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Review title: Rodents as pre-clinical models for predicting vaccine performance in humans

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Abstract

Vaccines represent a key building block for establishing a successful and sustainable control strategy against infectious diseases. Vaccine development often depends on the availability of correlates for protection and reliable animal models for the screening, selection and prioritization of potential vaccine candidates. This is performed according to their immunogenicity, efficacy and safety profiles in pre-clinical studies, which are also critical for identification of candidate antigens, selection of an optimal delivery system and design of appropriate vaccine formulations. Thus, pre-clinical studies in animal models are a prerequisite for addressing crucial issues and generating a solid pre-clinical package for the approval of clinical trials. This review addresses the strengths, limitations and perspectives of rodents as a vaccine development and pre-clinical validation tool.

Keywords: animal studies, *in vivo* testing, rodent models, pre-clinical validation, vaccine development

Introduction

Vaccination represents the most cost-efficient tool to prevent life-threatening infectious diseases. The present review provides an overview on the use of rodents in biomedical research, focusing on the advantages, disadvantages and limitations of the existing models with respect to vaccine development against human infectious diseases and future perspectives.

Vaccines act by mimicking infections, thereby stimulating the immune system to build up defense mechanisms, which in turn protect the host against disease upon contact with the targeted pathogen. Although vaccination is thought to be quite a new achievement, evidence exists that empiric active immunization was already employed by the Chinese as early as in the 10th century, when smallpox inoculation was practiced to protect individuals against the disease [1, 2]. This approach, variolation, was practiced in Africa and Turkey as well, before it was introduced in Europe and North and South America [3]. However, variolation was controversial as it not only triggered protective immunity, but also killed some individuals and contributed to smallpox outbreaks [4]. Only Edward Jenner's innovation in 1796, using cowpox material to promote immunity to smallpox, resulted in the propagation of vaccination due to the increased vaccine safety, and finally in the eradication of smallpox in 1980. Following its introduction, Jenner's method underwent considerable medical and technological improvements and vaccines against infectious diseases such as rabies, cholera, typhoid fever, diphtheria, tuberculosis and more recently influenza, poliomyelitis, meningitis caused by *Haemophilus influenza* type b or *Neisseria meningitidis*, and hepatitis B have been developed [5]. In all these cases, animal models helped to identify pathogens and to develop efficient vaccines. For example, in the 1940s, Enders *et al*. brought evidence using monkeys and mice that polio is caused by an infective agent [6]. Based on this knowledge, Sabin developed in the 1950s the first polio vaccine for mass

vaccination campaigns [7]. Worldwide implementation of polio vaccines almost led to disease eradication. However, the Nigerian boycott of the polio vaccination campaign in 2003 brought a re-emergence of this disease, which was again declared an international health emergency by the WHO in 2014 [8, 9]. Without animal research common childhood diseases such as whooping cough, measles, mumps, and rubella would not have been combated that efficiently. Nevertheless, despite these achievements existing vaccines still need to be optimized and continuously adapted to changing pathogen antigens. Furthermore, because of increasing antibiotic resistance, for example against *Mycobacterium* spp. and *Staphylococcus* spp., and the emergence or re-emergence of other diseases, such as AIDS (acquired immune deficiency syndrome), pandemic influenza, dengue or malaria, the search for improved and new vaccines is indispensable [10, 11]. Currently, vaccine development comprises years of discovery, drawbacks and optimization, whereupon only very few vaccine candidates progress to the market [12, 13]. For example, the transition probabilities of prophylactic vaccine candidates advancing through the pre-clinical into clinical phase 1, phase 2, phase 3 until licensure are around 0.48, 0.74, 0.58 and 0.61 respectively [14]. However, without animal research the success rate would be even lower and the progression of a candidate into the market would take much longer. This explains why in cases in which no appropriate animal models were available (*e.g.* hepatitis C virus, measles, respiratory syncytial virus) the development of interventions to control disease has been delayed. Hence, pre-clinical development is sustained by *in vitro* assays followed by *in vivo* studies using animal models. Within this phase of vaccine development, relevant antigens are identified, vaccine formulations are created, vaccine immunogenicity, efficacy and safety are evaluated and, finally, vaccine candidates are manufactured according to good manufacturing practice (GMP) standards. Subsequently, vaccine candidates can be tested in humans. It is therefore critical to develop tools and strategies encompassing robust and reliable animal models with high predictive value for

humans. This allows early pre-clinical screening of vaccine candidates in order to select those with the best chances of success, thereby accelerating the process and reducing costs during vaccine development.

Impact of animal models in vaccine development

Despite ethical and financial concerns that result in ongoing efforts aimed at incorporating *in vitro* and *in silico* models during pre-clinical development, cell culture and computer models cannot mimic the *in vivo* complexity of an intact immune system embedded in the host matrix. The host immune system consists of a well-coordinated and regulated functional network of many different cell types, which is governed by a myriad of long and short-range cell signaling interactions. According to the current knowledge, there is also a tight integration of the immune system with the endocrine and central nervous systems, thereby generating a true immune-neuro-endocrine synapsis [15, 16]. Therefore, animal models are used when intricate problems, which are far more complex than the sum of their parts, need to be addressed. Thus, complete replacement of animal models by alternative methods is not possible yet. The importance of animal models in biomedical research is strongly underscored by the fact that a considerable number of medical breakthroughs were based on animal studies [17]. Nobel prizes have been awarded, for example, to research addressing the development, control and activation of innate and adaptive immunity, and the underlying mechanism of the cross-talk between viral infected cells and immune cells [18, 19]. The findings of the principle for production of monoclonal antibodies represented a milestone in medical research [20]. These discoveries were mainly based on animal research and opened new avenues for the development of preventive and therapeutic measures against infections and other diseases[19]. In this context, pre-clinical animal models have also been instrumental for the development of vaccines against a number of lethal diseases. The microbiologist Robert Koch demonstrated for the first time the central role of animals for human vaccine design. Beside the isolation of microorganisms in culture, he postulated that a pure culture transferred to a suitable animal model should result in typical clinical signs of the disease. As a result, the development of human vaccines made great progress when appropriate

animal models were available. Several vaccines against diseases such as diphtheria, whopping cough, meningitis, influenza and cervical cancer have been developed using different animal models (Table 1) [21-31]. The development of the vaccine against diphtheria, a respiratory disease earlier with a mortality rate of about 40% that mainly affected young children, exemplifies the crucial contribution of animal research. Already by the end of the 19th century, guinea pigs, but also rabbits, mice and rats contributed to the isolation of the *Corynebacterium diphtheria*, the production of the exotoxin, and the evaluation of the antitoxic potential. Consequently, only a few years later von Behring developed the first vaccine using guinea pigs, monkeys and donkeys [32]. In contrast, the development of vaccines against diseases which cannot be mimicked easily by using animal models is still challenging (*e.g.* HCV). The establishment of an animal infection model that allows the characterization of the disease-causing pathogen represents a first important step in vaccine development. Thus, the use of suitable animal models will facilitate vaccine design. Using such models, it is possible to address the identification of the route of infection, pathogen targeted organs, incubation time, form of disease progression, and virulence factors. Furthermore, first hints concerning the immunogenicity and safety profiles of the selected vaccination strategy can be obtained. For example, subunit vaccines are often characterized by a good safety profile but decreased immunogenicity, making necessary the implementation of adjuvants to enhance or modulate the obtained immune response. In this regard, several vaccine adjuvants, such as agonists of pattern recognition receptors, cationic polysaccharides, ceramides or cytokines which were identified and characterized using animal models were transferred into the clinical development pipeline [33]. The same is true for novel antigen delivery systems which are already approved for humans (*e.g.* virosomes, viral like particles, liposomes) or are currently tested in clinical trials (*e.g.* nanoparticles, ISCOM) [34, 35].

Animal models also assisted to develop new application strategies, such as transdermal and mucosal vaccination [34, 36].

Thus, the knowledge gained through animal models provides the basis for developing new vaccines. However, ethical constraints render necessary a reduction in the number of animals needed, while improving scientific accuracy. In this regard, an appropriate study design constitutes a major factor allowing a decrease in animal numbers. Selecting the appropriate animal model and including proper controls and readout systems will result in robust data, even with modest animal numbers. For example, for transdermal vaccination the pig model represents a valuable tool, since porcine skin is more similar to human skin than the skin of mice or rats [37]. On the other hand, when investigating the potential of cytokines as vaccine adjuvants, one needs to consider functional differences between animals and humans. For example, in humans both Th1 and Th2 cells are able to produce IL-10, whereas in mice IL-10 is considered to be only a Th2 cytokine [38]. Diverse chemokines, which are crucial for the migration of immune cells into different tissues, have been identified in humans but not in mice and vice versa [39]. Technological developments also offer chances in terms of refinement. An example is that the use of multiplex readouts (*e.g.* multiparametric flow cytometry, magnetic bead–based assays) offers the possibility to collect a wealth of information about the stimulated immunological processes in different cell subpopulations. Furthermore, the implementation of advanced *in vivo* imaging systems allows a more accurate testing of vaccines, while at the same time reducing the required number of animals and their suffering [18]. Amongst other advantages, they offer the possibility to monitor the bio-distribution of vaccine components or pathogens following challenge, thereby providing critical information about vaccine safety and efficacy [40, 41].

Strengths and limitations of mouse models for the development of human vaccines

Rodents make up approximately 90% of the animals used in medical research and represent the main species for addressing numerous aspects of vaccine research and development, as well as regulatory guidelines. Among rodents, mice are the species primarily used (~60%) for pre-clinical research [42]. Mice are small in size, easy to handle, generated at relatively low costs, and can produce many offspring in a short time. Mice also represent one of the few available systems which are amenable for high-throughput antigen discovery (*e.g.* reverse vaccinology approaches) [43, 44]. The well-defined genotype and controlled environment in which they are breed allow a precise monitoring of the health conditions and restricts the variability of experiments to a minimum. Also, the short life span of mice enables studies covering the complete life cycle and renders them ideal to study age-related issues, such as vaccine efficacy in the elderly. Beside these qualities, the similarities between mice and human are even more important. The mouse and the human genomes display an exact consensus in 80% of the genes, whereas less than 1% shows no homology [45]. In addition, mice are susceptible to a number of human pathogens or there are homologous infectious agents to those causing disease in humans. Also, forward genetic approaches identifying the genotype responsible for the observed phenotype have resulted in crucial findings relevant for the understanding of human diseases [46]. Furthermore, the murine immune system operates quite similarly to the human one. Particularly in terms of innate and adaptive responses, which lead to the generation of humoral and cellular immunity. This renders mice a suitable model to study the immunological responses that need to be assessed in the process of vaccine development (Box 2). This encompasses (i) identification of promising candidate antigens, (ii) assessment of efficient vaccine formulations including adjuvants and delivery systems, and (iii) evaluation of the optimal delivery route. The investigation of these issues is not only of interest for research and development, but is also required by the legislative authority. The so called “proof of principle” is often a prerequisite

needed before potential vaccine candidates can be administered to larger mammals, such as primates and finally to humans. It mainly assesses the immunogenicity of a promising candidate, including the analysis of (i) humoral immune responses (*e.g*. level of antibody production, immunoglobulin [Ig] class switching, biological activity of antibodies), (ii) cellular immune responses (*e.g*. T helper cell characterization, cytokine production, stimulation of cytotoxic T lymphocytes), (iii) duration of the immune responses (*e.g*. memory), and, if possible, (iv) protective efficacy after challenge with the respective pathogen.

The vaccine against *Neisseria meningitidis* serogroup B, which was recently approved by the European Medicines Agency (Bexsero®, GlaxoSmithKline (GSK)), is an example of a vaccine that has been developed by using mouse models. More specifically, potential antigen candidates identified by reverse vaccinology were tested in three different mouse models for their capability to induce bactericidal antibodies [26]. A combination of antigens resulted in the generation of antibodies that had excellent bactericidal activity against three strains of *N. meningitidis* and was found to be effective against almost all strains, especially the most lethal ones. Rats treated with serum containing antibodies from vaccinated mice displayed protection against *N. meningitidis* infection [47]. The outcome of these studies in turn paved the road toward further clinical development. Other examples are the studies aimed at enhancing or modulating immune responses to already approved vaccines by implementing new adjuvants. For example, the first approved hepatitis B vaccine (Engerix®, GSK), which was adjuvanted with alum, has been continuously improved by different combinations of adjuvants. Mouse studies demonstrated the superiority of a new adjuvant formulation combining alum and monophosphoryl lipid A (MPL-A), as compared to alum alone, thereby providing the basis for the recently approved hepatitis B vaccine Fendrix® (GSK) [48]. However, species specific differences have to be considered when targeting TLR-mediated immune responses. For example, mouse and human cells differ in terms

of TLR-9 expression, rendering mice not always the most appropriate model for the assessment of TLR-9 based strategies [49, 50]. Mice also represent a good experimental model to address possible mechanisms of protection [51-54]. Accordingly, cell culture-derived rotavirus vaccines (RotaTeq®, Rotarix®, GSK) have been extensively studied in an adult mouse model permissive for rotavirus infection. These challenge studies revealed not only a protective mechanism due to neutralizing antibodies, but also highlighted the contribution of rotavirus specific CD4 and CD8 T cells [55].

However, it is important to be aware that mouse models also exhibit limitations (Box 2). The murine cytomegalovirus (MCMV) model constitutes a very efficient animal model for the study of human CMV (HCMV) pathogenesis and immunity. It enabled understanding several aspects of viral latency, reactivation and immune evasion, as well as to determine the need of both $CD8^+$ T cells and antibody responses for vaccine-mediated protection. However, in contrast to HCMV, the MCMV is unable to infect the fetus by transplacental route. Thus, vaccine development aimed at preventing congenital HCMV infection is still challenging [56]. The use of mouse specific and/or adapted pathogens to study closely related human pathogens is quite common. For example, the use of rodent-plasmodium species (*Plasmodium yoelii*, *P. chabaudi* and *P. berghei*) advanced the development of malaria vaccines targeting the sporozoite and liver-stages of the parasite [59, 60]. These vaccines are currently being tested in clinical trials. However, promising malaria vaccine candidates that act at the blood-stage infection and displayed protection in mice failed in clinical trials due to low efficacy, thereby stressing the translational bottlenecks of using mouse specific pathogens [60, 61].

To prevent and treat diseases it is indispensable to understand the underlying immunological mechanisms, both at steady state and under pathogenic conditions. A detailed knowledge of the effector function of the gene products involved in adaptive immune responses against pathogens

is thus a prerequisite for the establishment of an efficient immune intervention. Recent advances in gene technology have enabled the generation of a number of mouse strains in which the gene of interest has been either inserted or removed, even in well-defined cell subsets, or in which the gene of interest can be expressed at will in a conditional manner [57, 58]. For example, the insertion of human genes enables mice to develop human diseases that do not naturally affect them and thus, were previously difficult to study [59]. These advances, for instance, have resulted in experimental mouse models that offer unique opportunities to study hepatitis B virus (HBV) pathogenesis, as well as prophylactic and therapeutic strategies [60]. Transgenic mice that contain the HBV genome persistently express HBV antigens or produce infectious virions. These mice are utilized for studying HBV pathogenesis and to address the efficacy of anti-HBV drugs [61-63]. However, the constitutive expression of HBV antigen leading to central tolerance, together with the continuous production of virions hampering the monitoring of viral clearance render these models inappropriate for the development of vaccines. A new alternative approach is based on infection with a recombinant adenoviral vector carrying the HBV genome, which in turn results in HBV viremia which are detectable for more than 30 weeks [64]. This model resembles the clinical progression of HBV infection and it is therefore suitable to evaluate potential vaccine candidates able to break HBV-induced immune tolerance. Immunization studies in this model suggested the therapeutic potential of a vaccine containing the TLR-9 agonist CpG as adjuvant. A subsequent clinical trial of a conventional hepatitis B vaccine combined with CpG confirmed the potential of CpG as adjuvant, thereby highlighting the intrinsic value of this new mouse model [65]. Nevertheless, also these transgenic mouse models have significant limitations, since the full HBV life cycle does not take place and no liver inflammation can be observed [63]. Thus, a chronic infection in animal models might be different from the clinical course observed in humans, rendering difficult the translation of results obtained in animals into humans.

Despite similarities in the genomes of mice and humans, their development in different ecological niches and under exposure to different pathogens can result in distinct evolutionary adaptations of their immune systems [66]. The pathogen driven development of diverse major histocompatibility complexes (MHC) represents one prominent example [67, 68]. Therefore, the possibility to mimic human antigen processing and presentation in a mouse model seems critical for the development of human vaccines. Thus, efforts have been focused on the establishment of human leukocyte antigen (HLA) transgenic mice, which express the human equivalent of murine MHC. For example, the HHD mice represent the basis for the third generation of HLA transgenic mice, being negative for murine H-2 class I and II molecules, but transgenic for human HLA like HLA-A*0201and HLA-DP4 [69]. HLA transgenic mice are mainly utilized to evaluate peptide-based vaccine approaches where T cell epitopes are administered combined with an immune-stimulating adjuvant [70, 71]. Due to the human HLA expression, pathogen-derived peptides relevant for humans can be tested for their immunogenicity, including the potential to efficiently prime cytotoxic T cells. This in turn allows the selection of the most promising peptides to be included in the vaccine formulation. Furthermore, with HLA transgenic mice the immune monitoring can be performed applying tools used in human trials. Beside transgenic animals, also immunodeficient mice occurring either naturally or generated by specific gene knock-outs, represent suitable models. Thus, they are often used to study the role of specific immune system components during infections [72].

Another important aspect of animal research in the field of vaccine development is the evaluation of potential toxic properties of new vaccine candidates [73]. Monitoring the health status including temperature, weight, appearance, behavior, biochemical and metabolic parameters in mice allows not only to identify potential safety concerns, but also helps to determine the optimal

doses which need to be given to other animal species and later to volunteers in human trials. However, toxicology studies need to be performed also in other animal models, such as rabbits, pigs, ferrets or non-human primates, mimicking best the human situation in terms of dosage (effects might be dependent on formulation) or route of administration (specific devices might be needed).

As mentioned above, several tools, protocols and immunological reagents exist that enable an in-depth characterization of the murine immune response induced by a vaccine candidate. However, due to several biological features and technical circumstances, direct translation of the obtained pre-clinical knowledge to humans remains quite challenging. Different housing conditions (*e.g.* specific pathogen free, germ-free) render it sometimes difficult to generate comparable data. Aspects such as the potential contribution of the human microbiome to the elicited response cannot be easily addressed, but this is a common problem for all pre-clinical models. The excellent health status of laboratory animals generates to some extend a distorted image about the real situation, since vaccinations are performed in naïve mice free of other pathologies. For example, the impact of previous antigenic challenges (*e.g.* past infections, vaccinations) or co-morbidities (*e.g.* chronic infections, non-communicable diseases) on the final outcome of the immunization are not routinely assessed. However, murine systems represent a versatile tool that would enable the assessment of such parameters under extremely well-controlled conditions. Another aspect that has to be considered is the genetic background of the mouse model used, which is of biological relevance for data interpretation. Inbred strains represent genetic individual clones, which render the generated data more reliable and reproducible. Indeed, this does not reflect the true situation and variability in human outbred populations. One should be aware that inbred strains can change their natural susceptibility to specific infectious agents, which can lead

to distorted results. Furthermore, various inbred strains reveal strong differences with respect to their immune cell distribution that also has influence on the elicited immune response after infection or vaccination [74]. These drawbacks can be overcome by performing complementary studies using the available outbred mice. Although outbred strains mimic the situation in humans more closely, data are more difficult to interpret. However, this increased variability for the readouts can be addressed by using a greater number of animals for the subsequent analysis.

Moreover, some pathogens that are relevant for human diseases, such as the human immunodeficiency virus (HIV), Epstein-Barr virus, and different hepatitis viruses, do not infect rodents. This hampers studying the biology of such human pathogens, as well as the analysis of immune responses to the specific pathogen and the possibility of performing an evaluation of vaccine efficacy post challenge. These aspects are in turn crucial for the development of efficient vaccines. Therefore, several approaches are under investigation aimed at improving translation of pre-clinical data to humans. One such approach is based on the use of humanized mice, which allow investigating interactions between human cells and pathogens in a physiological intact environment.

Humanized mice

Immense efforts have been undertaken to generate improved systems that allow studying *in vivo* interactions between human pathogens and human immune cells. To this end, mice have been developed harboring a human immune system, thereby enabling the investigation of human immune cell differentiation as well as the study of pathogens infecting human leukocytes [75-77]. In brief, human hematopoietic stem cells (HSC) that subsequently differentiate into human immune cells are transplanted into mice lacking the adaptive immune system. However, also these advanced mouse models are still suboptimal for the evaluation of human vaccines. One

bottleneck is that the T cell education takes place in the murine thymus. Thus, T cells are more primed to recognize antigens presented in the murine MHC context rather than in the HLA system. This leads not only to impaired cellular responses, but also to inefficient antibody production. To overcome this problem, human HLA class I and/or class II transgenes have been introduced allowing the education of human T cells in the context of the human HLA, which results in the improved development of human T cells harboring a HLA-specific T cell receptor repertoire [78]. Other research groups have generated humanized mice by transplanting HSC together with human liver and thymus tissue, the so-called BLT (bone marrow-liver-thymus) mice. Human tissues are transplanted under the kidney capsule of adult mice, resulting in a better HLA-specific T cell response [79]. These mice are mainly used in HIV research and for studying virus-specific human immune responses [80, 81]. However, they are susceptible to thymic lymphoma (average lifespan ~8.5 months) and are therefore not suitable for long-term studies. Furthermore, the generation of BLT mice is a two-step operation that requires surgical skills and good quality tissues (*e.g*. thymus can be preserved for a limited time span), rendering the routine usage difficult.

All humanized mouse models depend on the availability of human HCS from different sources (*e.g*. fetal tissue, cord blood, G-CSF mobilized human CD34+ cells from peripheral blood), which limits their potential use for a broader research community. An alternative approach is based on the engraftment of adult immunodeficient mice with easily obtainable human mature peripheral blood leukocytes (PBL). This model can be used to monitor vaccine induced immune responses or to study human hematotropic pathogens and their impact on the human immune system [82, 83]. However, due to the lack of *de novo* hematopoiesis, primary immune responses cannot be assessed. Furthermore, engraftment with PBL is associated with the development of a severe graft versus host reaction within 4-6 weeks, allowing only short-term studies [84].

A common disadvantage of all above described humanized mouse strains is the lack of functional lymph nodes associated with an impaired formation of the germinal center. This is accompanied by incomplete isotype switching and affinity maturation of humoral immune responses, which are essential for the generation of efficient immune responses. This in turn limits their use for the evaluation of vaccine efficacy against infectious agents. Residual murine innate immunity and the in general low human T cell number represent further drawbacks. However, recent studies showed that following HSC transplantation the adoptive transfer of self-differentiated myeloid-derived lentivirus-induced dendritic cells co-expressing human IFN-α and GM-CSF resulted in partial regeneration of lymph nodes and induction of HCMV-specific IgG responses [85]. In addition, even models with suboptimal lymph node formation can be efficiently exploited for translational purposes, to study for example the effector functions of adjuvants, delivery systems or vaccine formulations on human cells from the innate and adaptive immune system [86-88].

For some human pathogens, vaccine efficacy studies not only need mouse models harboring human immune cells but also human tissue due to host-specific restriction. For example, hepatitis C virus (HCV) infections represent a major global health challenge, but the development of vaccines is hampered by the fact that this virus infects only human and primate hepatocytes. Thus, a great amount of effort has been invested in the generation of mice harboring both a human immune system and human liver cells to enable the study of host immune responses, immunopathogenesis, as well as prophylactic and therapeutic approaches. In this regard, a humanized mouse model was described that is based on the Balb/c *Rag2*$^{-/-}$ *IL-2Rγc*$^{-/-}$ mouse strain. The insertion of the FKBP-caspase 8 gene under the control of the albumin promoter resulted in a liver-specific transgenic mouse model with inducible liver suicidal activity, the so called AFC8 mouse. This humanized mouse model allows the co-engraftment of human immune and liver cells, thereby enabling the study of both hepatitis virus-induced liver diseases and virus-specific

immune responses. Therefore, the AFC8 mouse represents a promising model to assess immune interventions against HCV [89, 90]. However, the model is limited by the low infection level with HCV that hinders detecting the virus in sera. An alternative approach was fostered by A. Ploss and co-workers. They developed a mouse model based on fully immunocompetent inbred mice that express human factors required to render mouse cells permissive to HCV entry. The corresponding factors were provided by adenoviral expression, thereby generating an animal platform suitable for combined immunization and challenge studies. In fact, blocking HCV infection by passive immunization, as well as stimulation of humoral partial immunity against heterologous challenge have been demonstrated in this model [59, 91]. Another humanized liver mouse model (TK-NOG) has been demonstrated to be of great benefit for pre-clinical toxicology studies [92]. Nevertheless, a combination of human hepatocytes or hepatocytes expressing human HCV receptors from a transgene with functional human immune cells in a mouse model would be still highly desirable.

Therefore, several ongoing attempts focus on the improvement of the hematopoiesis of human immune cells and their interaction with the murine stroma. For this, human transgenes not only for HLA but also for different cytokines and growth factors have been introduced into different mouse strains [93-95]. It can be expected that this continuous incremental progress will result in the generation of a variety of different humanized mouse models suitable for addressing diverse scientific questions, such as the pre-clinical evaluation of human vaccine candidates and immune therapies.

Further rodent animal models used for human vaccine development

Although a number of vaccine-related issues can already be addressed by using the variety of available mouse models, in some cases other rodent models represent an additional or even better

alternative. The inclusion of more than one animal model in pre-clinical investigation of vaccine candidates further reduces the risk of vaccine failure during clinical development. After mice, rats are the second most commonly used animal species in medical research (20%) [42]. Rats possess numerous characteristics that make them a good model next to mice. Similar to mice they are commercially available and most of them are well characterized, possess a genetic uniformity (complete genome sequenced). Even if, compared to mice, the housing and handling of rats requires more attention. Further, rats are far less transgenic rat models available due to obstacles in their generation [96]. This might change with the implementation of new emerging technologies, such as the use of CRISPR-Cas-System [97, 98]. Nevertheless, rats still represent an important animal model to study human diseases, as well as to assess vaccine safety and efficacy [99].

Another rodent used for vaccine development is the cotton rat, as it is susceptible to a number of human pathogens, such as respiratory viruses, herpes simplex viruses, and hanta virus [100-103]. For example, it has been demonstrated that cotton rats immunized with an inactivated trivalent influenza vaccine exhibit homologous but not heterologous protection against challenge with the influenza A viruses of the vaccine [104]. Therefore, the cotton rat represents a suitable model to address influenza-specific questions which cannot be properly addressed in mice. In this regard, likewise ferrets show clinical symptoms that resemble more closely the human symptoms than mice. Influenza virus can also be more easily transmitted in ferrets than in mice, showing symptoms after infection with most human isolates [105]. Nevertheless, it is clear that all potential animal models for influenza have strengths and limitations [106], and that an optimal strategy should be based on their wise combination during pre-clinical development. In this regard, as compared to mice, the availability of reagents for cotton rats and ferrets is more limited. Also, costs are much higher which often results in small numbers per group and thus

does not allow obtaining large data sets with sufficient statistical power. This hampers the broader use of cotton rats and ferrets. Improved access to tools that will enable a detailed characterization of the induced immune responses may render cotton rats and ferrets promising tools to assess new vaccine candidates against different pathogens.

Further rodent species have fruitfully contributed towards the development of human vaccines. Already the German scientist Robert Koch used guinea pigs when he discovered that tuberculosis is caused by *Mycobacterium tuberculosis*. The susceptibility to tuberculosis and other infections and the similarities of its immune defense system to that of humans makes the guinea pig an important tool to study infectious diseases [107]. The guinea pig also played a crucial role in the development of vaccines against tuberculosis and diphtheria. Nowadays, they are mostly used to develop vaccines against anthrax and to target the multidrug-resistant *M. tuberculosis*. The Sereny test performed using guinea pigs was originally envisioned as a test to assess for invasiveness of enteropathogenic bacteria, such as *Escherichia coli*, *Listeria monocytogenes* and *Shigella* spp. [108-110]. However, it was also broadly used to test vaccine candidates, particularly against *Shigella* spp. [111, 112]. Furthermore, like mice and rats, guinea pigs are used to evaluate the toxicity of vaccine candidates. The longer gestation period renders guinea pigs a good model to perform safety tests in the course of pregnancy as well [113]. Finally, tissue and organs derived from guinea pigs are often used to study medical drugs [114]. Nevertheless, despite being a good model, guinea pigs are being replaced more and more by mice and rats. Today, they find an application in less than 1% of the *in vivo* studies performed in medical research [103].

Beside the above mentioned rodents, approximately 0.6% of the animals used in research are hamsters, and even less, gerbils. The most frequently used hamster in biomedical research is the Syrian golden hamster. Commercially available golden hamsters are the progeny of a few littermates collected in Syria in 1930, whereas the Mongolian gerbil is used in research since

1800 [42, 115]. As compared to mice and rats, both hamsters and gerbils do not display the wide range of spontaneous and latent diseases. However, due to their good health conditions as well as the susceptibility to induced disease conditions, they represent useful animal models to study vaccine efficacy and safety as well as infectious diseases [116, 117].

In addition to the traditional rodent laboratory models, some non-traditional rodent models are also common for specific applications. For example, the woodchuck is used to study hepatitis and hepatocellular carcinomas [118, 119]. The deer mouse, a natural host of the hanta virus, is useful to study the hanta viral pulmonary syndrome, and additionally represents a useful model for toxicological studies [120]. However, the lack of commercially available reagents and tools hampers their broader use in the field of vaccine development. In fact, some effort has been made with respect to the generation and establishment of tools to assess a wide range of immune responses at least for guinea pigs and hamsters. Nevertheless, as compared to tools available for mice, it is just a drop in the bucket.

Ethical aspects and safety concerns

The ethical aspects of using animal models for the pre-clinical evaluation of human vaccines are always controversially discussed. In general, animal studies require the official agreement of the local government and should only be accepted if (i) no alternatives exist, (ii) the suffering of animals is reduced to a best possible minimum and (iii) the benefit for humans morally justifies the use of animals. In this context, the principles of the three Rs (replacement, reduction, refinement) provide a rational approach to reduce the number of research animals (Box 1).

Researchers performing animal experiments are aware of the moral concerns such as animal rights, the causing of pain, and the reduction of life quality or time to the animals involved. However, more efforts need to be undertaken that precisely point out the necessity of animal

experiments. Common statements that claim animal studies are crucial to demonstrate vaccine efficacy and safety present only the half-truth and open the door for counter-arguments like that the benefit of animal experiments has not been proven for humans. Indeed, the use of animals cannot completely predict the outcome for humans, but animal studies help to decide whether it is safe and worth to test a vaccine candidate in humans. Thus, vaccine candidates that do not display the required safety profile and/or have not been proven to be efficient will never be assessed in clinical trials. This is often not taken into account by activists against animal experimentation, when they claim that candidate products considered safe according to pre-clinical studies can also lead to adverse events. In fact, a dramatic increase in the percentage of dropout candidates during clinical testing is likely, both in terms of efficacy and safety, if pre-clinical studies in animal models would be banned.

Beside the ethical concerns also safety issues of animal models have been raised. Thus, the generation of humanized mice that can be infected with pathogens that are not naturally pathogenic in mice might lead to pathogen adaptation, resulting in a new strain with a broader host spectrum. These thoughts are understandable, and scientists need to be prepared to address potential questions and to inform the public about why the use of animals represents a need for the development of human vaccines, whether an attached risk is real or just a matter of perception, and which mechanisms are in place to make negligible the potential impact of animal experimentation for the community. It also needs to be emphasized that the benefits outweigh potential risks.

Expert commentary

In the last centuries, the great achievements made in the battle against infectious diseases have illustrated the success story of vaccines. Nowadays, we have a longer life expectancy and a

higher quality of life than ever before. Undoubtedly, this would not have been possible without animal research, which played a pivotal role in the dissection of the pathogenesis processes underlying infectious diseases, as well as in the development of effective vaccines.

However, animal experiments have their limitations with respect to translation of the emerging knowledge into humans. Thus, it is critical to know the potential and limitations of each model to extract maximal information, minimizing the risk of being misled. In this regard, it is of paramount importance to be aware that "mice do not tell lies", but scientists should understand the strengths and limitations of murine models, and learn which questions can be properly addressed in each experimental system. In addition, several issues should be considered to maximize the output while minimizing the number of animals needed. For example, the use of laboratory inbred or genetically modified strains can result in responses that are strain-specific. In contrast, laboratory outbred animals (rodents and non-rodents) are in general more suitable to generate responses that mimic population variation, but limited tools and reagents might restrict the wealth of extracted information. On the other hand, associated costs or ethical concerns might represent a logistic bottleneck. To facilitate translation from animal models to clinical application, the model should mimic the anatomy and physiology of the human organs and tissues of interest, and recapitulate the morphological and biological aspects of the pathogenesis process as much as possible.

Taken together, although animal studies have limitations, they still remain a cornerstone for the development of vaccines. Further efforts need to be taken to generate improved and further refined models that have a high predictive value for humans, thereby increasing the translational meaningfulness of the gained knowledge (*e.g.* further development of models based on humanized mice). This is also expected to result in a reduction in the number of animals needed. The latter could be further supported by the establishment of alternative methods, such as

advanced *in vitro* or *in silico* approaches. In this regard, academic and industrial researchers should always keep in mind the concept of alternatives defined by the three Rs of animal models.

Five-year view

Great progress has been achieved in the evaluation of human vaccines by using rodents. However, the translation of pre-clinical data to humans aimed at validating the efficacy and safety of vaccines is still suboptimal and requires further refinement. Several new aspects emerged that might be beneficial to render the translation process more effective and reliable. One issue that impedes the translation of data is that today most *in vivo* experiments are performed under specific pathogen free conditions, a highly controlled environment. Results obtained under these conditions show less intra- and inter-laboratory variability. However, important biological facts are neglected, such as the importance of the microbial flora in the outcome of immune responses. For example, depending on the composition, microbiota can potentiate anti-inflammatory and immune regulatory processes or favor pro-inflammatory responses [121]. Wendy S. Garrett and co-workers reviewed host–microbiota interactions in different animal models showing the regulation of host genetic programs by the microbiome and the resulting influence on host immune defense [122]. These findings are further supported by studies showing that the intestinal microbiome not only affects gastrointestinal immunity, but also immune regulation at distal mucosal sites [123]. Furthermore, recent experimental studies suggest that the gut microbiome could have a considerable impact on host responsiveness to certain vaccines [124]. Taken together, advanced investigations of the complex host–microbiome interactions have to be done in order to develop optimal experimental conditions for more efficient translation of immunological data obtained using any animal model.

Another critical issue is that in general *in vivo* experiments are mainly performed with young or middle aged healthy rodents, thereby neglecting the impact of co-morbidities and the influence of aging in humans, which dramatically affect vaccine efficacy [125]. There is still a paucity of knowledge on the entity and exact causes of the functional alterations observed in the immune system of aging individuals. However, the dissection of the molecular and mechanistic events involved is a prerequisite for establishing effective intervention strategies for the elderly. Thus, a further aspect that needs to be systematically addressed by animal models is the impact of aging in the outcome of vaccination. Frequent co-morbidities that can also exhibit a crucial impact in vaccine efficacy, such as metabolic dysfunctions and chronic infections, can also be modeled in a cost-effective manner in rodents. For example, *in vivo* experiments using mice predisposed to metabolic dysfunction or affected by chronic infections (*e.g*. MCMV) would allow predicting for vaccine efficacy in high risk groups.

To complement this, systems biology-driven approaches comparing data generated in animal models and humans are also needed [126]. In this regard, it is important to understand how to utilize these models in order to access reliable information, which enables making accurate predictions. Interestingly, a recent study suggested that the murine model was not appropriate to evaluate parameters relevant for inflammatory diseases in humans, due to poor correlation between mouse and human transcriptomic profiles [127]. However, a subsequent study making use of the same dataset, but focusing on genes whose expression levels were significantly changed in both species, suggested that results in mouse models indeed mimic human inflammatory responses at the genomic level [128].

Animal studies not only enable the detailed phenotypic and functional characterization of initiated immune responses, but also allow addressing vaccine efficacy based on appropriate challenge models. Thus, immune parameters that might be critical for achieving protective

immunity can be investigated in detail in animal models. This in turn could provide initial hints on potential surrogate markers to be implemented in future clinical trials. Such biomarkers are indeed critical to increase the predictive value of pre-clinical and clinical studies. They will also contribute towards the identification of correlates for protection, which represent a true challenge for the development of human vaccines. The availability of reliable markers for responsiveness to vaccination (efficacy and safety) would also enable identification of those individuals who will maximally benefit from a particular vaccination, thereby reducing development costs and maximizing later the impact of vaccine implementation in the field.

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