ENOVA – European Network of Vaccine Adjuvants (Cost Action CA16231)

4th ENOVA Adjuvant Workshop

Hybrid event

20-22 September 2021, Genova, Italy

Meeting report

20 September 2021

Introduction to ENOVA and the 4th Adjuvant Workshop. Dennis Christensen (Staten Serum Institute, Denmark)

Dennis Christensen gave an introduction to ENOVA and program of the 4th ENOVA Adjuvant Workshop. This Workshop was planned for 23-25 March 2020, and due to COVID-19 pandemic have been moved to hybrid event which was held 20-22 September 2021. Dennis Christensen explained what impact has COVID-19 pandemic had on ENOVA activities. As a challenge in responding to pandemic diseases he emphasized why adjuvants and vaccines infrastructures are needed.

Keynote: Vaccine-controlled infective diseases and cancer burden: achievements and perspectives. Professor Alberto Izzotti, (Genova University, Italy)

Professor Izzotti made an introduction into adjuvant functions and types of vaccines, particularly cancer vaccines. Professor pointed out connection between cancer development and infection caused by Hepatitis B virus or *Helcobacter pylori* and how targeting of specific oncogenic factors of microorganisms can help to lower cancer rate. Use adjuvants to deliver artificial miRNAs into tumour cells and induce apoptosis was also covered within presentation.

Session 1: Improving existing adjuvant-based formulations

Using microcontainers for oral delivery of vaccines. Philip Hassing Ronøe Carlsen (PhD student DTU, Denmark)

Philiip Hassing discussed on disadvantages of vaccine administration by injection route (invasive method, medical personnel needed etc.). Alternative oral route, designated as non-invasive vaccination is more convenient for human and animal use and main advantage of the oral route is the activation of mucosal immune system, especially activation of immune response in intestine including Peyers patches. Philiip Hassing focused on his research based on using micro containers, little tubes that embed into the mucus and epithelium, for oral delivery of vaccines. The research includes preparation of mucoadhesive polymers as release

of the micro containers content can be targeted depending on coating, aiming to release at the start of small intestine. The system was tested in mice model and next step would include testing on larger animals (rabbits, pigs).

The adjuvant LT-K63 enhances B cell activation and survival factors expression in neonatal mice - comparative study of LT-K63 and other adjuvants on the induction of germinal center and ASC. Stefania P. Bjarnarson (University of Iceland/National University Hospital of Iceland)

Adjuvants are immune stimulating agents which enhance duration of protection induced by vaccines. The question was if safe and effective adjuvants in vaccine formulations can circumvent limited induction of the neonatal immune system. Initially, maturation status of different B cell populations was assessed in neonatal and adult mice and neonatal mice had dramatically fewer B cells than adult mice. The study showed if and how the adjuvant LT-K63 (non-toxic mutant of *Escherichia coli* heat labile enterotoxin) given with pneumococcal conjugate vaccine in neonatal mice, could affect the expression of tumour necrosis factor receptor (TNF-R) known to be involved in:

-maintenance of antibody responses,

-B cell activating factor receptor (BAFF-R)

-B cell maturation antigen (BCMA)

-and their ligands: BAFF and a proliferation inducing ligand (APRIL).

The conclusion was that LT-K63-enhanced expression of TNF receptors BAFF-R and BCMA, as well as their ligands BAFF and APRIL, are associated with persistent humoral immune responses in neonatal mice after only one immunization.

Also, comparative study of LT-K63 and other adjuvants (mmCT, MF59, IC31 and alum) on the induction of germinal centre and Antibody secreting cells (ACS) was performed. Study demonstrated that, in contrast to alum, LT-K63, mmCT, MF59 and IC31 can overcome limitations of the neonatal immune system and enhance both induction and persistence of protective immune response when administered with pneumococcal vaccine. These adjuvants are promising candidates for early life vaccination.

Session 2: Novel vaccine adjuvants

Development of new adjuvants / adjuvant formulations based on purified Astragalus saponin, Astragaloside VII (AST VII). Nilgün Yakuboğullari (İzmir Institute of Technology, Turkey)

Astragaloside VII (AST VII), a plant triterpenoid saponin isolated from *Astragalus* species, has been shown to possess potency to be utilized as a promising vaccine adjuvant, as it supports a balanced Th1/Th2 immune response.

Within presented study the immunomodulatory activities of the purified *Astragalus* saponin, Astragaloside VII (AST VII) and its newly synthesized analogues Dicarboxylic Astragaloside VII (DC-AST) and Dodecylamine Conjugated Astragaloside VII (DAC-AST) were investigated *in vitro* on human whole blood cells, as well as on mouse bone marrow-derived dendritic cells (BMDCs) and mouse bone marrow derived macrophages (BMDMs).

AST VII and its analogues (DC-AST VII and DAC-AST VII) increased the production of IL-1β in stimulated human whole blood cells comparing to QS-21, a triterpenoid saponin isolated from *Quillaja saponaria* stated as golden standard of saponin based adjuvants. In terms of IL-17A production, AST VII and QS-21 increased the production of IL-17A, opposite to the DC-AST VII and DAC-AST VII. Upon co-treatment with LPS, AST VII and its derivatives significantly boosted IL-1β secretion in BMDCs compared to LPS alone. In BMDMs, AST VII and DC-AST VII demonstrated slight increase in IL-1β compared to LPS alone, whereas IL-1β production after DAC-AST VII treatment was below the detection limit.

AST VII induced the maturation and activation of BMDCs in the presence of LPS, while AST II alone did not induce dendritic cell maturation.

DC-AST VII alone induced the maturation and activation of BMDCs in the absence of LPS. The study showed that AST VII derivatives in combination with LPS decrease the dendritic cell maturation via self-assembly micelle formation.

Conclusion: AST VII and its derivatives could be good alternatives to other saponin-based adjuvants as they induce cellular and humoral immune response, have high solubility in water, high isolation yield from plant, high stability and suitability to lyophilisation.

Protein corona and rigidity affect regulatory T-cells induction using liposomes. Bram Slütter (LUMC, NL)

Atherosclerosis is chronic inflammatory disease of the vessel wall initiated when cholesterol-carrying low-density lipoprotein (LDL) is retained in in the arterial wall. Apolipoprtoein B100 (ApoB100) is the primary protein in LDL. Subsequently, activated macrophages upregulate their endocytic pattern-recognition receptors and internalize oxidized LDL, which gives them their foam-cell appearance. Macrophage foam cells (FCs) play a crucial role in the initiation and progression of atherosclerosis.

T_{regs} are an essential part of the immune system and have indispensable functions in maintaining immune system homeostasis, mediating peripheral tolerance, preventing autoimmune diseases, and suppressing inflammatory and proatherogenic immune response. In addition, T_{regs} can suppress the activity of proatherogenic effector T cells, suggesting an atheroprotective role.

Current strategies for treatment of such disorders involve systemic suppression of inflammation with drugs or by selective cell depletion. However, these therapies can result in severe side effects, especially upon long-term treatment. A more specific strategy would be

to design a vaccine that induces specific tolerance through induction of T_{regs} that recognize the autoantigens involved in inflammatory diseases. LDL- derived ApoB100 has been identified as the most relevant antigen in atherosclerosis, as it is important in the initiation of atherosclerosis. Therefore, there is a need for vaccine formulations that induce tolerogenic DCs and ApoB100-specific T_{regs} *in vivo.*

Liposomes are delivery vehicles widely used for vaccination and their properties such as size, charge, shape/rigidity can influence T cell responses. The goal of presented study was to assess whether liposomal formulations can be used to induce antigen-specific Tregs, and subsequently use these liposomes to encapsulate a newly identified ApoB100-derived peptide to provide protection against atherosclerosis.

After screening for T_{reg} inducing liposomes, study revealed that 1,2-distearoyl-sn-glycero-3-phosphoglycerol (DSPG) containing liposomes show propensity to induce T_{reg} that are specific for liposomes' cargo. Study showed important role of the corona protein that forms on the liposomes in circulation, as uptake of DSPG-liposomes by antigen-presenting cells is mediated *via* complement component 1q (C1q) and scavenger receptors (SRs). It can be concluded that DSPG-liposomes have potential as a delivery system in vaccination against atherosclerosis.

Thermogelling and mucoadhesive hydrogels as sublingual boosters for obtaining mucosal immunity. Lorena Garcia del Rio, (STSM, PhD student, University Santiago de Compostella, Spain)

Despite the success of parenteral vaccination on controlling infectious diseases global spreading, elicit protective immune responses at mucosal surfaces still remains a challenge. This represents a serious drawback since mucosae are the main portals of entry of most pathogenic agents (e.g. oral cavity and respiratory, digestive and genitourinary tracts). Mucosal vaccination activates more efficiently the immune responses at mucosal sites than traditional routes, conferring also systemic protection. Thus, the main purpose of this research was to test, the immunogenicity and potential adjuvant properties of thermosensitive hydrogels, when sublingually administered in mice. Hydrogels present several advantages as ability to be distributed over a wide area of the oral cavity minimizing the risk of drug low retention and delivery deficiency. More specifically, thermosensitive mucoadhesive hydrogels can form hydrogen bonds with mucins, dramatically increasing the residence time of the formulation in contact with the mucosa. Antigen model was prepared using Chalmydia CTH522 subunit vaccine in combination with adjuvants CAF01 and OGEL. The immunization strategy was performed by systemic priming followed by sublingual boosting using *in vivo* mouse model. The obtained results showed that co-administration of CAF01 and OGEL loaded with CTH522 subunit vaccine tends to increase Th1 and Th17 responses.

In vitro investigations of adjuvant properties of selected novel PRR agonists. Samo Guzelj (STSM, PhD student, University of Ljubljana, Slovenia)

Nucleotide-binding oligomerization domain containing protein 2 (NOD2) detects and responds to peptidoglycan fragments from Gram-positive and Gram-negative bacteria.

Activation of NOD2 lead to inflammatory responses through the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and the Mitogen-Activated Protein Kinase (MAPK) pathways. Activation of Toll-like receptor 7 (TLR 7), endosomal PRR responsible for the detection of ssRNA, leads to production of type I interferons and other inflammatory cytokines through the NF-κB pathway. Muramyl dipeptide (MDP), the smallest fragment of peptidoglycan still capable of activating NOD2, comes with too many side-effects. Analogues have been generated targeting NOD2 and TLR7. Results obtained within the study showed that NOD2/TLR7 conjugated co-drugs are potent adjuvants *in vitro* and *in vivo* and have higher activity than an unconjugated mixture of NOD2 and TLR7 agonists.

The IC-tagging multipurpose platform. Tomas Boirazian (Center for Research in Biological Chemistry and Molecular Materials - CiQUS)

Protein microspheres are potent vaccine delivery system with adjuvant activity which can induce anti-viral immunity. The induction of anti-tumour immunity requires pattern as observed in model of anti-viral immunity induction with an emphasis on enabling cellular cytotoxic effector activities. The goal of the research was to explore potential of microspheres to be used as vaccine strategy in tumour immunotherapy. Automatic packaging into a microsphere by co-expression of previously tagged target protein with a sequence called intercoil (IC) and viral protein called protein muNS-Mi showed CTL-mediated citotoxity in tumour model in mice.

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Session 3: Assays for the characterization of antigen-adjuvant formulations

Investigating the role of antigen-adjuvant interaction. Katharina Wørzner (PhD Student, SSI, Denmark)

The degree of antigen adsorption to adjuvants may significantly influence the immune responses induced upon vaccination. The aim of this research was to investigate how changes in the adsorption properties of single antigen to an adjuvant can impact the vaccine induced immune response. The model antigen Lysozyme (Lys) with very high pI 11.1 (+) was succinylated to different extents, resulting in a reduction of the pI value to 4.4–5.9, depending on the degree of succinylation. The degree of succinylation can be controlled by varying the molar ratio of succinic anhydride to lysozyme used in the succinylation reaction. As succinylation is inflammatory itself, it was explored if this influence the potency of the antigen. Study showed that succinylation of lysozyme resulted in increased adsorption to CAF®01. Reducing the pI of LYS enhances CAF®01-induced LYS-specific Th1 and Th17 cell responses. Hence, it can be concluded that suucinylation could reduce the surface charge of Lys, increasing the interactions between antigen and CAF®01. On the other hand, there is a clear

inverse correlation between the antigen pI value and its adsorption to CAF®01. Increased adsorption prior to immunization resulted in an increased Th1/Th17 response.

Optimisation of a vaccine against Melioidosis. Siobhán McClean (University College Dublin, Ireland)

Melioidosis is an infection disease caused by *Burkholderia pseudomallei* and is associated with a range of clinical manifestations, including sepsis and fatal pneumonia. and is endemic in Southeast Asia and Northern Australia. Melioidosis is endemic in in Southeast Asia and Northern Australia and A fifth of infections reported in Thailand are among children under 14 years with an overall mortality of 51%. The aim of the study was to develop prophylactic vaccine candidate targeted against mileoidosis. The identified candidate was the outer membrane protein, OmpW previously demonstrated that this protein was immuno-protective in mouse models of *Burkholderia cepacia* complex (Bcc) infections. It has been shown that protection against *B. pseudomallei* relies on a cell-mediated immune response, and that is possible explanation why SAS was a much more effective adjuvant compared with alum in triggering BpOmpW induced protection in BALB/c mice. In conclusion, study demonstrated that BpOmpW was able to induce protective immunity against melioidosis.

Session 4: Selecting protocols for preclinical testing and down selection and scale-up procedures for adjuvant production

Vaccine Adjuvants Differentially Affect Kinetics of Antibody and Germinal Center Responses, thus Confounding Adjuvant Comparisons. Dennis Christensen (SSI, Denmark)

Within this presentation comparison of adjuvants (kinetics of immune response was examined. Comparing MF59 and CAF01adjuvants revealed that these adjuvants are very unlike adjuvants. CAF01 induces higher T cell response, and suggested CAF01 schedule was 6 weeks for boost, but more experiments should be set up. In conclusion, each adjuvant needs own regime to be able to prepare them.

Chitin derived polymers as vaccine adjuvants. Ed Lavelle(Trinity College Dublin, Ireland)

Chitosan has been widely investigated as an adjuvant for injectable and mucosal vaccines but its mode of action is not fully understood. Within the study relationship between chitin-derived polymer deacetylation and its ability to activate dendritic cells (DCs) was investigated, finding that the degree and pattern of deacetylation critically controls the ability of the polymer to activate DCs and its adjuvant properties. Polymer induced reactive oxygen species generation regulates activation of the cGAS-STING pathway and the NLRP3 inflammasome both of which are required for adjuvanticity.

Preparation of liposomal vaccine adjuvants with hydrodynamic flow-focusing microfluidics. Tandrup Schmidt, (SSI, Denmark)

Microfluidics is a promising new method for large-scale manufacturing of particle-based medicals which is scalable from laboratory to GMP production. Manufacture of liposomal formulation by using microfluidics has potential benefits for large-scale production as compared to the standard lipid rehydration methods and reduces loss of material. The liposomal vaccine adjuvant CAF09b (Statens Serum Institut, Copenhagen, Denmark) is capable of inducing robust cytotoxic T-lymphocyte (CTL) response. An *in vivo* comparison in mice of the immunogenicity to the cervical cancer peptide antigen HPV-16 E7 adjuvanted with CAF09b prepared by lipid film rehydration or microfluidics showed no difference between the formulations, indicating adjuvant activity is intact. Thus, it is possible to prepare suitable formulations of CAF09b by microfluidics.

Dennis Christensen – funding opportunities for further network activities

- Pitch of different funding opportunities to use to maintain the network;
- Working group was established to work on ideas.

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Summary of MC meeting discussion and network discussions

- Introduction of COST Virtual Networking tools: Virtual Networking Support (VNS) Grant and Virtual Mobility (VM) Grants. Virtual Mobility (VM) Grants aim at strengthening the existing networks by allowing scientists to foster collaboration in a virtual setting, to exchange knowledge, learn new techniques, disseminate Action results, etc. These activities may include surveys, questionnaires or preparation of protocols, virtual mentoring of activities that can generate capacity, build new skills, etc.
- Presentation of planned activities of Grant Period 5 (6-month extension period).
- Planning of 5th Adjuvant Workshop scheduled for March 2022 in Gallilee, Israel.