ImmuLiver: an immunologically-competent liver model to assess attenuation of yellow fever vaccines

Overall aim

The aim of this Challenge is to develop an *in vitro* model of the human liver that is metabolically and immunologically-competent for the routine assessment of Yellow Fever (YF) vaccine attenuation.

The vaccine is based on a live-attenuated virus derived from a pathogenic strain of YF virus and is currently assessed in non-human primates (NHPs), specifically cynomolgus macaques. Existing *in vitro* liver models are not fit for viral testing in a pharmaceutical context due to a lack of immune competency. A non-exhaustible or easily renewable source of cells, with a characterised origin is required. The model must be compatible with automated distribution and multiple sample analysis, as well as the constraints linked to manipulations of pathogenic agents (Biosafety Levels 2 and 3 (BSL2, BSL3)).

Duration

Phase 1: six months, Phase 2: Up to three years

Budget

Phase 1: Up to £100k, Phase 2: Up to £1 million

Sponsor(s)

Sanofi Pasteur EU

Background

Yellow fever is a mosquito-borne flavivirus disease affecting human populations in tropical areas of South America and Africa. The virus maintains an enzootic cycle in NHPs and is periodically re-introduced to humans through infected NHPs living nearby (Fernandes NCCA *et al.,* 2017, Moreno ES *et al.,* 2013). This means the global eradication of Yellow Fever virus (YFV) infection is not possible and it remains a major public health concern. YFV infection can cause severe illness characterised by the impairment of liver function, leading to acute hepatitis, severe hemorrhage and if it proceeds to infect the central nervous system, death (Monath TP, 2001, Monath TP *et al.,* 2015). YFV-infected cynomolgus macaques fully recapitulate the pathophysiology of human YF disease, and were central in the development of the first YFV vaccine strain, YF17D (Frierson JG, 2010), obtained by the prolonged cultivation of the wild type YFV-Asibi strain.

For the development and evaluation of vaccines, current regulatory guidelines (WHO Annex 5, 2013) recommend that appropriate safety characterisation studies should be conducted using the YF17D vaccines as a comparator. Due to the lack of suitable small animal models able to mimic YF pathophysiology, much weight is currently placed on cynomolgus macaque neurovirulence studies for vaccine safety evaluation (Levenbook IS *et al*.,1987). A typical safety test is described in “Recommendations to assure the quality, safety and efficacy of live attenuated yellow fever vaccines” (WHO Annex 5, 2013).

In this assay, neurovirulence, immunogenicity and viscerotropism are evaluated in the same macaques (n=10) through innoculation with a test dose in the frontal lobe followed by clinical observation, sampling and post-mortem histology. Neurotropism can also be evaluated through peripheral innoculation.

There is strong industry interest in replacing macaques in YFV vaccine attenuation assessment.

- Neurovirulence/ neurotropism: a recent pilot study conducted by Sanofi Pasteur, with wild type and attenuated YFV vaccine has shown that *in vitro* blood/brain barrier (BBB) mini-brains constitute a promising alternative to macaques for neurovirulence testing with the additional ability to detect neurotropism (da Costa A *et al*., 2018).

- Immunogenicity: studies can be performed in small rodent animal models and when combined with the product of this Challenge and the *in vitro* BBB assay developed by Sanofi, could lead to the replacement of studies in macaques.

- Viscerotropism: is currently assayed by measuring by blood viraemia in the innoculated macaques. An *in vitro* model, as outlined in this Challenge, has the potential to replace this assay.

An *in vitro* model of viscerotropism

The development of an immune competent *in vitro* liver model for use in the viscerotropic assessment of YF vaccine products has the potential, when combined with the *in vitro* neurovirulence model, to replace the use of macaques in safety testing of these products. The use of small animal models for assessment of viscerotropism is limited by their poor predictivity. For example, type I Interferon (IFN)-deficient mice succumb to infection with wild type virus and survive infection with the vaccine, but are immuno-compromised, and hamsters can develop viscerotropic symptoms and high levels of virus in the liver, but only when infected with a hamster-adapted virus strain (Julander JG, 2016). These have limited predictive value for viscerotropism and a human relevant *in vitro* model would have significant potential to replace the use of macaques.

Both the 17D vaccine and wild type (Asibi) viruses can productively infect various liver cells *in vitro* and elicit similar replication levels, making them indistinguishable by replication when used in the same model. However, Sanofi have recently demonstrated that human primary hepatocytes may be a valuable *in vitro* model to investigate innate and metabolic disorders elicited by YFV infection. The study was conducted in a commercial human liver organotypic model (InSphero™) based on primary hepatocytes. Using a transcriptomics screen, type III IFN was identified as a potential biomarker to differentiate between wild type and vaccine YFV. This observation is consistent with other reports describing the critical role of type III IFN in clearance of hepatic infections, like hepatitis C, *in vitro* and *in vivo*. Other potential biomarkers have been identified, suggesting that such an *in vitro* model could constitute an alternative to the macaques viscerotropic assay to predict YF liver disease (Masse-Deragon N *et al*., 2016, thesis work and manuscript in preparation).

A functional liver model that can recapitulate as much as possible the complexity of the organ, is needed to assess viscerotropism and replace macaques in the testing of new vaccine products. Besides its major role in metabolism, the liver is also a critical component of defence against blood-borne infections (Crispe IN, 2009). It contains both conventional and unconventional populations of resident antigen-presenting cells (APCs), like Kupffer cells, liver sinusoidal endothelial cells and hepatic stellate cells, which can support T cell activation. The presence of such cells in a model of liver infection is critical. 3D cell culture has been demonstrated to recreate the *in vivo* microenvironment and physiological relevance of tissues (Mehta G *et al.,* 2012). A number of multicellular liver models are commercially available but have not been proven to be suitable for routine assessment of YFV vaccines. The principal limitations are:

- A lack of well-defined models: there is no definition for “normal” functioning for a liver immune competent model, and a lack of biological markers to assess this status.

- A limited supply of primary cells makes them incompatible with routine testing for the lifetime of the vaccine (usually >30 years) and they are usually isolated from donors with a medical history of liver pathology (diabetes, cancer, alcohol abuse). The impact of the donor status may impact the outcome of an infection study.

- Culture medium formulations and extracellular matrix (ECM) components are not optimised for viral infection (e.g. interference with receptor fixation, reduced virus half-time, viral particle dissociation).

- Design of the models does not always permit BSL2 safety containment of the pathogen (protection of the operator and the environment, safe elimination of liquid and solid wastes).

- Insufficient assay throughput and limited number of readouts for assay validation.

3Rs benefits

All currently manufactured YF vaccines are based on two subtypes of the YF17D attenuated strain. WHO current guidelines recommend that attenuation in macaques is assessed for each new manufactured seed lot and for any new YFV attenuated strain (WHO Annex 5, 2013). A typical assay is described in Moulin *et al* (Moulin JC *et al.,* 2013). Depending on the manufacturing capacity, this assay is repeated two to four times a year. For each new working seed lot, at least two groups of ten cynomolgus macaques (both sexes), one test group and one reference group, are anesthasised and inoculated with viral suspension intracerebrally into the frontal lobe. Macaques are observed for 30 days and records of clinical observations (ranging from not eating to limb paralysis and death) are obtained according to WHO recommended scores.

With growing evidence that both the neurovirulence assay will be replaced by an *in vitro* assay in the future (da Costa A *et al*., 2018), there is an urgent need to find a substitute to the macaque viscerotropism test to enable replacement of this invasive and terminal procedure. A robust, biologically-relevant *in vitro* assay for vaccine attenuation assessment would provide a strong case to convince health authorities to remove the macaque assay from the YF vaccine guidelines. As an example, the US and Canadian Health Authorities (FDA and BGTD, respectively) have recently approved the replacement of the inactivated polivirus (IPV) inactivation test in Primary Monkey Kidney Cells (PMKC), by an *in vitro* assay in a mouse cell line genetically engineered to express human poliovirus receptor, CD155, based on a robust validated assay (da Costa A *et al*., 2018).

There are many forms of liver infection, classified according to the category of the infecting agent as viral (e.g. cytomegalovirus, HIV and herpes simplex), bacterial (e.g. acute bacterial hepatitis, bacterial liver abscesses and granulomatous liver disease) or parasitic (e.g. plasmodium P.vivax, liver flukes (trematoda) and tapeworms (cestoda)). Implementation in industrial and academic settings, of assays developed through this Challenge could be a viable and improved alternative to the current animal models used for studying these various hepatic pathogenes as well as for the development of vaccines and antivirals.

Need for collaboration

To solve this Challenge expertise in the following areas will be required including, but not limited to:

- Liver immunology and physiology (for selection and characterisation of appropriate cell types, identification of markers etc).

- *In vitro* cell biology.

- Tissue engineering.

- iPS cells, hepatic cell differentiation and/or cell lines.

- Cell banking.

Key deliverables

Phase 1 deliverables

- A list and evidence of production, of the cells used in the model with their characteristics, and a rationale for their inclusion.
    - The model should use hepatic cells derived from iPS cells. Primary cells will not be suitable for the reasons mentioned earlier in this document.
    - Hepatocytes, Kupffer cells and endothelial cells are a minimum requirement to mimic the basic liver unit (the liver sinusoid).
    - Stellate cells may also be considered although their role in YFV infection has not been extensively studied.
    - The model should mimic lymphocyte infiltration following liver infection with wild type YFV. Autologous responses between cells from different donors should be minimal due to the presence of Kupffer cells, known to be responsible for liver tolerance.
- Supportive data showing equivalence with primary human cells, alone or in co-culture for 12-15 days, minimum. This duration is based on the maximal duration of an infection experiment (eight to ten days) plus a margin of +/- two days to be able to choose the day of infection, plus an additional margin of two to three days to ensure that the physiological parameters of non-infected cells are maintained along the infection period and to avoid mis-interpretations. Assays based on stimulation of metabolic or immune functions will be appreciated.
- Evidence of the ability of the model to support YF virion production.
- A set of technical tools to routinely identify the cell populations and to monitor the evolution of their functionality over time. Quantitative assays are preferred.
- A list of specific markers and pathways of importance with regard to viral infection.
- Robust plans to deliver Phase 2 of the Challenge including commercialisation and dissemination.

Phase 2 deliverables

- It is possible that all the cells types needed will not be fully tested at the end of Phase I, and that additional work will be needed. For example, if an engineered-cell line is envisaged. Cell banks will need to be available before starting Phase 2.
- The compatibility of the cell culture conditions with virus infection should be evaluated. This could be done by using the YF17D-204 attenuated strain (available at the ATCC), to infect hepatocytes, which are known to replicate the virus. Infection readouts will include infectious titration assay and/or genomic quantification, and will have to be performed on different lots of cells. End points should include, but are not limited to: virus genome quantitation in the cell supernatant and the cellular fraction, cytokines dosage in the cell supernatant (protein dosage), quantification of the cell population producing specific mRNA/protein, transmigration through the endothelial barrier (quantitative, %), hepatic metabolism deregulations (CYP450 dosage, BSA, enzymes). The expected deliverable will be a statistical report summarising the different formulations tested.
- The assay design should permit the evaluation of 24 to 96 samples in parallel. The preferred format is 96-well plates, but 24 well plates are acceptable. The number of cells per well should be sufficient for protein quantification by ELISA or any sensitive equivalent method. Pooling of wells for some endpoints may be necessary.

A crucial aspect of the Challenge is to deliver a platform that is compatible with use in BSL2/3 laboratories. Key requirements include:

- Ability to be used in a confined space.
- Compliance with the constraints on types and specifications of equipment used (e.g. air and water-tight centrifuges, microscopes with a reader that does not need to be removed, etc).
- Contamination of reagents and media with viral particles (medium, extracellular matrix etc.) must be kept at minimal level.
- Design of the platform must permit safety containment of the pathogen (protection of the operator and the environment, safe elimination of liquid and solid wastes).

Equipment outside these areas could be used, but only after the samples have been decontaminated (formaldehyde, UV, heat) and proven not to be infectious. This usually takes one to three hours minimum and several days if residual infectivity has to be verified.

It is important to note that the CRACK IT Challenges competition is designed to support the development of new 3Rs technologies and approaches, which will improve business processes and/or lead to new marketable products. The application must include a plan to commercialise the results into a product or service. This should be taken into consideration when completing your application.

Sponsor in-kind contributions

The Sponsor will provide:

- Scientific expertise on YFV (and viruses in general).
- Feedback on previous internal experiments with YFV infection of microliver tissue and iPSC-derived hepatocytes.
- Infection protocols, sequences of primers for qPCR detection.
- In-house viral testing with the wild type YFV (BSL3 confinement).
- Premises: BSL2 and BSL3 laboratories are available onsite. Training will be provided.
- Equipment of interest for the work e.g. Luminex (multiplex ELISA), BioMark (gene profiling by qPCR), digital PCR, confocal microscopy, all available onsite.

References

Crispe IN (2009). The liver as a lymphoid organ. *Annu.Rev Immunol* 27:147-163.

da Costa A *et al*. (2018). Innovative in cellulo method as an alternative to *in vivo* neurovirulence test for the characterization and quality control of human live Yellow Fever virus vaccines: A pilot study. *Biologicals*.53:19-29.

Fernandes NCCA *et al.* (2017). Outbreak of Yellow Fever among Nonhuman Primates, Espirito Santo, Brazil, 2017. *Emerg Infect Dis* 23:2038-2041.

Frierson JG (2010). The yellow fever vaccine: a history. *Yale J Biol Med* 83:77-85.

Julander JG (2016). Animal models of yellow fever and their application in clinical research. *Curr Opin Virol* 18: 64-69.

Levenbook IS *et al.* (1987). The monkey safety test for neurovirulence of yellow fever vaccines: the utility of quantitative clinical evaluation and histological examination. *J Biol Stand* 15:305-313.

Masse-Deragon N *et al*. (2016). *New Insights into Yellow Fever Liver Pathogenesis Using a Liver Organotypic Model.* [Poster]. EC Environment Scientific Conference: Non-Animal Approaches-The Way Forward, 6-7 December 2016, The Egg, Brussels, Belgium.

Mehta G *et al.* (2012). Opportunities and challenges for use of tumor spheroids as models to test drug delivery and efficacy. *J Control Release* 164:192-204.

Monath TP (2001). Yellow fever: an update. *Lancet Infect Dis* 1:11-20.

Monath TP *et al*. (2015). Yellow fever. *J Clin Virol* 64:160-173.

Moreno ES *et al.* (2013). Yellow fever epizootics in non-human primates, Sao Paulo state, Brazil, 2008-2009. *Rev Inst Med Trop Sao Paulo* 55:45-50.

Moulin JC *et al.* (2013). Yellow fever vaccine: comparison of the neurovirulence of new 17D-204 Stamaril seed lots and RK 168-73 strain. *Biologicals* 41:238-246.

WHO, 2013. WHO Expert Committee on Biological Standardization. Geneva: WHO Press. Available at [pdf] Geneva, Switzerland: WHO. Available at http://www.who.int/biologicals/expert_committee/TRS_978_61st_report.pdf Annex 5 [Accessed 8 June 2018].

WHO, 2013. Recommendations to assure the quality, safety and efficacy of live attenuated yellow fever vaccines. Geneva: WHO Press. Available at http://www.who.int/biologicals/areas/vaccines/TRS_978_Annex_5.pdf Appendix 2 [Accessed 8 June 2018].