig. 6). Although HLA-DR$^+$ APCs in positive TST indurations have been previously shown (156, 160), the definition of DC subsets has not been reported. Our in situ analysis therefore showed for the first time that both PDCs and CD141$^+$ MDCs, which are otherwise rare cells in skin (164), infiltrate TST indurations.

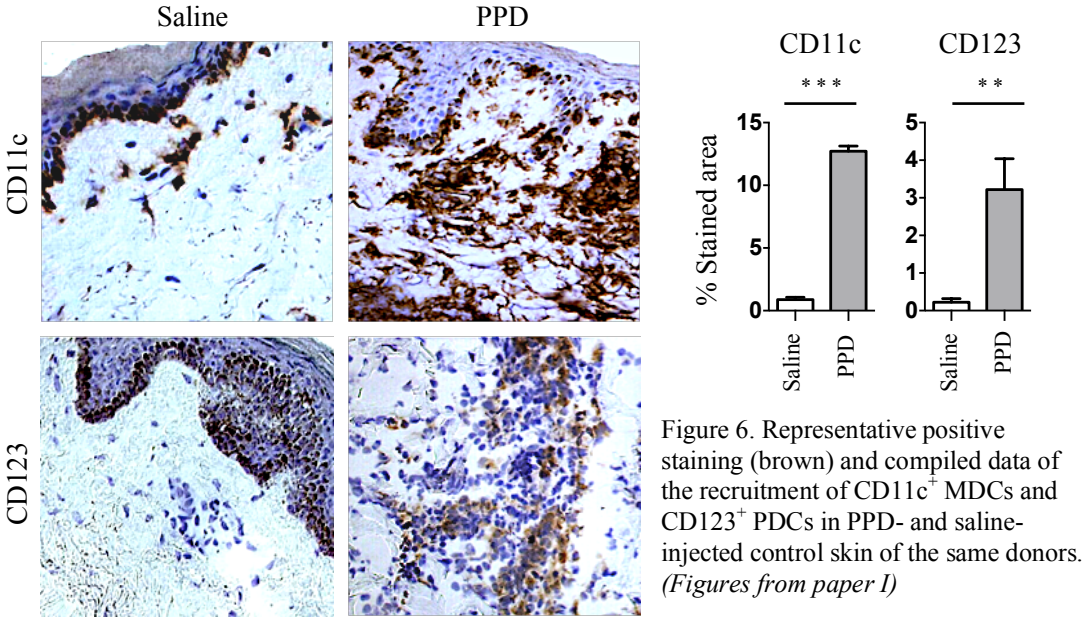

Figure 6. Representative positive staining (brown) and compiled data of the recruitment of CD11c$^+$ MDCs and CD123$^+$ PDCs in PPD- and saline-injected control skin of the same donors. *(Figures from paper I)*

As described above in section 1.4.3, CD141$^+$ MDCs have been reported to be superior in cross-presentation and PDCs excel in mounting anti-viral responses by secreting high levels of type I IFNs. Infiltration of CD141$^+$ MDCs plus IFN-α secreted by PDCs at the site of antigen delivery may influence the type of T cell response stimulated. Whether CD141$^+$ MDCs can enhance CD8$^+$ T cell responses or impact the DTH responses remains elusive while the role of PDCs is discussed below.

4.1.2 Immune activation of plasmacytoid dendritic cells in positive TST indurations

Since the finding that PDCs accumulated in positive skin indurations was intriguing, we further analyzed whether they were activated and contributed to inflammation. Since PDCs are efficient producers of type I IFNs such as IFN-α, we stained for the MxA protein, which is exclusively upregulated by cells exposed to type I or III IFNs (165). We found significant increase of MxA in the TST reactions compared to control skin. This is indicative of PDC

activation in the positive indurations. Although other cells also can produce type I IFNs and cause MxA expression, PDCs are able to rapidly secrete type IFNs at levels that are several magnitudes higher (166). IFN-α producing PDCs have been detected in autoimmune skin disorders like lupus and psoriasis (66, 166, 167). The shared feature of DTH reactions and autoimmune skin disorders is that immune activation is induced in the absence of microbial stimuli. We therefore explored the potential cause for the induction of type I IFNs in the positive TST induration. We confirmed that the PPD antigen was unable to directly activate PDCs in vitro, nor induce PDC infiltration in individuals with negative induration. In general, inflammation causes varying degrees of cell death induced by different pathways (168) and presence of self-nucleic acids derived from dead cells are a plausible source for PDC activation. In fact, neutrophils, which are commonly found in inflammatory tissues, have been proposed to release self-DNA that activates PDCs during lupus erythematous (169, 170). To this end, we also found that neutrophils were present in the positive indurations (F. Liang, unpublished data). Strictly intracellular antigens like self-nucleic acids in the extracellular environment act as danger signals and are referred to as DAMPs, which also include the anti-microbial peptide LL37. Complexes of self-nucleic acids and LL37 have been demonstrated to activate PDCs in psoriasis lesions and the complex formation rescued self-nucleic acids from extracellular degradation (65, 66). Indeed, we found increased staining of LL37 as well as markers associated with cell death (caspase-3) or lost cell integrity (HMGB-1 and lactate dehydrogenase) in the PPD-injected skin. We were not able to detect extracellular nucleic acids, which may relate to insufficient detection limit. However, the presence of extracellular staining of HMGB-1 protein that interacts with DNA in the cell nuclei, partially suggests that self-nucleic acids may be found outside the cell. This demonstrates that there are stimulatory complexes present in the TST induration that can activate PDCs. The data suggest that antigen injection sites with substantial inflammation including cell death facilitate DC recruitment and this milieu also contains stimulatory components that activate DCs and can induce a type I IFN response.

4.1.3 Dendritic cell recruitment to skin is compromised during HIV-1 infection

As mentioned in section 1.5.2, $CD4^+$ T cells and blood DCs are depleted during HIV-1 infection. Whether fundamental DC functions such as mobilization to antigen delivery sites and antigen presentation are intact in HIV-1 patients is not clear. In **paper II**, we studied if the robust DC infiltration to positive skin test indurations also was detected in HIV-1 patients. We compared the magnitude of DC infiltration in TST indurations of healthy, asymptomatic $HIV\text{-}1^+$ individuals and AIDS patients. As shown in **paper I**, accumulation of DCs in the positive TST induration. In **paper II** we also expanded the analyses to include other skin antigen tests to see whether DTH indurations in general would induce the same level of DC recruitment. We quantified DCs in skin indurations of healthy and $HIV\text{-}1^+$ individuals after injection with skin test antigens derived from mumps virus and the gut commensal yeast *C. albicans*. Immunity to these antigens is established by mumps vaccination and constant exposure to *C. albicans* respectively. In contrast to the well-established depletion of blood DCs during HIV-1 infection, we did not find reduced levels of DCs in skin biopsies from

HIV-1$^+$ individuals. The saline-injected control skin of healthy and HIV-1+ individuals had similar levels of staining representing MDCs (CD11c$^+$ total MDCs including CD141$^+$ MDCs) and CD123$^+$, CD303$^+$ PDCs (Fig. 7). Frequencies of MDC subsets and PDCs were higher in the positive indurations compared to the control skin in all donors, regardless of whether they were healthy or HIV-1$^+$. However, the accumulation of the MDC subsets and CD303$^+$ PDCs were significantly lower in the HIV-1$^+$ individuals and especially in those with AIDS. In fact, the level of DC infiltration correlated with the magnitude of CD3$^+$ T cell infiltration in the positive TST indurations.

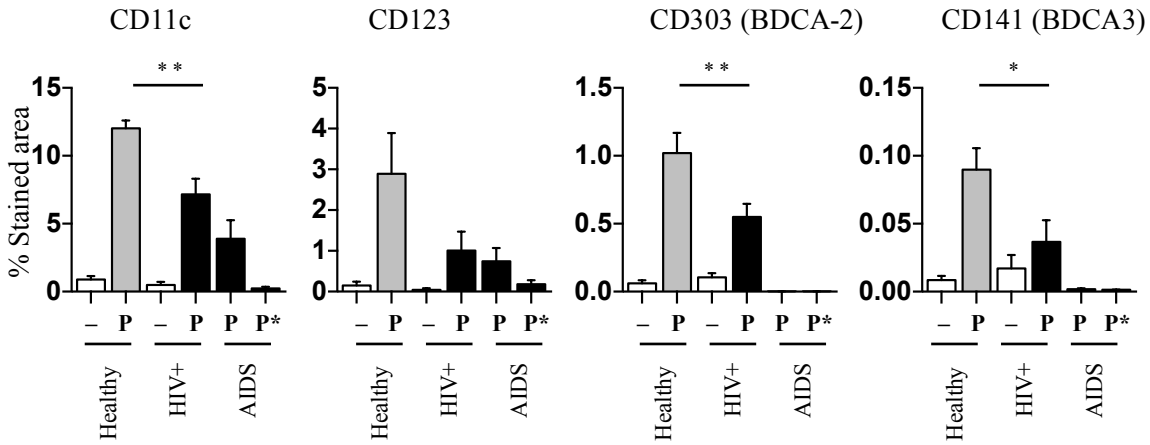

Figure 7. Compiled data on the frequencies of total CD11c$^+$ MDCs, CD123$^+$ or CD303$^+$ PDCs and the cross-presenting CD141$^+$ MDC subset in healthy versus HIV-1$^+$ individuals, including AIDS patients. Saline (–), positive TST (P) and negative TST (P*). *(Figures from paper II)*

As described above for the TST indurations, both healthy and HIV-1$^+$ individuals had higher recruitment of MDCs and PDCs in indurations caused by mumps and *C. albicans* antigens compared to control skin from the same donors. However, there was a differential pattern of DC recruitment depending on the type of skin test antigen and HIV-1 seropositivity. When comparing the level of DC accumulation in the indurations of HIV-1$^+$ and healthy individuals, we observed that *C. albicans* antigens induced a higher level of DC influx than mumps antigens in asymptomatic HIV-1$^+$ individuals. In contrast, mumps induced higher DC levels than *C. albicans* in the healthy subjects. In these study groups, the differential pattern of DC levels was related to the levels of T cell infiltration. The level of memory T cells specific for the respective antigens likely determine the level of DC recruitment. In this regard, high levels of CD45RO$^+$ cells (memory T cell marker) were found in healthy individuals after mumps-injection and in line with this, CD45RO$^+$ cells were especially elevated after *C. albicans*-injection in asymptomatic HIV-1$^+$ individuals. This could explain the more efficient *C. albicans*-specific T cell responses observed in HIV-1$^+$ individuals compared to healthy controls (171, 172). Thus, the level of T cell accumulation, which likely contains pre-existing antigen-specific memory T cells, influences the level of inflammation that in turn dictates the level of DC recruitment.

4.2 NONHUMAN PRIMATE MODEL FOR EARLY VACCINE RESPONSES

Increased knowledge of the innate immune mechanisms involved in initiating and regulating adaptive immunity after antigen administration is important for understanding how vaccine responses are elicited and can be manipulated for better efficacy. The observations of rapid and robust recruitment of several DC subsets to the site of skin test antigen administration as well as local inflammation in **paper I-II**, encouraged subsequent studies to determine how vaccine injection influences these processes. In the second part of my thesis, I therefore took another approach and in **paper III-IV** we established an animal model predictive of humans to be able to sample and comprehensively study innate immune stimulation over time following intramuscular injection of a protein vaccine antigen together with distinctly different adjuvants.

In the first part of my thesis, we studied the innate immune responses during recall of memory responses and the injected antigens in these studies were also different compared to those used in subsequent studies. The skin test antigens used in humans (**paper I- II**) stimulate pre-existing immunity and are not designed to prime immunity. In contrast, the antigens in (**paper III-IV**) i.e. adenoviral vaccine vector encoding Ebola glycoprotein (**paper III**) and the candidate vaccine antigen HIV-1 Env (**paper IV**) are designed to prime immune memory in antigen-naïve animals. Despite these fundamental differences, the distinct antigens may share some common features. First, they all have the capacity to promote local inflammatory responses either directly via adjuvants (**paper IV**) or intrinsic adenoviral PAMPs (**paper III**) or indirectly via pre-existing memory cells (**paper I-II**). Second, they all require antigen presentation to stimulate T cell responses. Thus, recruitment of immune cells to the site of antigen injection in either system likely precedes migration and antigen presentation in draining LNs.

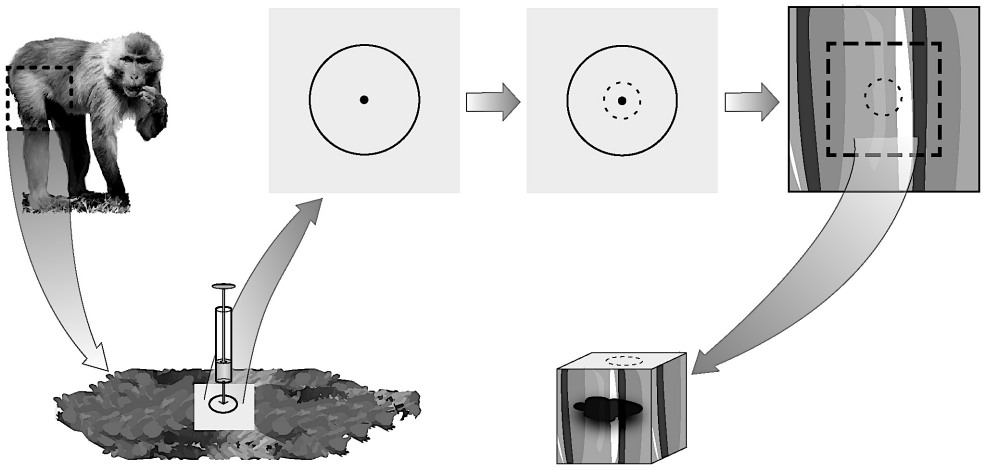

Figure 8. Schema of intramuscular antigen injection and tissue sampling. Injection area was encircled on shaved skin (solid circle) and antigen was administered at the midpoint. A biopsy punch pressed through the skin at the center of encircled area created a mark on the underlying muscle tissue and indicated the injection area (dotted circle, upper far right). Muscle tissue proximal to this mark (within the dashed square) was collected. *(Figure from paper III)*

As described in section 1.6, NHPs offer a powerful in vivo model for generating data on vaccine responses that may be translatable to humans. Although rhesus macaques are frequent in vivo models for vaccine studies, the end point of these studies are usually derived from analysis of post-challenge protection and/or adaptive responses in sera or PBMCs. The innate immune events such as recruitment of immune cells to the muscle, where most clinical vaccines are delivered, remain elusive. Performing comprehensive studies of immune cell infiltration and activation after vaccine administration in human muscle are challenged by the invasiveness of muscle biopsy collecting and the fact that muscle tissue contains relatively few immune cells (41), which, means that rather sizeable biopsies are required for reproducible data.

In **paper III**, we developed protocols for precise collection and dissociation of NHP muscle biopsies to be able to address questions related to the early local immune responses after vaccination. To mobilize immune cells, intramuscular injection with an adenoviral vaccine vector was performed 72 hrs prior to tissue sampling during necropsy. In **paper III**, much attention was focused on finding procedures for exact sampling of the tissue that was in closest proximity to the vaccine injection (Fig 8). These procedures are described in section 3.1.2 and 3.4.1. An overview of injection and tissue sampling is depicted above. The protocols developed in **paper III**, enabled detailed characterization of the innate immune cells present in rhesus macaque muscle after intramuscular injection with model vaccines. We found that the number of live immune cells per gram muscle that could be retrieved was around 7 x10^5. More than ten different immune cell subsets were identifiable using antibodies for classical human CD-markers. These cell subsets include; CD66abce$^+$ neutrophils, CD14$^+$ CD11b$^+$ monocytes, CD14$^+$ CD11b$^-$ macrophages, CD123$^+$ PDCs, CD1c$^+$ MDCs, CD16$^+$ MDCs, CD4$^+$ T cells, CD8$^+$ T cells, CD20$^+$ B cells, CD16$^+$ and CD16$^-$ NK cells. PBMCs from the same animals were also stained as controls and all subsets, except macrophages were detected (F. Liang, unpublished data). Immune cells in blood, lymphoid and mucosal tissue of rhesus macaques have been characterized to various extents (64, 135, 140, 173, 174). However, infiltrating immune cells in immunized muscle has mainly been studied in mice (35, 42). The protocol refinement and validation of the detection of rhesus immune cells in **paper III** were important for proceeding to subsequent studies of innate stimulation after vaccination performed in **paper IV**.

4.3 INNATE IMMUNE RESPONSES INDUCED BY DISTINCT ADJUVANTS

Capitalizing on the experience and method development from the earlier papers in the thesis, we were able to in perform an extensive project characterizing how early innate stimulation by different adjuvants shape vaccine responses in **paper IV**. In this study, we examined intramuscular injections of fluorescently labeled HIV-1 Env, co-delivered with distinct adjuvants, already approved for human use or on a clinical path. We investigated MF59 (oil-in water emulsion), alum (benchmark adjuvant) and a TLR7-agoinst adsorbed to alum (alum-TLR7). MF59 and alum-TLR7 were chosen since they are distinctly different in their

composition and they both have recently proven to induce superior vaccine responses over alum (175). The NHP model allowed us to simultaneously phenotype multiple immune cells in the muscle and draining LNs, assess vaccine-induced activation of specific cell subsets, track the distribution of labeled vaccine antigens and identify when and where adaptive T and B cell responses were formed. The animals were divided in four different groups receiving Env alone or together with either MF59, alum or alum-TLR7. We started by comparing the local immune responses in the vaccine-injected muscle to control muscles from the same animal that were injected with PBS and only adjuvant respectively.

4.3.1 Dendritic cells, neutrophils and monocytes infiltrated all adjuvant-injected muscles

As DCs are essential for priming of naïve T cell responses their recruitment is considered to be critical for priming of vaccine responses. We found that $CD11c^+$ MDCs and $CD123^+$ PDCs were significantly increased in adjuvant-injected muscle compared to PBS-injected muscle of the same animals. Both MF59 and alum-TLR7 induced higher recruitment of MDCs to the muscle compared to alum and PDCs were highest in alum-TLR7 group (Fig 9).

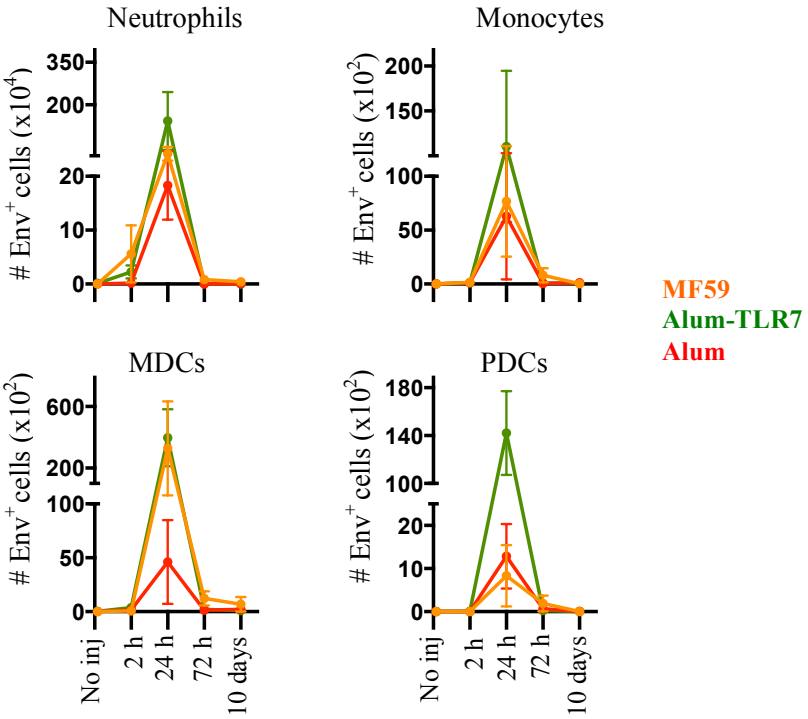

Figure 9. Rapid and transient immune cell infiltration and uptake of fluorescently labeled Env by indicated cells over time. *(Figure from paper IV).*

The majority of the MDCs comprised of the $CD1c^-$, $CD16^-$ population, which may indicate that infiltrating monocytes may also have differentiated to DCs as shown in mice (176). No or very few tissue resident $CD103^+$ DCs, or skin-resident $CD1a^+$ LCs or DC-$SIGN^+$ DDCs were detected in the muscle, which suggests that the DCs infiltrating vaccine-injected muscles came from the blood rather than from adjacent tissues. Regarding the $CD141^+$ MDCs, no optimal cross-reactive CD141 antibody exists. By using alternative markers expressed on human $CD141^+$ MDCs, this rare subset has recently been identifiable in rhesus macaques (177) although we did not have the reagents available at the time of our study.

Apart from DCs, we found a large infiltration of CD66abce$^+$ neutrophils and CD14$^+$ monocytes in muscles injected adjuvants (Fig 9). As previously discussed, monocytes and neutrophils are known to be the first cell types to arrive tissue exposed to inflammatory stimuli (159). Previous mouse studies have found that infiltrating monocytes and neutrophils were the predominant cell populations infiltrating the muscle after MF59 and alum injection (42). . In line with this we found that monocytes and neutrophils were the dominant cell types at the site injected with either of the adjuvants. The infiltration of DCs, monocytes and neutrophils into the muscle was detected already at 2 hrs and peaked at 24 hrs. Interestingly, this is usually the time soreness appears in the muscle after being vaccinated.

4.3.2 Mobilization of cells to draining lymph nodes

A large infiltration of DCs, monocytes and neutrophils to the muscle after vaccination followed by sustained migration to draining LNs may be advantageous for generation of strong adaptive responses. The accumulation of these cells in the LNs draining the injected muscle followed similar kinetics as the infiltration into the muscle and peaked at 24 hrs. In line with these findings, expression of CCR2, mediating transmigration of immune cells from the circulation to tissues, was most pronounced on these cells at 24 hrs. Similarly, CCR7, mediating migration to LNs, was highest at 24 hrs. While MDCs expressing higher levels of CCR2 and CCR7 were found in the MF59 group, PDCs expressing highest level of these chemokine receptors were found in the alum-TLR7 group, which may contribute to the respective DC recruitment patterns induced by these adjuvants. In the MF59 and alum-TLR7 groups, DCs were still elevated at 72 hrs and had not completely returned to baseline levels after 10 days. This suggests that the inflammation created by MF59 and alum-TLR7 at the injection site may be more persistent than with alum, which resulted prolonged cell infiltration.

The PDCs and CD1c$^+$ MDCs were proportionally more frequent in the draining LNs compared to the CD16$^+$ MDCs, which were very few in the draining LNs. This indicates that specific DC subsets have distinct migration pattern and also may reflect their distinct functions during early vaccine responses. As mentioned in section 1.4.3, CD1c$^+$ MDCs are especially efficient in stimulating CD4$^+$ T cell responses while CD16$^+$ MDCs have been described as pro-inflammatory. Thus, CD16$^+$ MDCs may be most useful for promoting an inflammatory milieu in the muscle to recruit and activate CD1c$^+$ MDCs in the muscle, which then migrate to LNs in order to present antigen to CD4$^+$ T cell responses.

Monocytes are frequently used for in vitro differentiation of DCs (MoDCs) and known for their ability to home to tissues and differentiate to macrophages or DCs (178). Monocytes stimulated in vitro with MF59 have been shown to differentiate to DCs reminiscent of moDCs and upregulated chemokine receptor CCR7, involved in migration to LNs (179). Mouse studies have shown that neutrophils can upregulate CCR7 and migrate to draining LNs (52) where antigen presentation occurs. In this regard, we found highest CCR7

upregulation on neutrophils and monocytes in the animals receiving MF59 injection, which suggests efficient LN homing of these cells in the MF59 group. We found that MF59 exclusively induced neutrophils to migrate to draining LNs. Mouse studies showed that neutrophils can upregulate MHC II and co-stimulatory molecules and present protein antigens to $CD4^+$ T cells (54, 180). Neutrophils have also been proposed to support B cell responses by secreting cytokines that promote B cell survival and differentiation (53).

4.4 FUNCTIONALITY OF IMMUNE CELLS IN MUSCLE AND LYMPH NODES

4.4.1 Adjuvants induce distinct innate activation profiles

The efficiency to induce DC activation directly or indirectly is likely an important property of an adjuvant (30, 181). We found that MF59 and alum occasionally induced upregulation of co-stimulatory molecules on infiltrating MDCs, PDCs, monocytes and neutrophils in the adjuvant-injected muscle compared to the donor-matched PBS-injected muscles. In contrast, alum-TLR7 consistently induced upregulation of co-stimulatory molecule in all animals and at all time points (2-72 hrs). TLR7 stimulation has previously been shown to induce strong activation of especially PDCs (64, 142, 182). We found that MDCs, monocytes and neutrophils were also able to phenotypically differentiate directly or indirectly by alum-TLR7.

Since PDCs express high levels of TLR7 and respond with abundant production of type IFNs, we analyzed MxA expression in the muscle and draining LNs of adjuvant- and PBS-injected muscle. As mentioned earlier, MxA is expressed only in cells responding to type I IFNs. We found MxA^+ cells only in the alum-TLR7- injected muscles and its draining LNs. A recent study in mice receiving intramuscular injection with TLR7 agonist (adsorbed to alum), resulted upregulation of multiple genes associated with type I IFNs in the injected muscle (37). However, MxA expression was absent in the injected muscle and draining LNs in alum or MF59 groups. Although alum-TLR7 was more efficient in inducing phenotypic maturation and type I IFNs, the other adjuvants and especially MF59, was also able to induce local cytokine response. In fact, we found that infiltrating APCs produced IL-8 and MCP-1 especially after administration of MF59 and alum-TLR7.

We found that neutrophils, which are not perceived as classical APCs, were able to upregulate HLA-DR (MHC II) in response to either adjuvant. However, the stimulated neutrophils expressed much lower levels of HLA-DR compared to the levels found expressed by MDCs and monocytes with or without adjuvant exposure. The reason why MF59 in particular induced neutrophil accumulation in LNs needs further investigation. It has been shown that MF59 adjuvanticity is likely independent of TLR signaling, as previously suggested for alum (183). Previous rabbit studies indicate that MF59 creates an “immune competent milieu” since MF59 delivered a day before antigen delivery at the same site still enabled induction of immunity, while the opposite order of delivery did not. This suggests that MF59 creates an environment that is beneficial to mount antigen-specific immune responses once the subsequent antigen is delivered. It was demonstrated in mice that potent

vaccine responses were induced when MF59 and antigen were co-delivered to the same site, but not when these components were delivered to separate sites (33). Other mouse studies showed that MF59 induces upregulation of genes involved in immune activation with higher magnitude compared to alum and many of these genes encoded cytokines, chemokines, cytokine receptors, proteins necessary for migration, and antigen presentation associated genes (35). The gene expression data confirmed that an immunocompetent environment was created at the MF59 injection site, which was also supported by the robust influx of e.g. monocytes, neutrophils and DCs, as well as cytokine production (35, 42). The cells initiating the immunocompetent environment may be resident macrophages and muscle cells that are activated by MF59 and this milieu promotes recruitment of immune cells.

4.4.2 Antigen uptake in muscle and draining lymph nodes

The generation of antigen-specific adaptive immunity requires internalization of antigens, processing to peptides for presentation via MHC molecules. Therefore, efficient delivery of vaccine antigen to cells is likely needed for induction of strong vaccine immunity. Fluorescent labeling of Env with AlexaFluor 680 enabled assessment of in vivo Env uptake and distribution in our studies. Since Env was adsorbed to alum and alum-TLR7, both vaccine protein antigen and adjuvant were tracked simultaneously. MF59 was labeled with lipophilic dye DiO and could therefore be separately detected when co-administered with the labeled Env.

In line with the cell infiltration kinetics, Env^+ as well as $MF59^+$ cells including, neutrophils, monocytes and DCs were the highest at 24 hrs in the muscle (Fig 9.). Since there were numerous infiltrating neutrophils and monocytes after injection with any of the adjuvants, a large proportion of the Env^+ cells were also represented by these cells. We selected the AlexaFluor-dye for labeling Env since it is stable and can be detected inside cells for a longer period. Importantly, the AlexaFluor-signal indicates Env uptake at some point but it does not mean that the Env protein is still intact. Low levels of MF59 were still detectable at day 10 and both MF59 and alum-TLR7 group had also low numbers of DCs at this time point. This is likely due to sustained low grade inflammation induced by these adjuvants during earlier time points rather than a depot effect. On this regard, one of the proposed mechanisms for alum is that it causes antigen retention at the delivery site, which allows extended antigen availability for sustained antigen uptake and stimulation (26). However, this concept has been challenged by independent studies showing efficient antigen clearance from the vaccination site as well as unchanged immune memory when tissue receiving the vaccine was surgically removed after immunization (26, 27). Increased antigen uptake by APCs has also been shown when antigens were adsorbed to alum (26) and this may lead to improved antigen processing and presentation.

Env^+ and/or $MF59^+$ cells were exclusively detected in the LNs that drained the muscles injected with vaccines and were not found in LNs draining PBS-injected muscle, which is in line with earlier mouse studies (42, 184). The Env^+ cells in the draining LNs are likely a

mixture of cells that acquired Env at the injection site or at the draining LN since injected antigens may also passively reach the LNs. The proportions of different types of Env^+ cells in the draining LNs were different compared to those detected in the muscle. A larger proportion of Env^+ MDCs and Env^+ PDCs were found in the alum and alum-TLR7 groups compared to the MF59 group where Env^+ cells were mainly monocytes and neutrophils.

4.4.3 Antigen presentation capacity of Env^+ cells in draining lymph nodes

Since we detected considerable numbers of Env^+ monocytes and neutrophils in the draining LNs, we compared their capacity to present Env to autologous $CD4^+$ T cells side-by-side with MDCs. To enable this comparison in our NHP model, we utilized a set of animals that were previously immunized with Env and adjuvant (about 2 years earlier) and had PBMCs stored from the time they had well-detectable Env-specific $CD4^+$ T cell responses. In this study, the same animals received an injection with fluorescently labeled Env together with either of the adjuvants and PBS was injected in the opposite arm. At 24 hrs, the draining LNs were collected for isolation of highly purified Env^+ MDCs, monocytes and neutrophils by flow cytometry. From LNs draining PBS injection, we isolated the same cell subsets, which were Env^-. The cells were then co-cultured with $CD4^+$ T cells isolated from the stored PBMCs. Proliferation of Env-specific $CD4^+$ T cells, as a result of antigen presentation, was assessed by CFSE dilution.

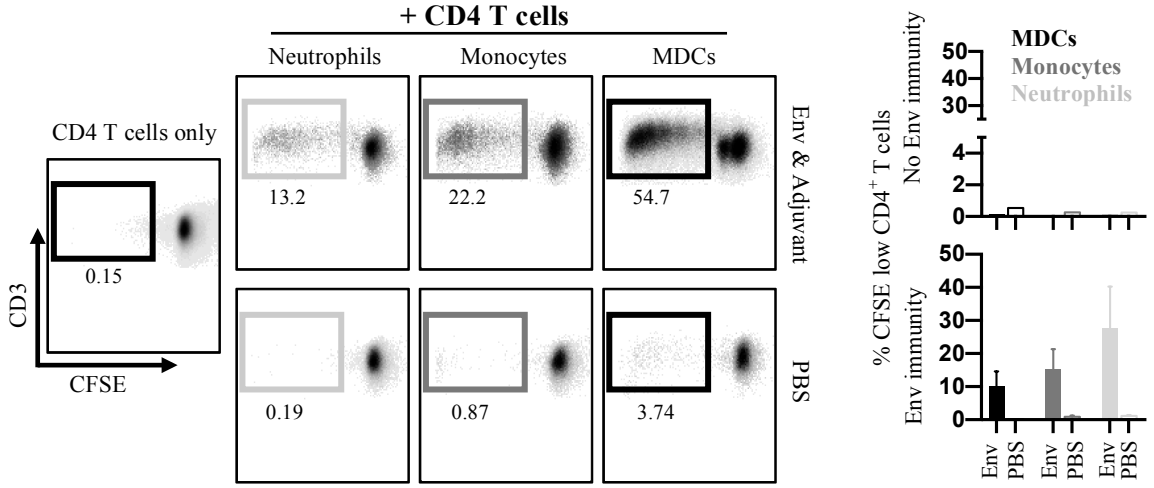

Figure 10. CFSE dilution of autologous CD4+ T cells for measuring antigen presentation capacity of different Env^+ APCs found in LN draining muscle injected Env plus adjuvant. The Env^- APCs were isolated from LN draining PBS-injected muscle in the same animals. *(Figure from paper IV).*

As expected, MDCs were found to be most efficient at presenting Env and inducing proliferation of responding Env-specific $CD4^+$ T cells (Fig 10). Proliferating T cells were also detected in co-cultures with monocytes. However, neutrophils were also able to present Env to a lower degree. As mentioned above, neutrophils in mice have been shown to present antigens to T cells (54, 180). However, other mouse studies suggested that antigen uptake by mouse neutrophils interfered with T cell responses by making antigens less available for DCs to present (55). Nevertheless, we found that the hierarchy of Env presentation capacity of MDCs, monocytes and neutrophils remained the same regardless of the adjuvant used.

Although neutrophils are considered to be rather short-lived cells, they have been reported to survive for five days (185) and T cells have also been shown to support neutrophil survival in co-cultures (186). Therefore, it is likely that some of the neutrophils in our co-culture system remained viable. By using highly purified human neutrophils, monocytes and DCs isolated from donors with chronic cytomegalovirus (CMV) infection, we confirmed that neutrophils were able to stimulate autologous $CD4^+$ T cell cytokine production after 24 hrs co-culture in presence of pp65 CMV protein (F. Liang, unpublished data). However, the T cell proliferation represents memory T cell responses and neutrophils may not have the capacity to prime T cell responses since their levels of HLA-DR and co-stimulatory molecules are not as high as on DCs. Nevertheless, this suggests that the mobilized neutrophils in the draining LNs, especially in the MF59 group, can present Env to already primed memory T cells during boost. The abundance of neutrophils in the immune system may also compensate for their lesser efficiency to present antigens.

4.5 GENERATION OF PRIMARY ENV-SPECIFIC IMMUNITY

4.5.1 Initiation of primary Env-specific CD4+ T cells

Further, in **paper IV** we analyzed whether the kinetics and magnitude of primary Env-specific $CD4^+$ T cell responses were different between the adjuvants. We immunized Env naïve animals at different sites and different time points and collected the draining LNs to evaluate Env-specific $CD4^+$ T cell responses. The LN cells were CFSE-labeled, re-stimulated in vitro with Env protein or peptides and cultured for five days.

We found no Env-specific $CD4^+$ T cells in LNs draining muscle that was immunized 2 hrs or 24 hrs before LN collection. Low but detectable levels of responding T cells were found at 72 hrs post-injection, but the Env-specific responses were most pronounced in LNs draining muscle immunized 10 days earlier (Fig. 11). The responses were antigen-specific since there was no proliferation in absence of Env. Interestingly, LNs that did not drain any of the immunization sites, such as mesenteric LNs lacked responding $CD4^+$ T cells at day 10. This suggests that the generation of responses occurs primarily in the LNs draining the vaccine administration site and that dissemination of Env-specific T cells to other LNs are not detectable level during the first 10 days of priming. Animals receiving either MF59 or alum-TLR7 showed higher proliferation compared to the animals in the alum group (Fig. 11). This could relate to the higher mobilization and activation of APCs observed in MF59 and alum-TLR7, which in turn suggest that more efficient antigen presentation was induced by these adjuvants.

To measure whether Env-specific T cells are disseminated to non-draining LNs after boosts, we analyzed responses in LNs draining muscles receiving the fifth immunization with Env and adjuvants 10 days earlier. We observed again that Env-specific T cells were predominantly found in LNs draining the muscle injected with Env and adjuvant. However,

low amounts of $CD4^+$ T cell responses were found in LNs draining PBS injection, which suggests that Env-specific T cells are disseminated to other LNs after boost vaccination.

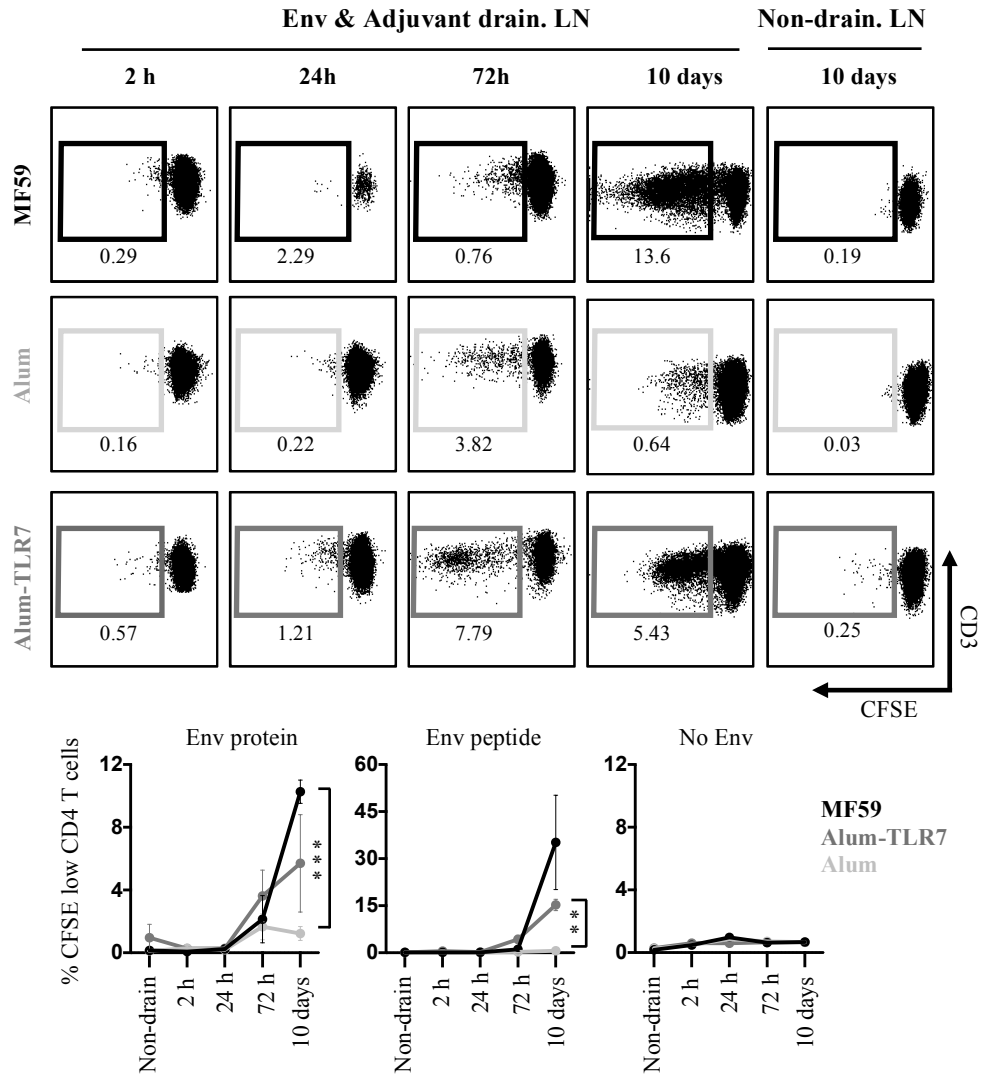

Figure 11. Presentation of Env protein or peptides in LNs draining muscle injected with Env plus indicated adjuvant at different time points. *(Figure from paper IV).*

4.5.2 Accumulation of T follicular helper cells

The quality of the antibodies has been reported to correlate with levels of Tfh cells (187, 188), which are essential for generation of GC reaction that give rise to neutralizing antibodies and long-lived plasma cells. Tfh cells highly express Bcl6 (transcription factor), CXCR5 (chemokine receptor for homing to the B cell follicles and germinal centers) and PD-1 (receptor involved in attenuation of immune responses) (19, 96). Lack of Tfh cells in mice is associated with both the absence of GCs and durable antibody responses (189). Since we detected Env-specific T cell responses 10 days after prime, we estimated the levels of Tfh in the same draining LNs. The Tfh cells were identified as $CD3^+$ $CD4^+$ T cells with high expression of CXCR5 and PD-1 among the central memory T cells ($CD28^{high}$ and $CD95^{high}$) as described earlier (136, 187) (Fig. 12). We found significantly higher numbers of Tfh cells in draining LNs of muscles that received MF59 and alum-TLR7 compared to alum (Fig 12).

This is supported by recent mouse studies that also showed that MF59 induced higher Tfh numbers 10 days after prime compared to alum (190, 191).

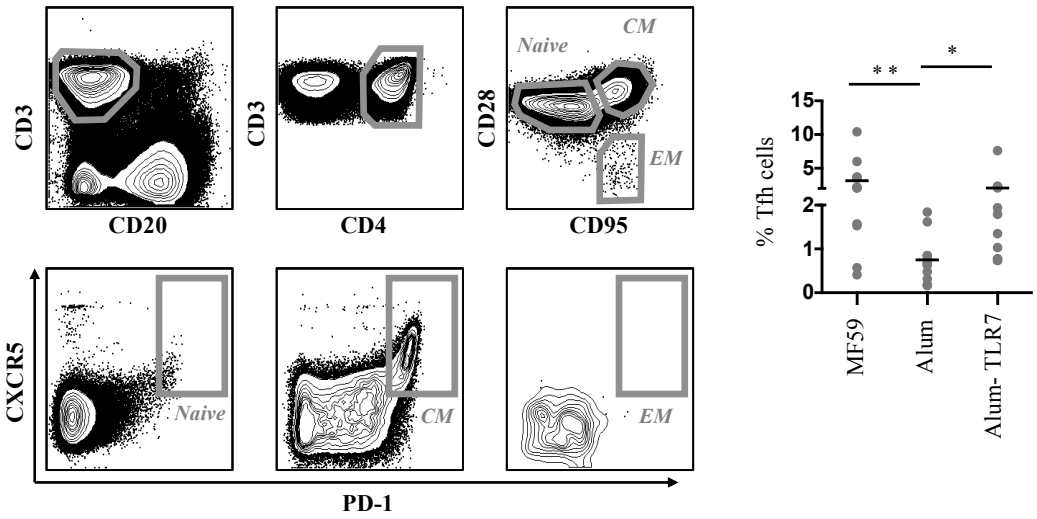

Figure 12. . Gating strategy of Tfh cells in LN at day 10 and compiled data on Tfh cells frequencies in the indicated adjuvant groups. *(Figure from paper IV).*

As mentioned earlier, neutrophils have been shown to support B cell responses by secretion of BAFF, APRIL and IL-21 (53). It has also been reported that IL-21 support Tfh differentiation (192). MF59 also induced homing of monocytes to the draining LNs and activated monocytes are able to upregulate the ICOS-ligand (193, 194), which is essential for differentiation of Tfh cells (192, 195-197). Therefore, the preferential mobilization of neutrophils and monocytes to draining LNs induced by MF59 may support the differentiation of Tfh cells. Further, influenza hemagglutinin (HA) with MF59 induced higher levels of both Tfh cells and GC B cells compared to unadjuvanted HA in adult and infant mice (191). As mentioned earlier, the adjuvant effect of MF59 has been proposed to be independent of TLRs (183). In contrast, the TLR7 agonist adsorbed to alum in our studies activates through TLR7 (37). Since the highest levels of PDCs and MxA expression were found in LNs draining muscle injected with alum-TLR7, it is likely that type I IFNs play a role in the adjuvanticity of alum-TLR7. Indeed, IFN-α supports both B cell and T cell responses (69, 198). In addition, DCs including PDCs are capable of producing IL-6 (67), which is also important for Tfh differentiation (189, 195). In this regard, mice that received intramuscular injections of the same TLR7 agonist used in our studies induced significantly higher IL-6 serum levels compared to alum (37). We also found that MF59 or alum-TLR7, but not alum, resulted in elevated serum IL-6 levels, and that only alum-TLR7 induced IL-6 secretion by highly purified MDCs, PDCs and neutrophils in vitro (F. Liang, unpublished data). Since B cells are also APCs expressing TLR7, they may also contribute to Tfh differentiation. In line with previous studies (42), Env^+ B cells were most frequent in the draining LNs at 24 hrs in all adjuvant groups. This suggests that B cells, like DCs, can take up Env, become activated by the adjuvants and stimulate the differentiation of Tfh cells via CD40 and ICOS-ligand co-stimulation during antigen presentation. However, the APC population that is most influential

for Tfh differentiation is still unclear. Studies in mice with $CD4^+$ T cells incapable of having sustained contact-dependent interaction with B cells, but interacts as normal with DCs, could still express Bcl6, CXCR5 and migrate to B cell follicles like Tfh cells (189). This was also observed in mice where HLA-DR was expressed by DCs and not by B cells (199). Nevertheless, differentiation of Tfh cells and their long-term maintenance are diminished in the absence of B cells (189) or when B cells do not express MHC II (199), which is likely due to loss of co-stimulation from B cells. It has also been suggested that DCs prime naïve T cells into Tfh cells and antigen presentation by B cells are important once Tfh are located in B cell areas (200). Thus, multiple APCs are likely needed for the complete differentiation of Tfh cells and more investigations are needed to elucidate the mechanisms by which alum-TLR7 and MF59 support these events.

4.5.3 Formation of germinal centers

Tfh cells are essential for the GC reaction (95) and the number of Tfh cells correlated with the frequencies of GC B cells in immunized mice (191, 197). Importantly, our collaborators recently reported that NHPs immunized Env with MF59 or alum-TLR7 induced significantly higher HIV-1 specific neutralizing antibodies compared to those given Env alone or together with alum (175). They also found higher levels of Env-specific memory B cells and IgG titers. This clearly demonstrates that different adjuvants have different potency. In this regard, the more pronounced innate activation induced by MF59 and alum-TLR7 in our study likely play a key role behind their findings. Our observation of higher levels of Tfh cells after prime in the MF59 and alum-TLR7 groups, prompted us to evaluate whether the number and size of GCs were affected. To define GCs structures, we stained for PD-1^+ Tfh cells and $Ki67^+$ proliferating GC B cells in cryosections of LNs draining muscle injected with Env and adjuvant 10 days earlier (Fig. 13). These markers readily defined GCs both in human and rhesus macaque tonsil and LNs (136, 201, 202). The clusters of $Ki67^+$ and PD-1^+ cells were located opposite to each other, which indicated the dark and light zone of the GCs respectively, and were found mainly in the B cell areas. We found the highest number of GCs in the MF59 group followed by the alum-TLR7 group (Fig. 13). Similarly, MF59 was shown to induce higher GC numbers in mice at 10 days (190, 191) and at one month after prime (190). We also found that the frequencies of $Ki67^+$ cells in the B cell areas significantly correlated with the size of the GCs (F. Liang unpublished data), which is in line with previous reports (202, 203). It is possible that the rapid turn over of proliferating $Ki67^+$ B cells, increases the size of the GGs. In fact, higher GC numbers and larger GC area have been reported to correlate with amounts of antigen-specific IgG titers in immunized mice (204) as well as controlling SIV disease (202). To this end, we found that as with GC numbers, the area of individual GCs, with or without normalizing for entire LN section area, were larger in MF59 and alum-TLR7 group compared to the alum group (Fig. 13).

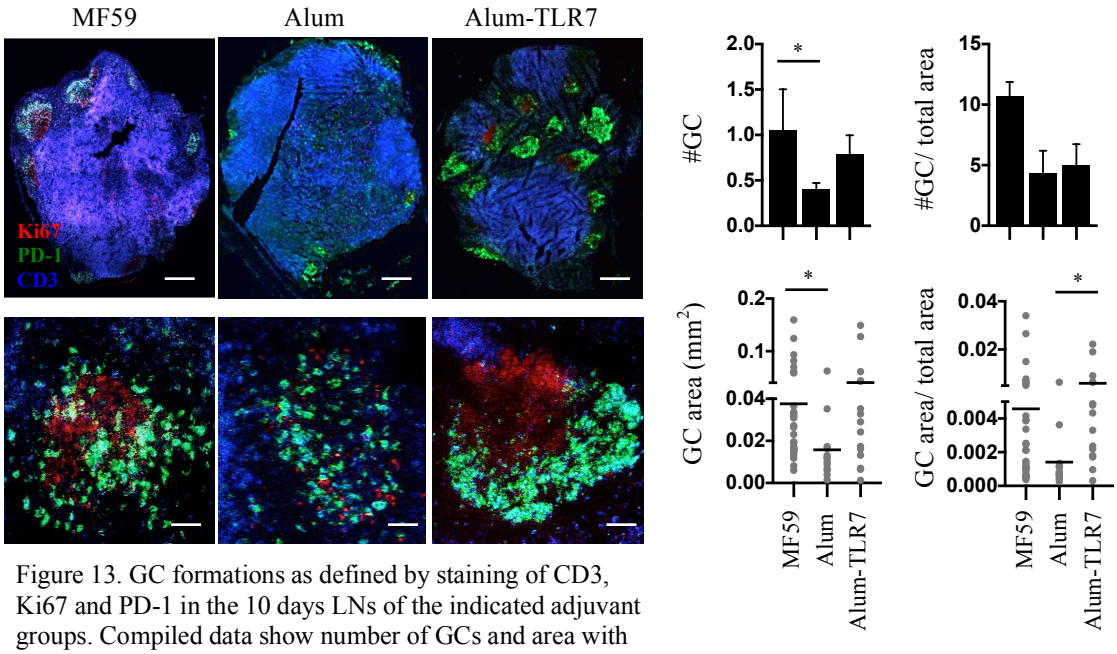

Figure 13. GC formations as defined by staining of CD3, Ki67 and PD-1 in the 10 days LNs of the indicated adjuvant groups. Compiled data show number of GCs and area with or without normalization with the tissue section area. *(Figure from paper IV).*

As mentioned, proliferating Ki67$^+$ B cells correlated with size of GGs. In fact, the GC B cell is the most rapidly dividing mammalian cell type (104). B cells express TLR7 and have been shown to proliferate efficiently after TLR7 stimulation (64, 205), which was further enhanced by IFN-α (64). This suggests that alum-TLR7 may influence the proliferation of GC B cells. Our in situ analysis showed that Env$^+$ cells were found in non-B cell areas at 24 hrs after immunization but no longer detectable at the 10 days time point. However, Env$^+$ B cells were detected at 24 hrs and they have likely acquired antigen in the T cell areas, including the T cell-B cell border. Thus, alum-TLR7 presumably supports transient proliferation of antigen-specific naïve B cells at the T cell-B cell border rather than GC B cells in B cell areas. However, the contribution of direct activation of B cells via TLRs in vaccine responses is controversial. In mice where only the B cells lack MyD88 (an essential protein for conveying activation via most TLRs, including TLR7 and TLR9) had unchanged antigen-specific IgG titers compared to wildtype mice after immunization with protein antigen adjuvanted by TLR9 agonist (206). In the same study, deficiency of MyD88 only in DCs resulted in significantly reduced IgG titers. This suggests that B cell mediated responses to protein-based vaccines are likely due to bystander activation rather than direct B cell activation via TLRs (205). This would mean that the strong DC activation and type I IFN responses in LNs draining alum-TLR7-injected muscle are important for B cell responses. However, other mouse studies have also shown that TLR activation in B cells is essential (205) and B cell responses by the extrafollicular pathway (GC independent) are enhanced by TLR activation in B cells (205, 206). TLR activation of human B cells in vitro has also been reported to support isotype-switching (207). Nevertheless, the durability of GCs may also lead to qualitatively better antibody responses since SHM and isotype-switch may occur for an extended time. The kinetics of GC has primarily been studied in mice and the GC numbers peak around day 10 after immunization and remains consistent for up to 3 weeks, while the

GC area gradually decreased (203). Whether the GC numbers and size in NHPs are altered beyond 10 days after prime need further investigations. Since adjuvants have shown to increase both GC numbers (190, 204, 208) and size in mice (204), they may also influence the durability of GC formations. Persistent antigen availability has been shown to play a role in the maintenance of Tfh cells within GCs (197), which in turn may sustain the number and size of GCs. In a previous mouse study, MF59-adjuvanted proteins were found retained in GCs as immune complexes (antibody-antigen aggregates) for up to 7 days after boost (209). We did not detect extracellular or cell-associated Env in draining LNs at day 10 after prime but it is possible that Env immune complexes can be found after boost.

In summary, our findings on the influence of antigen and adjuvant components on the early innate responses after immunizations illustrate several critical steps on how responses to vaccines are developed and regulated. More knowledge on how the immune system can be manipulated to enhance the quality of adaptive responses is still needed.

5 CONCLUDING REMARKS

The recent swine-flu pandemic, outbreaks of Ebola virus disease and the prevalence of malaria, tuberculosis and HIV infection are constant reminders of the need of novel vaccines as well as strategies to develop new vaccine platforms. Understanding how vaccines utilize the immune system to induce protective responses is central in vaccine development. Vaccines interact with the immune system for the first time at the site of administration and the immune responses initiated at the site most likely dictate the quantity and quality of adaptive immunity. In the two initial studies, we used clinical skin tests to model the local immune responses occurring after antigen delivery. By using, biopsies from positive indurations we found robust DC recruitment. Among the infiltrating DCs were the cross-presenting $CD141^+$ MDCs and PDCs, which are usually low or absent in normal skin. We found that HIV-1 infection influenced the magnitude of DC recruitment, which suggests dysfunctional mobilization of DCs to the skin. In the subsequent studies, we established a NHP model to study the local immune responses in vivo after intramuscular vaccination. This model enabled multiparametric phenotyping and enumeration of several immune cells including neutrophils and DCs after injection of fluorescently labeled HIV-1 Env together with the adjuvants MF59, alum or alum-TR7. We found distinct innate immune activation by these adjuvants. Although all adjuvants induced a rapid and transient mobilization of neutrophils, monocytes and DCs, alum-TLR7 induced robust DC activation and MF59 specifically induced neutrophil homing to the LNs. Env^+ DCs, monocytes and neutrophils were detected in the immunized muscle plus draining LNs. Side-by-side comparison of antigen presentation capacity ex vivo showed that Env^+ MDCs were superior at inducing Env-specific $CD4^+$ T cell proliferation. However, neutrophils were also capable of antigen presentation. Furthermore, both MF59 and alum-TLR7 were more efficient in priming of Env-specific T cells and inducing differentiation of Tfh cells and GC formations compared to the benchmark adjuvant alum. In summary, our approach to investigate the initial immune events at the site of antigen administration provide understanding of the basic mechanisms of vaccine responses, which may help tailoring of better vaccines

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