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EDITORIAL
Exploitation of immunological approaches for the quality testing of human vaccines to phase out the use of animals

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In Europe, the legislation on animals used in biomedical and regulatory research found an important milestone on the Council Directive 86/609/CEE of June 1986 (unfortunately implemented nationally only in 1992) and the more recent text of the Directive 2010/63/EU, which represents a very significant step forward in the protection of animals used in scientific procedures. A nodal issue in those progressive legislative efforts was aimed at reducing the total number of vertebrate nonhuman subjects (and from 2010 of cephalopod molluscs) in experimental studies, while substituting the use animal testing with alternative solutions and procedures.

Indeed, in addition to the use of animal models in fundamental and translational research, it has been estimated that more than 10 million animals per year are used for the development, production and quality assurance of biologicals, namely products that are produced from living organisms or contain components of living organisms, including vaccines, blood components, cells, allergens, tissues, and recombinant proteins [1]. Several concerns have been, thus, raised on a more ethical use of animals employed for scientific purposes leading to different actions worldwide. In this context, National Control Laboratories (NCLs), World Health Organization, European Directorate for the Quality of Medicines and vaccine manufacturers are highly committed in the development of alternative *in vitro* methods complying with the 3Rs Principle, introduced by Russell and Burch in 1959 [2]. Notably, the 3Rs refer to:

a. replacement of the use of animal with *in vitro* technologies or approaches;
b. reduction of animal numbers through appropriate experimental design and statistical evaluation;
c. refinement of husbandry and experimental conditions to minimize animal pain and distress.

In this context, the Istituto Superiore di Sanità (ISS, Italian National Institute of Health) since 1986 has been playing a pivotal role in controlling and monitoring animal experimentation at the national level. Moreover, ISS personnel has been and is constantly involved in educational efforts aiming at fostering the implementation of the 3Rs Principle both within and outside the biomedical community also at international level. Moreover, ISS researchers have been committed in establishing and employing innovative methodological approaches and experimental models alternative to animals, thus becoming a reference institution especially in biomedical research and regulatory activities. As a non-exhaustive example, within the collaborative framework of a European project (VAC2VAC,https://www.imi.europa.eu/projects-results/project-factsheets/vac2vac), ISS was actively involved in the optimization of tests used for the evaluation of pyrogens, fever inducing molecules whose presence or contamination in medicinal products for parenteral use results in unwanted symptoms and noxious side-effects, as fever, myalgia, headache, fatigue, and soreness at the injection site. In particular, the Monocyte Activation Test (MAT, present in the European Pharmacopoeia (Ph. Eur.) as chapter 2.6.30) was applied, as an alternative to the conventional rabbit pyrogen test (RPT, Ph. Eur. 2.6.8), for the pyrogenicity testing of human vaccines against bacterial and viral infections [3, 4]. At variance of RPT, relying on the administration of a product in the rabbit ear vein followed by rectal temperature level measurement to assess any sign of fever, the MAT is carried out with human primary immune cells obtained from blood donation, which

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are fully equipped to recognize endotoxin and non-endotoxin pyrogens, thus assuring a high-sensitive and species-specific test. For instance, even if the reaction to endotoxins is similar between rabbits and humans, the response to non-endotoxin pyrogens is stronger in humans than rabbits [5].

The successful replacement of the RPT with the MAT for several human vaccines boosted the scientific discussion on the applicability of MAT also for the testing of other biopharmaceuticals [4], thus accomplishing a reduced use of rabbits. This research activity, pioneeringly conducted at ISS, is in line with the vision of the European Pharmacopoeia (Ph. Eur.) Commission to phase out RPT from the Ph. Eur. within 2026 [6] and endorsed ISS as one of the few European NCLs authorized to conduct this not animal test for regulatory purposes. Last but not least, the experience and know-how acquired on this matter allowed the appointment of a delegation of ISS researchers as Italian representatives to the panel of expert for the Ph. Eur. BET (bacterial endotoxin test) working party, involved in the development and modernization of analytical methods for bacterial endotoxin and pyrogen testing as part of the quality control of medicinal products.

Under this umbrella, the BET is currently under discussion at the Ph. Eur. since it is performed with the Limulus Amebocyte Lysate obtained from horseshoe crabs. Indeed, blood sampling required to produce BET reagents causes an estimated dead rate of roughly 150,000 animals per year and exposes the natural *Limulus* population to a high risk of extinction. To accomplish 3Rs principle, a new test for evaluation of endotoxin content is now available and included in the Ph. Eur. (2.6.32), namely the recombinant Factor C test (rFC) and whose reagents are completely synthetic thus, avoiding procedures carried out in *Limulus*.

Moving from the fruitful MAT experience, ISS researchers decided to utilize human primary immune cells to test the immunogenicity of vaccine for human use. Thus, the immunological competences and know-how were exploited for setting a new *in vitro* cell-based model to predict or test vaccine immunogenic potential as alternative non animal method. Indeed, for most of human vaccines, potency test is still conducted through *in vivo* immunization of small laboratory animals followed by lethal challenge with toxin/virus/bacteria or titration of specific antibody in immune sera. Nevertheless, in addition to ethical reasons, animal-based methods are costly, exhibit high inherent variability, poor robustness and limited functional relevance given the physiological inter-species differences in immune responses. Besides causing significant pain, suffering and distress to many sentient animals (i.e., potency testing of viral vaccines requires roughly 80 mice per batch) and showing potency variations of up to 300%, the phylogenetic distance between laboratory animals and humans may limit the predictive value of such *in vivo* potency tests [7]. Considering these differences, the wide portfolio of molecules expressed by pathogens – the so-called pathogen associated molecular patterns (PAMPs), which are included in vaccines as subunit or as component of the inactivated or attenuated version of the pathogen – as well as the exploitation of novel vaccine adjuvants have raised concerns about the reliable applicability of animal-based assays for the testing of the present and forthcoming vaccine formulations.

Interesting data have been recently generated in the context of human peripheral blood mononuclear cells (PBMC) stimulated *in vitro* with the inactivated viral vaccine against the tick-borne encephalitis virus [8] shedding light on the possibility to use the innate immune signature driven by the type I Interferon (IFN) as read-out to monitor vaccine potency or as new correlates of vaccine protection. Being inspired by system vaccinology data revealing the fundamental mechanisms by which the immune system orchestrates protective responses to vaccination, the immunological power of the main immune cells present in blood was analyzed and exploited to facilitate a successful implementation of *in vitro* testing alternatives. By using a selected panel of type I IFN stimulated factors as biomarkers, the high sensitivity of an *in vitro* PBMC model to discriminate between conforming and non-conforming drug substance batches of an anti-TBEV vaccine was demonstrated [8].

Understanding the mechanism through which vaccines interact with the immune system can indeed offer several intriguing cues that could be translated into the quality control testing of vaccines such as the identification of critical microbial components that needs to be retained during the manufacturing process. In addition, the identification of key cellular pathways engaged by the interaction of pathogen structures with the blood immune cells might support the design and/or the selection of potent immunogenic vaccine candidates during the research and development phase.

In conclusion, human PBMC-based assays are promising and malleable experimental setting that can be successfully implemented as *in vitro* testing alternatives given their intrinsic and dynamic biological properties ranging from the capacity to sense all PAMPs, to predict the immunostimulatory potentials as well as possible bias related to inflammatory nature of vaccine formulations.

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Conflict of interest statement

The Authors declare that there are no conflicts of interest.

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