Human cell-based in vitro systems for vaccine evaluation

Tapia Calle, María Gabriela

DOI: 10.33612/diss.100812074

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date: 2019

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Download date: 02-02-2020
Chapter 1

General introduction and scope of this thesis
Introduction

Vaccines

Vaccines are probably one of the biggest triumphs of human medicine; they enable a simple yet efficient way to prevent infectious diseases. Vaccines are also the most cost-effective way to prevent the spread of diseases as vaccinated individuals can halt the transmission of the infection, and thus their effect also reflects on a broader community [1]. Vaccination can contribute to a large extent to the improvement of health and thereby to economic growth [2]. As proof, during the last century, vaccines have eradicated and extensively diminished major diseases like smallpox and poliomyelitis. They also had a substantial effect on the incidence of other diseases like pertussis, tetanus, yellow fever, measles, and, diphtheria. And recently, extraordinary successes have been accomplished in the prevention of hepatitis (A and B), meningitis and pneumonia which resulted in a decrease of mortality caused by these maladies [3]. However, vaccine development is a time-consuming and expensive process widely known for its trial and error approach. Consequently, many vaccines that succeed in animal testing fail in clinical trials; therefore, additional ways to screen vaccine candidates before human trials are necessary.

Recently, systems vaccinology has enabled the identification of early innate signatures from blood of vaccinated individuals that can predict later immune responses to vaccines [4-6]. This new approach is taking us closer to what we would like to call; “a rational vaccine design”. Noteworthy, systems vaccinology has demonstrated that responses measured early after vaccination in peripheral blood mononuclear cells (PBMCs) are predictive of later secondary responses in terms of antibody titers and T cell responses. Hence, responses of PBMCs to vaccines could potentially give information on immunological properties of the vaccines in more general terms. In this thesis, we have used this rationale to establish an in vitro platform based on human primary cells to evaluate vaccine candidates using influenza vaccine formulations as the model antigen. This in vitro system can potentially assist in the selection of promising candidates for clinical evaluation.

Vaccine development

Vaccine development is a collaborative process comprised of 3 main research stages: 1) fundamental research; 2) translational research, and 3) clinical evaluation [7].

Fundamental research is a critical part of vaccine development, it aims
Introduction

at understanding the mechanism of action of pathogens and how the immune system can mount a proper response to prevent the infection. Additionally, it deals with the development of reliable animal models, the identification and characterization of vaccine targets and finding potential key components that can trigger an immune response.

Translational research turns all the basic concepts into vaccine candidates. It also aims at bridging the gap between fundamental research and the development of clinical products. This research line includes the \textit{in vitro} and \textit{in vivo} evaluation of vaccine candidates in a preclinical setting, development of assays, safety, toxicity and product optimization. Furthermore, it includes the assessment of novel adjuvants required to trigger the initial activation of the immune response.

Clinical evaluation aims at using the knowledge gathered during fundamental and translational stages and convert it into products that will potentially affect public health. To do this, early clinical trials are performed to evaluate novel vaccine candidates for safety, immunogenicity, and efficacy.

Overall, the vaccine development process is long, expensive and risky. Multiple aspects are influencing its outcome such as; the limited knowledge of the disease and the required immune response, the inadequate design of clinical trials for general and also specific populations, and high costs of vaccine development. All of these factors can affect the probability of a vaccine to move from one phase to the next during the development process \cite{8,9} (Figure 1).

Vaccines are commonly considered as the most expensive and risky type of drug to be developed. However, a study recently revealed that the probability of a vaccine to find the way throughout all the different developmental stages up until licensing and marketing is about 0.11%, and that of other pharmaceuticals is about 0.12% \cite{10}. From this study, it was also concluded that although vaccines have been considered riskier investments than other medicines, this is indeed not the case. Only the length of the pre-clinical stage appeared to be significantly longer than for other medicines \cite{10}. On average, the estimated development time of a vaccine is between 8 – 18.5 years (other pharmaceuticals take between 10 – 12.5 years) with an estimated cost between US$ 200 – 900 million \cite{10-13}. As by 2018, the WHO reported 240 vaccines in development of which 59% were in Phase I, 30% in Phase II and 10% in Phase III \cite{14}. These were all vaccines against infectious diseases from which the most common targets were influenza, human immunodeficiency virus (HIV), respiratory syncytial virus (RSV) and Ebola virus \cite{15}.
Relevance of animal models

Throughout the history of vaccine development, there has been a large number of cases in which vaccine candidates succeeded in animal experiments but failed in clinical trials. Well-known examples that hampered the development of vaccines are the formalin-inactivated RSV vaccine \cite{16,17}, the recombinant adenovirus 5 vector-based HIV vaccine \cite{18–21} and the MVA85A (modified vaccinia Ankara 85A) vaccine against Mycobacterium tuberculosis \cite{22}. All of these vaccines managed to succeed in Phase I and II clinical trials but failed to meet the efficacy end-points in the subsequent Phase IIb and III trials.

Failure of vaccines questions the relevance of animal models and their capacity in mimicking the human setting. Notwithstanding, the use of animal models has been paramount in the vaccine development process. This is not surprising given the fact that immunological studies using mice have yielded impressive insights into the mechanism of action of the immune system \cite{23}. Animals are also pivotal in the licensing process of new vaccines and lot release testing of commercially marketed vaccines. Among the animals used in vaccine development, mice are the most important model. From a practical perspective, mice are easy and quick to breed, antibodies and reagents are readily available, genome manipulation is relatively smooth and compared to larger animals they are economical to buy and to house in animal facilities. However, in many other ways, there are concerns about the suitability and robustness of this model.
Introduction

Multiple studies have shown vaccine candidates working well in mice but failing to achieve comparable responses in humans [24-27]. Hence, although the contribution of mice to our understanding of the immune system is indisputable, the use of mice as animal model has different drawbacks;

**Immunological and genetic discrepancies**

Differences between mice and humans in the innate and adaptive immune system are one of the critical reasons for the poor translation of animal experiments into clinical trials. To start, percentages of lymphocytes in peripheral blood are estimated to be around 30-50% in humans and in mice between 75-90% [28]. There is also evidence of differential responses of human and murine macrophages to LPS, driven mainly by the type of receptor on each species [29]. Differences in the display of Toll-like receptors (TLRs) in terms of their abundance and the cells on which they are being expressed [30-32]; the T helper 1 cells (Th1) differentiation in response to type I IFN (interferon) in humans but not in mice [33]; and the expression of IL-10 by Th1 (T helper) cells, which is limited to Th2 (T helper 2) cells in mice, but in humans both Th1 and Th2 can express it [34]. Furthermore, at gene level, murine immune inflammatory signatures have shown to poorly correlate with that of humans [35]. Animal models cannot also mimic the immunological variation that occurs in humans (i.e., variation in availability of TLRs, susceptibility to endotoxins, the difference in the ratios of immune cell population [6,36,37]).

**Mice are not natural hosts of most common human pathogens**

Pathogens display strict species-specific tropisms due to host co-evolution; hence, genetic modifications to mice or the pathogen have to be performed to use them as models. These manipulations increase the differences from the real infection/disease setting. For example, there are different alternatives to overcome the human tropism of viruses. When viruses cannot infect animals due to lack of receptors, the use of transgenic (Tg) mice expressing human receptors enables a proper infection. This technique has been used to generate mouse models for infection of several viruses including hepatitis viruses [38], polio virus [39], HIV-1 [40], and measles [41]. One of the first transgenic (Tg) mouse models was engineered for poliovirus (PV), just after the elucidation of its receptor (PVR/CD155) [42]. The biggest discrepancy between this model and the human situation is the route of infection; in humans PV is transmitted orally, nevertheless, oral administration of PV into polio Tg mice does not lead to infection [43]. In other cases, human viruses can be adapted in such a way that they can infect mice. This approach, however, does not necessarily ensure a better translation of experimental findings into the human setting because disease kinetics and symptoms are different from those in the
human setting. For example, mice are not a natural host for influenza, thus to enable pathogenesis studies influenza viruses are adapted to mice. Adaptation is performed by serial lung-to-lung passages, which results in changes in amino acids that can improve receptor binding, replication and virulence of the virus in mice. Mouse-adapted influenza results in the selection of mutants that replicate faster and are more virulent than other adapted viruses. These adapted strains however, may be very different from their wildtype counterpart in terms of antigenicity and/or phenotype.

**Animal models do not represent the reality**

Animal experiments are an ideal tool to understand basic immune process. One of the main advantages of this approach is the possibility to use genetically homogenous (inbred) mice kept under heavily hygienic and strict conditions. However, this type or reductionist experiments cannot be translated to the human setting, where inter-individual diversity of the immune system is an intrinsic process that allows the evolution of mechanisms of protection against pathogens. Laboratory mice are kept under unnaturally hygienic conditions (i.e., specific pathogen-free facilities), which has been shown to influence their type of response towards stimuli. Additionally, they cannot really mirror the immune system in humans which has been shaped by the exposition to different microorganisms throughout the lifetime. As an example, a study showed that by altering the husbandry conditions of laboratory mice to a more natural state, immune signatures reflected more closely the situation in human adults. Overall, the study showed that T cell frequencies in mice were fewer and phenotypically different from those in adult men. Others have also highlighted the lack of predictive power of laboratory mice for the situation in humans. In an interesting study, Reese and colleagues, demonstrated how previous infections can shape the responses to vaccines. By infecting SPF mice with common pathogens, they described changes in the pre- and post-vaccination gene profiles. They additionally compared SPF and pet shop mice and found that sequential infection of SPF mice recapitulated the gene expression with that of the pet shop mice.

**A “rational” approach for vaccine development**

Traditionally, the approach to make vaccines involved the identification of the etiological agent, its inactivation or attenuation, and its inoculation to generate an immune response. However, the complexity of several important diseases for which we desperately need protection, like AIDS (acquired immune deficiency syndrome), influenza, malaria, dengue, and tuberculosis, does not allow this traditional approach. These diseases
integrate complex microorganisms and/or mechanisms and hence, require a more comprehensive and rational vaccine design \[^{[54,55]}\].

Currently, there are different approaches to rational vaccine design, two important ones being structural vaccinology and systems vaccinology. The first is a recent approach focusing on the determination of the molecular structure of microbial proteins and carbohydrates \[^{[56–58]}\]. By high resolution analysis of the three-dimensional structure of antigen/antibody complexes, structural vaccinology provides detailed information on the tertiary structure of important antigens and the position of relevant epitopes. This information enables the development of promising vaccines even for challenging infectious diseases \[^{[59]}\].

Systems vaccinology can be defined as a “comprehensive analysis of the manner in which all the components of a biological system interact functionally over time” \[^{[60]}\]. This interdisciplinary approach intends to provide a holistic view of biological responses to vaccines. By making use of transcriptomics and proteomics it pinpoints the most relevant genes, proteins or interactions taking place during the generation of the immune response \[^{[61]}\]. Systems vaccinology comes as an essential tool to understand the immunological mechanism of vaccination and as such, is a critical approach in the rational vaccine design of future vaccines. So far, systems vaccinology has profoundly increased the knowledge of the innate immune system and the mechanisms by which protective immune responses are generated \[^{[62]}\].

One of the first and most essential studies displaying the potential of systems vaccinology aimed at understanding immune reactions to the yellow fever vaccine, YF-17D. In the history of vaccines, the YF-17D is probably the most successful and efficacious human vaccine. With a single immunization, it confers protection in almost 90% of vaccinees. The YF-17D vaccine is a live attenuated vaccine generated through serial passaging of the pathogenic yellow fever virus strain, Asibi \[^{[63]}\]. Interestingly enough, it was not until 2005 that the specific mechanisms by which this vaccine exerted protection were unveiled —using a systems vaccinology approach Querec and colleagues \[^{[64]}\] succeeded in identifying the molecular signatures induced early after vaccination which could predict later immune responses. Also, they could pinpoint biomarkers for vaccine efficacy and yielded insights in understanding the underlying mechanisms of action of the yellow fever vaccine. Amongst the identified genes were: innate sensing receptors like TLR7 (Toll-like receptor 7) and RIG-I (retinoic acid-inducible gene I), and transcription factors like IRF7 (Interferon Regulatory Factor 7) and signal transducers like STAT1 (Signal Transducer And Activator Of Transcription 1)
Follow up studies on this vaccine revealed that immunization induced the early expression of GCN2 (general control nonderepressible 2) in blood which strongly correlated with the magnitude of later CD8 T cell responses. The authors pointed towards the critical role of vaccine-induced GCN2 activation in enhanced antigen presentation in dendritic cells (DCs) to both CD4 and CD8 T cells. This data gave further understanding of the mechanism of action of the yellow fever vaccine.

A significant amount of work has also been done in the field of influenza vaccines. Nakaya and colleagues collected blood and serum samples from a cohort of vaccinated individuals with inactivated influenza vaccine at different time points. They found that the transcriptional signatures of these individuals correlated with increased HA antibody responses in serum, which are the gold standard of protection in the context of influenza vaccination. Other studies have also identified early gene signatures (i.e., IFN (interferon) transcriptional signatures) in blood to correlate with high antibody responses. Also, very recently it has been described the correlation of early activation of follicular T helper cells (T<sub>FH</sub>) responses with the magnitude of the B-cell mediated antibody responses in adults. In an additional study by Nakaya, it was found that adjuvanted influenza (MF59) vaccines induce a more potent antibody response in infants than that of non-adjuvanted ones. These responses showed to strongly correlate with blood transcriptional signatures related to IFN networks. Interestingly, this signature response is similar to that in vaccinates adults with non-adjuvanted influenza vaccine.

Knowing that early immune signatures from PBMCs of vaccinated individuals correlate with later antibody and T cell responses implies that PBMCs could be useful to dissect vaccine-related immune responses. In this thesis, we have worked under this premise and have exploited this knowledge to establish a PBMC-based platform to evaluate vaccine candidates in vitro and to elucidate immunological mechanisms behind vaccine-induced responses.

**Innate and adaptive immune responses**

To establish a PBMC-based in vitro system to assess vaccines, it is necessary to understand the different players involved in the generation of an immune response, which is mainly divided in innate and adaptive responses. Innate players are mainly antigen presenting cells (APCs) like dendritic cells and macrophages (and B cells) but also monocytes and natural killer cells (NKs). Adaptive players are represented mainly by lymphocytes; T and B cells. Here the most critical difference is that innate immune cells are
activated by pathogens or signals through a very rich array of receptors, whereas adaptive immune cells are restricted to the activation of unique and specific antigen receptors, which can only be engaged with a single antigen[74].

To generate an appropriate immune response, signals from different sources need to be integrated in DCs in a complex process. Immune responses start by the recognition of pattern-associated molecular pattern (PAMPs); conserved molecular structures found in microorganisms) or damage-associated molecular patterns (DAMPs) by pattern-recognition receptors (PRRs). As of today, 6 families of PRRs have been described (Box 1); these can be classified on basis of the type of ligands recognized, the signaling pathway they trigger and the downstream cascade they activate. Multiple PRRs can be triggered by the same ligand, which is an interesting evolutionary strategy to back-up pathogen sensing. Thus;

**Box 1. Pattern recognition receptors, their signaling pathways and the adaptive immune response they induce.** Abbreviations TRIF: TIR-domain-containing adapter-inducing interferon-β; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; LGP2: Laboratory of Genetics and Physiology 2; MAVS: Mitochondrial antiviral-signaling protein; VISA: Virus-induced signaling adapter; NOD-1/2: Nucleotide-binding oligomerization domain-containing protein; NLRP3: NLR family pyrin domain containing 3; DC-SIGN: Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin; BDC2: Blood dendritic cell antigen 2; ITAM: immunoreceptor tyrosine-based activation motif; NFAT: Nuclear factor of activated T-cells; IFI16: Gamma-interferon-inducible protein; DAI: DNA-dependent activator of IRFs; AIM2: Absent in Melanoma 2; STING: Stimulator of interferon genes; ASC: Apoptosis-associated Speck-like protein containing C-terminal caspase recruitment domain [CARD]; OAS: oligoadenylate synthase; cGAS: cyclic GMP–AMP (cGAMP) synthase; cGAMP: Cyclic guanosine monophosphate–adenosine monophosphate. References used for each PPR; TLRs: [75–79]; RLRs: [80–86]; NLRs: [87–91]; CLRs: [92–97]; ALRs: [98–102]; OLRs: [103–108].

<table>
<thead>
<tr>
<th>Location</th>
<th>Toll-like Receptors (TLRs)</th>
<th>RIG-1-like Receptors (RLRs)</th>
<th>NOD-like Receptors (NLRs)</th>
<th>C-type lectin Receptors (CLRs)</th>
<th>AIM2-like Receptors (ALRs)</th>
<th>OAS-like Receptors (OLRs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipopolysaccharide (TLR2)</td>
<td>Lipoprotein (TLR2)</td>
<td>dsRNA (TLR3)</td>
<td>lipopolysaccharide (TLR4)</td>
<td>Flagellin (TLR5)</td>
<td>CPPG DNA (TLR9)</td>
<td>Prolin (TLR11)</td>
</tr>
<tr>
<td>Ligand</td>
<td>Lipoprotein (TLR2)</td>
<td>Lipoprotein (TLR4)</td>
<td>dsRNA (TLR3)</td>
<td>Lipopolysaccharide (TLR4)</td>
<td>Flagellin (TLR5)</td>
<td>CPPG DNA (TLR9)</td>
</tr>
<tr>
<td>Response</td>
<td>NF-κB Type I IFN</td>
<td>NF-κB Type I IFN</td>
<td>NF-κB Type I IFN</td>
<td>NF-κB Type I IFN</td>
<td>NF-κB Type I IFN</td>
<td>NF-κB Type I IFN</td>
</tr>
<tr>
<td>Activated cells</td>
<td>CD8 T cell response</td>
<td>CD8 T cell response</td>
<td>CD8 T cell response</td>
<td>CD8 T cell response</td>
<td>CD8 T cell response</td>
<td>CD8 T cell response</td>
</tr>
</tbody>
</table>
signaling pathways triggered by the same ligand can be independent of each other. A typical example is the flagellin in bacteria, which can activate both TLR5 and NLRC4 (NOD-Like Receptor Family CARD Domain Containing 4) [109,110]. Activation through TLR5 induces MyD88-dependent NF-κB translocation while NLRC4 activation induces inflammasome formation and caspase activation which leads to the cleavage of pro-IL-1β and pro-IL-18. Another interesting example is the sensing of the yellow fever vaccine YF-17D through TLR2, 7, 8, and 9 also the triggering of genes related with inflammasome, RIG-1, and MDA5 (Melanoma Differentiation-Associated protein 5) which ensure a powerful activation of the innate immune response and guarantees the potency and efficiency of this vaccine [5,64,111].

Signals derived from PRRs (input signals) can induce the activation, maturation and subsequent migration of DCs to the lymph nodes. For this to happen, multiple cascade signals are integrated to steer the induction of the immune response. As a result, output signals turn into the induction of transcription factors, co-stimulatory molecules and cytokines (Figure 2).
Once in the lymph nodes, DCs can present antigens to T cells, in a process that relies on the expression of co-stimulatory molecules and signals in both DCs and T cells. Furthermore, the cytokines in the milieu dictate the fate of the T cell differentiation. CD4 T cells can differentiate into various T helper subtypes (T\(_{H1}\), T\(_{H2}\), T\(_{H17}\), T\(_{FH}\)) and exert critical functions like the activation of CTLs and macrophages and the activation and differentiation of B cells into plasma and memory B cells. As for the CD8 T cells, depending on the balance of cytokines, they can differentiate into effector cytotoxic or memory CD8 T cells (Figure 3).

The resolution of an infection is characterized by the expansion and differentiation of T cells into effector T cells which are pivotal in clearing pathogens. Subsequently, a contraction phase takes place, in which the majority of effector cells die by apoptosis, and finally, a memory phase is reached in which a small fraction of the primed T cells remains and differentiates into long-term memory T cells that protect against future infections. These memory cell pools enable improved responses reflected in enhanced speed, magnitude, sensitivity and efficiency as compared to primary responses\(^\text{[112-114]}\).

Memory T cells are classified in different subsets; effector-memory (T\(_{EM}\)), central memory (T\(_{CM}\)), and terminally differentiated T cells (T\(_{EMRA}\)) from which multiple subcategories have been described depending on two basic parameters; longevity and proliferation capacity\(^\text{[115]}\). T\(_{CM}\) cells are proposed to be an “ideal” memory T cell subset as they can persist in the periphery longer than other subsets like T\(_{EM}\) (in mice)\(^\text{[112,116]}\). In human studies, T\(_{EM}\) cells have shown to be the most predominant memory T cell population in tissues and peripheral blood\(^\text{[117-119]}\). Also, T\(_{EM}\) cells are very important for sustaining the frequency of T resident memory (T\(_{RM}\)) CD8 T cells in the lungs following influenza infection\(^\text{[120]}\). This implies that T\(_{EM}\) have a critical role in sustaining the immune defense. Both T\(_{CM}\) and T\(_{EM}\) can produce IL-2 and effector cytokines upon stimulation, however, T\(_{CM}\) display homing receptors for lymph nodes (CCR7) and a high proliferative capacity contrary to T\(_{EM}\) which in turn can produce effector cytokines more avidly than T\(_{CM}\) with a lower proliferative capacity\(^\text{[121,122]}\). T\(_{EMRA}\) is an interesting subtype as these cells possess an effector phenotype, yet during the contraction phase, they persist in circulation despite their expression of homing molecules (CCR7). These cells are more prevalent in the CD8 subsets and have a high capacity to produce IFN\(\gamma\) but low proliferative potential\(^\text{[117,123,124]}\). Expansion of T\(_{EMRA}\) in CD4 and CD8 subsets was observed in individuals infected with Dengue virus\(^\text{[125]}\), which suggest that some viruses can trigger T\(_{EMRA}\) formation.
Chapter 1

Figure 3. Innate and adaptive response in the context of viral infection. DCs play a key role in the initiation of the adaptive immune response. In the context of viral infection, viruses are sensed by PRRs located on DCs; like TLR2, TLR3, TLR4, TLR7/8, TLR9, RIG-1, MDA5, and AIM2. Once DCs get activated they will secrete different cytokines to steer the T cell differentiation fate into what is best to tackle a specific microorganism. During viral infection, DCs are poised to secrete different cytokines like type I IFN and IL-12; IL-12 is known to be one of the hallmarks during viral infection as it steers the T cell response into a Th1 phenotype. Likewise, IL-12 (together with IRF-4) is also involved in determining the fate of T cells towards a Th1 phenotype. A Th1 phenotype is characterized by the upregulation of CCR7 as a surface marker, expression of the transcription factor T-bet and the production of IFN-γ. Protection during viral infection is also characterized by the induction of cytotoxic CD8 T cells (CTLs), these cells are distinguished by the upregulation of CD107 and their ability to efficiently kill infected cells through the production of granzyme, perforin and IFN-γ. A counterpart is the recently characterized CD4 T cell subtype with effector cytotoxic properties. These MHCI-restricted cytotoxic CD4 T cells can originate from Th0 or Th1 cells when the transcription factor ThPOK is inhibited. These cytotoxic cells have shown to be critical in the resolution of infections like; influenza, dengue, hepatitis, and HIV. Upon differentiation, ThFH cells upregulate ICOS, CXCR5, Bcl-6 and produce IL-21. These cells provide B cells with the co-stimulatory signals, CD40L and ICOS; Together with the production of IL-21, ThFH cells enable the generation of high affinity-matured, long-lived plasma cells and memory B cells. Underlined molecules were used in the experiments presented in this thesis. Image created with BioRender.
**Influenza vaccine as a model**

Influenza virus is a widely known pathogen; infection can result in fever, cough, headache, and sore throat. Influenza A and B virus constantly circulate among humans causing seasonal epidemics and occasional pandemics \[^{143-146}\]. This results in 3 – 5 million infected people every year, and leaves between 290.000 and 650.000 deaths around the world \[^{146}\]. Due to its high prevalence, the prevention of infection is pivotal, and vaccination has shown to be the best method for the protection and control of influenza \[^{146}\].

Influenza vaccines have played an important role in the history of the vaccines’ development. In 1930, a whole inactivated virus (WIV) influenza vaccine was the first successful inactivated vaccine obtained \[^{147}\]; and its establishment served Jonas Salk to develop an inactivated polio vaccine \[^{148}\]. Throughout history, different influenza vaccine formulations have been developed like; split, subunit, live attenuated (LAIV), recombinant vaccines, WIV, and “add-on peptide vaccines”. As of today, all of these vaccine formulations (except “add-on” peptides) are licensed as seasonal influenza vaccines, although the inactivated vaccines (subunit and split) are predominantly used worldwide \[^{149,150}\]. In the following, we will focus on four of them as they were used in the studies presented in this thesis.

**Whole inactivated virus (WIV) vaccine.**

WIV vaccine is prepared by propagating the virus in embryonated chicken eggs or cells and then harvesting the allantoic fluid or cell supernatant for later purification and inactivation of the virus using formaldehyde or β-propiolactone \[^{151,152}\]. Recent studies have pointed out that inactivation with β-propiolactone is a better alternative than formaldehyde as the latter cannot fully inactivate the virus \[^{152}\] and affects the yield of the final product \[^{151}\]. β-propiolactone modifies nucleic acid bases in the viral RNA and therefore blocks viral replication. For decades the immunological correlates of protection of influenza vaccines have been related to humoral responses; hence during clinical trials the ability of these vaccines to exert protection has been limited to assess antibodies-related responses. WIV have shown to induce humoral responses in clinical trials \[^{153-157}\]; however T cell responses have so far not been investigated. In mice, WIV does induce both, humoral and cellular immunity \[^{158,159}\].

Next to inducing potent hemagglutinin (HA)-binding antibodies, WIV has displayed the ability to induce antibodies targeting the viral neuraminidase (NA), and to do so more efficiently than other vaccine formulations \[^{160}\]. These antibodies (previously neglected) have recently shown to be of
great importance in the context of influenza as they can provide potent and broad cross-reactive protection \[^{160,161}\]. In the ‘60s, WIV vaccines were removed from the market due to reports claiming local reactogenicity and side effects -especially observed in children. These reactions were thought to be caused by egg contaminants in the vaccines \[^{162}\], which was mainly due to suboptimal vaccine production guidelines. The later introduction of zonal centrifugation allowed a highly purified influenza vaccine preparation with a significant reduction of vaccine reactogenicity \[^{163}\]. Currently, an adjuvanted WIV vaccine is commercially used in some countries \[^{150}\].

**Split vaccine.**

Split vaccine is a widely used vaccine formulation. This vaccine is produced by treating inactivated virus with detergents; Tween80 and cetyl trimethylammonium bromide (CTAB) \[^{151}\]. During this process the membrane is disrupted; this causes the loss of the viral structure and remnant RNA to be quickly degraded. Despite this, all of the structural proteins of the influenza virus remain intact. Split vaccine triggers humoral responses by inducing the production of antibodies against HA and NA \[^{164-166}\], but it induces poor cellular immunity \[^{167-170}\].

**Subunit vaccine.**

Subunit vaccine is a highly pure vaccine formulation consisting of HA and NA. To obtain a subunit formulation, the inactivated virus is also disrupted with detergents followed by ultracentrifugation to remove the viral core \[^{171}\]. As with split vaccine, subunit induces a humoral response represented in the production of antibodies mainly against HA \[^{172-174}\].

**“Add on” Peptide vaccine.**

This new vaccine approach is aiming at broadening the T cell responses in influenza vaccine strategies. Current influenza vaccines (i.e., split and subunit) exert their protective effect through the induction of virus-specific neutralizing antibodies targeting the surface proteins of the influenza virus \[^{175}\]. But due to mutational changes (antigenic drift and shift) in the surface proteins, the virus can easily evade these antibodies. Peptide vaccines are an innovative approach that relies on activation of cellular immune components; like CD4 and CD8 T cells. These cells can recognize highly conserved epitopes of the virus; hence allowing T cells to cross-react with different influenza strains and even subtypes \[^{128,176,177}\]. Peptides representing conserved T cell epitopes can be used to induce influenza-specific CTLs which can clear infected host cells; thus controlling the infection by inhibiting virus replication and limiting the viral spread \[^{178}\]. One limiting factor with peptides is their low immunogenicity; given
their lack of PAMPS, they cannot actively stimulate APCs. To overcome this issue, antigenic peptides are combined with current vaccine formulations (adjuvanted subunit \[^{179,180}\], WIV \[^{181}\]) in such a way that APCs can properly process peptides. Using highly conserved peptides as “add ons” together with standard influenza vaccine formulations it could be possible to induce both; B cell and T cell responses. M-001, the first peptide influenza vaccine has recently entered phase III evaluation; its goal is to perform as a broadly cross-protective universal influenza \[^{182}\].

**Outline of this thesis**

In the first part of this introduction, we highlighted vaccine development as a time-consuming and expensive process. Vaccine candidates often succeed in animal experiments but tend to fail during clinical trials, this highlights two essential issues: problems in translating results obtained in animal experiments into humans and gaps in the understanding of mechanisms of action of vaccines. To understand infections and immune responses it is necessary to walk away from animal models and take one step further into the “human model”. Hence, human PBMCs have shown to be a potential source to understand and to assess vaccines.

The primary aim of this work was to establish an *in vitro* modular system to assess responses and elucidate immunological mechanisms of vaccines using human cells. We envisioned an *in vitro* vaccine evaluation system consisting of PBMCs able to recreate responses towards vaccines. Here we present two different platforms to assess innate and adaptive responses. The first platform consists of mono cultures of monocyte-derived DCs (MoDCs) and enables a detailed assessment of the properties of vaccines to activate innate immune responses. The second platform makes use of whole PBMCs and focusses on dissecting T-cell induced responses by vaccines. The use of *in vitro* systems to evaluate responses towards vaccines has been recently explored by others \[^{183-186}\]. However, these studies have been limited to innate players: DCs and has used different approaches like cell lines or murine DCs.

To establish this *in vitro* system, influenza vaccines were used as our main antigen as there are various vaccine formulations which show well-characterized differences in immunogenicity in animals and in clinical trials. As previously mentioned, WIV, split and subunit vaccines undergo different processes during vaccine production; these processes lead to important physico-chemical differences between the vaccines that are reflected in distinct immune stimulatory capacities on cells.
To build the modular *in vitro* system to assess vaccine candidates we first established the innate module in which we mainly focused on the evaluation of DCs *(Chapter 2)*. From mechanistic studies in animal models, it is known that a proper stimulation of PRRs like TLRs in DCs can shape the desired type of response to mount protective immunity against a given pathogen. However, humans and animal models differ in the expression and specificity of TLRs [30–32]. In this study we first set out to identify the best platform to assess stimulatory properties of vaccines on APCs *in vitro*; hence, we compared the suitability of two platforms: the DC-cell line MUTZ-3 and primary monocyte-derived DCs (MoDCs). Secondly, we assessed whether the system was capable of discriminating between highly immunogenic and low immunogenic vaccines using WIV and subunit influenza vaccines as models. Lastly, we evaluated whether freshly isolated and frozen/thawed PBMCs were equally suitable for generation of Mo-DCs and whether Mo-DCs derived from fresh and frozen PBMCs respond to vaccines and do so in a similar way.

Since vaccines exert important effects in DCs but also downstream in T cells, understanding these response mechanisms upon vaccination is important to fully visualize the mechanisms of action of vaccines. In *Chapter 3* we focused on establishing an experimental system that allows a detailed characterization of vaccine-induced responses in CD4 and CD8 T cells. Here, we first determine immune memory responses. Previous *in vitro* approaches to assess T cell responses aim at the determination of antigen-specific memory responses induced by previous infection or vaccination [187–191]. To determine memory T cell responses, cells are isolated from blood and pulsed for a short period of time with antigen or stimuli. Then, responses are evaluated using proliferation assays, ELISpot, or intracellular cytokine staining. Induction of antigen-specific responses makes use of purified co-cultures of DC and T cells [187–189,191,192], in a rather laborious and time-consuming process. Different from this approach, we present an *in vitro* system using long-term cultures of unfractionated PBMCs to assess T cell responses (Chapter 3). Using this approach together with flow cytometry staining, we characterized T cell-mediated immune responses involving T helper, CTLs, T_{FH} and memory T cell subsets to different influenza vaccine formulations (WIV, split and, “add-on” peptides).

After having established these two immune modules, we set out to test their robustness. Different influenza subtypes differ in immunogenicity in preclinical and clinical studies. However, a proper head-to-head comparison of vaccines derived from different influenza subtypes is still lacking. In *Chapter 4* we have performed a systematic comparison of vaccines derived from four different influenza virus strains using *in vitro* and *in vivo*
Introduction

approaches. We have first assessed the physicochemical properties of H1N1, H3N2, H5N1, and H7N9 WIV vaccines and then evaluated the stimulatory capacities of the different influenza subtypes on the DC module presented in Chapter 2 and assessed the antigen-specific T-cell responses (Chapter 3) stimulated by each virus subtype in vitro. In parallel, we evaluated the immunological responses to the different influenza vaccine subtypes in mice in vivo. These experiments enabled us to visualize the correlation between our in vitro approach and the in vivo setting.

Lastly, in Chapter 5 we exploited the DC and T cell platforms (Chapter 2 and 3) to assess the effect of size in the magnitude of the immune response. For this we used two different subunit formulations: influenza and Hepatitis B and coupled them to nano- and micro-particles (sizes 0.5 and 3 μm). Each vaccine formulation was then used to stimulate MoDCs and T cells; in the MoDCs we assessed their ability to be up taken my microscopy while in the T cells we evaluated the induction of cytokine-induced responses by flow cytometry.

In Chapter 6 we summarize the findings of this thesis and discuss the implications of the work in the light of vaccine development. We also examine the future perspectives regarding the expansion of the in vitro system with a B cell module and the integration of modern technologies to better dissect and understand immune responses induced by vaccines. We further examine the possibilities to exploit this in vitro system approach to better understand differences in immune responses between young and old individuals.
References


Introduction


[34] Del Prete G, De Carli M, Almerigogna F, Giudizi MG, Biagiotti R, Romagnani S. Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production. J Immunol 1993;150:353–60.


[44] Sakabe S, Ozawa M, Takano R, Iwastuki-Horimoto K, Kawoaka Y. Mutations in PA, NP, and HA of a pandemic (H1N1) 2009 influenza virus contribute to its adaptation


[67] Bucases KL, Franco LM, Shaw CA, Bray...


[88] van Beelen AJ, Zelinkova Z, Taanman-


Introduction


Introduction


undefined. Split-virus influenza vaccines: do they provide adequate immunity in the elderly? AcademicOupCom n.d.


Introduction


