In vitro approaches for the evaluation of human vaccines
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DOI:
10.33612/diss.166150822

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

Publication date:
2021

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Chapter 1

General introduction and scope of this thesis
A modern approach to vaccine development

Vaccination is one of the most important advancements in history; thanks to vaccines, the eradication of diseases that have plagued humanity for millennia or the swift defeat of a global pandemic are finally realistic prospects [1,2]. Ancient techniques have long been exploring the fascinating interaction between human health and microorganisms – even before anyone postulated their existence. Already since the mid-1500s, material from patients recovered from mild forms of smallpox was given to healthy individuals as protection from a more severe course of the disease, a practice known as variolation [3]. In the 18th century, what started as a simple observation – the apparent resistance of dairy workers from serious forms of smallpox – led a number of individuals, among which Edward Jenner, to study the protection conferred by the closely related, lowly pathogenic animal infection, the cowpox [4]. The empirical investigation of this principle resulted in the first vaccination (from the Latin word vacca, cow, referring to the bovine pathogen used for the immunization), and ultimately gave rise to the development of more sophisticated and safer vaccines. Vaccination is regarded as one of the most far-reaching and cost-effective methods of medical intervention, due to its direct protection of immunized individuals as well as its indirect effect on the transmission of pathogens in the community [5]. The impact of vaccines on human longevity and global health in the last centuries has been estimated as secondary only to the availability of clean water [6,7].

Traditionally, vaccine development has been dominated by trial and error. Thanks to the increasing knowledge and insights into biological and immunological mechanisms, vaccines have been improved and their production perfected. Still, the development of vaccines is a long and complex process, often lasting more than a decade and with a success rate of as low as 6% [8]. A large number of vaccine candidates fails in the transition from pre-clinical to clinical stages, often due to the low predictive value of animal-based data for the immunogenicity of the vaccine in question in humans [9]. Nevertheless, animal-based readouts are still the gold standard in vaccine development. In addition to their use in the research phase, animal-based in vivo methods are also extensively employed during post-marketing surveillance for batch release testing of commercially marketed vaccines. While animal studies have contributed greatly to immunological research, growing evidence supports the need for a more rational vaccine design and assessment [10]. Systems vaccinology has led to the identification of vaccine-induced immune signatures and of correlates of protection [11–15], offering a comprehensive picture of the immune responses to vaccination [16,17]. The application of the knowledge derived from a systems biology approach to vaccine research and the use of novel omics techniques allow us to consider more relevant human-derived platforms as alternative to the current animal models [18].
In this thesis, we aimed at developing \textit{in vitro} assays suitable for analyzing the characteristics and assessing the consistency across batches of viral vaccine formulations. These methods should ultimately help select promising vaccine candidates, replace \textit{in vivo} potency evaluation of existing vaccines and elucidate molecular mechanisms in the interaction between vaccines and human immune cells.

This introduction will first summarize the mechanisms of action of viral vaccines in their interaction with the immune system, to then focus on the role of animal models in vaccine research and development, the shortcomings derived from their use and the possible alternatives. Furthermore, the vaccine models used in this thesis will be described and an outline of the various chapters will be presented.

\textbf{Vaccines: mechanism of action}

Prophylactic vaccines are most commonly described as substances that provide acquired immunity to infectious diseases – without causing the disease itself. Vaccines are traditionally prepared using the causative agent of the disease, such as bacteria or viruses, inactivated or adapted to be lowly pathogenic [6]. In this thesis, we will focus on viral vaccines.

Empirical development methods – with little or no understanding of the immunological principles behind vaccination – have been successful for most vaccines [19]. Yet, the development of vaccines against certain pathogens (e.g. human immunodeficiency virus or respiratory syncytial virus) has so far been unsuccessful despite decades of effort, due to the pathogens’ evasion strategies or to complex antigen conformations [20,21]. To tackle vaccine development and assessment in a more rational way, it is necessary to understand the immunological mechanisms by which vaccines confer protective immunity.

The hallmark of a successful vaccine is, from an immunological standpoint, its ability to trigger potent antigen-specific responses, that include the production of neutralizing antibodies – also referred to as humoral immunity – and/or the induction of cellular (T cell-based) immunity [22–24]. Antibodies prevent microbes from binding and infecting the host’s cells, as well as allow the opsonization and phagocytosis of the antibody-coated pathogen [25,26]. Cellular immunity is mediated by T lymphocytes that, once activated, recruit phagocytes to eliminate the pathogens, stimulate the proliferation of antibody-producing plasma cells and long-lived memory cells, and – in the case of CD8$^+$ T cells – differentiate into cytotoxic cells that kill microbe-harboring host cells [26].
These are features of the adaptive immune response, a form of immunity that develops and adapts in response to infection or immunization, characterized by the specificity to the pathogen and the ability to establish an immune memory [27]. To develop an adaptive response, the immune system must first be activated; this action is performed by the innate immune system, capable of sensing pathogens and readily respond to their presence.

The mechanisms underlying innate immunity are aspecific and not based on an immune memory; among them, the recognition of microbes by antigen-presenting cells (APCs) is one of the most important steps [28]. APCs, the 'sentinels' of the immune system, sense pathogens and cell damage through pattern recognition receptors (PRRs) that recognize pathogen- and damage-associated molecular patterns (PAMPs, conserved molecular structures found in microorganisms, and DAMPs, host-derived molecules released by damaged or dying cells) [29,30]. Several families of PRRs have evolved to sense a diverse range of microbes and their components.

While not pathogenic, vaccines contain PAMPs that – together with DAMPs released by cells damaged in/by the injection – can initiate the induction of immune responses through the activation of APCs [31]. Depending on the formulation, vaccines can contain several PAMPs derived from the pathogen’s components [32]. For what concerns viral vaccines, the object of this thesis, various types of vaccines have been developed and they can be broadly categorized as whole virus particles, split or subunit vaccines and reassortant vaccines [33]. Whole inactivated vaccines – the kind investigated in the next chapters – are one of the most traditional types of vaccine, and are prepared using physicochemical treatments that impair virus replication. The composition of whole inactivated vaccines includes structural proteins and viral genome, which are sensed by the innate immune cells through several PRRs (Figure 1).

In addition to the pathogen-specific components, vaccines can include adjuvants – substances that can enhance or modulate the vaccine-induced immune response, such as aluminum salts [34]. All these components in a vaccine contribute to the induction of innate immune responses. The innate immune cells, once activated by PRR-triggering PAMPs and DAMPs, can initiate antigen presentation. This process, mediated by professional APCs and CD4+ T cells [35], ultimately results in the generation of the desired adaptive response (Figure 2). The nature of the responses elicited by a viral vaccine depends on several aspects, such as the antigen concentration, the specific type of components present in the formulation and the presence of adjuvants [36].
Figure 1. Interaction of virus particles with innate immune cells. ① As virus particles bind to host receptors in the plasma membrane, they can be sensed by PRRs such as plasma membrane-bound Toll-like receptors (TLRs) [37]. ② Receptor binding is followed by the internalization through endocytosis [38–40]. ③ For a majority of animal viruses, entry into the cytosol is triggered by low pH (that for enveloped viruses induces membrane fusion) [41,42]. ④ TLRs in the endosomal compartment are triggered by viral nucleic acids, which after uncoating can also activate cytosolic sensors such as RIG-I-, OAS-, AIM2- and NOD-like receptors (RLRs, OLRs, ALRs and NLRs) [43–52]. After triggering of any of these receptors a signaling cascade follows, with the engagement of receptor-specific adaptor proteins and kinases that culminates in the activation of transcription factors [53–55]. Once activated, these factors translocate to the nucleus to promote transcription of genes involved in different downstream responses [56–58]. Image created with BioRender.

For the purpose of vaccine assessment, innate immune responses are relatively easy to study – as they require no prior encounter with the pathogen and are among the most conserved immunological features intra- and interspecies [59]. Additionally, the successful induction of an innate immune response has been shown to correlate with the potency of several vaccines [11,12,60]. Therefore, in the investigation of alternative methods to in vivo assays for viral vaccine testing, this thesis will focus on the early interactions of vaccine components with target cells and cells involved in innate immunity. In this introduction, we will first present the current vaccine evaluation strategies and then examine suitable alternative methods.
Figure 2. Vaccine-induced immune responses. ① PAMPs and DAMPs are sensed by antigen-presenting cells, which can be further stimulated by bystander cells (such as phagocytes) through the secretion of chemical signals, such as interferons (IFNs), chemokines and cytokines [32]. ② APCs become mature and activated, thus able to secrete chemical signals and express co-stimulatory molecules [61]. ③ Activated APCs migrate to the lymph node, where they present antigens to CD4+ T cells through a so-called immunological synapse [62]. Their interaction steers the differentiation of T cells towards various subtypes, that ultimately drive the generation and proliferation of plasma and memory B cells from B cells, and of effector and memory T cells from T cells [63]. Differentiated effector lymphocytes can migrate into the bloodstream, while pools of long-lived memory cells are differentiated and maintained to protect against future encounters with the pathogen [64]. Image created with BioRender.

In vivo assays in vaccine development and production

The development of a vaccine is a collaborative effort that involves public and private organizations, and includes basic, translational and clinical research stages [65]. Basic laboratory research focuses on the pathogen and its interaction with the host’s immune system. In this exploratory stage, proof-of-concept studies aim to identify natural or synthetic antigens that can ultimately serve as vaccine candidates. Basic research is also necessary for the development and assessment of reliable animal models.

In the next phase, translational research uses cell- or tissue-culture systems and animal testing to evaluate the safety and immunogenicity of a vaccine candidate. This pre-clinical step includes the selection of adjuvants, the development and performance of in vitro or in vivo potency assays, the analysis of toxicity at various doses and the assessment of several animal models as in vivo readout systems.
This stage involves highly diverse and iterative research, in which candidate vaccine formulations are continually tested and improved. The outcome of these studies will potentially suggest specific responses to be expected in humans and safe starting doses for the next phase of development. Many vaccine candidates never progress past this stage as they fail to induce the desired immune response.

Subsequent clinical studies aim to turn the outcomes of pre-clinical research into safe and effective products that will protect individuals and benefit public health. This stage is divided into 3 phases that test increasingly larger groups of people to assess the efficacy of the vaccine in the general (or target) population and to investigate possible rare side effects. Even once a vaccine is successfully developed, its assessment continues: post-licensure monitoring is in place to observe potential safety issues in the long term, as well as to ensure consistency in the quality during product manufacturing. At this stage, animal experiments are used to evaluate the potency and safety of newly produced batches.

The road to a vaccine is a highly regulated, costly and (in non-pandemic times) long endeavor (Figure 3). Between the initial investigation of possible antigens in basic research stages and the release of a vaccine to the market, an operation costing 150-450 million € and lasting – on average – more than 15 years takes place [65]. Scientific advances, such as those in immunology and systems biology, can reduce the time required to a certain extent. However, vaccine development remains a lengthy process, largely due to the steady increase of regulatory requirements for vaccines and to the challenging aspects of assessing and manufacturing complex formulations with multiple components [66].

![Figure 3. The vaccine development process.](image)

Several vaccine candidates are identified in pre-clinical research, but as they move along the clinical trials their number diminishes. The length of each phase is shown, together with the probability of a vaccine candidate to advance into the next stage. Adapted from GlaxoSmithKline and updated from Wong et al. [67].
Animal tests are used, in the design and development of new vaccines, for analyzing the type and extent of the elicited immune responses, as well as for determining safe and effective routes and doses of administration [68]. Several readouts can be used as indicators of infection in challenged animals, such as pathogen recovery, clinical symptoms, morbidity and death. Moreover, the quality and magnitude of the induced immune response in immunized animals can be studied by evaluating antibody production, T cell responses and expression of relevant biomarkers identified during the basic research stage.

Animal testing is also heavily employed during the production of already existing vaccines, with batch release safety and potency being the two main aspects requiring in vivo studies [69]. Safety testing ensures the lack of toxicity and the absence of extraneous agents that could induce adverse effects, whereas efficacy (assessed on the target species) or potency (evaluated based on an animal model) assays are used to determine the protective capacity of the vaccine. International guidelines (such as regulations from the European Medicine Agency and the Food and Drug Administration, Pharmacopoeia monographs and WHO documents) indicate the requirements vaccines need to meet in such tests. Generally, safety tests assess clinical manifestations of toxicity and/or contamination through injection of animals, followed by the observation of changes in weight, body temperature, or of signs of illness or virulence.

Potency tests evaluate the immunological properties of a vaccine; this can be achieved indirectly or directly. Indirect tests, used for vaccines with neutralizing antibodies as correlate of protection, determine the potency through mixing of the sera from immunized animals with the pathogen. The mixtures are then transferred to a group of naïve animals, in which lethality, cytopathogenesis or clinical signs are assessed to estimate the potency. Indirect potency tests are frequently employed for veterinary vaccines, such as Clostridium toxoid vaccines [70]. For direct tests, a traditional immunization is performed followed by an (often lethal) challenge: the number of animals that survives is used to estimate the potency. This design is applied for widely used inactivated human vaccines, such as the Bordetella pertussis vaccine or the Tick-Borne Encephalitis vaccine [71,72].

In vivo testing is therefore a well-established and often mandatory practice in R&D, preclinical studies and routine monitoring of vaccines. In a 2019 report, it was estimated that about 10 million animals were used each year for scientific research across the 28 EU member states [73], 15% of which accounted for vaccine assessment in an earlier study [74]. However, the disputed relevance of animal models, the ethical issues and the economic burden associated with animal testing are increasingly highlighting the need to develop and validate in vitro assays to possibly replace in vivo tests.
The scientific relevance of animal models, among which mice are paramount, is questioned for several reasons:

- **The animals present immunological and genetic differences with humans.** Well-known discrepancies between mice and humans include, among others, the ratio of leukocyte subsets, the expression of antimicrobial peptides and pattern recognition receptors, and the differentiation and polarization of T helper cells in adaptive immunity [75]. Even among primates, differences in the mounting of an innate immune response were found to be species-specific – particularly for viral response genes [76].

- **The animal models are not always natural hosts to human pathogens.** To circumvent this problem, transgenic models expressing receptors that allow the animals to be susceptible are sometimes developed. Alternatively, the pathogens are adapted to the animals, which are often more resistant than humans. Yet, such manipulations could increase the distance from the model to the real infection or immunization setting [77]. Even when a natural pathogen exists or the animal model is susceptible to a human microbe, the pathophysiology of the infection might differ, thus hindering extrapolation of data from animal-based studies for human-targeted vaccines [78].

- **The models are not representative of a diverse and “dirty” reality.** To allow for repeatable analyses of vaccine-specific responses, genetically homogeneous animals are kept in highly hygienic environments. These unnatural conditions influence their immune responses [79], and reduce the possibility to observe inter-individual variability [80]. Additionally, naïve animals can hardly reproduce the complexity of immune systems shaped by their immunological history such as those of humans [81].

Ethically, the use of *in vivo* tests is an issue due to the large numbers of animals often required – especially for lot release testing, mandatory for each newly produced batch. Additionally, pain and distress in animals are inherently present in lethal challenge assays or safety tests revealing the presence of residual active pathogens or of contaminants.

Last but not least, animal-based tests are cumbersome and expensive: animal housing, husbandry and handling requires specific conditions as well as personnel training. A rough estimate for the use of animals in toxicity and safety assessments suggests costs of 620 million € in Europe per year [74].
Moving away from animal testing: the 3R concept and the consistency approach

In the vaccine industry and in regulatory agencies, four main drivers warrant the use of alternatives to animal-based tests: the animals’ welfare, the economic advantage in using in vitro assays, the scientific knowledge offered by more relevant platforms and technologies, and the compliance to regulatory guidelines [82].

The 3R concept aims at addressing these issues through:

- **Refinement**: adoption of procedures that minimize the pain and distress and improve the welfare of the animals.

- **Reduction**: use of strategies that reduce the number of animals needed per experiment.

- **Replacement**: implementation of methods that exclude the need for animal experiments.

Several 3R strategies are already defined and in use in the European Pharmacopoeia and vaccine industry (Table 1).

Vaccines are complex formulations whose quality depends on a variety of factors, ranging from starting materials (such as media, cells, bacterial and viral seed lots) to equipment and manufacturing methods. Therefore, each batch produced is considered as a unique drug product, for which the quality must be verified.

The standardization and optimization of the production process, together with the implementation of Good Manufacturing Practice (GMP) and Quality Assurance (QA) procedures [83], has led to significant improvements in vaccine quality [84]. At the same time, several non-animal methods have been developed and fine-tuned for the characterization of intermediate and final vaccine products. The combination of these elements allows the design of a lot-release strategy, called consistency approach, in which animal-based tests may only be needed for the first vaccine lots; once a product profile is set up, non-animal-based assays can be used to assess the conformity of the new batches to lots of proven safety and efficacy [85–87]. The successful application of this approach relies on the availability of suitable alternatives, such as analytical or cellular assays (Table 2).
Table 1. Use of alternative methods to *in vivo* safety and potency tests for vaccines. The alternative methods are accepted ("√"), partially substitutive (e.g. alongside *in vivo* methods), in validation ("ongoing") or development. Adapted from [82].

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Type of test</th>
<th>Alternative method</th>
<th>Ph. Eur.</th>
<th>Vaccine industry</th>
</tr>
</thead>
<tbody>
<tr>
<td>All vaccines</td>
<td>Safety:</td>
<td>Omission</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td></td>
<td>• abnormal toxicity test</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• general safety test</td>
<td>Replace in vivo test with molecular methods</td>
<td></td>
<td>partial</td>
</tr>
<tr>
<td></td>
<td>• adventitious agents test</td>
<td></td>
<td></td>
<td>development</td>
</tr>
<tr>
<td>Diphtheria</td>
<td>Safety:</td>
<td>VERO-cell based method at Drug Substance stage;</td>
<td>√</td>
<td>partial</td>
</tr>
<tr>
<td></td>
<td>• specific toxicity</td>
<td>removed test at Drug Product stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pertussis</td>
<td>Safety:</td>
<td>CHO-cell based method at Drug Substance and Drug Product stage</td>
<td></td>
<td>partial</td>
</tr>
<tr>
<td></td>
<td>• specific toxicity</td>
<td></td>
<td></td>
<td>development</td>
</tr>
<tr>
<td>Oral Polio</td>
<td>Safety:</td>
<td>Switch from non-human primate to transgenic mice</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td></td>
<td>neurovirulence</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inactivated Polio</td>
<td>Safety: inactivation</td>
<td>Replacement primary monkey cells with cell line</td>
<td>√</td>
<td>partial</td>
</tr>
<tr>
<td>Inactivated Polio</td>
<td>Potency</td>
<td>Switch to <em>in vitro</em></td>
<td>√</td>
<td>partial</td>
</tr>
<tr>
<td>Inactivated Rabies</td>
<td>Potency</td>
<td>Switch to <em>in vitro</em></td>
<td>√</td>
<td>development</td>
</tr>
<tr>
<td>Inactivated Hep. A</td>
<td>Potency</td>
<td>Switch to <em>in vitro</em></td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>Inactivated Hep. B</td>
<td>Potency</td>
<td>Switch to <em>in vitro</em></td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>Inactivated H. influenzae</td>
<td>Potency</td>
<td>Switch to <em>in vitro</em></td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>Human Papilloma</td>
<td>Potency</td>
<td>Switch to <em>in vitro</em></td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>Diphtheria and Tetanus</td>
<td>Potency</td>
<td>Serology instead of lethal endpoints</td>
<td>√</td>
<td>partial</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Use single dilution assay</td>
<td>√</td>
<td>partial</td>
</tr>
</tbody>
</table>
Table 2. Examples of in vitro methods for the analysis of vaccines/antigens. The application of these tests in accordance to the consistency approach can replace, reduce or refine the use of animal-based methods. Adapted from [86].

**Physicochemical**
- Chromatography
- Electrophoresis
- Tryptic digest analysis
- Mass/fluorescence spectrometry
- Circular dichroism

**Immunoochemical**
- Biosensor analysis
- ELISA
- Immunoblotting

**In vitro immunological**
- Antibody production
- Cytokine production
- Lymphocyte activation/proliferation

Analytical methods allow the characterization and quantification of intermediate and final products [88]. Colorimetric and separation techniques (such as mass spectrometry, electrophoresis and chromatography) are used to measure the concentration of polysaccharides, proteins and nucleic acids. Immunochemical assays, such as ELISA and immunoblotting, can assess the presence, conformation and characteristics of the antigens. A limitation of many of these methods is that adjuvants present in the formulations can cause interference. In these cases, the analysis of adjuvanted vaccines requires a desorption step in which the adjuvant is removed, a process that can diminish the quantity or quality of the antigen [89].

Cellular methods include in vitro assays that can determine the immunological and adverse properties of a vaccine or antigen – without the use of animals [90]. These assays fall in two categories: (i) those assessing the activation of an innate immune response – through PRR stimulation, cytokine production or expression of costimulatory molecules – and (ii) those evaluating the induction of an adaptive response – through evaluation of antibody production or of T cell functionality. Naturally, the complexity of the immune systems in an organism can hardly be replicated. However, the use of several in vitro modules allows us to mimic parts of the immune response and study their interaction following stimulation with the vaccine [90–97].
**Viral vaccine models**

In this thesis, we explored the applicability of *in vitro* immunochemical techniques and cell-based immunological assays for the assessment of viral vaccines in the development, production and batch release phases, using Tick-Borne Encephalitis vaccine and whole inactivated influenza vaccine as models.

**Tick-Borne Encephalitis**

Tick-borne encephalitis (TBE) is the most important tick-transmitted neurological disease in Central and Eastern European countries and in Russia. TBE has a biphasic course: first, an influenza-like stage arises, usually followed by an asymptomatic period. In 33% of the cases, the disease progresses due to the infection of the central nervous system, which can lead to long-term sequelae or even death [98–100]. TBE is caused by the tick borne encephalitis virus (TBEV), belonging to the *Flaviviridae* family and transmitted to humans predominantly by bites from infected ticks [101]. TBEV is an enveloped virus with 3 structural proteins, of which two are surface proteins: the large envelope protein E and the small membrane protein M. Inside the viral envelope is the nucleocapsid, which includes the capsid protein C and the viral genome, consisting of a single-stranded positive-sense RNA molecule of about 11 kb [102] (Figure 4).

In Europe and Asia, the 3 subtypes of the virus are reported to cause about 13,000 human cases of TBE annually, although this number is probably underestimated [103]. No specific antiviral treatment is available to date, and active immunization is the most effective way to prevent the disease. In Europe, two vaccines are available: FSME-IMMUN (Pfizer) and Encepur (GlaxoSmithKline). They both contain an inactivated European subtype of TBEV: the Neudörfl strain for the FSME-IMMUN and the K23 strain for Encepur. Both vaccines confer cross-protection, preventing not only the disease by the European subtype but also by the other subtypes. The production method is the same for both vaccines: the virus is grown on primary chicken embryo fibroblasts, purified by centrifugation and ultrafiltration and inactivated by formaldehyde. The final formulation contains the adjuvant aluminum hydroxide [104]. For a complete vaccination, three doses are required. The TBE vaccine is an example of successful empirical vaccine design: its immunogenicity is proven by the near eradication of the disease in countries where mass vaccination was implemented [105,106]; yet, besides the induction of a humoral response, relatively little is known about how the vaccine interacts with the immune system [107]. This is in part due to the fact that the sensing of the live virus itself is poorly understood, with unclear involvement of the different PRRs [108,109].
Currently, lot-release testing for the TBE vaccine requires extensive use of animal-based assays, in particular of lethal challenge tests: 4-5 dilutions of each new vaccine batch are prepared and administered to groups of mice (minimum 6 animals per group), which are then challenged. The animals are observed for 3 weeks, and the number of mice that die in the period between 7 and 21 days after the challenge is recorded to calculate the potency of the vaccine; this must fall within a range defined by a reference batch of proven effectiveness. Even for the determination of the challenge dose, a large number of animals (at least 4 groups of 10 mice) is required [71]. The implementation of the consistency approach, involving several in vitro assays – including cell-based tests – would comply with the 3R concepts of replacement or reduction of animal-based testing. To do so, however, it is necessary to understand the interaction of the vaccine with cells of the innate immune system.

![Figure 4. Schematic structure of the tick-borne encephalitis virus.](image)

**Figure 4. Schematic structure of the tick-borne encephalitis virus.** The main antigen consists of the envelope protein, which is involved in receptor binding (to candidate receptors laminin-binding protein and αVβ3 integrin), fusion with endosomal membranes and entry, resulting in the release of the genomic RNA into the cytosol [110,111]. The viral genome is proposed to trigger the activation of several PRRs [109]. Image created with BioRender.

**Influenza**

Influenza virus is responsible for acute respiratory epidemics, resulting in 290,000 to 650,000 deaths annually worldwide, and occasional pandemics, associated with high mortality rates [112,113]. The virus belongs to the Orthomyxoviridae family, and presents a lipid envelope and a genome consisting of 8 single-stranded, negative-sensed RNA segments (viral ribonucleoproteins, vRNPs). Transmission occurs by direct contact with infected secretions or contaminated surfaces, or inhalation of aerosols. The virus replicates in the upper respiratory tract, with epithelial cells being the main targets [114].
Influenza viruses are divided in types A, B, C, and D; only A and B types infect humans, and the influenza A virus accounts for the majority of infections and the severest disease. Type A is furthermore classifiable in several subtypes, characterized by different combinations of the principal antigens, hemagglutinin (HA) and neuraminidase (NA), two membrane-bound glycoproteins [115]. The high mutation frequency of the virus accounts for the need of yearly vaccinations.

Currently, influenza vaccine is available in two forms: an inactivated preparation and a live-attenuated influenza vaccine [116]. Several types of inactivated vaccines are produced: whole virus vaccines, split virus vaccines and subunit vaccines (Figure 5). Whole inactivated virus vaccines consist of virus culture subsequently inactivated by chemical agents or physical treatments. In split vaccines the virus has been disrupted by a detergent, while in subunit vaccines HA and NA have been further purified by removal of other viral components. Live-attenuated influenza vaccines, instead, are based on temperature-sensitive variant strains [117].

**Figure 5. Influenza vaccine formulations.** Influenza vaccines currently licensed are predominantly inactivated (whole, split or subunit) or live attenuated [118] (see text for detail). Image created with BioRender.
Contrary to TBEV, influenza virus has been studied for more than a century [119], and its interaction with the innate and adaptive immune system has been extensively investigated – resulting in tens of thousands of research articles, compared to (only) hundreds for TBEV. Yet again, some of the processes involved in influenza vaccine development and production are based on common practices instead of scientifically based analyses. Such is the case for a crucial step in influenza vaccine preparation, the chemical inactivation of virus particles: a systematic characterization of inactivation-induced modifications to the immunochemistry of the vaccine is yet to be presented. International guidelines specify the limits of concentration of the inactivating agents to be used, but leave the optimization of important parameters (whose effect on the inactivation process is unclear) to the manufacturers [120–122]. Analytical and cell-based assays could provide insights for influenza vaccine development and production, yielding more informative results than animal-based data alone.

**Outline of this thesis**

In this introduction, the main themes of this thesis were presented: the understanding of the vaccine-host interaction and its exploitation for the study, development and production of safer and more potent vaccines – without the use of animals.

In the first three experimental chapters, we studied the responses of the human immune system to TBE vaccine formulations, whose mechanisms of interaction with the host remain unclear.

In **Chapter 2**, we characterized the responses of primary human immune cells to adjuvanted and non-adjuvanted TBE vaccine formulations. Furthermore, we assessed the cell subsets responsible for vaccine sensing and the components of the TBE vaccine required to induce the observed responses.

In **Chapter 3**, we applied the knowledge gained in Chapter 2 on the interaction between the TBE vaccine and human immune cells to develop a cell-based assay that could be used in a panel of *in vitro* methods to replace *in vivo* potency testing in a quality control setting. To do so, we first aimed to establish a suitable cellular platform, for which primary immune cells and cells derived from an immune cell line were considered and compared in their responses to the vaccine formulations. Secondly, we evaluated potential biomarkers for their ability to distinguish between high- and low-quality batches. Finally, the robustness and sensitivity of the assay were tested.
In **Chapter 4**, we used the optimized cellular platform described in Chapter 3 to further analyze the TBE vaccine-innate immune system interaction, with the objective of identifying molecular pathways and pattern recognition receptors involved in the sensing of the vaccine. Furthermore, to define components the integrity of which should be retained upon inactivation to preserve the immunogenicity of the preparation, we compared the innate responses to the replicating and the inactivated virus.

In **Chapter 5**, we compared the properties of replicating and inactivated influenza virus *in vitro* to optimize the inactivation procedure for vaccine production. In this study, we assessed the effect of different inactivation methods on several influenza virus strains by characterizing the immunochemical properties of the viral particles.

Lastly, in **Chapter 6** the findings of this thesis are summarized and discussed in the context of vaccine development and implementation of the 3R approach, with the perspective of delivering improved vaccines and reducing the need of animal-based tests.
References


Introduction


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