UniVax

Publishable summary at project month 54

A "Universal" Influenza Vaccine through Synthetic, Dendritic Cell-Targeted, Self-Replicating RNA Vaccines
FP7 Collaborative Project no. 601738

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Background

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 defences. 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.

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 – 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 Dendritic cells 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 c-di-AMP, potentiates robust humoral and cellular immune responses, including cytotoxic and multifunctional T cells – the latter related to robust protective T-lymphocyte immunity.

Description of Work and main results until month 54

1. Replicon Constructs

The active component of the multimeric influenza vaccine being developed in this project consists of replicon RNA (RepRNA) based on a pestivirus (CSFV) genome. Replicons are genetically modified viral genomes; they are self-amplifying (self-replicating) vaccines, incapable of generating infectious progeny. This is achieved by deleting genes encoding for essential viral structural proteins. Their translation capacity to promote this self-replication also produces the vaccine antigens from inserted genes encoding for these antigens.

The pestivirus replicon has major advantages over other replicons. Pestivirus RepRNA is non-cytopathogenic, unlike the majority of replicons in use today – primarily alphavirus or flavivirus derived – which are cytopathogenic. Moreover, pestivirus RepRNA is well supported by dendritic cells for its translation and replication. Importantly, the pestivrus RepRNA will not kill dendritic cells in which it is translating and replicating, rendering it more ideal as a vaccine for

targeting dendritic cells. The second important characteristic is that the leader protein encoded by the pestivrus genome interferes with the cell-signalling pathway leading to the interferon induction that can impair replicon function. Moreover, the pestivrus genome and replicon do not carry 5'-triphosphates, which would signal a cell's innate defences to attack the replicon. Alphaviruses do carry 5'-triphosphates, and can signal the cell innate defence mechanism.

The characteristics of the pestivirus replicon facilitate its retention in dendritic cells for prolonged periods, which fits well to the manner by which dendritic cells slowly process antigen for presenting to the adaptive immune system over a prolonged period, thus promoting robust immune defences. Initially existing constructs encoding reporter genes and influenza virus haemagglutinin (HA (H5)) were produced as starting material to test with different biodegradable, nanoparticulate delivery technologies. Additional constructs have been generated, encoding for influenza virus HA (H1), neuraminidase (NA (N1)), M protein, PB1 protein and nucleoprotein (NP).

For more details, see *Démoulins et al 2017 Self-Replicating RNA Delivery to Dendritic Cells", in "RNA Vaccines: Methods and Protocols", ed. T. Kramp & K Elbers, Chapter 5, Methods in Molecular Biology vol 1499, Springer Science+Business Media, New York.*

2. Multimeric Potential of RepRNA Replicon Constructs

As described in the previous section, RepRNA constructs encoding a series of antigens can created. In turn, these can translate to provide the means for inducing both humoral and cell-mediated arms of immune defence. Such a multimeric Flu vaccine can be formulated for delivery, with particular emphasis on interaction with dendritic cells, a major contribution of the UniVax project.

During the first part of the project, a series of new RepRNA prototypes encoding some of these Flu antigens were generated. This has been elaborated to include all the aforementioned Flu antigens. Moreover, replicons have been constructed to lack one, several or all the structural proteins of the original pestivirus sequence. The expression of the encoded Flu proteins has been assessed alongside expression of certain pestivrus proteins still encoded by the replicon. By such means the relative efficiency of translating the "gene of interest" – encoding the Flu antigen – can be compared with the endogenous genes, which are translated via different ribosomal entry sites in the 5'-NTR or inserted after the gene of interest. This allows determination of the efficiency with which the gene of interest near the 5' end of the RepRNA can be recognised and translated compared with the genes downstream of this.

In addition, this assessment of translation can determine how the mode of replicon delivery influences the ultimate interaction with the cellular ribosomal translational machinery. By such evaluation, efficacious formulations of the RepRNA with the delivery system are readily identified and selected as prototypes for testing *in vitro* and *in vivo*. Accordingly, most of the partners in UniVax have now been trained by the lead partner in replicon generation, as well as producing and quality assessing the RepRNA. These partners can then associate the RepRNA with particular delivery vehicle formulations, for assessment of the capacity to promote translation of the encoded Flu antigens. The partners having expertise with *in vivo* evaluations take the process to the next level for evaluating induction of both humoral and cell-mediated compartments of immune defence.

For more details, see the above reference of *Démoulins et al 2017.*

3. Formulation for delivery to dendritic cells

The various partners of the UniVax Consortium have assessed several different biodegradable delivery systems, and modifications therein. Assessment characterizes their capacity to package RepRNA and protect from RNase, together with delivery of the cargo to dendritic cells for promoting translation of the encoded Flu antigens or reporter genes. Selected cell lines were also employed for comparative purposes and also to facilitate rapid identification of candidates for testing in dendritic cells. This also assisted those partners unable to employ dendritic cells, thus focusing their attention on cell lines capable of providing meaningful information on the efficiency of formulation and RepRNA delivery leading to translation.

The main criteria for the delivery vehicles were biodegradability, readily tolerated by cells and host, capable of interacting efficiently with dendritic cells to promote cargo uptake, efficient at complexing RepRNA, and efficient also at promoting RepRNA release within cells to facilitate translation of the encoded Flu vaccine antigens. For this purpose, the delivery vehicle components were classified as (i) cationic lipids (lipoplexes), (ii) PEI-based polymers (polyplexes), (iii) polymers combined with lipids (lipopolyplexes), and (iv) chitosan-based complexes and nanoparticles. In addition, due to the success observed with virosome vaccines by consortium partners, a new virosome-like delivery system termed “HA-nanoparticle” has been evaluated. A large number of each compound employed in the delivery vehicle formulation has been assessed, together with modification of formulations employing variations of each compound. The main aim was to identify efficient formulation with the RepRNA encoding different genes of interest, including reporter genes and the genes encoding Flu antigens.

All biodegradable nanoparticulate delivery systems were physico-chemically characterized, and selected on their capacity to deliver functional nucleic acid, either to a cell-line model or primary dendritic cells. While many of the delivery formulations could deliver DNA or small RNA molecules, such as siRNA or mRNA, this was not necessarily indicative of efficient RepRNA delivery, probably due to the larger size of the RepRNA and therefore its more complex interaction with the delivery vehicle components. Nonetheless, in a number of cases, initial assessment with DNA or small RNA molecules could identify formulations which would not work, or formulations which might prove of value. Similarly, delivery to cell lines did not guarantee delivery to dendritic cells with the same efficiency. Nonetheless, the use of appropriate cell lines could prove of value for determining the potential for delivery to the dendritic cells. Certainly, the aim had been to assess the formulations showing potential with cell lines for delivery to primary dendritic cells. This greatly simplified the screening process, especially when new modifications or new formulations were under investigation.

For more details, see

1. Démoulins et al 2016 Polyethylenimine-based polyplex delivery of self-replicating RNA vaccines Nanomedicine 12:711-722;
2. Démoulins et al 2017 Self-Replicating RNA Delivery to Dendritic Cells”, in “RNA Vaccines: Methods and Protocols”, ed. T. Kramp & K Elbers, Chapter 5, Methods in Molecular Biology vol 1499, Springer Science+Business Media, New York;
3. Démoulins et al 2017 Self-replicating RNA vaccine functionality modulated by fine-tuning of polyplex delivery vehicle structure. J Control Release 28;266:256-271;
4. Englezou et al 2018, Self-amplifying Replicon RNA delivery to Dendritic Cells by Cationic Lipids, Molecular Therapy Nucleic Acids, in press.

4. Targeting

The nanoparticulate delivery vehicles were also modified to target ligands on dendritic cells. On the one side, this employed ligands such as hyaluronic acid, well known for targeting the CD44 found on dendritic cells and indeed many cell types. The other approach was to employ specific glycan structures that should bind to members of different families of dendritic cells, such as C-type lectins, SigLecs and galectins. The approach with hyaluronic acid proved particularly successful. There was no toxicity from these formulations, and delivery to cells proved most efficient. Translation of the delivered RepRNA was also observed, and this also proved successful *in vivo*, whereby these delivery vehicles were among the most successful for inducing immune responses (see sections 6 and 7 below).

For the glycans, it was necessary to determine their relative capacity for binding to dendritic cells compared with other mononuclear cells. Over 300 glycoconjugates were assessed on mononuclear cells obtained from porcine and human donors, and clustered into different groups dependent on their relative binding to the different cell populations present. This clustering facilitated a definition of glycan binding capacity of the different mononuclear cells, with particular emphasis on the dendritic cells. The analyses permitted the identification of species-common and species-specific glycan-binding receptors. A selection of the probes could be selected on the basis of highly efficient interaction with different human blood cell populations, in particular differentiating the degree of binding to dendritic cell subsets and monocytes. These efficiently-binding glycans have been further analysed by microscopy to define binding capacity and internalisation efficiency, as well as the endocytic route employed by the cell. The latter relates to the manner by which the cell will process the material, in turn impacting on the release of the RepRNA for translation. Certain of these probes have been modified to interact with the delivery vehicles, to assess their influence on RepRNA cargo delivery and translation efficiency.

For more details see

> Rapoport et al, 2018, “Glycan recognition by human blood mononuclear cells with an emphasis on dendritic cells”, Glycoconjugate Journal, doi.org/10.1007/s10719-017-9811

5. Adjuvants

While the characteristics of the pestivirus replicon facilitate retention by dendritic cells and therefore increase the chances for developing robust immunity, the aforementioned characteristics render it is less likely to activate the innate immune mechanisms required for maturation of the dendritic cells. Accordingly, the delivery vehicles employed with the pestivrus replicon require a potent adjuvant. In this context, adjuvants for parenteral or mucosal immunisation are being studied. The most promising candidates were MALP-2 and cyclic-di-AMP, both having a distinct mode of action. These offer the further advantage of being manufactured synthetically and applicable via parenteral or mucosal routes.

The adjuvants were formulated with a number of the delivery vehicles carrying RepRNA mentioned under section 3. These delivery vehicles carrying the RepRNA were selected in the most part from the *in vitro* evaluations (see Section 6 below). The final formulation with adjuvant was assessed by both mucosal and parenteral immunisations; this work is described under Section 7 below. Effectively, pulmonic or subcutaneous administration employed RepRNA alone or in delivery vehicle formulation, co-administered with c-di-AMP, comparing

adjuvanted with non-adjuvanted groups for induction of humoral immunity, T-lymphocyte profiling, T-lymphocyte activities, and cytokine profiling.

Additional experiments compared several different adjuvants, for enhanced induction of humoral and cellular immune responses against the RepRNA-encoded vaccine antigen. Importantly, the cyclic-di-AMP proved to be the most efficacious, by both parenteral and mucosal routes of immunisation.

For more details, see

> Ebensen et al 2017 Mucosal Administration of Cycle-Di-Nucleotide-Adjuvanted Virosomes Efficiently Induces Protection against Influenza H5N1 in Mice. Front Immunol. 28;8:1223

6. *In vitro Evaluation*

6.1. Interaction with cells.

Both primary dendritic cells (murine, porcine, and human) and cell lines relevant for either replicon translation or as models for dendritic cells were employed. Certain delivery formulations were more efficient than others at delivering the RepRNA leading to translation of the encoded genes. Neither the efficiency of associating the RepRNA with the delivery vehicle, nor the efficiency of the RepRNA delivery into the cells could be related to this translation. Certainly, clear delivery of the RepRNA was essential, but only certain formulations facilitated the apparent release of the RepRNA for translation of the encoded antigens.

For comparative purposes, DNA and small RNA molecules such as siRNA and mRNA were also employed, particularly when the partner was unable to use the RepRNA. While this proved a good model for the chitosan delivery, it was not always reliable for the other delivery vehicle formulations. As observed by partners using the UniVax RepRNA, delivery leading to translation was seen to be dependent on both the delivery formulation and the cells employed for assessment. Indeed, certain cell lines proved much less efficient for assessment of the translation. Moreover, the results demonstrated the importance for screening with primary dendritic cells.

6.2. Targeting different cells for RepRNA translation.

These results showing how different cell lines can vary considerably in their support of RepRNA delivery/translation can be related to the analyses on the glycan targeting ligands. A number of these have been selected on the basis of binding to human and porcine dendritic cells. Yet, it is now clear that the binding patterns obtained with cell lines, including cell lines supposedly related to dendritic cells, were not the same as the binding observed with the primary dendritic cells or even monocytes.

While different delivery vehicle formulations were shown capable of delivering to both dendritic cells and cell lines, this was dependent on the components of the delivery formulations. Moreover, the efficiency of delivery did not relate to efficiency at promoting translation of the delivered RepRNA. Modification of these delivery vehicle components has allowed delivery leading to increased translation efficiency of the delivered RepRNA. This was particularly notable with certain PEI-based polyplexes, newly developed PTG-lipopolyplexes, and modified chitosan-based delivery vehicles. In addition, the new virosome-like HA-nanoparticle delivery vehicle also showed *in vitro* delivery promoting translation. These new formulations were therefore selected for evaluation by mucosal (intra-pulmonary) and parenteral vaccination *in vivo* (see Section 7).

6.3. Influence of delivery vehicle complexing with the RepRNA on translation efficiency.

It was considered that the RepRNA may have been non-functional in certain complexes or compacted to a degree that would not be reversed adequately for ribosomal entry and translation of RepRNA-encoded antigens. Thus, the functionality of the RepRNA was assessed using virus replicon particles (VRP). These are constructed in complementing cell lines to create the original virus-like particles, but carrying the replicon in place of the virus genome. VRPs were efficiently delivered to cells, promoting efficient translation of the encoded antigens, including the Flu antigens. These results clearly demonstrated that the RepRNA was indeed translation- and replication-competent.

Analyses with different PEI-based formulations may add some clarity to the situation. While certain formulations were efficient at promoting translation of all encoded antigens, other formulations provided for an imbalanced translation, often favouring translation of the endogenous replicon genes with little or no read-out from the gene of interest. This would imply a differential degree of compaction along the RepRNA, either preventing ribosomal entry or inefficiently protecting from RNase.

It is now clear that careful selection of the delivery vehicle components permits efficient formulation with RepRNA to protect from RNase, promotes delivery to dendritic cells, and facilitate translation of the vaccine antigens leading to induction of immune responses. This work is continuing to determine the variability between experiments and further modify the structures to improve the translation efficiency.

For more details, see the publications referenced at the end of Section 7 below.

7. In vivo Evaluation

7.1. In vivo evaluation models

Following the *in vitro* identification of the most potentially efficacious delivery formulations delivering RepRNA for translation in dendritic cells, the prototypes were assessed by immunogenicity studies in mice and pigs. Certain delivery vehicle formulations were identified as showing promise for efficacious immunogenicity. Interestingly, this was noted with examples of each type of delivery system initially under investigation – lipoplex, polyplex, lipopolyplex and chitosan-based nanoparticle formulations.

This extensive work employed a number of *in vivo* experiments. The evaluations employed conventional immunogenicity studies with RepRNA encoding Flu antigens. In addition to vaccination of non-immune animals, pre-immune mice also employed, as well as the TCR Ova model (OTI and II) together with a RepRNA encoding the Ova antigen. Assessment using the TCR Ova model offers a means of crossing *in vitro* with *in vivo* assessment. $CD8^+$ and $CD4^+$ ovalbumin-specific T cells were isolated from OTI and OTII mice, respectively. Naïve TCR transgenic CD8+ and CD4+ T cells were enriched, labelled with CFSE, and injected intravenously into C57BL6 mice. After 24h, the animals were vaccinated with the prototype vaccine formulations, carrying RepRNA encoding for ovalbumin, selected as described under section 6; the only exception were the lipoplexes, only some of which were pre-evaluated with dendritic cells *in vitro*. The proliferative capacity of the injected ovalbumin-specific T cells was analysed after a further 5 days.

In-depth analysis assessed most aspects of immune responsiveness, with both the conventional vaccination models in mice and pigs, and the TCR Ova model. This included

antibody induction, T-lymphocyte subset induction, cytokine profiling and assessment of individual lymphocyte activities.

7.2. Evaluation of initial delivery vehicle formulations

The initial lipoplex, polyplex and lipopolyplex formulations did not promote the clear specific immune response anticipated from the *in vitro* evaluations. However, modifications of the polyplex and lipopolyplex formulations showed increased efficiency *in vitro* for delivery to dendritic cells and cell lines leading to translation of the encoded gene of interest. This included assessment of different PEI molecular weights and formulation modifications. In addition, modifications to the chitosan-based nanoparticles in terms of molecular weight and formulation were brought into the evaluations, again based on initial *in vitro* assessment (see section 6 above).

These approaches identified new polyplexes, lipopolyplexes and chitosan-based vehicles with high promise for increased translation of the delivered RepRNA. To this group were added the new virosome-like HA nanoparticles, again showing high promise from the *in vitro* evaluations in dendritic cells and cell lines. New *in vivo* assessments were employed. On the one side, this employed both murine and porcine models, while on the other side comparison was made in mice of a mucosal route (intra pulmonary) and parenteral route of injection. These experiments confirmed the capacity of the new formulations to induce immune responses against the Flu antigens encoded by the RepRNA. Particularly interesting is the efficiency of the chitosan-hyaluronic acid nanoparticles for inducing both humoral and cell-mediated immune responses. Moreover, the power of the cyclic-di-AMP, in particular as a mucosal adjuvant, has been further evidenced. Overall, this demonstrates that particular formulations will facilitate delivery of the RepRNA for translation in the manner observed with RepRNA delivery in a single shot by VRPs, although this may prove more efficacious dependent on the route of administration.

7.3. Further assessment of delivery vehicle formulations

The above experiments were extended to assess delivery vehicle formulations with RepRNA in terms of relating vehicle modifications to increased efficacy at inducing immune defences. Accordingly, lipoplex formulations – assessed with dendritic cells or with the replicon from the third party outside the consortium using cell lines – as well as polyplex, lipopolyplex, chitosan-based nanoparticle and virosome-like HA-nanoparticle formulations selected using dendritic cells and cell lines, were assessed.

A number of *in vivo* immunisations have been performed, using either mucosal delivery or parenteral delivery (sub-cutaneous or intra-muscular). These formulations displayed clear efficacy for delivery, especially after intra-pulmonary injection, resulting in clear induction of specific immune responses. These *in vivo* experiments provided clear evidence of immunogenicity. An important discovery was the particularly powerful immunomodulatory capacity of cyclic-di-AMP, both parenterally and mucosally. Moreover, these results have allowed the selection of the delivery formulations showing the greatest promise for efficacy at inducing influenza virus-specific immune responses. This evaluation will now continue with this selected delivery formulation.

For more details see

1. Démoulins et al 2016 Polyethylenimine-based polyplex delivery of self-replicating RNA vaccines Nanomedicine 12:711-722;
2. Démoulins et al 2017 Self-Replicating RNA Delivery to Dendritic Cells", in "RNA Vaccines: Methods and Protocols", ed. T. Kramp & K Elbers, Chapter 5, Methods in Molecular Biology vol 1499, Springer Science+Business Media, New York;

3. Démoulins et al 2017 Self-replicating RNA vaccine functionality modulated by fine-tuning of polyplex delivery vehicle structure. J Control Release 28;266:256-271;
4. Ebensen et al 2017 Mucosal Administration of Cycle-Di-Nucleotide-Adjuvanted Virosomes Efficiently Induces Protection against Influenza H5N1 in Mice. Front Immunol. 28;8:1223
5. Englezou et al 2018, Self-amplifying Replicon RNA delivery to Dendritic Cells by Cationic Lipids, Molecular Therapy Nucleic Acids, in press.

8. *Immunological Evaluation of Biobank material in relation to Clinical Trial*

A clinical trial at Haukeland University Hospital in Bergen, Norway was used to evaluate local and systemic immune responses after LAIV in children and adults. Clinical trial samples have been stored in a biobank of blood and saliva samples collected at different time points.

Overall, the biobank samples provide material from influenza H1, H5 and H7 infections or vaccinations, including seasonal vaccine studies. These have been employed to dissect the immune responses, in terms of human B and T cell epitopes, particularly potential universal epitopes. The HA stalk is highly conserved allowing the influenza A viruses to be divided into two groups. HA head and stalk specific antibody binding has been assessed, together with the avidity of binding and the functionality of stalk-specific antibodies using virus neutralization and antibody-dependent cellular cytotoxicity (ADCC). HA stalk-specific antibodies may have an important role in protection through neutralization and ADCC in people who respond poorly to traditional inactivated vaccines.

The data obtained to date shows that particularly young children respond well to LAIV with serological responses to H3N2 and B strains, along with local and systemic antibody secreting and memory B cell responses. The H1N1 strain did not elicit antibody responses, although T cell responses were detected in blood and tonsils. LAIV induces systemic and local T cellular responses including protection associated cross reactive T cells which may provide heterologous protection in children. This information is especially valuable to the RepRNA vaccine of UniVax, which possesses at least similar characteristics of immunogenicity to an LAIV. In particular, this concerns the ability of RepRNA to self-replicate and more closely mimic the situation with an lnfluenza virus infection, and therefore the type of immune responses induced.

For more details see

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3. Olberg H, Eide G, Cox RJ, Jul-Larsen, Å, Lartey S, Vedeler C, Myhr KM. Antibody response to seasonal influenza vaccination in multiple sclerosis patients receiving immunomodulatory therapy. European Journal of Neurology 2018 Mar;25(3):527-534
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5. Andersen TK, Zhou F, Cox R, Bogen B, Grødeland G.. A DNA Vaccine That Targets Hemagglutinin to Antigen-Presenting Cells Protects Mice against H7 Influenza December 2017 Volume 91 Issue 23 e01340-17
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immunity to influenza viruses in pre-school children living in Addis Ababa, Ethiopia. Open Forum Infect Dis. 2017 Feb 11;4(1):ofx026. doi: 10.1093/ofid/ofx026.

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11. Trieu MC, Zhou F, Lartey S, Jul-Larsen Å, Mjaaland S, Sridhar S, Cox RJ. Long-term maintenance of the influenza-specific cross-reactive memory CD4+ T-cell responses following repeated annual influenza vaccination. J Infect Dis (2017) 215 (5): 740-749.

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 to dendritic cells, glycoconjugate-based targeting of dendritic cells, and mucosal adjuvants. The first ever prototype synthetic RepRNA vaccines with innovative new generation mucosal adjuvants are being evaluated pre-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

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