UniVax **Publishable summary at project month 18**

A "Universal" Influenza Vaccine through Synthetic, Dendritic Cell-Targeted, Self-Replicating RNA Vaccines
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Summary project context and objectives

While vaccination is the cornerstone of influenza (Flu) prophylaxis, current inactivated vaccines provide only moderate protection, requiring annual updating due to poor long-term immunity and antigenic drift of the virus. Efficacious, protective immunity requires humoral and cell-mediated defenses.
The UniVax goal is to develop the first multimeric and synthetic universal Flu vaccine based on self-replicating RNA replicons targeted to dendritic cell receptors by synthetic delivery vehicles, inducing humoral and cell-mediated immunity for broad, long-lasting protection. The project will include a clinical phase I study assessing safety, tolerability and initial immunogenicity of the vaccine.

Replicons are derived from defective virus genomes, from which at least one structural protein-encoding gene has been deleted – replicons replicate and translate, but cannot produce virus. Inherent problems with many current replicons are their cytopathic nature (reducing the duration of immune response induction), the need for virus-like particles for delivery, and their derivation from human pathogens. Moreover, they are not targeted to dendritic cells (DCs) – critical cells for immune response induction.
UniVax employs replicons (RepRNA) derived from a non-cytopathic porcine pestivirus, which is non-infectious for humans. The RNA is delivered into human DCs by synthetic means, wherein the RepRNA efficiently translates and replicates. Our current RepRNA carries insertion sites that efficiently facilitate the accommodation of Flu genes of interest for broad universal protection against Flu. Co-formulation with novel mucosal adjuvants, such as MALP-2 or c-di-AMP, potentiates robust humoral and cellular immune responses, including cytotoxic and multifunctional T cells – the latter have been related to robust protective T-lymphocyte immunity.

Description of Work and main results until month 18

Delivery and targeting

In the first period of this project one of the main goals was to generate and identify synthetic formulations of nanoparticles that are able to efficiently transport RepRNA into antigen-presenting cells.

Different biodegradable delivery systems were tested and compared for their capacity to package RepRNA and deliver this cargo in a functional manner into cells. Three main categories of delivery vehicles have been used by the partners involved in this task: (i) cationic lipids (lipoplexes), (ii) cationic lipids combined with polymers (lipopolyplexes), and (iii) chitosan-based nanoparticles.

Variations of each compound were formulated in a number of different ways to ultimately package, protect and deliver RepRNA. In a first step, promising formulations were tested with readily available nucleic acids other than RepRNA to assess the initial proof-of-concept to package and deliver nucleic acids. All resulting nanoparticles were physico-chemically characterized with regards to their shape, structure, charge, zeta-potential and stability. Secondly these particles were tested for their capacity to deliver functional nucleic acid to a relevant cell culture model. Here the expression of reporter genes (e.g. GFP, luciferase) facilitated evaluation of efficacy.

After having identified the most promising and potent formulations, these were combined with RepRNA in a variety of ways while taking into account the larger size and reduced stability of the RepRNA compared to the DNA test constructs. There was an obvious difference in designing these nanoparticles for RepRNA compared to other nucleic acid compounds related to the differing characteristics of large RNA from DNA. By employing RepRNA constructs that were also encoding reporter genes (e.g. GFP, luciferase), the efficacy of packaging, protection and delivery to cells could be monitored. Of each category of delivery vehicles promising formulations could be identified that facilitated efficient delivery of RepRNA to cells and expression of encoded genes. Development of RepRNA delivery systems by synergizing technologies leading to functional read-out has been accomplished. During this process also the first vaccine prototype of RepRNA, encoding influenza antigens, was used and analyzed. Due to some variability in the results and subsequent analysis of this effect, it became clear that rigorous quality controls are needed along the process to keep it under good control. Several SOPs were generated and circulated among the partners to ensure standardized settings and monitored quality.

As a further optimization of the nanoparticles the idea was to combine these nanoparticles with specific glycan structures that efficiently bind to dendritic cells *in vivo*, as the main antigen presenting cell, and, thus, enhance vaccine delivery specifically to these cells. From a collection of over 250 glycoconjugates the most efficient ones have been identified during several rounds of screening. Glycoconjugates were labelled with fluorescein and the binding characteristics to dendritic cells were analyzed via flow cytometry. Using PBMCs as cell pool for the analysis even allowed discriminating specific affinities of glycans to dendritic cells in comparison to other immune/blood cells; in this way another layer of specificity was added to the selection process. A selection of 10-20 very promising probes could be identified in a first screen, resulting in a selection of four probes after another round of screening to proceed with towards targeting of nanoparticles. The next step in the process will be to combine these glycans efficiently with the most promising nanoparticle technologies selected before to enhance nanoparticle delivery *in vitro* and *in vivo*.

Replicon Constructs and Adjuvants

The active component of the multimeric influenza vaccine being developed in this project consists of replicon RNA based on a pestivirus genome that has been genetically modified to not generate any progeny viral particles by deleting essential viral structural proteins. Initially existing constructs encoding reporter genes and influenza hemagglutinin (HA) were produced as starting material to test the different delivery technologies described above.

Additional constructs

In order to generate a multimeric Flu vaccine having the capacity to protect against different influenza strains at the same time, a selection of influenza antigens have to be included in this vaccine. During the first part of the project a series of new replicon prototypes encoding influenza antigen was generated via cloning procedures and subsequently quality controlled and tested for functionality. During this course the optimization of some constructs were tested as a potential concept to be translated to other constructs. For example, the expression of the influenza HA protein was tested in different conformations including or excluding the cytosolic and transmembrane domain and using codon-optimized sequences vs. normal.

After having generated a set of new constructs encoding influenza HA proteins of different strains, NA and NP proteins, these constructs were used as prototypes for testing the vaccination approach *in vitro* and *in vivo*.

The ultimate vaccine containing replicon RNA in targeted nanoparticles shall be combined with a potent adjuvant in order to assure proper immune stimulation and efficacy. For this different novel adjuvant concepts were made available by partners of the UniVax consortium. The most promising candidates were MALP-2 and cyclic-di-AMP, both having a distinct mode of action, can be manufactured synthetically and are applicable via parenteral or mucosal routes.

The adjuvants were manufactured in a preclinical scale and provided to the different partners for evaluation of different tasks of the project: (i) co-formulation of delivery technologies with adjuvants, (ii) *in vitro* efficacy of nanoparticle delivery after co-application with adjuvants, (iii) *in vitro* and *in vivo* efficacy of RepRNA delivery in the presence of adjuvants, and (iv) enhancing immunogenicity of RepRNA *in vivo*.

In vivo Testing

In order to test *in vivo* efficacy of vaccination using RepRNA, combined with different delivery technologies, DC targeting moieties and adjuvants, *in vivo* screenings in mice were set up. Several delivery platforms could show promising results after formulation with RepRNA and *in vitro* testing (see above). The next step was to translate these approaches into an *in vivo* setting and evaluate their capacity to stimulate specific immune responses. During a first *in vivo* study applying 18 different formulations delivering RepRNA into mice, four of these could be identified as promising vaccine candidates. This initial proof-of-concept was an encouraging first result and gave rise to an extended screening of many combinations of formulations with different delivery technologies and adjuvants.

To properly handle this large number of samples in an economical way and to perform these tests in a comparable manner a transgenic animal model was introduced into the project. CD8+ and CD4+ ovalbumin-specific T cells were isolated from OTI and OTII mice, respectively. Naïve TCR transgenic CD8+ and CD4+ T cells were enriched using a CD8+ T and a CD4+ T cells isolation kit, respectively, and subsequently labeled with 1 μM CFSE. Afterwards, these cells were injected in C57BL6 mice by i.v. route. Mice were vaccinated 24 h later with the different vaccine candidates and the proliferative capacity of the injected ovalbumin-specific CD4+ or CD8+ T cells was analyzed after another 5 days. The outcome of this high-throughput analysis system is the identification of novel promising vaccine candidates, such as a novel lipid emulsion, lipopolyplexes and chitosan nanoparticles co-administered with replicon and adjuvant. In ongoing studies we have used our large human biobanks collected after seasonal and pandemic vaccination to further dissect the immune response seeking universal human B and T cell epitopes. We have found that pandemic H1, H7 or H5 vaccination induces antibodies to the conserved stalk of the virus and that the stalk is a promising candidate for universal vaccines.

Expected final results and potential impact and use

Influenza is a serious public health problem affecting more than 100,000,000 people per year. Most recover from the symptoms within a week without requiring attention, but there is a high risk for severe illness (3-5 million cases) or death (250,000 to 500,000) (WHO); most deaths occur among the elderly. The most effective way to prevent the disease is vaccination, although the vaccine among the elderly reduces severe illness and complication by only up to 60%. Annual vaccination is currently recommended for pregnant women, children 6 to 59 months of age, healthcare workers with patient contact, the elderly and people with underlying chronic health conditions such as respiratory, cardiac, metabolic, neurological and immunosuppressive diseases.

The outcome of the UniVax project will be the first synthetic replicating RNA vaccine against Flu. UniVax will generate essential data on integrating innovative technologies for RepRNA, synthetic delivery, glycoconjugate-based targeting of DCs and mucosal adjuvanting. The data integrates for the first time polysaccharide and lipid technologies, providing a most powerful integrated technology for delivering functional RNA into DCs. The first ever prototype synthetic RepRNA vaccines with innovative new generation mucosal adjuvants will be evaluated pre-clinically and clinically, providing data on enhancing efficacy of vaccine delivery for breadth and duration of protection. UniVax has as a direct outcome a novel clinical technology with great benefits for population's health; this also provides tools in basic research (RNA delivery system to DC, new adjuvant, replicative RNA technologies). It will enable the widespread use of nucleic acid delivery to dendritic cells and other "immune cells" such as monocytes, macrophages, natural killer cells or even lymphocytes with the purpose of investigating cellular functions and overexpressing or silencing genes in therapeutic strategies.

Contact and further information: www.UniVax-FP7.eu