PhD Program in Biomolecular Sciences

Department of Cellular, Computational and Integrative Biology – CIBIO

35th Cycle

"Analysis and optimization of immune responses elicited by Outer Membrane Vesicles"

Tutor

Prof. Guido Grandi

University of Trento

Ph.D. Thesis of

Lorenzo Croia

University of Trento

Academic Year 2021-2022

Declaration of authorship

I, Lorenzo Croia, confirm that this is my own work or work I have done together with other members of our group and the use of all material from other sources has been properly and fully acknowledged.

Contents

1 Abstract

Outer membrane vesicles (OMVs) are spheroidal particles released from all gram-negative bacteria and exert different biological functions. OMVs carry many microbe-associated molecular patterns (MAMPs) such as peptidoglycan, lipopolysaccharide (LPS), and lipoproteins and can be decorated with foreign antigens. Thanks to these properties OMVs are becoming an attractive vaccine platform.

However, a systematic investigation on the potentiality of OMVs to design vaccines to elicit protective antigen-specific T and B cell responses is missing.

In this study we systematically investigated the antigen-specific B and T cell responses elicited by OMVs decorated with heterologous antigens.

Regarding the B cell induction by OMVs, we selected and purified 16 OMV endogenous proteins, studying aspects of their immunogenicity and their concentration within OMVs. We also used these proteins to analyze other aspects of the immunogenicity of OMVs such as the minimum amount of protein, decorated to OMVs, sufficient to induce a protein-specific antibody response or the minimum amount of protein to achieve saturating protein-specific antibody titers. We also analysed the influence of physical association of antigens to OMVs in antigen-specific antibody titers, comparing the immunogenicity of OMVs expressing B cell antigens on their surface with the immunogenicity of OMVs absorbed with the same antigens.

We also investigated the immunogenicity of OMVs decorated with T cell epitopes in inducing epitope-specific $IFN\gamma^+$ CD4+ and CD8+ T cell responses. In particular, we analysed important aspects such as the amounts of OMVs and heterologous antigen necessary and sufficient to reach the plateau of antigen-specific T cell responses. We studied the effect of immunization schedule and route of immunization on antigen-specific T cell response and the influence of physical association of peptide epitopes to OMVs in epitope-specific T cell frequencies.

2 Introduction

2.1 The immune system

The development of new laboratory techniques, genetic approaches and animal models have grown tremendously our knowledge of the immune system. The classic division of the immune system into two different arms, innate and adaptive immunity that work together to fight pathogens, has become more complex (*1*). In fact, the immune system establishes cross-talk not only between immune components but also with host microorganisms and with the nervous and endocrine systems (*2*). In the past, the difference between innate and adaptive defences was based on the ability to remember a pathogen, for adaptive immune defences, and the absence of this ability for innate immunity. However, the discovery of "trained innate" immunity (or innate immune memory) has provoked a shift in our understanding of the immune response (*1*).

Innate immunity constitutes the first line of defense against pathogens. The innate immune system includes granulocytes (neutrophils, eosinophils and basophils), mast cells, macrophages, monocytes, the complement system, dendritic cells (DCs) and innate lymphoid cells (ILCs), including natural killer (NK) cells. The adaptive immune system is composed of T and B lymphocytes that recognize specific antigens, with T cell receptors (TCR) and B cell receptors (BCR) respectively.

2.2 Innate immunity

Innate immunity comprise four different defensive barriers: anatomic (skin and mucous membrane), physiologic (low pH (3-5) and temperature), phagocytic and endocytic and lastly inflammatory (*3*).

More in the detail, skin and mucosa constitute an immune barrier between the internal and external environment. The most external layer of the skin mainly consists of keratinocytes, able to produce antimicrobial peptides and cytokines that induce an inflammatory cascade. Physiological barrier is a defence system that includes: acidity of the stomach, presence of lysozyme, sweat and nasal hair.

Inflammatory barrier is constituted by humoral immunity and its main component is the complement system that promotes inflammation, and attacks the pathogen's cell membrane. The complement system consists of about 50 proteins and protein fragments that are synthesized by the liver, and circulate in the blood as inactive precursors. When activated by pathogens or activating molecules (such as toxins), proteases in the system cleave specific proteins to release cytokines and initiate an amplifying cascade of further cleavages. This process leads to the stimulation of phagocytes, inflammation and activation of the cell-killing membrane attack complex (*4*, *5*).

Cells of innate immunity, recruited to the site of infection by the inflammatory molecules recognize pathogens via Pattern Recognition Receptors (PRRs). These receptors recognize Microbe-Associated-Molecular Patterns (MAMPs), common structures like flagellin, peptidoglycan, lipopolysaccharide (LPS), double stranded ribonucleic acid (dsRNA) and DNA expressed by bacteria (*6*)(*7*).

There are four different families of PRR receptors expressed by cells of innate immunity: Toll-like receptor (TLRs), NOD like receptors (NLRs), peptidoglycan recognition proteins (PGRPs) and C-type lectin receptors (CLRs) (*8*).

Members of the TLR family are major PRRs in cells. Those proteins are type I transmembrane proteins containing leucine-rich repeats (LRRs). Each TLR is able to recognize a different molecular pattern. TLR4 recognizes lipopolysaccharides (LPS), TLR3 recognizes ds-RNA, TLR5 recognizes flagellin, TLRs 1/2/6 recognize lipoproteins, TLRs 7/8 recognize ssRNA and TLR9 recognizes CpG DNA.

Instead, NLR family contains a pyrin domain at the amino terminus, a central nucleotide-binding oligomerization NACHT domain, and carboxy-terminal LRRs. Of particular interest are NOD1 and NOD2 that recognize different bacterial peptidoglycan components (*9*, *10*).

Interaction between PRRs and MAMPs triggers a number of different signaling pathways. Signal transduction is operated initially by a family of adaptor molecules, which determines the specificity of the response by activation of downstream signal transduction pathways via phosphorylation, ubiquitination, or protein-protein interactions. Subsequently, signal transduction culminates in activation of transcription factors that regulate the expression of genes involved in inflammation and antimicrobial host defenses, activating the cells of innate immunity (*11*).

Once activated, these cells release cytokines such interleukin 1 (IL-1), interleukin 6 (IL-6) and tumour necrosis factor (TNF) (*3*).

The cells of innate immunity perform different tasks. In particular, macrophages play a key role in in phagocytizing pathogens and subsequently, presenting pathogen antigens to T cells, thus functioning as Antigen Presenting Cells (APCs) (*12*). Instead, mast cells and basophils are critical for generating a rapid acute inflammatory response. Eosinophiles, a subset of granulocytes, play a key role in the destruction of parasites that are too large to be phagocytized. Regarding the destruction of cancer cells or virus-infected cells, natural killer cells (NKs) play a key role through the release of perforins and granzymes (*13*).

Beyond this function, NKs, releasing interferon-gamma (IFN-γ), helps to mobilize APCs. Finally, the ILCs play a more regulatory role by releasing interleukin-4 (IL-4), interleukin 17 (IL-17) and IFN-γ and therefore help to direct the appropriate immune response to specific pathogens (*3*).

Also the cells of innate immunity possess a memory which consist essentially of epigenetic modifications that may last up to several months. Beyond that, innate immunity performs nonspecific protection to reinfection (*14*, *15*).

The discovery of immunological memory, by the innate immune system, needs further knowledge to allow future development of new vaccine strategies that also stimulate this type of memory (*16*).

2.3 Adaptive immunity

Unlike the innate immunity system, the adaptive arm is able to provide a long lasting specific protection against cancer cells, viruses, bacteria and toxins. The cells of the adaptive immunity are T cells and B cells. T cells are subdivided in two different cell types, CD4+ T cells and CD8+ T cells and provide cell-mediated immunity while B cells are involved in the secretion of antibodies (*17*).

2.3.1 B cells: activation, differentiation and functions

B cells are able to recognize pathogens directly, utilizing receptors (BCRs) expressed on their surface.

Once the binding between the BCR and an exogenous antigen has occurred, B cells, with the help of CD4+ T cells, proliferate and differentiate in both plasma cells and memory B cells. Plasma cells secrete specific antibodies against the antigen, while memory B cells are long-lived cells that can

be rapidly activated to produce antibodies in the event of a future encounter with the antigen (*3*, *18*).

More specifically, B cells originate from hematopoietic stem cells in the bone marrow, and mature in the secondary lymphoid organs. Here, T follicular helper cells (Tfh cells), activated by a given antigen (epitope), migrate from the T cell zone to the edge of the B cell follicle, where they activate those B cells that have recognized the antigen with their BCR and present the same epitope in the context of MHC II. These cells produce both Immunoglobulins M and D (IgM and IgD), which are the first two heavy chain segments in the immunoglobulin locus. After activation by T cells, these B cells proliferate, undergo isotype switching and affinity maturation (*19*).

Unlike the primary immune response, more antibodies are produced during secondary responses which are of switched isotypes and higher affinity (*20*).

An antibody is a heterodimeric molecule that is composed of two light, heavy chains linked by covalent disulphide bonds and non-covalent interactions. Different classes of antibodies can be distinguished on the basis of differences in the constant region of the heavy chain: IgG, IgE, IgD, IgM, and IgA.

- IgG is the most abundant isotype in the body. They are mainly produced during secondary immune response, they neutralize toxins and viruses and opsonize antigen for destruction. In humans, immunoglobulins are divided into four subclasses: IgG1, IgG2, IgG3 and IgG4 with different functions. s.

- IgE is associated with allergic reactions and hypersensitivity and it is involved in immune response to parasites.

- IgD are present at a low level in the body and are co-expressed with IgM. These immunoglobulins are an auxiliary receptor for antigen-naïve B cells.

- IgM is the first immunoglobulin expressed during B cell development. They are involved in opsonization antigen for destruction and complement fixation. These immunoglobulins can form pentameric structure through disulphide bonds in the CH4 region.

- IgA can multimerize like IgM. They protect mucosal surfaces from bacteria, viruses and toxins and form dimers without activating the complement system (*21*).

B cells, through cytokine production, can positively influence memory and effector CD4+ T cell responses. Indeed, naïve B cells can differentiate into distinct cytokine-producing B cell subsets (Figure 1). More specifically, effector B cells producing TNF and CC- chemokine ligand 3 (CCL3)

promote Th1 cell differentiation. IFN-γ-producing B cells can also induce Th1 cell differentiation (*22*). IL-6-producing B cells, displaying a $CD19^{-}$ CD86+ phenotype, promote Tfh, Th1 and Th17 cells differentiation. In mice infected with *Heligmosomoides polygyrus* it was demonstrated that IL-2-producing B cells are essential for the development of effector and memory Th2 cells (*23*). Some B cells differentiated into granulocyte–macrophage colony-stimulating factor (GM-CSF)-secreting. It was found that the increase production of GM-CSF is closely correlated with increase of interleukin-12 (IL-12) production by DCs (*22*).

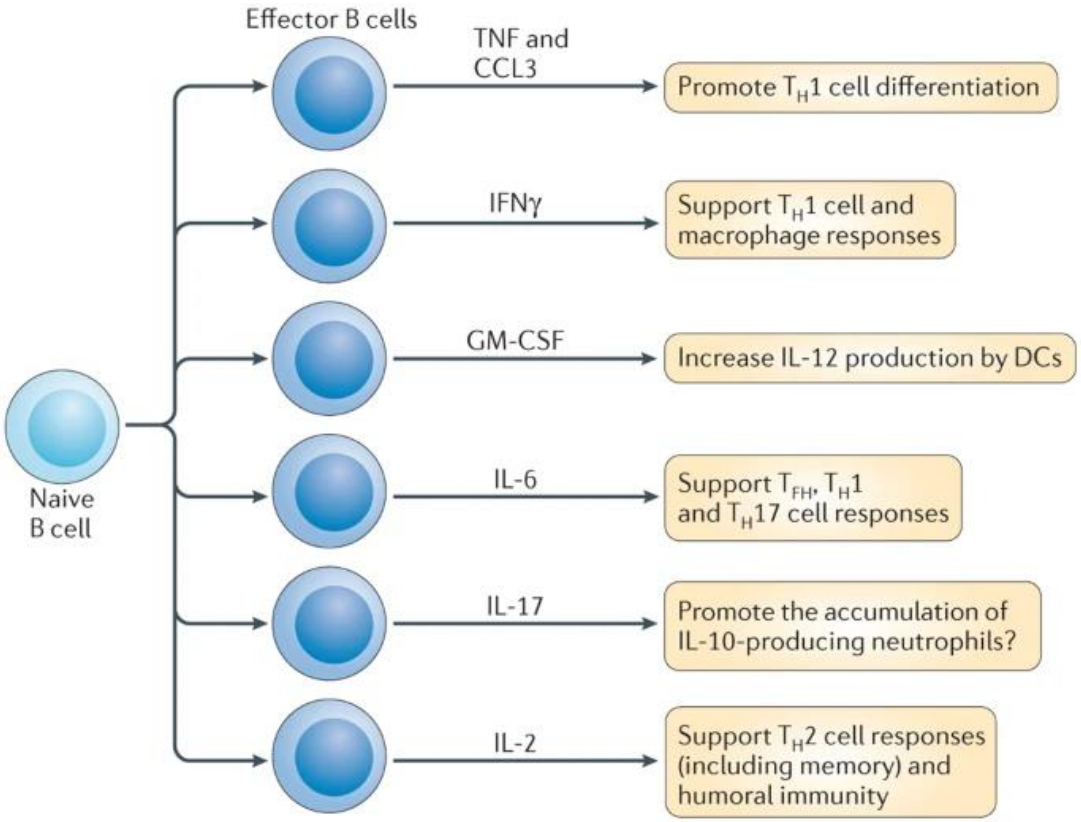

Figure 1. Role of cytokine-producing B cells in the polarization of CD4+ T cells. *This image shows schematically how B cells according to their differentiation into cytokine-producing subsets drastically affect CD4+ T cell responses. TNF- and CCL3-expressing B cells promote TH1 cell differentiation. Also IFNγ-producing B cells induce the TH1 cell differentiation. B cells can differentiate in GM-CSF-secreting that increase IL-12 production by DCs. IL-6-producing B cells promote the differentiation of TH1 cells and Th17 cells. IL-17A-secreting B cells promote the accumulation of IL-10-expressing neutrophils. IL-2-producing B cells are required for optimal development of effector and memory TH2 cells. Figure modified from Ping Shen et al., 2015* (*22*).

2.3.2 CD4+ T cells: activation, differentiation and functions

T cells derive from hematopoietic stem cells in the bone marrow and mature in the thymus. Each T cell expresses a single type of antigen-binding receptor, called T-cell receptor (TCR), on their

surface and after binding to the target peptide in the context of the MHC molecules these cells can rapidly proliferate and differentiate (*3*). CD4+ T cells can recognize the target peptide presented to them by APCs, DCs, macrophages, and B cells via the Major Histocompatibility complex (MHC) class II molecules (Figure 2). This protein complex consists of α and β chains. The amino-terminal domain of each chain, called α1 and β1, contain polymorphic residues and form a sufficiently large pocket to accommodate peptides composed of 10-30 residues. These residues are accommodated in an open binding groove, with the peptide N-terminus usually extruding from the pocket.

The non-polymorphic β2 domain contains the binding site for the CD4 coreceptor (expressed by CD4+ T cells). Each MHC molecule is capable of presenting many different peptides, one at a time.

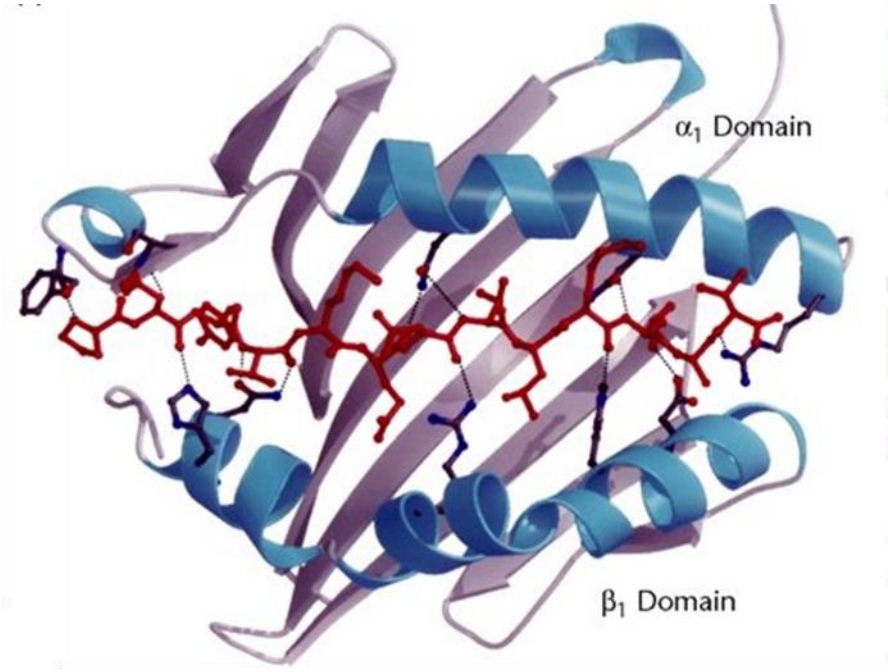

Figure 2. MHC class II molecule structure. *Peptide atoms form hydrogen bonds with residues stored in the pocket of the MHC class II molecule. Peptide is localized in an open binding groove. Hydrogen bonds are depicted as dashed lines. Figure modified from Adnane Achour 2001* (*24*).

To better understand how CD4+ T cells work, it is important to describe how MHC II loading occurs within APCs. In fact, APCs can internalize extracellular pathogens via endosomes, which will later fuse with lysosomes. Within these vesicles, proteins are cut into peptides of varying length and sequence by proteolytic enzymes. APCs continuously synthesize class II molecules in the endoplasmic reticulum (RE). Each neo synthesized MHC II molecule is associated with a class II Invariant Chain Peptide (CLIP) that blocks access to the MHC II pocket. These molecules are transported through an exocytic vesicle into the endosomal vesicle containing the peptides. Here,

by means of the HLA-DM protein, the CLIP peptide is removed and the pocket of the MHC II is made accessible for epitopes with high affinity for it. Subsequently, vesicles containing the peptide-loaded MHCII will fuse with the plasma membrane of the APC (Figure 3) (*25*, *26*). According to several studies, MHCs present on the plasma membrane that are still loaded with the CLIP peptide could interact directly with an extracellular epitope (by removing the CLIP peptide) without the internalization of the peptide (*27*).

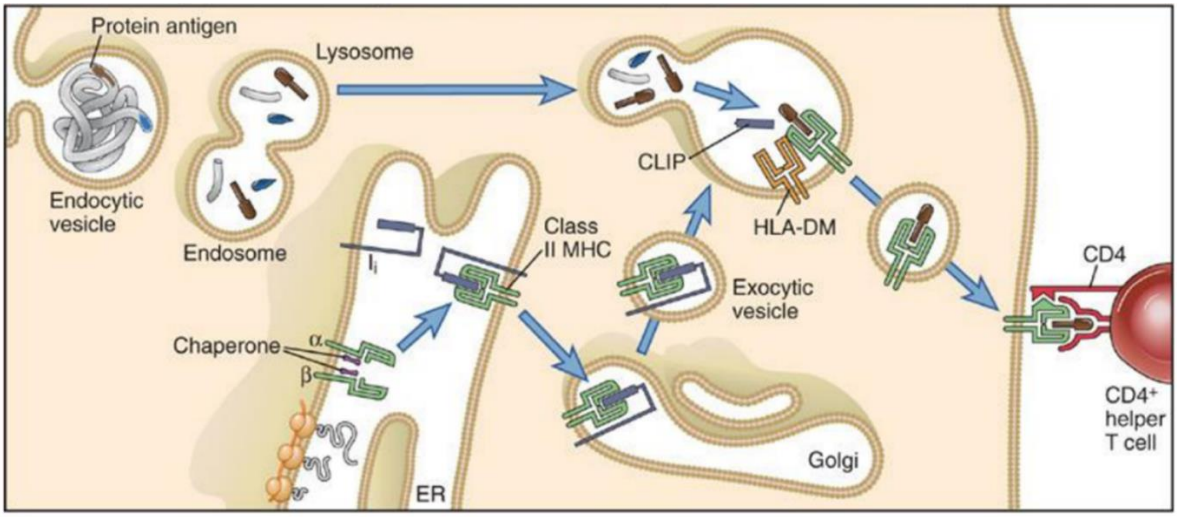

Figure 3. Schematic representation of the loading of peptides into MHC class II molecules in APCs*. Proteins are internalized into vesicular compartments of APC. Subsequently these vesicles fuse with lysosomal vesicles. At the same time in ER, MHC II are biosynthesized and transported to endosomes. Later, within lysosomes, MHC II comes into contact with peptides formed by cutting the protein. Subsequently peptide-MHC II complexes are expressed on the cell surface. Figure modified from Abbas et al., 2007* (*28*)*.*

Triggering the T lymphocyte responses requires the intervention of several membrane receptors capable of recognizing specific ligands expressed by APCs. Indeed, at the same time, TCR recognizes both the peptide and MHC II. Two or more TCRs on the same T cell, together with the CD4+ coreceptor, must be aggregated for signal transduction to be activated.

Signal transduction in CD4+ T cells is activated by the interaction of CD3 and CD28 complexes expressed by CD4+T cells and APCs expressed molecules such as B7-1 and B7-2. With regard to interaction between the lymphocyte and APC, the molecules LFA-1 and VLA-1, expressed by lymphocytes, and the molecules ICAM-1 and VCAM-1, expressed by APCs, are crucial. Beyond these molecules, chemokine released by APCs are equally crucial in inducing CD4+ T cell activation (*29*).

CD4+ T cells, due to their ability to activate CD8+ T cells and B cells, are also called T helper cells (Th) (*30*).

In the past, Th cells were classified only in Th1, Th2 cells and regulatory T cells (TREG) (*31*). Today we know that at least six human Th cells subsets exist: Th1, Th2, Th9, Th17, Th22 and TfH cells (*32*). Th1 cells are differentiated by IL12 secreted by APCs, which is also able to stimulate NKs to produce IFNγ that is also an essential molecule that allows Th cells differentiation into this subgroup (*33*, *34*). These cells, expressing T-box transcription factor (T-bet), are capable of releasing IFNγ. Th1 cells are critical to activate humoral and cellular immunity, macrophages as well as kill intracellular pathogens. On the other hand, IL-2 and IL-4 are critical to differentiate Th cells into a Th2 cellular profile. These cells produce IL-4, IL-5, IL-13 and express GATA-3 (*35*, *36*). Th2 cells are specialized in activating B cells (and the consequent production of IgA or IgE), and in activating eosinophils against parasite helminths (*37*, *38*). IL-6, IL-21, IL-23 and TGF-β are cytokines involved in Th17 cells differentiation. Th17 cells produce IL-17A, IL-17F and IL-22 and express retinoic acid receptor-related orphan receptor gamma-T (RORγt) (*39*, *40*). These cells are involved in mobilizing neutrophiles for the clearance of extracellular bacteria and fungi as well as in mucosal protection. Th9 cells express IL-9 and are involved in mucosal immunity and in protection against parasites. Th22 cells produce IL-22 and are involved both in mucosal protection and promote wound healing. Tfh cells express CXCR5 and chemokine receptor CXCL13 (expressed on B-cell follicles). These cells reside in the germinal center of lymph nodes which are crucial for antibody class switching, affinity maturation and development of B cells (*41*–*43*). Tregs cells are involved in inflammation reduction. These cells release molecules such as IL-10, IL35 and TGFβ, that suppress proinflammatory responses, thus limiting tissue damage by the inflammatory process (*44*).

2.3.3 CD8+ T cells: activation, differentiation and functions

Like CD4+ T cells, CD8+ T cells mature in the thymus after being produced in the bone marrow. These cells are so called because they express on their surface the dimeric CD8 marker. Naive CD8+ T cells require the help of APCs to be activated and to differentiate into CTLs. Through a molecular complex called MHC I, any nucleated cell in the organism can present an antigen to CD8+ T cells. MHC I consists of two subunits, an α chain noncovalently linked to a β2 chain. The amino-terminal portions of the α1 and α2 heterodimer of the MHC class I molecules form a pocket that contains 8-10 amino acid peptides. The binding groove is closed at both ends by conserved tyrosine residues leading to a size restriction of the bound with its C-terminal end docking into the pocket (Figure 4).

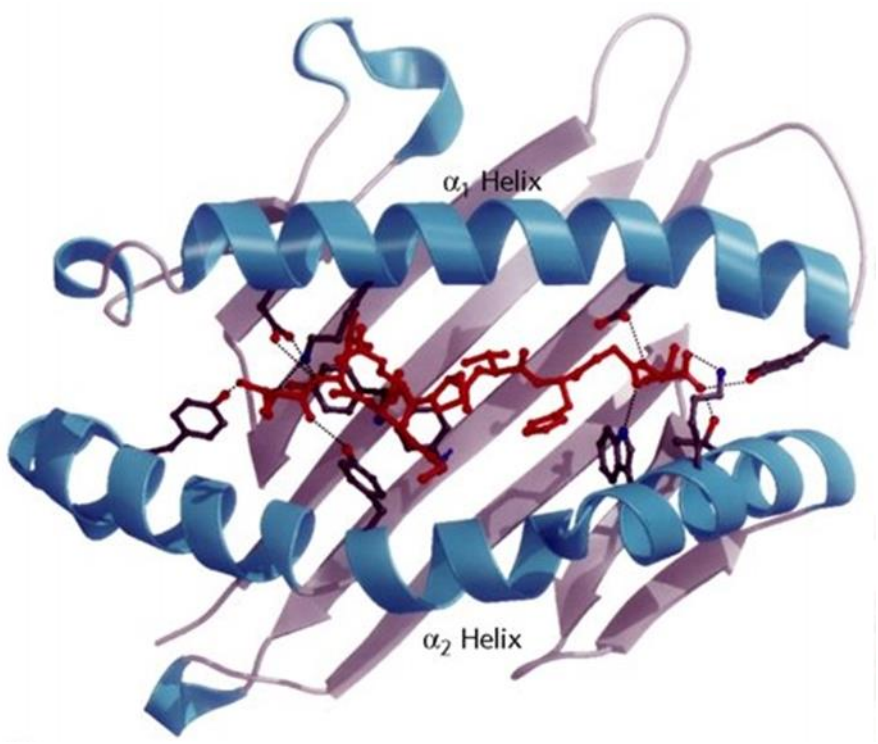

Figure 4. MHC class I molecule structure. *Peptide atoms form hydrogen bonds with residues stored in the pocket of the MHC class II molecule. The binding groove, where peptide is localized, is closed at both ends. Hydrogen bonds are depicted as dashed lines. Figure modified from Adnane Achour 2001* (*24*).

MHC I binds peptides derived from intracellular antigens (e.g. viral proteins), and other cytosolic proteins (e.g. mutated self-proteins). However, DCs can also take up, process and present extracellular antigens with MHC class I molecules to CD8+ T cells by cross-presentation. More in detail, within the cell, when proteins bind to the proteasome, they are degraded and cut into peptides of suitable size so that they can be bound by MHC class I molecules (Figure 5). Peptides, resulting from the action of the proteasome, are localized in the cytoplasm while MHC I molecules are synthesized in the endoplasmic reticulum (ER). A specialized transport machinery, called transporter associated with antigen processing (TAP), located on the membrane of the ER, actively binds and pumps cytoplasmic peptides into the ER. The new synthesized MHC I molecules are weakly bound to the inner end of the TAP molecule. Peptides transported inside RE can be captured by class I molecules.

After binding between MHC I and the related peptide, the complex is stabilized and carried out of the ER and via vesicles can reach the plasma membrane of the cell. At this point, CD8+ T cells can recognize the peptide presented by MHC I (by a mechanism quite similar to that described for CD4+ T cell activation), and also as a result of receiving other stimuli from the APC become activated and differentiate into CTLs.

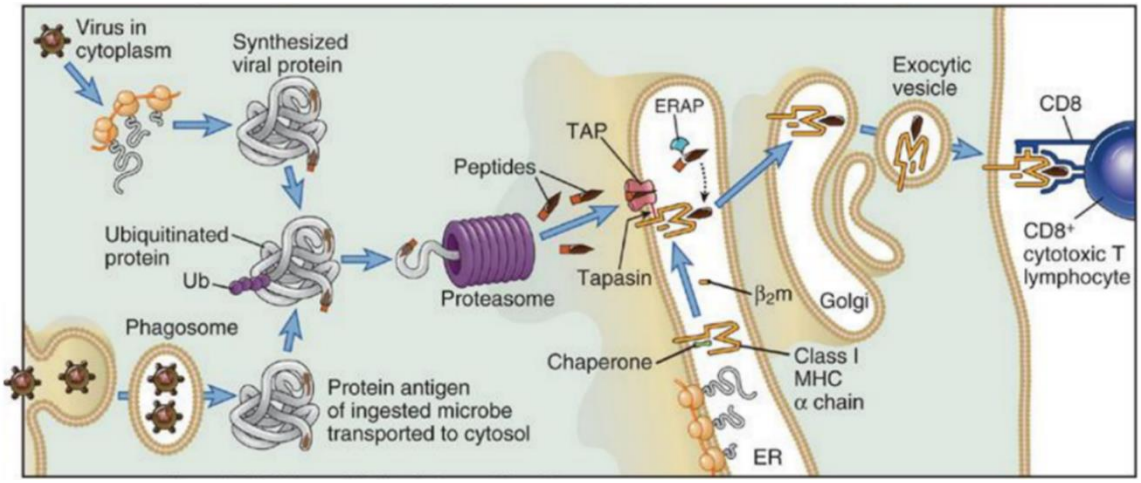

Figure 5. Schematic representation of the loading of MHC class I molecules in APCs. *On the left side of this image proteins (tumoural, viral or exogenous proteins) are ubiquitinated and proteolytically degraded by the proteasome. Peptides are transported from cytosol to ER and within ER there is assembly of peptide-class I. Via exocytic vesicles, peptide-class I complexes are expressed on the surface of APC. Figure modified from Abbas et al., 2007* (*28*).

Within the first 2 days after activation, T lymphocytes begin to proliferate within the lymph nodes causing antigen-specific clone expansion. Once released into the bloodstream, CTLs can interact with the target cell through binding between integrins (FAS) expressed on the surface of the lymphocyte and FAS receptors (CD95) expressed on the surface of the target cell. Prolonged interaction with the target cell induces the death of the latter. This is possible mainly through the use of two proteins released by CTLs: granzymes and perforin. Granzymes are enzymes that cause proteolysis to the target cell, leading it to the activation of caspases that induce apoptosis of the cell. Instead perforin is required to deliver granzymes within the cytoplasm of the target cell. Perforin and granzymes can penetrate the target cell by binding to a sulfated glycoprotein called serglycin (*25*, *26*, *45*, *46*).

Once they have performed their function of eliminating target cells, CTLs will undergo two different fates depending on the expression of two markers: IL-2Rα and CD25. CTLs showing high levels of CD25 are more sensitive to IL-2, so they proliferate more but are more prone to apoptosis. In contrast, cells expressing less level of CD25 will be less sensitive to IL-2 and subsequently will increase the expression of CD127. This marker is typical of those lymphocytes that will survive apoptosis the most to go into the memory CD8+ T cell population (these cells are called memory precursor (MPs)) (*47*).

These cells have the ability to generate secondary immune responses, which occur when the organism is re-exposed to the same pathogen and the immune system will be able to respond in both a fast and strong manner. Instead, effector memory CD8+ T cells are characterized by lack of

expression of those markers, display receptors that promote the migration back and from body tissues to secondary lymphoid organs and are more reactive to produce cytokines.

While the presence of proinflammatory cytokines (IL-2, IFN-γ, IL-12) stimulate CD8+ T cells to maintain a cytotoxic profile, reduced TCR signal strength and low presence of inflammatory cytokines induce the switch of CD8+ T cell to memory precursor (*48*).

2.4 How vaccines work and approved adjuvants

After Edward Jenner reported the first ever human vaccine trial against smallpox in his 1798 publication, terms such as ''vaccine'' and ''vaccinology'' started to be used. For this reason Jenner, together with Pasteur, share the appellation of fathers of vaccinology (*49*). Vaccinology is defined as the science and engineering of developing vaccines to prevent infectious diseases (*50*).

The aim of vaccination is to protect healthy people but especially physically fragile people (e.g. the children, the elders, immune-compromised individuals, people living with chronic diseases) from the risk of developing the disease caused by a particular pathogen. Through the use of vaccines it is possible to contain and sometimes eradicate infectious diseases (*51*). In fact, vaccines have eliminated dangerous diseases (such as smallpox caused by the variola virus (VARV) and rinderpest caused by the rinderpest virus (RPV)) in countries that have implemented a strong program of vaccination (*52*).

At the present time, 11 vaccines against bacterial pathogens and 16 against viral pathogens are available on the market (for this count, different serotypes isolates belonging to the same species are considered as a single pathogen).

A vaccine to be effective must contain:

- Antigen(s);
- Adjuvant component (molecules capable of enhancing and stimulating the immune response);
- A vehicle that allows co-delivery of the previous components to the same APC (*53*, *54*).

Vaccines are divided into two broad categories: live vaccines and non-live vaccines. Live vaccines contain attenuated replicating pathogens (the pathogens contain all the "ingredients" to stimulate immune responses, e.g. measles, mumps, rubella and rotavirus, oral polio vaccine, the Mycobacterium bovis bacillus Calmette–Guérin (BCG) vaccine for TB and live attenuated influenza

vaccine). Instead, the antigenic component of non-live vaccines can be killed whole organisms, purified proteins from the organism, recombinant proteins or polysaccharides. When necessary, non-live vaccines are combined with adjuvant(s) to improve their ability to induce an immune response (e.g. pertussis vaccine, inactivated polio vaccine, haemophilus influenzae type B (Hib) vaccine, pneumococcal vaccine, shingles vaccine, hepatitis B) (*52*, *55*).

Whenever a vaccine is formulated with killed or attenuated pathogens, the addition of an adjuvant is generally not necessary because the pathogen itself contains the molecules to activate the immune system. However, when a vaccine is formulated with other components, as recombinant proteins (hepatitis B virus (HBV) vaccine), proteins isolated from organism (acellular pertussis vaccine), viral vectors (Ebola and COVID-19 vaccines) or polysaccharides (pneumococcal vaccine against *S. Pneumoniae*), the addition of an adjuvant is generally necessary to activate the immune response (*52*, *54*).

The search for new and effective adjuvants is an extremely slow process, and this is due to the special characteristics that these molecules must possess. In fact, if on the one hand the function of adjuvants is to enhance the immune response, on the other hand the risk could lead to excessive inflammatory reaction and possible autoimmunity (Table 1) (*53*, *54*).

Most of the early adjuvants were mainly aimed at inducing protective antibody responses (*56*).

The first adjuvant to be used, as well as the only one on the market until 30 years ago was insoluble aluminum salts (alum) that is currently the most widely used adjuvant in vaccines. Alum works as a delivery system, indeed the adjuvanticity of alum is mediated through a “depot effect”. This mechanism consists in a slow release of antigen administered with alum. More recently it has been discovered that Alum activates NLRP3 inflammasome and caspase-1 in DCs, promoting the release of inflammatory cytokines (IL1β and IL18) which stimulate the production of antibodies. Examples of vaccines containing alum are vaccines against hepatitis B, diphtheria, tetanus and pertussis or human papilloma virus (*56*–*59*).

In the last 30 years, five other adjuvants have been approved for human use. These are the oil-in-water emulsion adjuvants.

Since 1997 in more than 30 countries 100 million doses of the influenza vaccine containing a new adjuvant, MF59, have been inoculated. MF59 is an oil-in-water, biodegradable, and biocompatible emulsion adjuvant that is a normal component of the human body. Like alum, MF59 is a delivery system. Injections of MF59 are able to upregulate the production of cytokines and chemokines

which attracts APCs to the vaccination site increasing antigen uptake. These APCs are able to mediate the induction of Th1, Th2 and Th17 cells (*56*, *60*).

Recently, the AS0 adjuvant series has been developed by GlaxoSmithKline. AS03 is a squalene-based emulsion with the addition of Alpha-tocopherol (vitamin E) licensed in 2009. Publications in both humans and mice have shown that AS03 enhances the magnitude of CD4+ T cell and antibody responses (*56*).

Alum, MF59 and AS03 are not potent inducers of cytotoxic T cell responses and therefore they are not very efficient against intracellular pathogens and cancer (*56*). The discovery of PRRs, such as TLRs and NOD-like receptors, changed the game of adjuvant formulation. Indeed, this discovery has boosted research into vaccine adjuvants aiming to induce cellular immune responses that are essential to fight intracellular pathogens and cancer cells (*61*).

Thus, agonists of hTLR4 have become important adjuvants also used for cancer therapy. 3-O-desacyl-4'monophosphoryl lipid A (MPL), LPS, detoxified extracted from *Salmonella Minnesota*, mixed with alum were combined to formulate AS04. This adjuvant is actually used for the hepatitis B vaccine (Fendrix) and the human papilloma vaccine (Cervarix). AS04 boosts both effector and memory B-cell immunity. In fact, AS04, activating TLR4, leads to the rapid production of cytokines and cell recruitment in the injected site (*62*, *63*).

Very similar in terms of composition and mechanism of action is AS01 formulated with MPL and purified saponin fraction (QS-21), a triterpene glycoside purified from the bark extract of the tree Quillaja Saponaria Molina, together in liposomes and in small concentration of cholesterol. AS01 is used as an adjuvant in the vaccine against varicella zoster (Shingrix) (*64–67*).

Other PRR agonists have been studied, such Cytosine phosphoguanosine 1018 (CpG), a 22-mer unmethylated CpG-B class oligonucleotide, TLR9 agonist. This powerful adjuvant stimulates a strong Th1, B cell and NK cell response. Has been developed recently as a vaccine adjuvant for Dynavax's hepatitis B vaccine, HEPLISAV-B(*66*, *67*).

Currently under study are other adjuvants capable of inducing a Th1-type response. For example, polyinosinic-polycytidylic acid (Poly(I:C)) is a synthetic analog of double-stranded RNA recognized by TLR3 or STimulator of INterferon Genes (STING), an endoplasmic reticulum transmembrane protein belonging to the family of nucleic acid sensors (*68*, *69*).

In summary, despite the recent introduction of novel adjuvants, the identification of immunostimulatory molecules capable of promoting potent and long lasting Th1-skewed responses remains a field of intensive research.

Adjuvant	Major Immunostimulatory Component(s)	Innate Receptors or Pathway Activated	Principal Immune Responses Stimulated
Licensed Adjuvants			
Alum	aluminum salts	NLRP3 inflammasome (?)	Ab, Th2 (+ Th1 in humans)
MF59 and AS03	squalene-in-water emulsions	tissue inflammation (no receptors defined)	Ab, Th1 + Th2
AS04	MPL plus alum	TLR4 and inflammasome (?)	Ab, Th1
Adjuvants in Widespread Experimental Use or in Late Stage Clinical Development			
Poly-IC (also Poly-ICLC)	synthetic derivatives of dsRNA	TLR3, MDA5	Ab, Th1, $CD8^+$ T cells
MPL and formulations (AS01, AS02)	MPL and QS-21	TLR4 (MPL), ? (QS21)	Ab, Th1
Flagellin, flagellin-Ag fusion proteins	Flagellin from *S. typhimurium*	TLR5	Ab, Th1 + Th2
Imiquimods	imidazoquinoline derivatives	TLR7, TLR8 or both	Ab, Th1, $CD8^+$ T cells (when conjugated)
CpG oligodeoxynuceotides and formulations (IC31, QB10)	synthetic phophorothioate-linked DNA oligonucleotides with optimized CpG motifs	TLR9	Ab, Th1, $CD8^+$ T cells (when conjugated)
CAF01	trehalose dimycolate (cord factor)	Mincle	Ab, Th1, Th17
ISCOMS and ISCOMATRIX	saponins	mechanism undefined	Ab, Th1+ Th2, $CD8^+$ T cells
IFA (and Montanide formulations)	mineral or paraffin oil + surfactant	mechanism undefined	Ab, Th1 + Th2
CFA	IFA + peptidoglycan, trehalose dimycolate	NLR, inflammasome, Mincle, TLR?	Ab, Th1, Th17

Table 1. Triggering of the innate and adaptive components of the Immune system by major adjuvants (*70*)

2.5 Characteristics and biological functions of Outer Membrane Vesicles (OMVs)

Cells of all domains of life, Bacteria, Eukarya and Archaea, can produce and release parts of membrane, such as membrane vesicles, microvesicles, tolerasomes and exosomes. Outer membrane vesicles (OMVs) are spheroid bilayer vesicles released by all pathogenic and non-pathogenic gram negative bacteria outer membranes. OMVs range in size from 20 to 300 nm and are released during bacterial growth (*71*, *72*). OMVs were described in the literature for the first time nearly 50 years ago and were initially studied in *vibrio cholerae* (*73*) and *Neisseria meningitidis* (*74*). They carry many MAMPs like lipopolysaccharides (LPS, also known as endotoxin), peptidoglycan and nucleic acids (DNA, RNA) (Figure 6) (*75*).

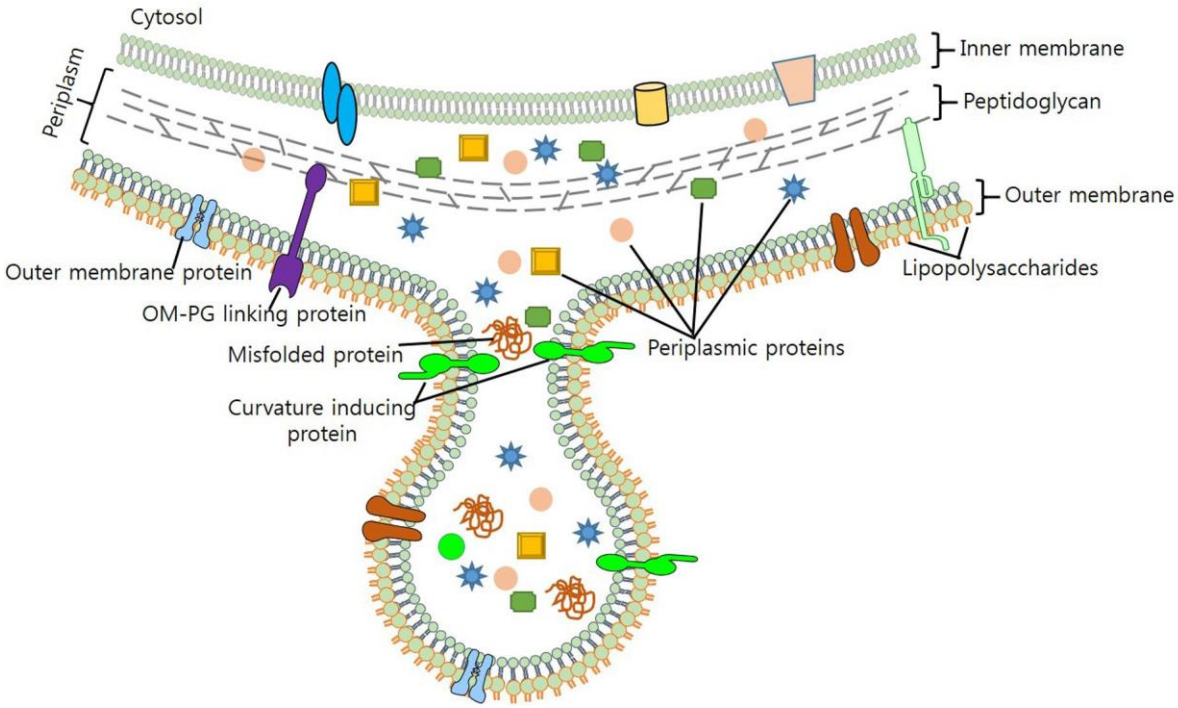

Figure 6. Biogenesis of OMV production in gram-negative bacteria. *Biogenesis of OMV production in bacteria. Figure describes the composition of OMV, cargo selection and loading as part of OMVs Figure modified from Jan. A 2017* (*76*).

OMVs have several functions, including stress responses, biofilm formation, inter- and intra-species delivery of molecules, modulation of the host immune responses and delivery of virulence factors. Bacteria can produce OMVs also to improve their chances for survival and, more in general, to modify the environment around them (*77*, *78*). Biogenesis of OMVs is a deliberate process rather than a stochastic event. The OMV production by gram-negative bacteria can be altered by multiple external factors like oxidation, pH, nutrient availability, temperature, quorum sensing and envelope-targeting antibiotics (*79*, *80*). In addition to these factors that stress for the bacterium generally correlates closely with increased production of OMVs (*81*, *82*). Vesiculation is also used by the bacterium as a way of constitutively expelling substances such as toxic molecules or misfolded proteins (*71*).

Based on mass spectrometry-proteomic profiling technologies, it was demonstrated that OMVs released by gram-negative bacteria contain different types, different amounts and different numbers of proteins. However, some proteins among them, playing a key role in the biogenesis of OMVs, are quite conserved (*76*).

Of particular interest are Outer membrane protein A (OmpA), Braun's Lipoprotein (Lpp) and peptidoglycan associated lipoprotein (Pal). Indeed, OmpA, containing a periplasmic binding site for peptidoglycan, functions as a bridge for this structure and the outer membrane. Instead, the

protein Pal, binding to the peptidoglycan-associated protein TolB (Tol-Pal complex) is critical for cell division of the bacterium and to give stability to the outer membrane-peptidoglycan bond (*83*). Finally, Lpp is one of the most abundant lipoproteins. It has a critical role in binding peptidoglycan to the outer membrane (*84*). Given the importance of these proteins in keeping the outer membrane connected to the peptidoglycan, mutations in them destabilize the outer membrane and lead the bacterium to assume a hypervesicular phenotype, this happens especially for the OmpA protein of *E. coli, Acinetobacter baumannii and salmonella* (*85*).

Another of the main constituents of OMVs are lipids that play a key role in OMVs biogenesis. Membrane lipids have biophysical characteristics that allow the outer membrane (and therefore also OMVs) to assume a certain curvature and fluidity (*71*).

2.6 Immune modulation by OMVs

OMVs released by bacteria induce the activation of innate and adaptive immunity in the host. In fact, OMVs, containing many MAMPs, mainly induce the activation of TLR2, TLR4 and NOD1 in host immune cells. OMVs can interact with a very large number of cells in the organism including endothelial and immune cells (*86*). In the first case, in fact, mucosal epithelial cells, after contact with the OMVs release proinflammatory molecules such as cytokines and chemokines. One of the earliest studies on this concerned OMVs released by *H. pylori,* which demonstrated that OMVs could modulate pro-inflammatory epithelial cell responses, causing the release of the CXCL8 (*87*), while *in vitro,* OMVs of *Legionella pneumophila* induced the production of IFNβ, IL-6, IL-7, IL13, CCL2,CXCL8 and G-CSF (*88*).

Once injected (or naturally released by the bacterium) OMVs are processed by innate immune cells. The interaction between neutrophils and OMVs released by *N. meningitidis,* induce in these cells the release of IL-1β and TNF-α and the upregulation of CXCL8, CCL3 and CCL4. It was also observed *in vivo* that the same OMVs induced in macrophages the release of several pro-inflammatory cytokines (*89*).

Activation of innate immune cells and the following release of inflammatory molecules, allows subsequent activation of adaptive immunity. OMVs, are promptly phagocytosed by professional APCs, which get activated and present OMV-derived peptides on MHC class II and MHC class I, and, at the same time, they are also recognized directly by the BCR expressed on the surface of B cells (*90*). For instance, Alaniz R. C. and colleagues demonstrated that mice immunized with Salmonella OMVs developed robust Salmonella-specific B and T cell responses. In addition, they observed that OMVs stimulated IFN-γ production by a large proportion of T cells from mice

previously infected with Salmonella, showing that OMVs are an abundant source of antigens recognized by Salmonella-specific T cells (*91*).

OMVs can be internalized through different pathways, such as through the so-called “lipid rafts” (*92*) or through dynamin-dependent cholesterol-independent endocytosis (*93*).

2.7 OMVs as a vaccine platform

The interest of the scientific community regarding the study of OMVs as an adjuvant in vaccines is increasing. Carrying many MAMPs, OMVs have a built-in adjuvanticity and they are able to elicit potent Th1 and Th17 immune responses without the need of additional adjuvants or delivery systems (*54*, *94*). This means that OMVs are able to activate antigen specific T and B cell responses, as has been amply demonstrated by our group and by many other works in literature (*94–96*). Indeed, the abilities of these vesicles to work as an adjuvant and antigen delivery system and to allow the release of pro-inflammatory molecules, make them a weapon of unique efficacy. OMVs have already been approved in humans: anti-Neisseria OMV-based vaccines are currently on the market for the prevention of Neisseria meningitidis serogroup B infections (*91*, *97*).

Another attractive feature of OMVs that make them an effective, versatile and promising vaccine platform for the future is that they can be easily decorated with exogenous antigens. Kesty and Kuhen in the early 2000s were the first to demonstrate that exogenous proteins could be compartmentalized in OMVs (*98*).

In addition, outer membrane vesicles can be manipulated to enhance their immunogenicity and reduce their endotoxicity (*99*, *100*). Our group created a new strain E. coli BL21(DE3)Δ60 derived from E. coli BL21(DE3)ΔOmpA. The strain features the release of OMVs deprived of 59 endogenous proteins and carrying a “detoxified” penta-acylated lipopolysaccharide (*101*).

Production costs of vaccines containing OMVs decorated with exogenous antigens are extremely low. OMVs are naturally released from bacteria and can be purified in large quantities from the culture supernatant (*102*, *103*). Considering that the final vaccine formulations would not exceed 10 ug to 50 ug OMVs, 2000 to 10000 doses per liter of culture can be produced.

Finally, OMVs are stable under different temperatures and treatments(*104*). Therefore, they can be easily stored and distributed without the need of the cold chain.

Thanks to these features, OMVs turn out to be an ideal platform to be used in vaccinology.

2.8 OMVs purified from several pathogens induce potent protective immune responses against the pathogens they derive from

OMVs derived from pathogens have been used to produce vaccines against the respective pathogens from which they were isolated and the effectiveness of these types of vaccines in stimulating T and B cell responses and in neutralizing the pathogen has been demonstrated. The most important works are those involving OMV-based vaccines against: *Vibrio cholerae*, *Burkholderia pseudomallei*, *Bordetella pertussis*, *Brucella melitensis*, *Escherichia coli*, *Francisella tularensis, Porphyromonas gingivalis.* OMVs-based vaccines against *Shigella* and *Salmonella typhimurium* are actually at an advanced clinical stage (*101*, *105*, *106*).

Moreover, OMV-based vaccines against *Neisseria meningitidis B* (MenB) are available for human use. Three vaccines based on OMVs isolated directly from the supernatant of MenB have been developed and used during MenB outbreaks (*107*):

1) The Cuban VA-MENGOC-BC® (that showed 83% effectiveness (over 16 months) in young and adults), is a vaccine consisting of OMVs (50 µg) from the epidemic strain B:4:P1.19,15 and the capsular polysaccharide of meningococcal serogroup C (50 µg), using a two-dose schedule (*107*, *108*).
2) The Norwegian MenBVac® (that showed 87% effectiveness (over 10 months) in young and adults), is composed from: deoxycholate 25µg (DOC)-extracted OMVs from meningococcal strain 44/76 (B:15:P1.7,16:P5.5:L3,7,9) adsorbed to 1.67µg aluminum hydroxide and 2µg of LPS and requires three doses (*107*, *109*).
3) MeNZB® administered in New Zealand (that showed 73% effectiveness in the young and adults) consist in an OMV vaccine (25µg) prepared from the B:4:P1.7-2,4 (NZ 98/254) strain (New Zealand strain) with 1.65 mg of aluminum hydroxide and requires four doses administered approximately six weeks apart (*107*, *110*, *111*).

Meningococcal B OMV-based vaccines are effective and have been of paramount importance in reducing the diseases associated with meningococcal infections. For instance, in New Zealand a dramatic reduction in the incidence of meningococcal infections following the vaccination campaign was reported (figure 7) (*111*).

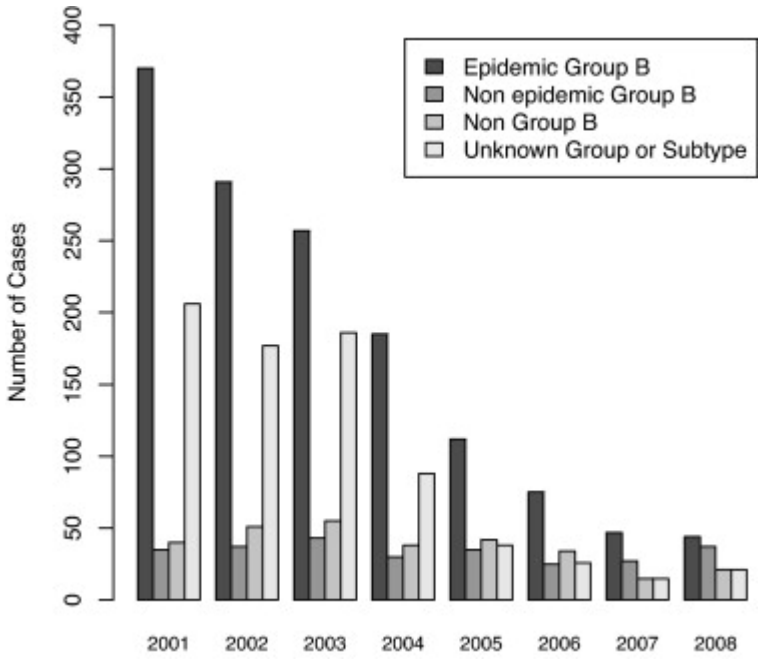

Figure 7. Counts of meningococcal disease cases, classified into 4 types*. This figure clearly shows how in New Zealand the cases of people with meningococcal disease dropped from 2001 to 2008. This decrease is due to the strong vaccination plan exercised by the New Zealand government with MeNZB. Figure modified from Arnold R., et al 2011* (*111*)**.**

However, the vaccines previously mentioned were effective for the strain from which they were isolated and not very effective for the other strains. This is due to the fact that the immunodominant antigens expressed on *N. Meningitidis* OMVs surface are porins (PorA and PorB) that present a high variability between strains of the same serogroup.

To overcome this problem recently Novartis developed the Bexsero vaccine that uses non-porin proteins to construct a more universal vaccine.

Bexsero vaccine is composed by 25 μg detoxified OMVs from *N. meningitidis* strain NZ 98/254 (B:4:P1.7-2,4: ST-42 (cc41/44)), absorbed with 50 μg of five recombinant antigens identified by reverse vaccinology (factor H binding protein (fHbp) fused with genome-derived Neisseria antigen (GNA 2091), Neisseria heparin-binding antigen (NHBA) fused with GNA1030 and Neisseria adhesin A (NadA) used alone). Proteins were fused to increase their immunogenicity and stability. The recombinant antigens induce strong immune responses against a high number of serogroups B strains. The vaccine is administered by intramuscular injection (two-four doses depending on the age of the patient) (*107*, *112*).

Beyond MenB vaccines that have already been approved for human use and are actually on the market, there is an OMV-based vaccine in advanced clinical stage (Phase II) against *shigella*. This vaccine (altSonflex1-2-3) formulated by GlaxoSmithKline (GSK) is a quadrivalent vaccine consisting

of OMV from *S. flexneri* 1b, 2a and 3a and *S. sonnei* (with higher O-antigen expression than 1790GAHB)(*113*).

In addition to a Shigella vaccine, promising results have been obtained with OMV-based vaccines against *Salmonella Typhimurium*. For example, it was demonstrated that 20 µg detoxified OMVs derived from *S. Typhimurium* flagellin-deficient mutants administered per via intraperitoneal or intramucosal provided protection against oral challenge with wild-type *S. Typhimurium* in BALB/c mice (*114*).

OMVs based vaccines against *Bordetella pertussis* and *vibrio cholerae* have also shown promising results.

A study published in 2013 demonstrated that BALB/c mice challenged with *B. pertussis* 18323, were protected by the s.c. vaccination (two doses) with 20 µg *B. pertussis* strain 165 proteoliposomes (or OMV (dOMVBP)) detoxified with Sodium deoxycholate absorbed with 0.25 mg of aluminum hydroxide (*115*). Moreover, Cristian J A Asensio et al., showed that mice immunized intranasally twice with 3 µg or 20 µg of OMVs from *B. pertussis Tohama I* strain (CIP 8132) were protected from *B. pertussis* challenge (*116*).

A study published by Stefan Schild et al., showed interesting results regarding a possible OMV-based vaccine against *V. cholerae*. In this experiment researchers purified *V. cholerae* strain AC108 OMVs (detoxified by formalin treatment) and tested different OMVs concentrations, from 0.0025 µg up to 25 µg administered per intranasal (i.n.) or intragastric (i.g.), or intraperitoneal (i.p.) routes on days 0, 14, and 28. Only mice immunized with at least 0.025 µg OMVs produced antibody titers against OMVs. Antibody titers increased in a dose-dependent manner. Importantly, OMVs were able to protect mice from the pathogen, 0.25 µg of OMVs being the protective amount using a maternal mouse model challenged with *V. cholerae* AC53 (*117*).

Finally, Oh Youn Kim and colleagues, before carrying out a challenge with a pathogenic strain of *E. coli,* immunized groups of mice with different concentrations of *E. coli* OMVs and demonstrated that 1 µg of OMVs were sufficient to induce protection in mouse models (*118*).

2.9 Heterologous OMV vaccines

OMVs can be decorated (engineered or absorbed) with heterologous proteins or peptides and this is a particularly attractive feature in that it makes OMVs a versatile vaccine platform potentially usable for any type of pathogen and even for cancer immunotherapy (*98*). The rational is to exploit OMVs as adjuvant and delivery system, taking advantage of their potent immunostimulatory

properties and ease with which they can be purified. Because of such versatility the use of OMVs to formulate heterologous vaccines is growing exponentially (*119*).

The easiest way to make "OMV-based heterologous vaccines" is to mix the vesicles with purified exogenous antigens. In this case, the preparation of the final vaccine formulation involves two production processes, one for the OMVs purification and the other for the synthesis and purification of the heterologous antigen. Although potentially expensive and time consuming, the advantage is that it is possible to optimize vaccine-induced immune responses by fine-tuning the amount of both antigen and OMVs (*98*).

Alternatively, genetic engineering is used to introduce heterologous antigens into OMVs, and the engineered OMVs, purified from the culture supernatant, are directly used as vaccines. Three main strategies are used for delivering protein antigens to the OMVs:

- N-terminal fusion of secretory leader sequence to the protein of interest (e.g. *E. coli* OmpA leader sequence for secretion). With this method, the protein enters the secretory pathway and reaches the periplasm. In so doing, the antigen is incorporated into the lumen of the OMVs (*120*);
- N-terminal fusion of a lipoprotein leader sequence (for instance, the Lpp leader sequence (Braun's lipoprotein)) to the protein of interest. In this way, the heterologous antigen is expressed as a lipoprotein precursor carrying a cysteine-containing "lipobox". At the level of the inner membrane the cysteine is diacylated and the lipoprotein precursor is cut upstream of the diacylated cysteines. Subsequently, the free amino group of the cysteine is acylated and the resulting mature triacylated lipoproteins are either transferred to the outer membrane (OM) or remain in the Inner membrane (depending on the sorting signal). Lipoproteins destinated to the OM are translocated by the Lol transport machinery. Importantly, lipoproteins are TLR2 ligands and therefore further potentiate the adjuvanticity of the OMVs (*121*);
- Construction of chimeras between heterologous proteins and proteins that are naturally transported to the outer membrane. For instance, ClyA is an autotransporter that assembles in the *E. coli* OM and OMVs. ClyA has been used to localize a large variety of structurally diverse fusion partners in *E. coli* OMVs (*122*).

Vaccines formulated with engineered OMVs have lower production costs with respect to vaccines formulated with OMVs absorbed with the same antigens (*96*, *123*). However, heterologous antigen concentration can only be modulated by optimizing its expression and/or compartmentalization in the OMVs (*95*, *124*).

Several groups have reported the successful engineering of OMVs.

David J. Chen and colleagues demonstrated that OMVs-overproducing *E. coli* strain JC8031 engineered with green-fluorescent protein (GFP) fused to bacterial hemolysin (ClyA) elicited strong anti-GFP antibody titers in mice immunized with the engineered OMVs (*125*).

Fantappiè et al., engineered *E. coli* BL21 (DE3) ΔompA with 6 different *Group A Streptococcus (GAS)* heterologous proteins. It was demonstrated that the OMVs expressing antigens not only induced antibody titers against those antigens in mice but also protected mice from the challenge with lethal dose *GAS* (*120*).

Bartolini and coworker engineered OMVs *E. coli* BL21(DE3) *ΔtolR* mutant strain with *Chlamydia muridarum* DO serine protease HtrA fused to OmpA leader sequence. They demonstrated that mice immunized with engineered OMVs induced antibodies that neutralize Chlamydial invasion as judged by an *in vitro* infectivity assay (*126*).

C. Garret Rappazzo and colleagues demonstrated that *E. coli Nissle* 1917 OMVs engineered with several antigens of the ectodomain of the influenza A matrix protein 2 (M2) elicited in ice a strong Th1 response and mice vaccinated with M2e-OMVs were protected against lethal influenza A challenge (*127*).

Schetters S.T.T. and coworkers demonstrated that OMVs purified from *Salmonella typhimurium* strain engineered with OVA peptide, induced high frequencies of peptide-specific CD8+ T cells (*128*).

Finally, M. Muralinath and coworkers vaccinated mice with *Salmonella enterica* serovar typhimurium-derived OMVs engineered with the Pneumococcal Protein PspA. The authors demonstrated that the vaccine elicited antigen-specific antibodies (IgG2a and IgG1) and conferred protection against the challenge with *S. pneumoniae* (*129*).

Over the last few years, our laboratory has been working on the optimization of vaccine design using heterologous antigen expression. In particular, we have recently generated a novel *E. coli* strain, named *E. coli* BL21 (DE3)Δ60, releasing OMVs (OMVs Δ60) deprived of 59 endogenous proteins and expressing a detoxified LPS. Such strain offers a number of advantages including (114):

- higher loading capacity of heterologous antigens in the OMVs;
- higher immunogenicity
- higher OMV productivity

2.10 Full exploitation of OMVs as vaccines: what is still missing

Despite the growing interest in the use of OMVs in vaccination, it is somehow surprising to observe that a systematic analysis aimed at determining the optimal conditions to elicit antigen-specific immune responses is still missing. In particular, the elucidation of the proper antigen and OMV concentrations necessary and sufficient to formulate a safe and effective vaccine requires a substantial amount of additional experimental effort.

From the inspection of the works published so far it emerges that the experimental data which describe the parameters that influence the OMV-mediated immune responses are still limited.

With regard to humoral responses, Weiwei Huang and colleagues published an article where they engineered *E. coli* DH5α OMVs with *A. baumannii* Omp22 protein and compared the immune responses elicited in mice by purified Omp22 + alum with the responses induced by different concentrations of engineered Omp22-OMVs (containing approximately 1% of heterologous protein). They demonstrated that mice vaccinated with 50 μg Omp22-OMVs produced high Omp22-specific antibody titers ($3x10^4$) while mice vaccinated with 50 μg of recombinant Omp22 did not elicit anti OmpA22 antibodies. The authors also performed a dose-response experiment, immunizing mice with different concentrations of engineered OMVs (from 5 μg to 50 μg) and they clearly demonstrated that 50 μg of Omp22-OMVs (containing approximately 0.5 μg of Omp22 protein) produced the highest antibody titers against Omp22. Antibody titers decreased until they reached a value of $3x10^2$ in mice immunized with 5 μg of Omp22-OMVs (0.05 μg of antigen)(*130*).

Kirsten Kuipers and colleagues engineered *Salmonella* OMVs with two *Streptococcus pneumoniae* proteins and immunized intranasally groups of mice with 4 μg of OMVs. They observed that two or three immunizations induced strong protection in a murine model of pneumococcal colonization (*131*).

David J. Chen and colleagues Immunized groups of mice with 2.5ug of purified ClyA + Alum, with 2.5ug of purified ClyA + OMVs or with engineered OMVs containing 2.5ug ClyA. All formulations induced specific ClyA antibodies with non-statistically significant differences(*132*).

Erika Bartolini and colleagues vaccinated groups of mice with 50 μg of engineered rHtrA-OMV or with 50 μg of CM rHtrA + 1 μg OMVs. The two formulations showed a very similar elicitation of anti-HtrA antibodies (*126*).

Anne L. Bishop and coworkers immunized two groups of mice with OMVs derived from *V. cholerae* using two different routes of administration: intranasal (i.n.) and oral. They showed that the i.n.

route of immunization induced approximately 10-fold higher antibody titers against the selected proteins than oral immunization(*133*).

Finally, in our work published by C. Irene et al., we tested the immunogenicity of five different lipidated *S. aureus* Antigens (FhuD2, Csa1A, LukE, Hla_{H35L} and SpA_{KKAA}) engineered in *E. coli* OMVs (the level of heterologous antigen expression ranged from 10% to 20% of total OMV proteins). We demonstrated that 0.2 µg of engineered OMVs formulated with Alum were sufficient to reach a plateau of heterologous antigen-specific antibody titers (*121*).

The number of publications providing a quantitative investigation of the cell-mediated immunity elicited by OMVs is also limited.

Zhang and coworkers used the Salmonella autotransporter protein MisL to display a specific MHC class II ovalbumin peptide (OVA-CD4) on the surface of a Salmonella vaccine vector. The OVA-CD4 peptide was fused through genetic engineering to the N-terminus of the translocator domain of MisL. Expression of the OVA-CD4-MisL fusion protein was evaluated with Salmonella strain SL7207. They showed that engineered OMVs induced OVA-specific T cell responses (*134*).

Jie Liang et al., and Keman Cheng and coworkers, in two different works, challenged mice B16-OVA melanoma cells and subsequently vaccinated the animals with *E. coli* (Rosetta (DE3)) OMVs engineered with different model epitopes including $OVA_{257-264}$. The results showed a strong protective capacity of OVA-OMVs with B16 epitopes, but no quantitative analysis to elucidate frequencies of OVA-specific T cells as a function of OMV concentration was reported (*132*, *135*).

V. Durand and colleagues immunized subcutaneously or intramuscularly groups of mice with *N. meningitidis* OMVs absorbed with 50 µg of OVA peptide. They showed a comparable ability to induce OVA-specific CD8+ T cells between the two groups (*136*).

Finally, we recently showed that 20 µg of OMVs mixed with five synthetic peptides (20 µg each) corresponding to CT26-specific T cell epitopes (“pentatope”) (*137*) protected mice from the challenge with CT26 tumour cells (*95*).

3 Aim of the thesis

OMVs are emerging as a promising vaccine platform against infectious diseases and cancer. However, information on if and how OMVs can be broadly used to design vaccines tailored for the specific needs remain elusive.

The aim of my experimental work is to thoroughly investigate the antibody and cell-mediated immune responses elicited by *E. coli*-derived OMV vaccine.

To do so, my objectives are to select a panel of different antigens and MHC II/MHC I epitopes and to analyze the parameters that affect the efficiency with which antigen-specific immune responses are induced in experimental animals.

In particular, the experimental work envisages the use of different formulations of vaccines in which the amount of OMVs and antigens are modified in order to establish the optimal conditions for the elicitations of immune responses.

My objective is also to analyze how the route of vaccine administration might influence the effectiveness of the OMV-based vaccine.

To quantify the level of immune responses, different analytical methods are used, including Western Blot and ELISA to follow humoral responses, and flow cytometry and ELISpot to follow epitope-specific T cell frequencies.

Ultimate ambitious goal of my work is to provide the scientific community with “general guidelines” to design effective OMV-based vaccines.

4 Results

4.1 Humoral responses elicited by OMV immunization

OMVs carry endogenous proteins, mostly belonging to the outer membrane and periplasmic compartments. Different groups have applied mass spectrometry to characterize the whole proteome of OMVs (*76*). For instance, more than 150-200 major proteins have been characterized in the OMVs isolated from *E. coli* strains (*99*, *138*). Since most of the OMV proteins are expected to be immunogenic, upon vaccination they should give rise to a large population of B and T cells. Moreover, they should "immunologically compete" with each other, thus diluting the immune response against each specific component. This could be an issue for vaccines based on OMVs engineered with heterologous antigens since the diluting effect could bring the immune response against the engineered antigens down to a level which is below the optimal protective threshold.

However, for humoral immunity, the presence in OMVs of several proteins is not necessarily a negative condition. Indeed, altogether the OMVs proteins carry a plethora of MHC II epitopes, which can generate a large pool of CD4+ T cells, each of which potentially capable to provide help for antibody production to any B cell that recognizes the OMV components with its TCR. Consistently, it is predictable that even those proteins that are expressed in OMVs at relatively low concentrations should elicit sufficiently high antibody titers (Figure 8).

This part of the experimental work aims at providing an extensive and systematic analysis addressed to establish the amounts of an OMV protein necessary and sufficient to induce antibodies and to reach the plateau of antibody titers.

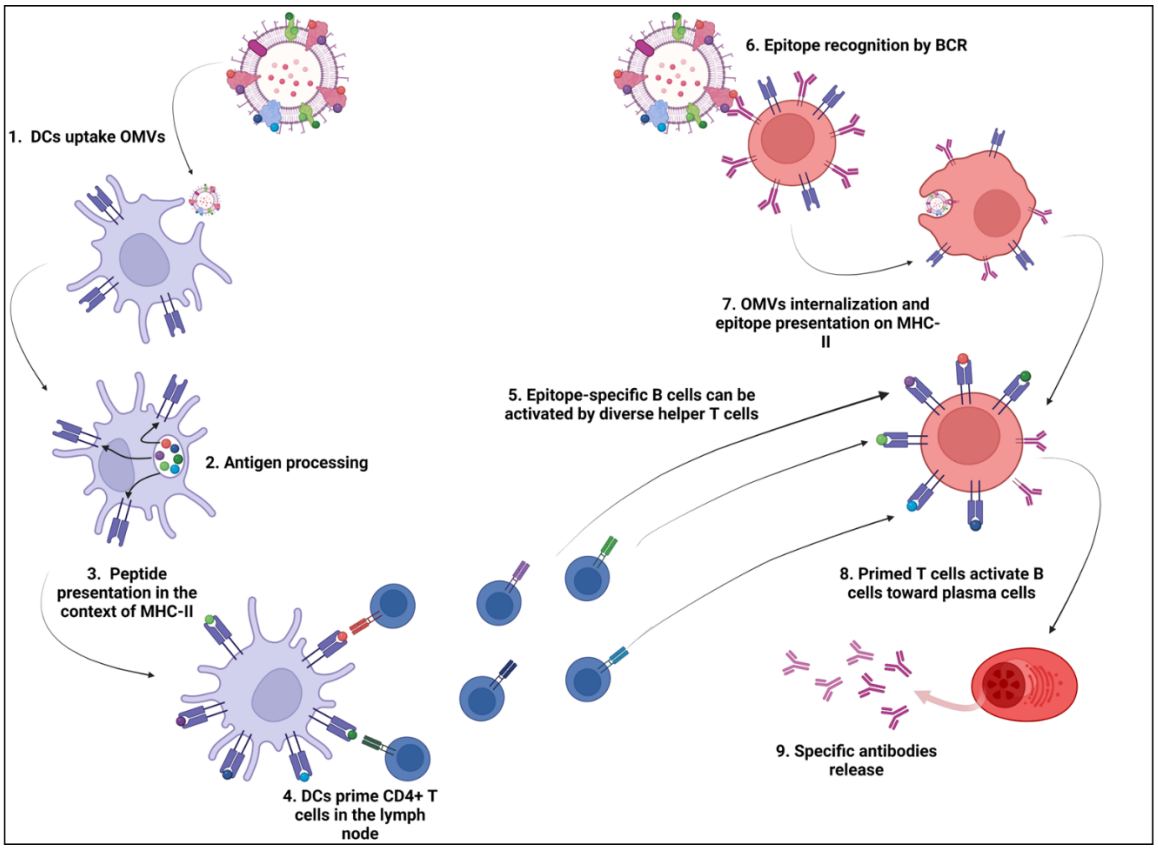

Figure 8. Activation of B cells by OMVs. *1) OMVs are internalized in DCs. 2) Within DCs, antigens are processed and MHC II loaded with peptides from OMVs. 3) DCs, expressing on the surface MHC II loaded with different CD4+ T cell peptides from OMVs, 4) activate diverse CD4+ T cells in the secondary lymphoid organs. 6) At the same time, other OMVs are recognized by BCRs expressed on the surface of B cells, internalized and processed. 7) Within B cells, MHC II are loaded with the epitopes contained in OMVs. 5-8-9) Once activated, CD4+ T cells produce cytokines that stimulate B lymphocyte, loaded with the antigens of OMVs, to plasma cell differentiation and subsequent antibodies production. Created with BioRender.com*

4.1.1 Determination of the minimal amount of OMV endogenous proteins necessary to elicit antibodies

To define how much a protein should accumulate in the OMVs to elicit an appreciable amount of antibodies, we took a *brute force* approach consisting in the following steps:

1. selection of a group of endogenous proteins known to be present in the OMVs from *E. coli* BL21(DE3)*ΔompA*
2. expression and purification of the selected proteins
3. immunization of mice with the OMVs from *E. coli* BL21(DE3)*ΔompA* and analysis of protein-specific-antibodies by Western Blot and ELISA, coating the plates with the purified antigens

4. determination of the amount of selected proteins in OMVs by quantitative Western Blot
5. correlation between protein-specific ELISA titers and amount of protein expressed in OMVs.

The data obtained in the course of this activity are reported below.

4.1.1.1 Selection of endogenous proteins

In our previous work (*101*) we managed to characterize the proteome of the OMVs from *E. coli* BL21(DE3)*ΔompA* strain by 2D-electrophoresis followed by mass spectrometry analysis. The 2D-mass spec analysis led to the unequivocal identification of 90 protein species belonging to the outer membrane and periplasmic compartments. On the basis of this work, we selected sixteen OMV proteins, four belonging to the family of lipoproteins, three to the outer membrane, and the remaining nine are periplasmic proteins (see table 2).

Gene name	Annotation	Compartment	Solubility	MW [kDa]
lpoA*	Penicillin-binding protein activator LpoA	LP	soluble	70
pal*	Peptidoglycan-associated lipoprotein	LP	soluble	16.6
rlpA*	Endolytic peptidoglycan transglycosylase RlpA	LP	soluble	35,7
bamA*	β-barrel assembly machinery	OM	insoluble	88,4
fhuE*	Fhue receptor	OM	insoluble	77,4
ompF*	Outer membrane porin F	OM	insoluble	37
cpoB*	Cell division coordinator CpoB	PP	soluble	25,4
surA*	Chaperone SurA	PP	soluble	45
yncE*	Uncharacterized protein YncE	PP	soluble	35,3
ydcL	Uncharacterized lipoprotein YdcL	LP	soluble	22,4
ybiS	Probable L,D-transpeptidase YbiS	PP	soluble (partially)	30,8
fkpA	FKBP-type peptidyl-prolyl cis-trans isomerase FkpA	PP	soluble	26,2
glpQ	Glycerophosphodiester phosphodiesterase, periplasmic	PP	soluble	38.2
malE	Maltose/maltodextrin-binding periplasmic protein	PP	soluble	40,7
oppA	Periplasmic oligopeptide-binding protein	PP	soluble (partially)	58,4
hisJ	Histidine-binding periplasmic protein	PP	soluble	26,2

Table 2. Purified OMV endogenous proteins. *The table shows the OMV endogenous proteins purified and used to answer biological questions. Specifically marked with an asterisk are the proteins present in both the E. coli BL21 (DE3) ΔompA and E. coli BL21 (DE3) Δ60 strains. Without an asterisk are shown delete proteins in E. coli BL21 (DE3) Δ60 strain. The compartment of the protein (lipoprotein (LP), outer membrane (OM) periplasmic (PP)), solubility and mass expressed in kDa is also reported*

4.1.1.2 Expression and purification of selected proteins

The selected proteins were expressed in *E. coli* and subsequently were highly purified following a two-step affinity strategy. First, the sequences of the selected proteins were amplified from the chromosomal DNA of *E. coli* BL21(DE3)*ΔompA* and inserted into the pET-15 plasmid using the

Polymerase Incomplete Primer Extension (PIPE) (described in Materials and Methods). The insertions were done to create fusion proteins carrying a HisTAG and the TEV cleavage site at the N-terminus of the proteins. The plasmids encoding the fusion proteins were used to transform *E. coli* strain BL21(DE3) *ΔompA*. Each recombinant clone was grown in LB, bacterial cells were collected and from the biomass the proteins were purified by IMAC affinity chromatography. Subsequently, the proteins were subjected to TEV protease cleavage and a final purification step was performed by a second passage through an IMAC Affinity Column (the details of the protein purification are reported in Materials and Methods). It has to be pointed out that 13 of these proteins were expressed in *E. coli* as soluble proteins while the other three (all belonging to the outer membrane compartment) as inclusion bodies. Therefore, their purification was carried out in the presence of Urea as a chaotropic agent. The quality of the purified proteins was finally analyzed by SDS-PAGE (figure 9).

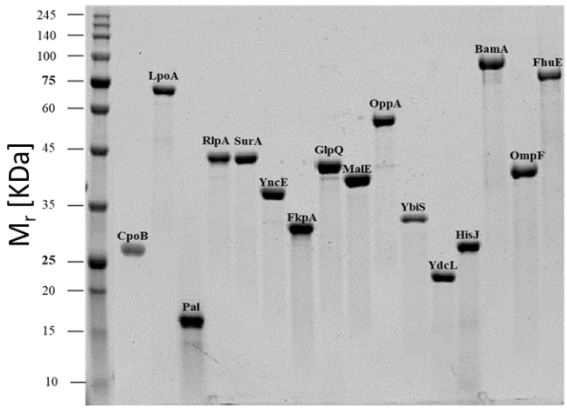

Figure 9. **SDS-PAGE analysis of the 16 selected OMVs proteins expressed in *E. coli* and purified using a two-step affinity purification strategy** (see text for details). 2 μg of each purified recombinant protein were run on a polyacrylamide gel and the proteins were visualized by Coomassie Blue staining.

4.1.1.3 Mice immunization with OMVs and analysis of protein-specific antibodies by Western Blot and ELISA

Next, we investigated whether the immunization of mice with OMVs$_{\Delta ompA}$ elicited antibodies against the selected 16 proteins. To this aim, a group of CD1 mice was immunized three times at two-week intervals with 10 μg of OMVs$_{\Delta ompA}$ in the presence of Alum. After 10 days the sera were collected, pooled together and used to establish the presence of antibodies against the 16 selected proteins by Western Blot. In more detail, the 16 purified proteins were run on a SDS-polyacrylamide gel, transferred to a cellulose membrane and the membrane was treated with the anti-OMVs$_{\Delta ompA}$ serum. As shown in Figure 10A, 9 proteins were well recognized by the anti-

OMVs$_{\Delta ompA}$ serum, indicating that their expression in the OMVs was sufficiently high to elicit specific antibodies. Three other proteins, YdcL, CpoB and OmpF, were also recognized but the band intensities were not high enough to exclude non-specific recognition.

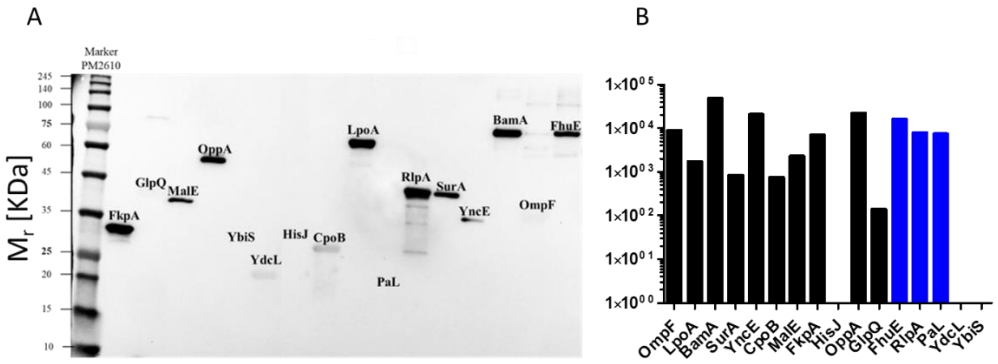

Figure 10. Immunogenicity of OMV endogenous proteins. A) *The sixteen OMV proteins were separated by SDS-PAGE, tansferred onto a cellulose membrane and visualized using the serum of mice immunized with 10µg OMVs$_{\Delta ompA}$.* ***B)*** *IgG titers in mice immunized with "empty" OMVs$_{\Delta ompA}$. 5 CD1 female mice were immunized i.p. 3 times at two-week intervals with 10µg OMVs$_{\Delta ompA}$ in Alum. Sera were collected 10 days after the third immunization and IgG titers were measured in duplicate by ELISA, coating the plates with the protein of interest (200 ng/well). Protein-specific antibody titers are sorted by decreasing protein concentration relative to the total proteins in the OMVs. Proteins for which the concentration could not be determined are shown in blue.*

To further confirm the immunogenicity of the OMV selected proteins, the ELISA assay was carried out coating the plates with the purified antigens and measuring the antibody titers in the anti-OMVs$_{\Delta ompA}$ serum. As shown in Figure 10B, the ELISA assay showed that, in addition to the 9 proteins recognized by the anti-OMVs$_{\Delta ompA}$ antibodies in Western Blot, 4 proteins gave appreciable titers. Such discrepancies can be due to the difference of the protein conformation when analyzed in Western Blot and ELISA.

In conclusion, 9 of the 16 proteins turned out to be immunogenic upon OMV immunization as judged by both Western Blot and ELISA.

4.1.1.4 Determination of the amount of selected proteins in OMVs by quantitative Western Blot

Having demonstrated that the OMV immunization elicited antibodies against several, but not all, OMV proteins, we investigated the level of expression of each selected protein in the OMVs. This information would allow to establish the existence, if any, of a concentration threshold below

which OMV proteins are immunologically "silent", and above which they elicit antibody responses after OMV immunization.

In order to accurately determine the quantity of each protein component in OMVs, techniques such as quantitative mass spectrometry would be required (*139*). Such chemical-physical analysis was out of the scope of this experimental work. Our approach was to use the semi-quantitative Western Blot analysis, an experimental procedure which is much less sophisticated, but has the advantages to be relatively rapid and inexpensive and to provide a sufficiently reliable information on the amount of specific proteins present in OMVs. According to this method, different quantities of the purified protein of interest are run on a polyacrylamide gel together with fixed quantities of OMVs. Next, the separated proteins are transferred onto a nitrocellulose membrane and the protein of interest is visualized with specific antibodies. The visual comparison of the band intensities between the OMV sample and the purified protein allows to determine, with good approximation, the amount of protein present in the OMVs.

Following the semi-quantitative Western Blot analysis, we managed to determine the amount of eleven out of sixteen proteins in the OMVs. As shown in Figure 11, the estimated quantities ranged from 25% in the case of OmpF, to as low as the 0.1% of GlpQ. Of the remaining five proteins, four of them (RlpA, Pal, YdcL and Ybis) failed to be quantified since the sera from mice immunized with the corresponding purified recombinant proteins cross-reacted with protein species having similar molecular weight and therefore they could not be resolved on SDS-PAGE. The analysis of the remaining protein, FhuE, is still in progress.

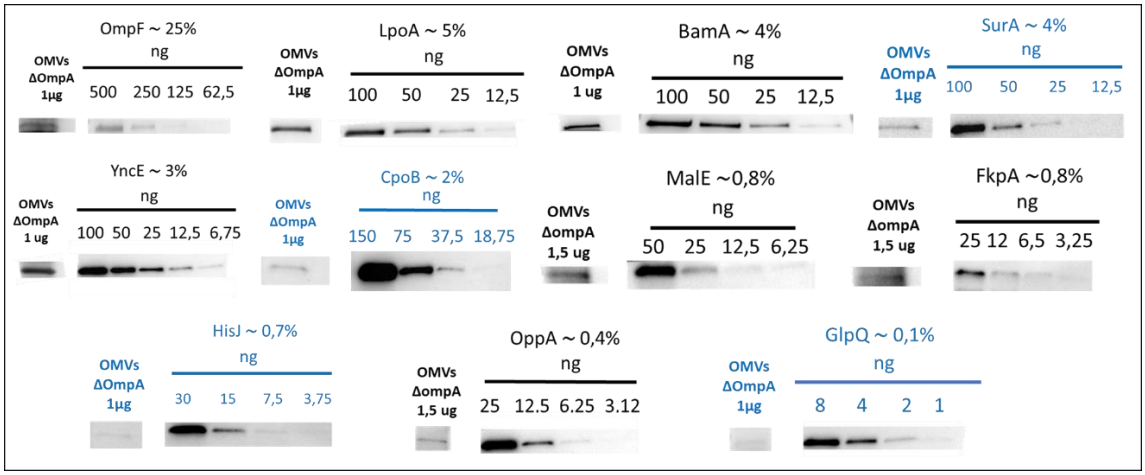

Figure 11. Quantization of OMV endogenous proteins by semi-quantitative Western Blot. *The amount of OMV endogenous proteins was estimated by loading OMVs and different quantities of purified recombinant proteins on western-blot and by comparing band intensities of endogenous proteins in OMVs and of the corresponding purified proteins. In black are concentrations obtained using serum of mice immunized with 10 µg OMVs$_{\Delta OmpA}$, in blue are concentrations obtained using serum of mice immunized with alum + 10µg recombinant protein.*

4.1.1.5 Correlation between protein immunogenicity and level of expression in OMVs

Having established that 9 out of the 16 selected proteins elicited antibodies upon OMV immunization (5.5), as judged by both WB and ELISA, and having determined the concentration of 7 of these 9 proteins in the OMVs, we asked the question whether it is possible to assign a protein concentration threshold, above which OMV proteins are likely to elicit specific antibodies upon OMV immunization in mice. As it emerges from the inspection of Table 2, all proteins that were classified as immunogenic on the basis of both Western Blot and ELISA were present in the OMVs at a concentration $\geq$ 0.8%. Therefore, it is reasonable to conclude that any protein that represents more than 0.8% of total OMV proteins will induce specific antibodies in OMV-immunized mice. This is not to say that lower concentrations are not immunogenic. Indeed, OppA which accounts for only 0.4% of total OMVs proteins, is immunogenic. However, HisJ, expressed at an estimated concentration of 0.7%, is negative at both Western Blot and ELISA assays and therefore the immunogenicity of poorly expressed proteins (< 1%) may vary depending upon their intrinsic immunogenicity properties and, to some extent, their compartmentalization in the OMVs. As far as protein compartmentalization is concerned, theoretically it should have a relevant influence on protein immunogenicity. However, as previously published by our group (*96*) and somehow unexpectedly, luminal OMV proteins can induce high antibody titers. This is likely due to the fact that at the site of injection the vesicles are partially destroyed, thus releasing their content, which becomes visible to the immune system.

One comment deserves the Outer membrane protein OmpF. OmpF is the most abundant protein in OMVs$_{\Delta ompA}$. However, when the anti- OMVs$_{\Delta ompA}$ serum is used, it is barely seen in Western Blot, while it appears immunogenic in ELISA. This is likely due to the fact that the OmpF-specific antibodies elicited by OMVs$_{\Delta ompA}$ immunization recognize the properly folded protein embedded in the membrane and therefore a fully denatured protein (as it is OmpF in the acrylamide gel) is expected to be invisible to conformational antibodies.

Protein name	Immunogenicity		Concentration (%)
	WB	ELISA	
OmpF	(-/+)	+	25
LpoA	+	+	5
BamA	+	+	4
SurA	+	+	4
YncE	+	+	3
CpoB	(-/+)	+	2
MalE	+	+	0,8
FkpA	+	+	0,8
HisJ	-	-	0,7
OppA	+	+	0,4
GlpQ	-	+	0,1
RlpA	+	+	N.D
FhuE	+	+	N.D
YbiS	-	-	N.D
YdcL	(-/+)	-	N.D
PaL	-	+	N.D

Table 3. Shows proteins sorted on the basis of their concentration. *The symbol (+) represents proteins that showed a signal on western blot or were positive in ELISA. The symbol (-) represents proteins that did not show a signal on western blot or were negative in ELISA. Instead, the symbol (-/+) represents proteins weakly positive on western blot.*

4.1.2 Determination of the OMV protein concentration necessary to elicit saturating antibody titers

To formulate a vaccine, it is important to establish the amount of antigen(s) and adjuvant necessary and sufficient to induce an effective (protective) immune response. Therefore, we next asked the question at which doses of OMVs a saturating level of antigen-specific antibodies is reached. It is predictable that such doses will depend upon the nature of the antigen and, in particular, on the antigen concentration in the OMVs.

To address this question, we immunized mice with escalating doses of OMVs and we followed the antibody titers against the 10 endogenous proteins that were positive in ELISA and for which their concentration in the OMVs was determined (Table 2). CD1 mice were immunized 3 times with 0.2 μg, 1 μg, 5 μg and 10 μg of $OMVs_{\Delta ompA}$ and anti-protein specific antibody titers were measured 10 days after the last immunization by ELISA. From the data reported in figure 12, it appears that the optimal amount of OMVs can vary from protein to protein. In general, for those proteins expressed at concentrations higher than 4-5%, 5 μg of OMVs is more than enough to elicit the highest possible levels of protein-specific antibodies. However, there are poorly expressed proteins such as FkpA for which even 1 μg of OMVs seems to be sufficient. It is anyway fair to say that proteins expressed under a concentration of 1% require 10 μg of OMVs to induce a sufficiently high level of specific antibodies.

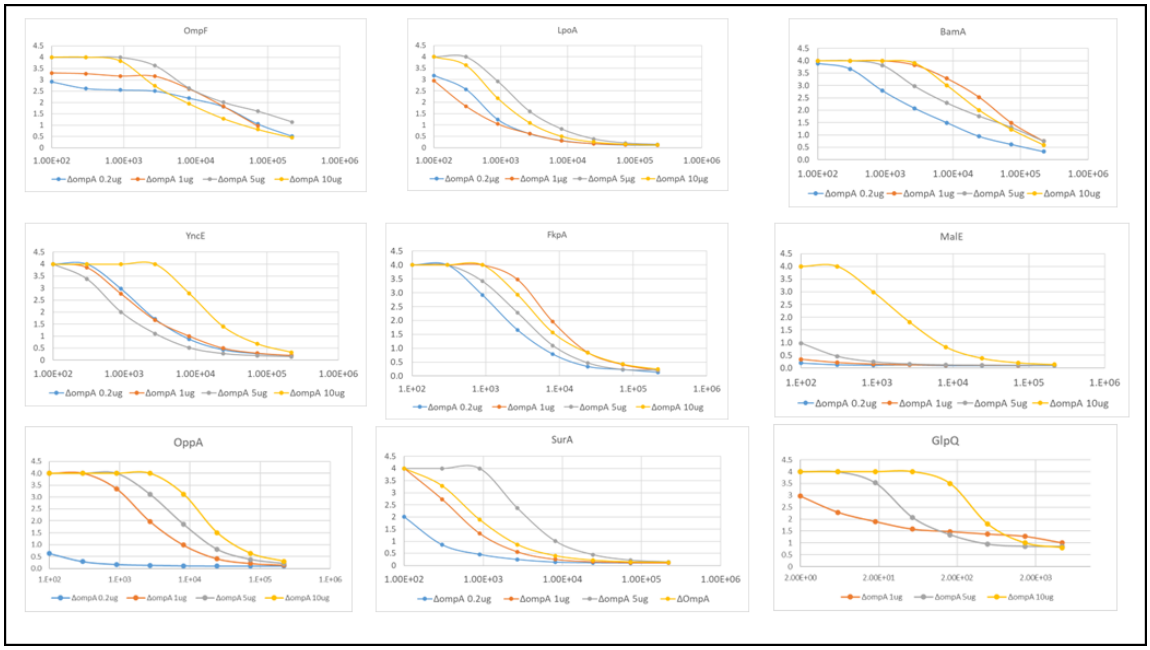

Figure 12. ***IgG titers in mice immunized with increasing doses of "empty" OMVs$_{\Delta ompA}$.*** *Groups of CD1 female mice (four mice per group) were immunized three times at 2-week intervals with 0,2μg, 1μg, 5μg or 10μg "empty" OMVs$_{\Delta ompA}$ formulated with alum. Ten days after the last immunization sera were collected and IgG titers were analyzed by ELISA, using plates coated with recombinant proteins.*

4.1.3 Influence of physical association of antigen to OMVs on antigen-specific antibody titers

In the previous sections we determined the concentrations of antigens, physically associated to OMVs, necessary to induce humoral responses upon OMV vaccination. The results led us to conclude that (i) OMV immunization elicits specific antibodies against OMV-associated proteins expressed at concentrations as low as 1%, and (ii) immunization with 1 to 5 μg of OMVs is sufficient to induce saturating levels of antibodies against OMV proteins present in the vesicles at concentrations higher than 4-5% of total proteins.

A still unanswered question is whether the same conclusions described above can still be applied when antigens are not physically associated to OMVs. For the sake of clarity, physically associated antigens are (i) components that are naturally embedded in the OMVs as a consequence of the vesiculation process, such as periplasmic proteins and outer membrane proteins, (ii) heterologous antigens which are properly engineered in the OMV-producing strains to reach the vesicular compartment (*96*, *98*, *123*), and (iii) components that are artificially linked to the OMVs using specific chemical-physical processes (*132*). On the other hand, non-physically associated antigens are those which do not have any specific linkage to the OMVs, as it is for

instance the case of antigens which are simply mixed to an OMV suspension before immunization.

The interest in comparing the immunogenicity of antigens when they are associated or not associated to the OMVs stems from the fact that associating certain antigens to the OMVs might be very complex, time consuming and expensive. For instance, eukaryotic proteins are often difficult to be expressed in OMVs and therefore if physical association to OMVs is strictly needed to elicit effective antibody responses, the development of vaccines constituted by some eukaryotic antigens and OMVs as adjuvant might be precluded.

From a theoretical standpoint, the elicitation of adaptive immunity against a specific antigen requires that the same antigen presenting cell (dendritic cell) that internalizes the antigen and presents it in the context of the MHC molecules is simultaneously activated by one or more agonists of the innate immune receptors. Such concomitant events efficiently occur when antigen and adjuvant are linked together. Therefore, only antigens coupled to OMVs are expected to elicit high antibody titers.

In this part of the work we compared the immunogenicity of a group of selected proteins which were either physically associated to OMVs by genetic engineering or were first purified from bacterial cultures and subsequently mixed to OMVs. Somehow differently with what theoretically predicted, we show that some OMV-antigen mixtures can be as effective as engineered OMVs in inducing elevated antigen-specific antibody titers.

4.1.3.1 Antigen selection

To study the role of OMV-antigen association in antibody-dependent immunogenicity, we selected three *Staphylococcus aureus* and two Group A Streptococcus (GAS) proteins we have been studying in our laboratories as potential vaccine candidates (*96*, *123*). The three *S. aureus* proteins are: the pore forming toxin Hla (*140*), the iron transporter FhuD2 (*141*) and the virulence factor Protein A (SpA) (*142*) instead the two GAS proteins are: streptolysin O (SLO) (*143*) and *Streptococcus pyogenes* cell envelope protease (SpyCEP) (*144*). We previously showed that the *s. aureus* antigens can be successfully incorporated in the OMV membrane by expressing them in the OMV-producing strain as lipoproteins (*123*) or that GAS antigens can be integrated in the OMV lumen as periplasmic proteins (*96*) (Figure 13). By using the five proteins purified from recombinant *E. coli* strains following the same procedure previously described (see Section 4.2.2)

and densitometry of SDS-page (Figure 13), we also established that the concentration of FhuD2, Hla, SpA, SLO and spyCEP was 28%, 10%, 15%, 19% and 5% respectively, corresponding to 2.8, 1, 1.5, 1.9 and 0.5 μg/10 μg of total OMV proteins.

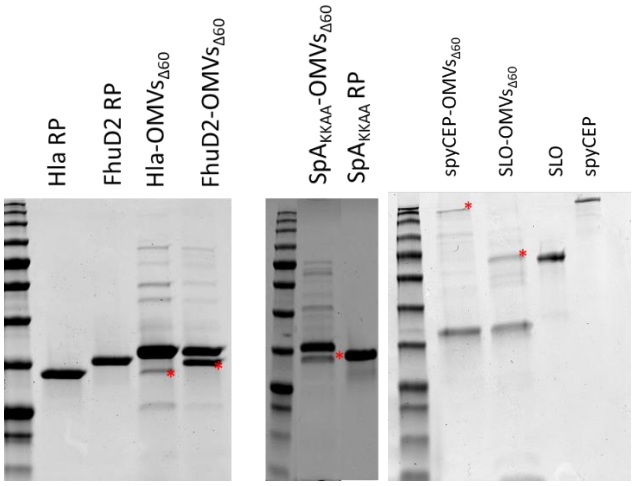

Figure 13. Purified recombinant proteins and OMVs$_{\Delta 60}$ engineered with heterologous proteins*. SDS-PAGE analysis of 10 μg of purified HlA-OMVs$_{\Delta 60}$, FhuD2-OMVs$_{\Delta 60}$, SpA$_{kkaa}$-OMVs$_{\Delta 60}$ SLO-OMVs$_{\Delta 60}$, spyCEP-OMVs$_{\Delta 60}$. Engineered proteins are indicated by a red star. Next to it are the respective recombinant proteins (2μg) expressed in E. coli BL21(DE3) and purified by IMAC.*

4.1.3.2 Antigen-specific antibody titers

Groups of four CD1 mice were immunized three times, two weeks apart, with 1 μg of FhuD2-OMVs$_{\Delta 60}$, Hla-OMVs$_{\Delta 60}$, SpA-OMVsΔ60, SLO-OMVs$_{\Delta 60}$ and spyCEP-OMVs$_{\Delta 60}$. Considering the high concentration of the five antigens in the OMVs, 1 μg of engineered OMVs should be sufficient to elicit a saturating level of antigen-specific antibodies (see above). In parallel, three groups of mice were immunized with 1 μg of "Empty" OMVs$_{\Delta 60}$ mixed with 0.28, 0.1, 0.15, 0.19 and 0.05 μg of purified FhuD2, Hla, SpA, SLO and spyCEP respectively. Such amounts correspond to the amounts of each protein expressed in 1 μg of engineered OMVs (see above). Ten days after the third immunization the blood from each group of animals was collected and pooled and antibody titers against each antigen were determined by ELISA using plates coated with corresponding purified proteins. As shown in Figure 15A, the Hla and SLO-specific antibody titers from mice immunized with Hla-OMVs$_{\Delta 60}$ and SLO-OMVs$_{\Delta 60}$ were essentially superimposable with the titers in mice immunized with the OMVs$_{\Delta 60}$ + Hla or SLO mixtures. By contrast, the immunization with the OMVs$_{\Delta 60}$ + FhuD2 mixture did not elicit appreciable levels of anti-FhuD2 antibodies. Finally, the

immunization with the $OMVs_{\Delta 60}$ + SpA or spyCEP mixtures induced antibody titers approximately 1 Log lower than the titers induced by SpA-$OMVs_{\Delta 60}$ or spyCEP-$OMVs_{\Delta 60}$. We also immunized the animals with OMVs-proteins mixtures containing a five-fold higher concentration of recombinant proteins (1.4, 0.5 and 0.75 μg of purified FhuD2, Hla and SpA, respectively). The titers slightly increased in the case of the $OMVs_{\Delta 60}$ + SpA mixture while remained basically unchanged for the $OMVs_{\Delta 60}$ + Hla mixture. Finally, the $OMVs_{\Delta 60}$ + FhuD2 mixture continued to induce very little amounts of anti-FhuD2 antibodies.

One possible explanation of these results is that SpA and SLO and particularly Hla and spyCEP can form a sufficiently stable association with the OMV membrane through electrostatic and/or hydrophobic interactions, and such association allows the antigen-adjuvant co-delivery to the dendritic cells. This is apparently not the case for fhuD2.

We also wanted to test higher doses of peptide to understand both whether it was possible to induce FhuD2-specific antibody titers and whether it was possible to increase antigen-specific titers. To demonstrate this hypothesis, we also immunized mice with OMVs-proteins mixtures containing a one hundred-fold higher concentration of recombinant antigens (1ug $OMVs_{\Delta 60}$ + 28, 19 and 4.9 μg of FhuD2, SLO and spyCEP, respectively). As can be appreciated from image 14A, by increasing the concentration of FhuD2 it is possible to obtain FhuD2-specific antibody titers and this is probably due to the fact that at the same APC it is able to internalize both adjuvant and antigen despite a possible physical non-association. High doses of SLO induce the same titers obtained with lower doses of antigen, instead high doses of spyCEP are able to induce 1 Log more titers of the OMVs absorbed with the same amount of antigen present in the engineered OMVs.

To support the importance of the association between adjuvant and antigen, we immunized mice with the three engineered OMVs and three OMV-protein mixtures (FhuD2, HlA and SPA) in the presence of alum. Alum has the capacity to form particulates which can trap both OMVs and proteins, thus facilitating the internalization of OMVs and proteins by the same antigen presenting cells. As shown in Figure 14B, the addition of Alum, which tends to synergize with the adjuvanticity properties of the OMVs, promoted the elicitation of anti-FhuD2 antibodies by the OMVs-FhuD2 mixture to a level approaching the titers obtained with FhuD2-OMVs.

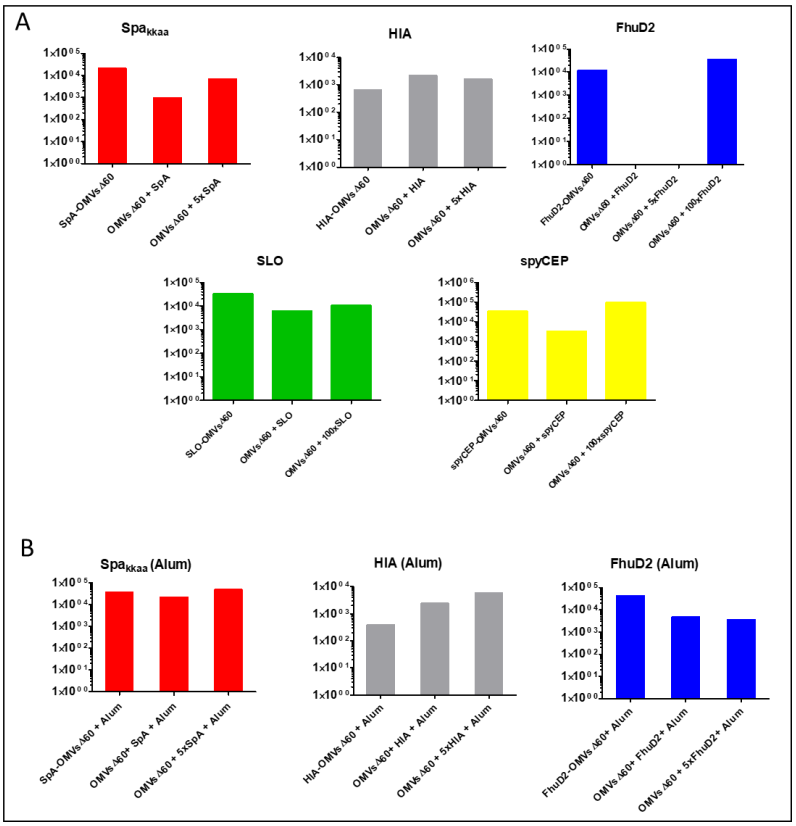

Figure 14. IgG titers in mice immunized with* OMVs$_{\Delta 60}$ *expressing heterologous antigens or* OMVs$_{\Delta 60}$ *absorbed with heterologous antigens. *Groups of 4 CD1 female mice were immunized i.p. 3 times at 2-wk interval with 1 µg OMVs expressing FhuD2, SpA$_{kkaa}$, HlA, SLO or spyCEP antigens or with 1 µg OMVs absorbed with the same amount, 5 times or 100 times the amount of antigens present in engineered OMVs, formulated without* ***(A)*** *or with* ***(B)*** *alum (only FhuD2, SpA$_{kkaa}$, HlA) .*

4.1.4 CONCLUSIONS

The ability of OMVs to elicit effective humoral responses was unequivocally demonstrated by the successful development of the OMV-based vaccines against Meningococcus B tested in human trials from 1987 to 2008 in different Countries. These vaccines showed efficacies ranging from 70 to 85% and protection was mediated by the elicitation of bactericidal antibodies against meningococcal antigens present in the OMVs.

Numerous studies in both the preclinical and clinical setting subsequently confirmed the unique capacity of OMVs to stimulate potent antibody responses. However, and somehow surprisingly, the detailed characterization of the OMV-mediated immune responses has not been thoroughly investigated so far. In particular, little is known about the immunogenicity of the different OMV components, particularly protein components, and how their concentration and localization

within the vesicular compartment influence their immunogenicity. This aspect has scientific and practical relevance, in view of the fact that vaccines based on OMVs engineered with heterologous antigens are being developed. For such vaccines the knowledge of the amount of heterologous antigens necessary and sufficient to induce antigen-specific immune responses becomes of paramount importance. Also important, is the information on how the OMVs components may negatively or positively affect the immunogenicity of the heterologous antigens.

In the experimental work described above we have thoroughly investigated the humoral responses of *E. coli* OMVs in the mouse model and our results lead to a few interesting conclusions.

First, we showed that, when 10 µg of OMVs (determined as total OMV proteins) are used as vaccine dose (for regulatory reasons we consider this dosage as a benchmark since it corresponds to half of the OMV quantity used in the human vaccine Bexsero), antibody responses are detectable against all proteins present in the OMVs at concentrations $\geq$ 1%. This result should be taken into account in designing OMV-based vaccines, considering that proteomic data indicate that a few tens of endogenous proteins are expressed in OMVs at concentrations above this "immunogenic" threshold.

A second important result from our work is that whenever a protein is expressed in the OMVs at concentrations $\geq$ 4-5%, the protein specific antibody titers reach a plateau with OMV doses between 1 to 5 µg. In other words, if a protein is well represented in the total OMV protein pool, and this is for instance the case of the majority of heterologous proteins expressed in OMVs with our engineering strategies, there is no need to formulate vaccines with an amount of OMVs higher than a few micrograms. This result has three important favorable implications. First, the use of limited amounts of OMVs substantially reduces possible reactogenicity issues due to the strong adjuvant properties of OMVs. Second, at vaccine doses containing less than 5 µg of OMVs the poorly expressed endogenous proteins are expected to become "immunologically silent" (see above), thus limiting the production of antibody responses not relevant for vaccine efficacy. Third, the use of minute amounts of OMVs reduces the overall vaccine costs.

The third interesting result regards the influence of antigen-OMVs physical association in determining the strength of the humoral response. It is known that to trigger the production of effector T cells, APCs have to present the antigen in the context of the MHC molecules and at the same time have to receive a stimulatory signal. This occurs at best if the antigen and the stimulatory molecule (adjuvant) are physically associated (cross-linked). However, to what extent OMV-antigen physical association ultimately affects antibody titers has not been investigated in

detail. We therefore compared the antibody titers obtained immunizing mice with either OMVs engineered with selected antigens or with mixtures of OMVs and purified antigens. The result of this study led to two main conclusions. 1) The need of OMV-antigen cross-link varies from antigen to antigen and in many cases it is not necessary. This is likely due to the fact that, upon mixing, OMVs and antigen interact with each other and such interactions are sufficiently stable to allow their concomitant internalization by the same APC. 2) The addition of Alum enhances the antibody responses of the poorly immunogenic OMV-antigen mixtures. This is most likely due to the formation of Alum-mediated aggregates which allow the OMV-antigen co-delivery to the APC.

4.2 Cell-mediated immune responses elicited by OMV immunization

In the first part of this work we have shown data which strongly support the notion that the OMV platform is highly competitive when, as it is the case for a large number of pathogens, vaccine efficacy requires the elicitation of high titers of functional antibodies.

A second objective of my experimental work has been to analyze the cell-mediated immunity induced by OMVs.

As pointed out in the Introduction, a number of publications describe the capacity of OMVs decorated with heterologous peptides to elicit peptide-specific CD4+ Th1/Th17 cells and CD8+ T cells in experimental animals (*118*, *134–136*). However, the conditions necessary to induce an effective peptide-specific cell-mediated immunity both in qualitative and quantitative terms have not been thoroughly investigated so far. In particular, two important questions remain to be addressed:

1) which are the amounts of OMVs and heterologous peptide necessary and sufficient to reach the plateau of peptide-specific T cell responses?

2) which are the levels of peptide-specific T cell frequencies elicited by OMV immunization and how do such levels compare with other adjuvants/vaccine platforms?

Answering these questions would be instrumental to establish the potentiality of the OMVs platform in developing vaccines whose efficacy depends on their capacity to induce a potent cell-mediated immunity. This is for instance the case of prophylactic vaccines against a number of intracellular pathogens and of therapeutic cancer vaccines.

In this Section the experimental work carried out to shed light on the effectiveness of OMVs to elicit cell-mediated immunity is described.

4.2.1 Epitope-specific IFNγ⁺- CD4⁺ T cells induced by OMVs

Interferon γ producing CD4+T cells play a key role in establishing a Th1-skewed immune response necessary to fight intracellular pathogens and to counteract tumor growth. Thanks to the nature of their immunostimulatory components, OMVs have an intrinsic capacity to direct the immune system toward a Th1-type of response. However, the open question is whether and to what extent CD4+ T cells can be elicited against the specific peptide used to engineer OMVs. Since OMVs carry numerous endogenous proteins, each of which possessing several potential MHC II epitopes (approximately 1 epitope per 100 amino acids for any MHC II allele), our prediction is that the frequency of peptide-specific CD4+ T cells should be relatively low unless the amount of heterologous peptide represents a large fraction of total OMV proteins. (see figure 15). To investigate the peptide-specific CD4+T cell responses induced by OMVs, we first selected a number of MHC II epitopes described in the literature as being immunogenic in laboratory inbred mice and subsequently (i) we tested whether OMVs decorated with such peptides can induce epitope-specific T cells in immunized mice, (ii) we quantitated the frequencies the epitope-specific T cells as a function of OMV/epitope concentration, and (iii) we investigated how T cell frequencies in different compartments (spleen, lymph node and blood) are affected by the route of immunization.

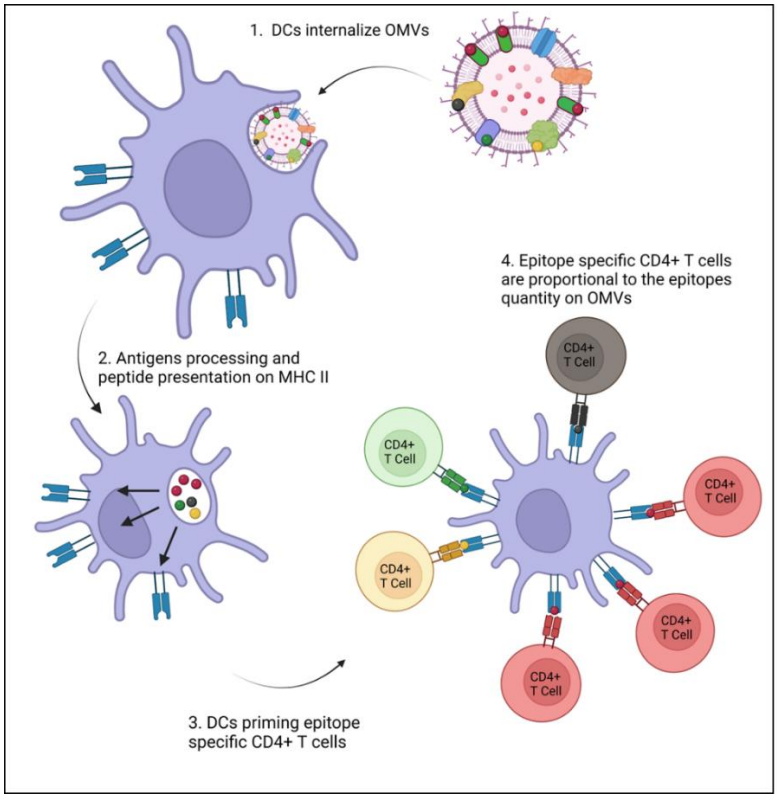

Figure 15. Frequencies of epitope-specific CD4+ T cell activation depends on the concentration of the peptide in the OMVs. *1) The image shows the uptake of OMVs by DCs (the red balls represent the epitope of interest). 2) Once internalized, OMV proteins are processed and peptides present to MHC II. 3) This leads to activation of a heterogeneous CD4+ T cell population, diluting the response against the epitope of interest. To achieve good frequencies of activation of CD4+ T cells specific for the peptide of interest it may be necessary to immunize with a high concentration of the selected epitope. Created with BioRender.com*

4.2.1.1 MHC II epitope selection

To investigate the capacity of OMVs to induce epitope-specific CD4+ T cells, we selected a group of MHC II epitopes reported to be immunogenic in inbred mice (Table 4). In particular, the following epitopes were selected:

- CT26-M03, CT26-M20, CT26-M27, CT26-M68 (called for simplicity M03, M20, M27, M68): these peptides were identified by Kreiter and co-workers after a thorough analysis of all the neo-epitopes generated by the 1680 non-synonymous mutations present in the CT26 tumour model. The epitopes in question all have a length of 27 amino acids and differ from wild-type sequences by one amino acid. To investigate their immunogenicity, BALB/c mice were vaccinated three times with a mixture of five RNA monotopes (7 μg each), consisting of the four epitopes mentioned above plus M26, a CD8+ T cell epitope, administered via intravenous. Spleens were collected and 5 x 10^5 splenocytes were stimulated in vitro with ELISpot assay individually using each of the epitopes used to immunise. More in particular, about 30 splenocytes of mice immunised with five RNA monotopes and re-stimulated *in vitro* with M03 released IFN-γ, about 60 splenocytes released IFN-γ when re-stimulated with M20, about 50 splenocytes released IFN-γ when re-stimulated with M27 and finally about 10 splenocytes released IFN-γ when re-stimulated with M68 (*137*).
- Ts-PmyP2, Ts-PmyP5: Based on the BALB/c mouse model, these two H-2^d restricted CD4+ T cell epitopes (I-A^d) of *Trichinella spiralis* paramyosin were predicted using the SYFPEI-THI database. BALB/c mice were s.c. immunized with the adjuvant ISA50 V2 emulsified with 25 μg of Ts-PmyP2 or Ts-PmyP5 peptides. 5 x 10^5 splenocytes were re-stimulated *in vitro* with Ts-PmyP2 or Ts-PmyP5 and results, analyzed with ELISpot, showed that about 30 CD4+ T cells release IFN-γ when stimulated with Ts-PmyP2 and about 40 CD4+ T cells release IFN-γ when stimulated with Ts-PmyP5(*145*).

- IsdB P4: This epitope was isolated from iron-regulated surface determinant B (IsdB) of *S. aureus.* Three programs were used to identify the candidate H-2^d and H-2^b restricted CD4+ T-cell epitopes on IsdB protein. BALB/c mice were i.m. injected twice with complete Freund's adjuvant absorbed with 100µg IsdB P4 peptide. Splenocytes were re-stimulated *in vitro* with the same peptide used to immunize and results, analyzed with ELISpot, showed that about 100 CD4+ T cells/10^6 splenocytes released IFN-γ(*146*).

EPITOPE NAME	ORGANISM NAME	GENE NAME	AMINO ACIDC SEQUENCE	EPITOPE LENGTH
CT26-M03	colon carcinoma cell line	Slc20a1	DKPLRRNNSYTSYIMAICGMPLDSFRA	27
CT26-M20	colon carcinoma cell line	Slc4a3	PLLPFYPPDEALEIGLELNSSALPPTE	27
CT26-M27	Colon carcinoma cell line	Agxt2l2	EHIHRAGGLFVADAIQVGFGRIGKHFW	27
CT26-M68	Colon carcinoma cell line	Steap2	VTSIPSVSNALNWKEFSFIQSTLGYVA	27
Ts-PmyP2	*Trichinella spiralis*	Ts-Pmy	QFEIDRLAAALADAE	15
Ts-PmyP2 long	"	"	LRKNMQFEIDRLAAALADAEARMKA	25
Ts-PmyP5	*Trichinella spiralis*	Ts-Pmy	AIAQRKLSALSAELE	15
Ts-PmyP5 long	"	"	TLDQYAIAQRKLSALSAELEDCKSA	25
IsdB P4	*Staphylococcus aureus*	IsdB	TLERQVYELNKIQDKLPEKL	20
IsdB P4 long	"	"	KAKTLERQVYELNKIQDKLPEKLKA	25

Table 4. Outline of the main information of CD4+ T cell epitopes used in the experiments

4.2.1.2 Epitope-specific IFNγ⁺ CD4⁺ T cell responses elicited by OMVs

Once selected the immunogenic epitopes, we asked the question whether OMVs formulated with such epitopes could elicit epitope-specific IFNγ^+ CD4$^+$ T cells. To this aim, 10 µg of purified OMVs$_{\Delta 60}$ were individually mixed with the four CD4+ T cell synthetic peptides M03, M20, M27, M68 (50 µg each) and BALB/C mice (5 mice per group) were intraperitoneally immunized on day 0 and day 7**.** On day 12, the spleens were collected and splenocytes were stimulated with either 5 µg/ml of each CD4+ T cell peptide or 5 µg/ml of unrelated negative control peptide and the frequency of IFN-γ-producing CD4+ T cells were analysed using the ELISpot assay.

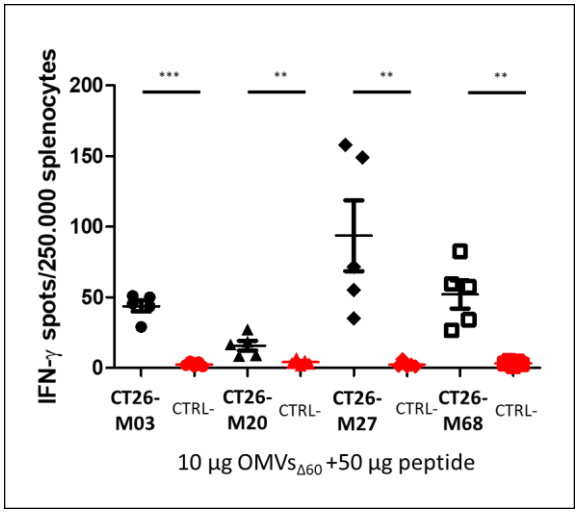

Figure 16. Frequencies of peptide-specific CD4+ T cell obtained immunizing BALB/C mice with OMVs$_{\Delta 60}$ absorbed with CD4+ T cell peptides. *Mice were immunized with 10 µg OMVs$_{\Delta 60}$ absorbed with 50 µg peptides, the mixtures were injected intraperitoneally on day 0 and day 7. Splenocytes harvested on day 12 were re-stimulated with either 5 µg/ml of the vaccination peptides (Black) or 5 µg/ml of unrelated peptide (red). Statistical significance was calculated using an unpaired two-tailed T-test. Not significant (ns)= P>0.1; *= P<0.1; **= P<0.01; ***= P<0.001.*

As shown in Figure 16, in the presence of OMVs the peptide mixture elicited T cells specific for each epitope, with frequencies ranging from 200 to 800 IFNγ$^+$ CD4$^+$ T cells /10^6 splenocytes. M27 and M68 appeared to be the most immunogenic epitopes of the mixture.

Although it is not appropriate to make head-to-head comparison of immunogenicity data obtained in different laboratories, it is interesting to observe that Kreiter et al. using the same epitopes formulated as RNA vaccines and peptide + Hiltonol vaccines, reported CD4+ T cell frequencies of 100-200 T cells/10^6 splenocytes (*137*). Therefore, it is tempting to say that vaccines constituted by appropriate mixtures of OMVs and synthetic peptides can be at least as good as other platform technologies, currently in clinical trials, to elicit epitope-specific IFNγ$^+$ CD4$^+$ T cells (*147*, *148*).

To further support the capacity of OMVs to elicit IFNγ$^+$ CD4$^+$ T cells, we investigated the T cell responses elicited by the mixtures of three additional H-2^d MHC II epitopes: the TsPmyP2 and TsPmyP5 epitopes from *Trichinella spiralis* and the IsdB epitope from *S. aureus* (Table 4). In particular, to have indications on how epitope processing could affect T cell frequency, two mixtures were used, one constituted by 15 amino acid peptides corresponding to the predicted length of the epitopes (short peptides), and the other formulated with 25 amino acid peptides (long peptides), thus prolonging the two epitopes for a few amino acids at both sides (Table 4). BALB/c mice were immunized twice at one-week intervals with mixtures containing 10 µg of OMVs and 33 µg of each peptide and 5 days after the second immunization splenocytes were analyzed for the presence of epitope-specific T cells by ELISpot. As shown in Figure 17, both mixtures

elicited epitope-specific T cells at frequencies ranging from 50 to 150 IFNγ$^+$ CD4$^+$ T cells/10^6 splenocytes. Although a larger number of mice should be used for statistical analysis, the mixture including the longer peptides appeared to perform slightly better than the short peptide mixture.

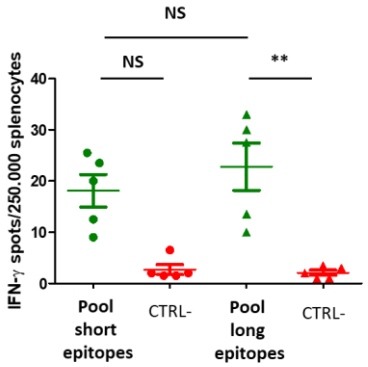

***Figure 17. Frequencies of peptide-specific CD4+ T cells obtained immunizing BALB/C mice with CD4+ T cell short or long peptides adsorbed to* OMVs$_{\Delta 60}$.** *Mice were immunized with 10 µg* OMVs$_{\Delta 60}$ *absorbed with 100 µg short or the corresponding long mix peptides (33 µg each). The mixtures were injected intraperitoneally on day 0 and day 7. Splenocytes harvested on day 12 were re-stimulated with either 5 µg/ml of the vaccination peptide mixtures (green) or 5 µg/ml of irrelevant peptide (red). Statistical significance was calculated using an unpaired two-tailed T-test. Not significant (ns)= P>0.1; *= P<0.1; **= P<0.01; ***= P<0.001.*

4.2.1.3 Analysis of CD4+ T cell frequencies as a function of different OMV and peptide concentration

Having demonstrated that the OMVs can elicit epitope-specific IFNγ$^+$ CD4+ T cells when mixed with peptide epitopes, we carried out a dose range study changing the concentrations of either OMVs or peptides.

We first analyzed how T cell frequencies vary as a function of different amounts of peptides. Increasing amounts of M27 (0,1 µg, 1 µg, 10 µg and 50 µg) were mixed to 10 µg of OMVs and BALB/C mice were immunized following the schedule described previously and T cell frequencies were analyzed by EliSpot. As shown in figure 12, the T cell frequencies varied in a dose-dependent manner up to 10 µg of peptide, with 0.1 µg being not sufficient to give an appreciable amount of T cells. Further increasing the peptide concentration did not result in a higher number of epitope-

specific T cells, whose frequencies appeared to slightly decrease. A very similar dose-response profile was obtained when the M68 epitope was mixed with 10 µg of OMVs (Figure 18).

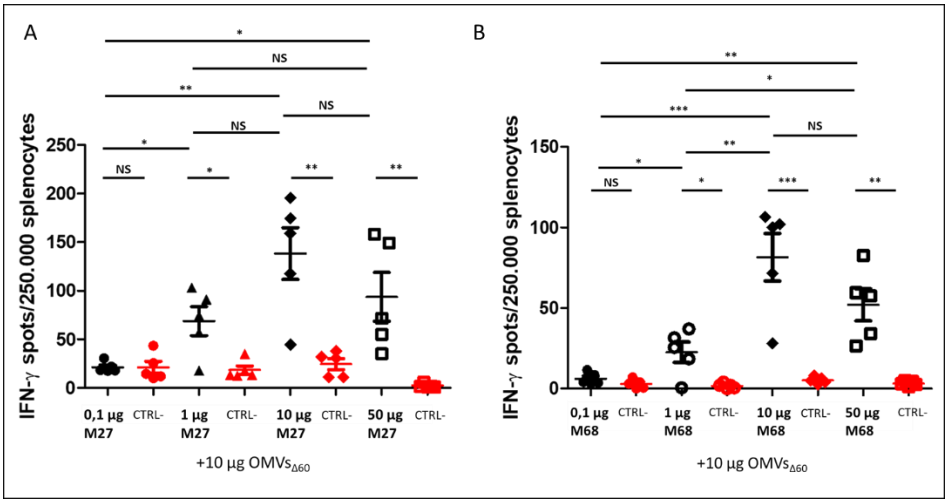

Figure 18. Influence of peptide epitope concentration on T cell frequencies. 0.1 µg, 1 µg, 10 µg or 50 µg of epitope peptide (M27 in **A** and M68 in **B**) were added to 10 µg OMVs and the mixtures were injected intraperitoneally in BALB/c mice on day 0 and day 7. Splenocytes were stimulated with either 5 µg/ml of peptide epitope (black) or 5 µg/ml of an irrelevant peptide as negative control (red). Statistical significance was calculated using an unpaired two-tailed T-test. Not significant (ns)= P>0.1; *= P<0.1; **= P<0.01; ***= P<0.001.

Next, we analyzed the influence of the quantity of OMV on T cell frequencies. In this experiment, we kept constant the amount of peptide using the optimal dose previously determined (10 µg) and we progressively reduced the amount of OMVs starting from 10 µg, the amount which is used in the OMV-based *Menigococcus* B vaccine (Bexsero). More specifically, 10 µg of M27 were mixed with 1, 5 or 10 µg of OMVs and epitope-specific T cells were determined following the immunization schedule and the ELISPOT assay previously described. As shown in Figure 19, even though 1 µg of OMVs was sufficient to elicit good frequencies of epitope-specific T cells, 10 µg of M27 appeared to be the optimal amount of T cell production.

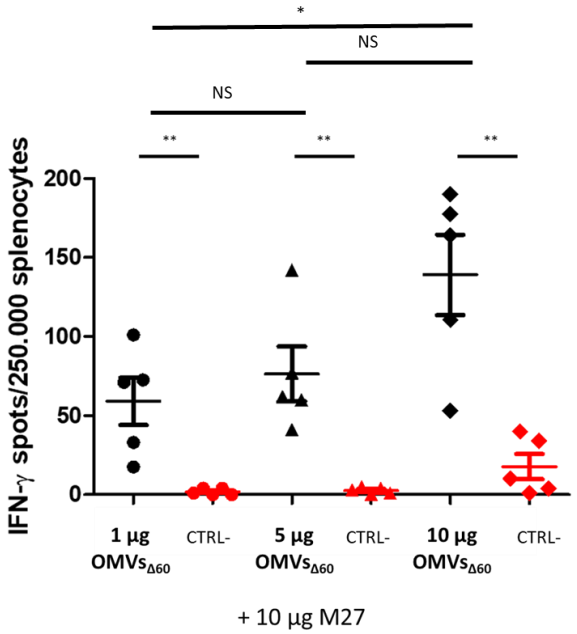

Figure 19. Influence of OMV concentration on T cell frequencies. *Mice were injected intraperitoneally on day 0 and day 7 and spleen were analized on day 12. Three groups of mice were immunized with 10 µg M27 peptide plus 1, 5, 10 µg of* $OMVs_{\Delta 60}$. *Splenocytes harvested on day 12 were stimulated with either 5 µg/ml of the M27 peptide (black) or 5 µg/ml of irrelevant peptide (red). Statistical significance was calculated using an unpaired two-tailed T-test. Not significant (ns)= P>0.1; *= P<0.1; **= P<0.01; ***= P<0.001.*

4.2.1.4 Effect of immunization schedule on antigen-specific T cell response

Next, we investigated how the schedule and the site of immunization could affect the frequency of epitope-specific IFNγ^+ CD4+ T cells.

To better understand why we focused our attention on this experimental activity it is useful to briefly summarize the mechanisms and kinetics of cell-mediated immunity (see Figure 20). Effector T cells take a few days to be generated in the draining secondary lymphoid organs and then they reach the "site of injury" (infection, vaccination) through the blood stream. In the absence of a repeated stimulation by the antigen, they tend to decrease and memory T cells are generated. It has been estimated that approximately 100 T cells in the naive T-cell repertoire expand in the lymphoid organs to about 100,000 effector cells by day 7, and then contract to about 7000 memory cells by day 25. Memory T cells reside either in the lymphoid organs and blood stream (central memory T cells and effector memory T cells), or in large part at the epithelial sites (tissue resident T cells).

Based on the above, the route of immunization and the immunization regime are expected to influence the frequency of T cells and the sites where T cells mostly reside.

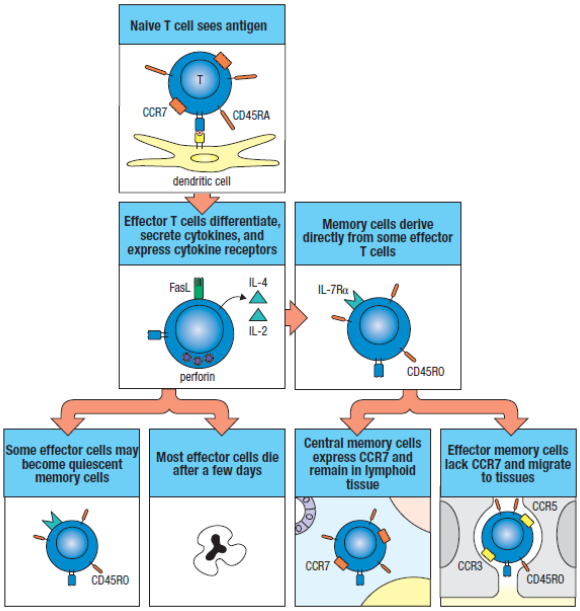

***Figure 20**. T cells differentiate into central memory and effector memory subsets, which are distinguished by the expression of the chemokine receptor CCR7. Quiescent memory cells bearing the characteristic CD45RO surface protein can arise from activated effector cells (right half of diagram) or directly from activated naive T cells (left half of diagram). Two types of quiescent memory T cells can derive from the primary T-cell response: central memory cells and effector memory cells. Central memory cells express CCR7 and remain in peripheral lymphoid tissues after restimulation. Memory cells of the other type—effector memory cells—mature rapidly into effector T cells after restimulation, and secrete large amounts of IFN-γ, IL-4, and IL-5. They do not express the receptor CCR7, but express receptors (CCR3 and CCR5) for inflammatory chemokines. (Taken from Janaway's Immunobiology, 9th Edition)*

In the experiments described so far, we injected the vaccine twice, at one-week intervals. Next, we tested the effect of the administration of a third vaccine dose one week after the second injection. Such regime should further increase the amount of epitope-specific effector T cells both in the splenocytes. As expected, the T cells frequencies almost doubled with respect to the two-dose regime (Figure 21).

Finally, we tested the frequency of T cells immunizing mice with one or two doses of M27-OMV vaccine followed by an additional immunization three weeks after the previous injection. Such experiment was designed to investigate the capacity of the OMV vaccine to elicit memory T cells since the additional dose given with an interval of 21 days should have activated these populations of T cells, while most of the original effector T cells should have declined (see Figure 21). The data reported in Figure 21 shows the presence of T cells in the spleen and, as expected, the frequencies

were lower than what detected following the regime of three doses given one week apart in which a high number of effector T cells have been produced.

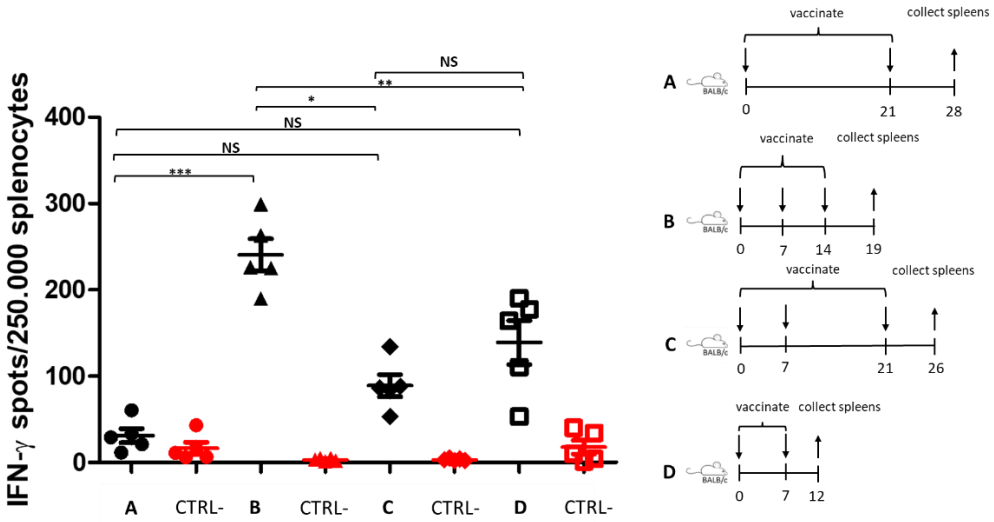

Figure 21. Frequencies of IFNγ+CD4+ T cells induced in BALB/C mice vaccinated with OMVs absorbed with M27 peptide using different immunization schedules. *Mice were injected intraperitoneally with 10 µg of* $OMV_{\Delta 60}$ + 10 µg of M27. *Splenocytes were restimulated in vitro with either 5 µg/ml of M27 peptide (black) or 5 µg/ml of negative control peptide (red). Statistical significance was calculated using an unpaired two-tailed T-test. Not significant (ns)= P>0.1; *= P<0.1; **= P<0.01; ***= P<0.001.*

Subsequently, we investigated how the site of immunization could affect the frequency of epitope-specific IFNγ+ CD4+ T cells. Indeed, when mice are vaccinated in the peritoneal cavity, the vaccine should partially reach the thoracic and mesenteric lymph nodes (the draining lymph nodes Figure 23) and in large part the spleen through the blood stream. Therefore, a large part of the effector T cells should be concentrated in the splenocytes and in the blood, while very few antigen-specific T cells should be detected in non-draining lymph nodes. Consistently, i.p. immunization with M27-OMVs and M68-OMVs elicited epitope-specific T cells which could be detected in the spleen and in the blood of immunized animals a few days after the third immunization (Figure 22).

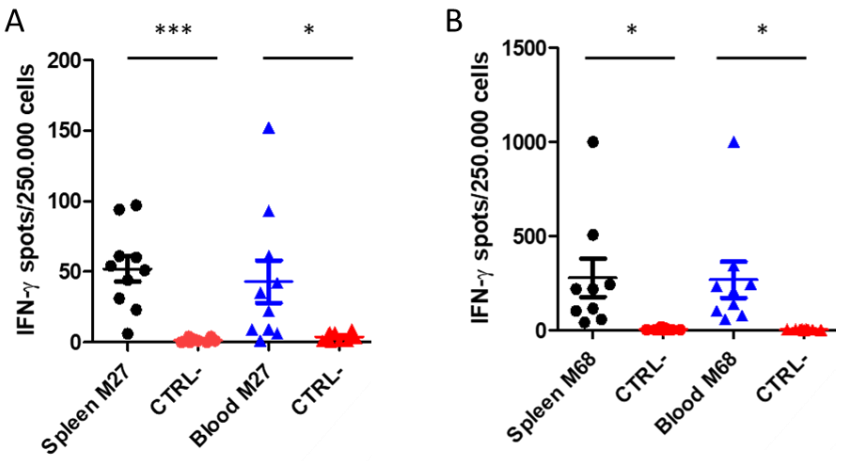

Figure 22. Number of peptide-specific IFNγ⁺-CD4+ T cells induced by vaccination with OMVs$_{\Delta 60}$ + M27 or M68 peptides immunizing mice with three doses one week apart administered intraperitoneally. A) *Mice were immunized i.p. with 10 µg OMV$_{\Delta 60}$ absorbed with 10 µg M27 in* ***A)*** *or M68* ***B)*** *in peptides. Splenocytes (Black) and PBMC (Blu) harvested were stimulated in vitro with either 5 µg/ml of the vaccination peptide (M27 or M68) or 5 µg/ml of negative control peptide (red). Statistical significance was calculated using an unpaired two-tailed T-test. Not significant (ns)= P>0.1; *= P<0.1; **= P<0.01; ***= P<0.001.*

In conclusion, i.p. immunization is an effective route to elicit epitope-specific T cells, which accumulate in the spleen and circulate in the blood stream. Although not confirmed by the analysis of specific markers (for instance surface expression of IL-7Rα and CD45RO), good levels of memory T cells are also produced, which can be activated by a vaccine dose given a few weeks after the primary immunization. Finally, as predictable, repeated injections at short intervals result in an amplification of the frequencies of the effector T cells.

The intraperitoneal immunization is not the common immunization route for humans where vaccines are usually given either i.m. or s.c.. We therefore investigated whether OMVs injected following these routes can elicit sufficiently high frequencies of epitope-specific T cells. It has to be pointed out that when given i.m. or s.c., the injected vaccine is drained to the proximal lymph nodes (also taken up by peripheral DCs), while does not enter the blood stream with high efficiency. Therefore, effector T cells are expected to be mostly generated in the draining lymph nodes, and not in the spleen. Memory T cells either remain in the lymph nodes or reside at the periphery transported by the blood stream.

BALB/C mice were vaccinated with three doses of 10 µg OMVs$_{\Delta 60}$ + 10 µg of either M27 or M68 peptides, and five days after the third dose epitope-specific T cells were analyzed in the draining lymph nodes, in the blood and in the spleen. As shown in Figure 23 and as expected, epitope-

specific T cells were detected in the lymph nodes and in the blood, while they were barely detected in the spleen.

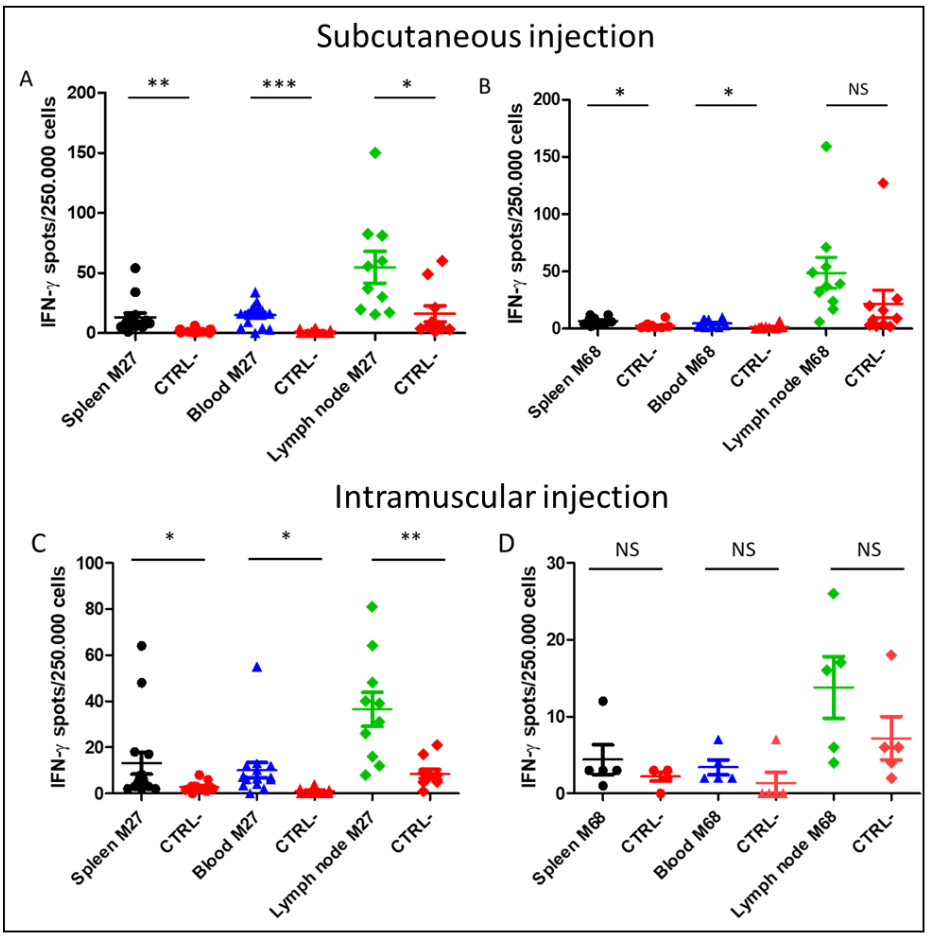

Figure 23. Number of peptide-specific IFNγ⁺- CD4⁺ T cells induced by vaccination with OMVs$_{\Delta 60}$+ peptide administered three times subcutaneously (s.c.) (A-B) or Intramuscularly (i.m.) (C-D). *Mice were immunized s.c. with 10 µg OMV$_{\Delta 60}$ absorbed with 10 µg M27 in **A)** or M68 in **B)**. Mice were immunized i.m. with 10 µg OMV$_{\Delta 60}$ absorbed with 10 µg M27 in **C)** or M68 in **D)**. Splenocytes (Black), PBMC (Blu) and lymph node (Green) harvested were stimulated in vitro with either 5 µg/ml of the vaccination peptide (M27 or M68) or 5 µg/ml of negative control peptide (red). Statistical significance was calculated using an unpaired two-tailed T-test. Not significant (ns)= P>0.1; *= P<0.1; **= P<0.01; ***= P<0.001.*

4.2.2 CONCLUSIONS

The capacity of OMVs to induce potent humoral responses and immunological memory implies that they are excellent activators of T cell-dependent adaptive immunity. This has been demonstrated by a number of publications which show that OMV immunization elicit OMV-

specific CD4+T cells and that such T cells predominantly belong to the Th1/Th17 subclasses, in line with the cytokine profile induced by the OMV-associated stimulatory molecules.

However, considering that the OMV proteome is constituted by tens of periplasmic and membrane proteins that can give rise to a plethora of MHC II epitopes, the OMV immunization is expected to elicit a highly diversified population of CD4+ T cell clones, each clone comprising a relatively small number of cells. Therefore, the open question is whether the OMV platform can be used to induce high frequencies of CD4+ T cells recognizing specific MHC II epitopes. This would be an important requirement for cancer vaccines constituted by mutation-derived MHC class II neoepitopes. Such vaccines have been recently shown to be effective in both the preclinical and clinical setting as long as a good number of neoepitope-specific CD4+ T cells are induced by vaccination (*137*).

To test the epitope-specific T cell responses induced by OMVs, we selected a group of murine MHC II epitopes and we immunized mice with vaccine formulations containing different amounts of OMVs and synthetic MHC II peptide epitopes. Our data indicate that good frequencies of epitope-specific CD4+ T cells can be obtained when the mixtures include 10 μg of OMVs and 10 μg of peptides. Under these conditions, the synthetic peptide epitopes were at least three orders of magnitude more abundant than any other OMV endogenous epitope (assuming that OMVs carry 100 proteins and that each protein containins an average of 10 epitopes). When lower doses of synthetic peptides were used, the CD4+ T cell frequencies dropped and became barely detectable when OMvs were mixed with 0.1 μg of peptide.

Overall, our results indicate that OMVs represent a good adjuvant platform for the production of epitope-specific IFNγ+ CD4+ T cells but, because of the diluting effect of the endogenous MHC II epitopes, detectable levels of specific CD4+ T cells are obtained as long as the epitope of interest is sufficiently concentrated in the vaccine formulation. This has to be considered in designing vaccines based on OMVs engineered with selected MHC II epitopes. Practically speaking, the epitope should represent a large fraction of the total OMVs proteins (> 10% w/w). At present, such expression levels of low molecular weight polypeptides (approx. 13-15 amino acids) are very difficult to achieve.

4.2.3 Epitope-specific CD8$^+$ T cells induced by OMVs

As already pointed out in the Introduction, the capacity of OMVs to induce epitope-specific CD8+ T cells has been described by a number of laboratories, including ours. In these studies, OMVs have been decorated with different MHC I epitopes by (i) genetic manipulation of the OMVs producing strains (*128*, *149*, *150*), (ii) chemical cross-linking of synthetic peptides to the OMVs (*134*, *135*, *151*), and (iii) simple peptide "absorption" to OMVs (mixing peptides with OMVs) (*136*). Altogether, the published data converge to the conclusion that OMVs do promote CD8+ T cell responses. However, these studies do not provide a systematic analysis on the optimal conditions necessary to elicit CD8+ T cells.

Theoretically, the use of OMVs as an adjuvant to promote CD8+ T cells against a specific MHC I epitope has potential advantages and drawbacks.

From the positive side, the immunization with OMV-MHC I epitope vaccine should result in the production of epitope-specific cytotoxic T cells in two ways: 1) Upon vaccination, the APCs internalize and present the vaccine epitope on MHC I, allowing the binding of epitope-specific naïve CD8+ T cells on the surface of the APC. At the same time, the OMV-mediated signaling of different innate immunity receptors (TLR4, TLR2, etc.) activates the APCs to a level sufficiently potent to induce the direct differentiation of the epitope-specific T cells into cytotoxic effector cells. 2) Upon vaccination, the APC internalizes the OMV-MHC I epitope vaccine and presents on its surface the MHC I epitope and the several OMV-associated T cell MHC II epitopes. This allows the concomitant binding on the same APC of the epitope-specific naive CD8+ T cells and of a population of effector CD4+ Th1 cells which can provide a strong stimulus for the vaccine-specific cytotoxic T cell activation.

On the other hand, there are two possible limitations in the use of OMVs for the production of epitope-specific CD8+ T cells. The first one is linked to the fact that OMV proteins carry their own MHC I epitopes and they can exert a "diluting" effect similar to the one described for CD4+ T cells. In other words, to elicit high frequencies of epitope-specific CD8+ T cells, the vaccine epitope has to be present at a concentration that dominates in the pool of all OMV-associated MHC I epitopes. The second possible limitation is the inefficiency with which OMV-associated CD8+ T cell epitopes can be presented on MHC I molecules. The mechanisms of cross-presentation remain to be fully elucidated and this is particularly true for the OMV-MHC I epitope vaccine. How and with which

efficiency the OMV-MHC I epitope vaccine is released from the endosome and processed by the immunoproteasome is basically unknown (figure 24).

In the attempt to better characterize the OMV-mediated activation of CD8+ T cells, we have investigated how different parameters determine the efficiency with which OMVs promote the production of epitope-specific cytotoxic T cells. In particular, we followed the influence of OMV and epitope concentrations, route of immunization and epitope/OMV formulations.

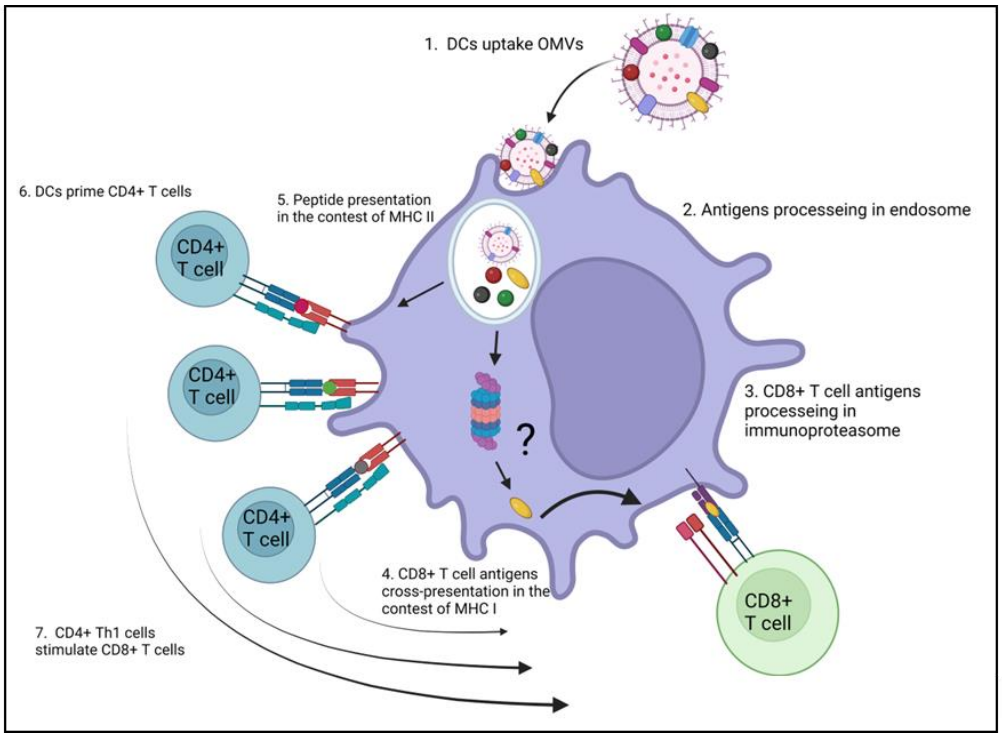

Figure 24. Cross-presentation by dendritic cells (DCs). *1) DCs uptake and internalize OMVs. 2) OMV antigens are processed in endosome. 3) OMV-MHC I epitopes are released from endosome and processed by immunoproteasome. 4) CD8+ T cell epitopes are released into cytosol where they are recognized by MHC I and transported to the surface of DCs to activate CD8+ T cells. 5) At the same time, MHC II recognizes OMV-MHC II epitopes and the CD4+ T cell epitope-MHC II complexes reach the membrane. 6) DCs activate a population of CD4+ T cells 7) which in turn help the activation of CD8+ T cells. Created with BioRender.com*

4.2.3.1 MHC I epitope selection

In order to study the CD8+ T cell responses by OMV immunization we selected three MHC I epitopes which could be conveniently used in murine models: $OVA_{257-264}$, SV40 $IV_{404-411}$ C411L and ADPGK.

OVA_{257}–SIINFEKL-$_{264}$ (OVA) is a CD8+ T cell epitope derived from the chicken ovalbumin protein and specific for the MHC I H2-K^b allotype expressed in C57BL6 mice. Kb-SIINFEKL is the most studied complex *in vitro* and *in vivo* thanks to its high immunogenicity and to the availability of

Kb-SIINFEKL specific reagents (*152*, *153*). Such reagents include B3Z hybridoma cells (*154*), OT-I TCR transgenic mice (*155*), and the 25D1.16 monoclonal antibody (*156*), which enables precise quantitation of complexes by flow cytometry. Immunization of C57BL6 mice with OVA synthetic peptide formulated with different adjuvants elicits high frequencies of OVA-specific T cells and therefore the epitope is an excellent model to follow the ability of OMVs to induce CD8+ T cells in a quantitative manner.

SV40 IV $_{404}$VVYDFLKL$_{-411}$ C411L (SV40) is an MHC I H2-K^{b} restricted epitope derived from the Simian Virus 40 (SV40) large tumor antigen. It has been extensively used to study T cell responses in the C57BL6 mouse model. For instance, C57BL6 mice immunized with B6/T116A1 cells express SV40 epitopes elicit around 8% frequencies of SV40-specific IFNγ^{+} CD8^{+} T cells (*157*, *158*).

ADPGK$_{ASMTNMELM}$ is an MHC I H2-D^{b} restricted neoepitope present in the murine MC38 tumor cell line and generated by an Arg>Met substitution (ASMTNRELM → ASMTNMELM) in the ADP-dependent glucokinase enzyme. C57BL6 mice immunized i.p. with poly(I:C) and 50 μg peptide showed IFNγ^{+}-CD8+ T cell frequencies of about 1% (*159*).

4.2.3.2 Epitope-specific CD8^{+} T cell responses elicited by OMVs

We previously showed that the immunization of C57BL6 mice with a mixture of OMVs and OVA and SV40 synthetic peptides elicited high frequency of OVA and SV40-specific CD8+ T cells (*149*). As a first experiment, we decided to expand our previous results by immunizing mice with 10 (Samine Jessica Isaac, PhD thesis, 2020) or 5 μg of OMVs mixed with 5 μg of OVA, SV40 or ADPGK. More specifically, groups of five C57BL6 mice were s.c. immunized twice one week apart and five days after the second immunization splenocytes were collected and epitope specific T cells were analyzed by either flow cytometry (for T cell analysis of OVA and SV40 T cells) or ELISpot (analysis of ADPGK T cells). As shown in Figure 25, the three vaccines induced epitope-specific T cells, the frequencies ranging from 0.5 % (ADPGK) to 4% and 11% (OVA and SV40, respectively) of total CD8+ T cells. The frequency of ADPGK-specific CD8+ T cells was estimated from the ELISpot analysis assuming that the CD8+ T cells in the spleen represent approximately 5-7 % of the total cell population.

The conclusions from the results described above is that the immunization with mixtures of OMVs and synthetic peptides corresponding to MHC I epitopes elicits epitope-specific CD8+ T cells. Although the comparison with published data has to be taken with caution, the frequencies

appear to be at least not inferior to what is described for the same epitopes using different adjuvants/vaccine formulations.

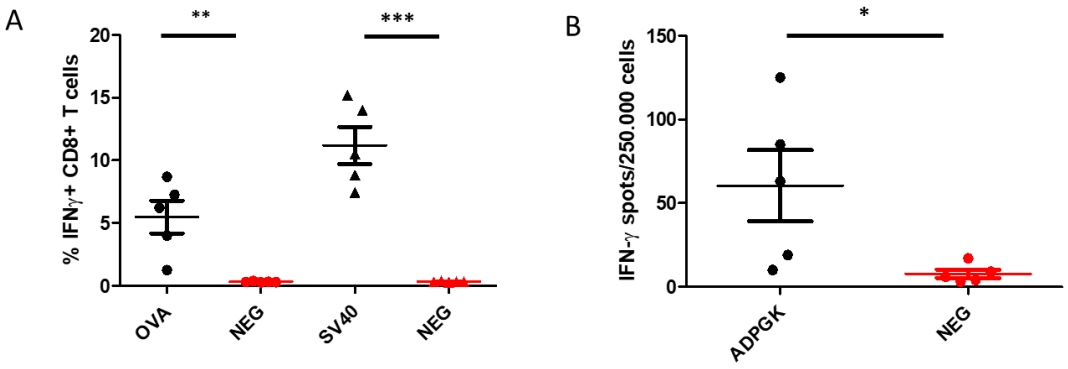

Figure 25. Frequencies/ number of peptide-specific CD8+ T cell obtained immunizing mice with OMVs$_{\Delta 60}$ absorbed with CD8+ T cell peptides. *Mice were immunized with 10 µg OMVs$_{\Delta 60}$ absorbed with 5 µg OVA or SV40 peptides and analyzed by FACS* ***(A)*** *(*Samine Jessica Isaac, PhD thesis, 2020*), or mice were immunized with 5 µg OMVs$_{\Delta 60}$ absorbed with 5 µg ADPGK peptide and analyzed by ELISpot* ***(B).*** *The mixtures were injected subcutaneously on day 0 and day 7. Splenocytes harvested on day 12 were re-stimulated with either 5 µg/ml of the vaccination peptides (Black) or 5 µg/ml of unreleated peptide (red). Statistical significance was calculated using an unpaired two-tailed T-test. Not significant (ns)= P>0.1; *= P<0.1; **= P<0.01; ***= P<0.001.*

4.2.3.3 Frequencies of epitope-specific CD8$^+$ T cells as a function of OMV and epitope concentrations

We next investigated the amount of OMVs (adjuvant) necessary and sufficient to elicit epitope-specific T cells in formulations constituted by mixtures of OMVs + synthetic peptides. This is an important question in consideration of the fact that, for safety reasons, the amount of adjuvants present in vaccines has to be carefully controlled and justified.

C57BL6 mice were immunized with three different formulations, each including 5 µg of OVA peptide and either 0.1, 1 or 10 µg of OMVs. Mice were immunized twice one week apart and OVA specific CD8+ T cells were counted by flow cytometry collecting the spleens five days after the second immunization. For such analysis the splenocytes were stimulated with 5 mg/ml of either OVA peptide or an irrelevant peptide as control. The data reported in Figure 26 show that all three formulations induced OVA-specific CD8+ T cells. While the repetition of the experiment is in progress to establish statistical significance using a larger number of animals, at this point in time it is fair to say that OMV concentrations ranging from 1 to 10 µg are sufficient to induce a saturating level of epitope-specific CD8+ T cell frequencies. This is a relevant piece of data,

considering that the reactogenicity studies carried out in our laboratories indicate that vaccine formulations containing up to 10 μg of *E. coli* OMVs are very well tolerated in mice.

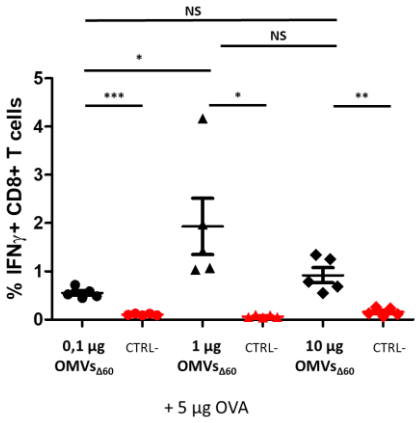

Figure 26. Percentage of epitope-specific IFNγ+ CD8+ T cells elicited vaccinating mice with OVA peptide + increasing amount of OMVs$_{\Delta 60}$. *Mice were injected subcutaneously on day 0 and day 7 and spleen were analized on day 12. Three groups of mice were immunized with 5 μg OVA peptide plus 0,1 μg, 1 μg, 10 μg of* OMVs$_{\Delta 60}$. *Splenocytes harvested on day 12 were stimulated with either 5 μg/ml of the OVA peptide (black) or 5 μg/ml of irrelevant peptide (red). Statistical significance was calculated using an unpaired two-tailed T-test. Not significant (ns)= P>0.1; *= P<0.1; **= P<0.01; ***= P<0.001* Samine Jessica Isaac, PhD thesis, 2020.

Next, we performed a dose-response experiment keeping the dose of OMVs fixed and testing different doses of peptides. More specifically, C57BL6 mice were immunized twice at one-week intervals with formulations containing 10 μg OMVs$_{\Delta 60}$ and increasing concentrations (0.5 μg, 5 μg and 50 μg) of either OVA or SV40 peptides. On Day 12, spleens were harvested and IFNγ^{+}CD8+ T cells were analysed by flow cytometry after splenocyte stimulation with the corresponding synthetic peptides (Samine Jessica Isaac, PhD thesis, 2020). As shown in Figure 26, 0.5 μg of both peptides were sufficient to elicit good frequencies of specific CD8+ T cells, frequencies that were essentially similar to those obtained with 5 μg of peptide. No further increase of OVA-specific T cells was observed when the formulations contained 50 μg of peptide. In fact, for both OVA and SV40 epitopes, the frequencies seemed to decrease with respect to the lower concentrations.

We repeated the same dose range experiment using the Adpgk peptide. In this case, the immunizations were done using 5 μg OMVs and the frequencies were analyzed by ELISpot. OMVs formulated with this peptide appeared to induce good activation frequencies of ADPGK-specific CD8+ T cells only when absorbed with 5μg of peptide.

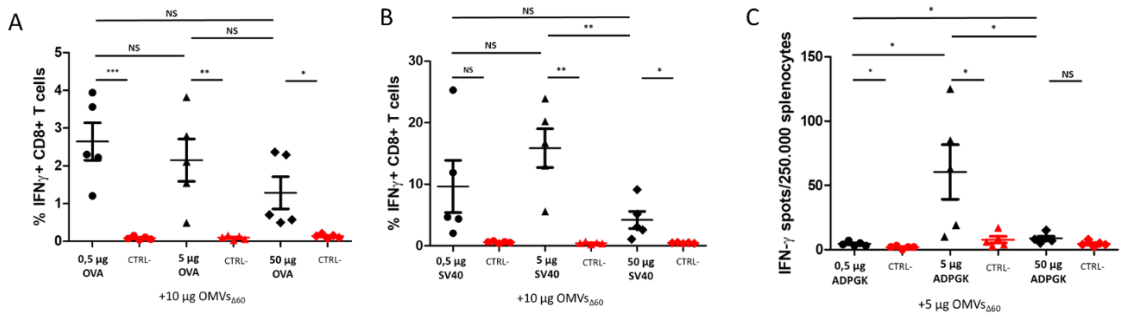

Figure 26. Percentage/number of epitope-specific IFNγ+ CD8+ T cells induced by immunization with OMVsΔ60 + increasing amount of epitopes. *0.5 µg, 5 µg, or 50 µg of epitope peptide (OVA in **A)** and SV40 in **B**) were added to 10 µg OMVs and 0.5 µg, 5 µg, or 50 µg of ADPGK epitope peptide **C**) was added to 5 µg OMVs. The mixtures were injected subcutaneously in CD1 mice on day 0 and day 7. On day 12, splenocytes were stimulated with either 5 µg/ml of peptide epitope (black) or 5 µg/ml of an irrelevant peptide as negative control (red). Statistical significance was calculated using an unpaired two-tailed T-test. Not significant (ns)= P>0.1; *= P<0.1; **= P<0.01; ***= P<0.001 (A and B* Samine Jessica Isaac, PhD thesis, 2020).

4.2.3.4 OVA-specific T cells induced by OMVs engineered with the OVA peptide

All experiments reported so far were carried out using formulations constituted by mixtures of OMVs and synthetic peptides. We next asked the question whether OMVs decorated with specific MHC I epitopes by genetic manipulation of the OMV-producing strain could also elicit epitope-specific T cells. From a theoretical standpoint, supported by several experimental evidences (*160–162*), since the physical association of the epitope to the adjuvant guarantees their co-delivery to the same APC, the frequencies of epitope-specific T cells elicited upon vaccination with engineered OMVs should be improved. On the other hand, to induce epitope-specific CD8+ T cells, phagocytosed OMVs have to enter the immune-proteasome degradation pathway and the engineered epitope has to be correctly processed and presented on the surface of the APC in the context of the MHC I molecules. The efficiency with which OMV-associated CD8+ T cell epitopes are "cross-presented" remains to be investigated.

To study the capacity of the engineered OMVs to elicit epitope-specific CD8+ T cells, we expressed the OVA peptide fused to the C-terminus of FhuD2, a protein that accumulates in the OMV membrane with high efficiency (*149*). More specifically, the peptide was fused in three copies, each copy separated from each other by a glycine-glycine flexible spacer (GG) (Michele Tomasi, PhD thesis, 2018). The construct was inserted in pET(21b+), generating the recombinant plasmid

(pET-LS-FhuD2-OVA3x). After transformation in the *E. coli* OMV-overproducing strain BL21(DE3)Δ60, OMVs were purified from the culture supernatant (see material and methods). As shown in Figure 27, the FhuD2-3x OVA fusion accumulated in the OMVs at a quantity that by densitometry analysis of the gel was estimated to be approximately 20% of total OMV proteins. Considering that the three copies of the OVA peptide account for approximately 10% of the fusion protein, we estimated that 5 μg of engineered OMVs included approximately 0.1-0.15 μg of OVA peptide.

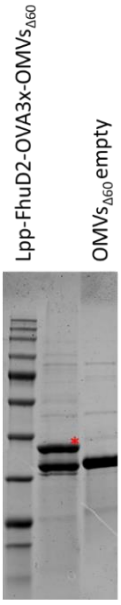

Figure 27. OMVs$_{\Delta60}$ were efficiently engineered with heterologous epitopes. SDS-PAGE analysis of 10 μg of OMVs purified from BL21(DE3)Δ60, Lpp-FhuD2-OVA3x-OMVs$_{\Delta60}$. OMVs purified from BL21(DE3)Δ60 strain transformed with the empty pET vector ("Empty" OMVs) were used as a control. Recombinant fusion protein is indicated by a red star.

Based on the above calculations, groups of C57BL6 mice were s.c. immunized with either 5 μg of FhuD2-3xOVA-OMVs$_{\Delta60}$ or 5μg empty OMVs$_{\Delta60}$ + 0.15 μg of OVA peptide on day 0 and day 7. On Day 12, OVA-specific CD8+ T cells were analyzed not only in the spleen but also in the blood and in the draining lymph node by ELISpot assay. From the data reported in Figure 28, we can draw two conclusions. First, OVA-specific T cells could be detected at appreciable frequencies in all three compartments, including blood. This is of interest since it is conceivable to believe that the effector CD8+ T cells elicited by the vaccines could potentially be disseminated in most tissues/organs. Second, the two formulations performed essentially in a similar manner. We are currently confirming this result using other epitopes, such as SV40 and ADPGK.

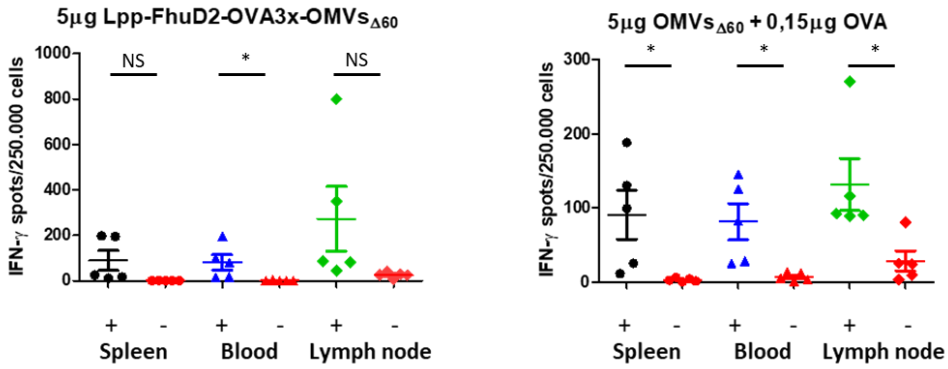

Figure 28. Immunizations with OMVs$_{\Delta 60}$ engineered with OVA3x and OMVs$_{\Delta 60}$ "absorbed" with the same amount of OVA peptide administered s.c. elicits peptide-specific IFNγ$^+$-CD8$^+$ T cell*. C57BL/6 mice were immunized with 5 µg of Lpp-FhuD2-OVA3x-OMVs$_{\Delta 60}$ and 5 µg OMVs$_{\Delta 60}$ absorbed with 0,15 µg of OVA peptide administered s.c.. After 5 days the second immunization, splenocytes/PBMC/ draining lymph nodes were collected from each mouse and peptide-specific IFNγ$^+$-CD8+ T cells were counted by ELISpot, after stimulation with the OVA peptide (black=spleen, blue=PBMC, green=lymph node) or an irrelevant peptide (red).*

4.2.4 CONCLUSIONS

While it has been demonstrated that OMVs can elicit CD8+ T cells, the efficiency with which they do so and how they compete with other adjuvants has been poorly investigated so far.

As already pointed out, the use of OMVs as an adjuvant/delivery system for cytotoxic CD8+ T cell responses might have potential advantages and drawbacks that need to be investigated. In particular, it is conceivable to believe that the efficiency of OMV-mediated CD8+ T cell responses is governed by two main mechanisms with possible opposite outcomes: co-presentation of MHC II and MHC I epitopes on APCs, and cross-presentation. From the one hand, once the OMV vaccine is phagocytosed, both the MHC I epitope(s) and the many OMV-associated MHC II epitopes will be presented on the surface of the same APC. This should guarantee a potent stimulation of effector CD8+ T cells mediated by a large population of CD4+ Th1 cells (see figure 25). On the other hand, the cross-presentation process with which the phagocytosed OMV vaccine is released into the cytoplasm and enters the MHC I presentation pathway might be too inefficient to promote a robust production of effector cytotoxic T cells.

To dissect the two opposite mechanisms, we first immunized animals with mixtures of OMVs and synthetic peptide epitopes. In so doing, once OMVs and peptides are simultaneously taken up by the same APC, the MHC I epitope can be cross-presented independently from the OMVs and therefore it should be promptly transported into the ER and loaded onto MHC I. In other words, should the OMV cross-presentation process be inefficient, this should not affect the cross-presentation of the MHC I epitope. It has to be pointed out that an alternative mechanism, with

which the synthetic epitope can be presented on the MHC I molecules of APCs, envisages its direct binding to MHC I molecules after the displacement of peptides bound with lower affinity. This mechanism is theoretically possible considering that the synthetic peptides used in a number of our experiments have the exact amino acid composition and length of the described OVA, SV40 and ADPGK epitopes.

Our data demonstrate that when OMVs and synthetic MHC I epitopes are mixed together, epitope-specific CD8+ T cells are induced with high efficiency. For all three model epitopes used in this study (OVA, SV40 and ADPGK), $\leq$5 μg of peptide were sufficient to elicit epitope-specific cytotoxic T cells with frequencies that, for the highly immunogenic OVA and SV40 epitopes, could be as high as 5 - 10% of total CD8+ T cells. To the best of our knowledge such frequencies are at least as good as those obtaining mixing 50 μg of the same peptides with other adjuvants, such as CpG and Poly I:C.

Our data also show that 5 μg of OMVs are more than enough to elicit saturating levels of peptide-specific cytotoxic T cells. In fact, in a particular experiment using OVA + OMV mixtures, 1 μg of OMVs gave excellent OVA-specific CD8+ T cell frequencies. These results are particularly relevant for future clinical studies in that they suggest that the use of low dosages of OMVs would avoid potential reactogenic reactions without impairing their adjuvanticity.

To investigate the efficiency of OMV cross-presentation, we engineered the OMVs with the OVA peptide and after immunization with 5 μg of OVA-OMVs, we followed the presence of OVA-specific CD8+ T cells in the spleen, in the draining lymph node and in blood. The engineering of OMVs was carried out by fusing three copies of the OVA epitope to the C-terminus of FhuD2, a protein we routinely use as a carrier to decorate OMVs with foreign polypeptides. Our data show that good frequencies of OVA-specific T cells could be measured in all three analyzed compartments. Our quantitative analysis of the OVA peptide in the engineered OMVs indicate that 5 μg of OMVs (the dose used for immunization) contain approximately 0.1-0.15 μg of OVA. Therefore, we compared the frequencies of OVA specific T cells elicited by OVA-OMVs with the mixture of 5 μg of OMVs and 0.15 μg of synthetic OVA peptide. Interestingly, the frequencies were comparable, suggesting that the cross-presentation of OMVs could be more efficient than what we originally thought. Should this result be confirmed with other MHC I epitopes, this should facilitate the development of OMV-based vaccines aiming at eliciting cytotoxic T cell responses.

5 FINAL CONCLUSIONS

The growing interest in the exploitation of OMVs as a vaccine platform can be appreciated by looking at the number of OMV-related publications. As of March 2023, the interrogation of the PubMed database using "OMVs [and] vaccines" as key words, results in a total of 950 papers retrieved, most of them published in the last 10 years. Such publications report the capacity of OMV-based vaccines to elicit protective immune responses against several bacterial and viral pathogens and against cancer.

However, only a few of the published papers (see Table 5) try to investigate, in quantitative terms, the conditions necessary to elicit optimal immune responses against specific antigens present in the OMVs. Such information is instrumental to establish the real potential of the OMV platform, considering the existence of several alternative platforms against which OMVs have to compete.

With this work we provide a thorough analysis of both the humoral and cell-mediated immune responses induced by OMVs. The data, which we believe are of particular interest for the scientific community working in the field, strengthen the attractive features of the OMVs and justify their use as prophylactic and therapeutic vaccines.

In essence, we show that minute amounts (1-5 μg) of OMVs decorated with a specific antigen are sufficient to elicit high titers of antigen-specific antibodies, as long as the antigen represents around 4-5% of the total OMV proteins. Considering that our strategies for OMV engineering usually result in the expression of the heterologous antigens at concentrations well above 10%, this makes the OMV platform particularly attractive. In this respect it is important to keep in mind that a number of commercially available vaccines (pertussis, meningococcus, HBV, HPV) are formulated with substantially higher amounts of antigens and adjuvants.

Moreover, our data described for the first time the conditions necessary to induce effective cell-mediated immunity. Considering that OMV vaccines have been shown to be protective in a number of tumor mouse models, our results are of paramount importance to optimally design novel cancer vaccines.

Overall, thanks to the results of this study our laboratory have demonstrated the effectiveness of OMV-based vaccines against a number of different pathogens and such vaccines are now ready to move the development phase.

	OMVs producing strain	Heterologous antigen(s)	Pathogen	Type of OMV-antigen association	Type of immune response analyzed	Ref
1	*E. coli*	ClyA-GFP	Proof of concept	Engineered: external (5%)	Antibody production	125
2	*E. coli*	S. aureus antigens (LukE, Hla, SpA, FhuD2, Csa1)	*Staphylococcus aureus*	Engineered: external (LukE and Hla: 2%-10%; SpA, FhuD2, Csa1: 10-20%)	Antibody production	121
3	*E. coli*	ClyA-Omp22	*Acinetobacter baumannii*	Engineered: external (1%)	Antibody production	130
4	*E. coli*	GAS/GBS antigens (Slo, SpyCEP, Spy, SAM)	Group A/B Streptococcus	Engineered: luminal (Slo: 5%, SpyCEP: 10%, Spy: 0.5%-1%, SAM: 0.5%)	Antibody production	96
5	*E. coli*	HtrA	*Chlamydia muridarum*	Engineered: luminal (0.16%-0.2%)	Antibody production	126
6	*S. enterica*	PspA	*Streptococcus pneumoniae*	Engineered: luminal (0.8%)	Antibody production	129
7	Salmonella	Hbp-PspA	*Streptococcus pneumoniae*	Engineered: external	Antibody production	131
8	*S. typhimurium* GMMA	fHbp	*N. meningitidis, Salmonella*	Engineered: luminal (3.9% in OAg-/2.2% in OAg+); Conjugated: external (11.2% in OAg+ conjugate/19.8% in OAg- conjugate)	Antibody production	(*163*)
9	*B. thetaiotaomicron*	F1, V	*Yersinia pestis*	Engineered: luminal (V antigen, 15 µg/mL total protein); Engineered: external (F1 antigen, 10	Antibody production	(*164*)

				µg/mL total protein)		
10	*S. typhimurium* GMMA	Hemagglutinin (HA)	Influenza A virus	Conjugated: external (19.8%); mixed	Antibody production	(*165*)
11	*S. typhimurium* GMMA	Rabies virus glycoprotein (RABVG)	Rabies virus	Conjugated: external (25%); mixed	Antibody production	(*165*)
12	*N. meningitidis*	HexaPro Spike	SARS-CoV-2 virus	Conjugated: external	Antibody production	(*166*)
13	*E. coli*	Influenza antigens (A/California/7/2009 (H1N1), A/Victoria/361/2011 (H3N2), and B/Massachusetts/2/2012)	Influenza A virus	Mixed	Antibody production	(*167*)
14	*E. coli*	H1N1 HA	Influenza A virus	Mixed	Antibody production	(*168*)
15	*N. meningitidis*	PhtD and PhtD-C	*Streptococcus pneumoniae*	Mixed	Antibody production	(*169*)
16	*E. coli* ClearColi	ClyA-M2e	Influenza A virus	Engineered: external (6%)	Antibody production	(*170*)
17	*S. typhimurium*	SaoA	*Streptococcus suis*	Engineered: external (10%)	Antibody production	(*171*)
18	*E. coli*	CPS14	*Streptococcus pneumoniae*	Engineered: external (1.3%)	Antibody production	(*172*)
19	*N. meningitidis*	RBD dimer	SARS-CoV-2 virus	Mixed	Antibody production	(*173*)
20	*E. coli*	RBD and core peptide NG06	SARS-CoV-2 virus	Engineered: luminal (RBD: 5%) external (ClyA-NG06: 0.5%)	Antibody production	(*174*)
21	*N. meningitidis*	PD5	Dengue virus	Mixed	Antibody production	(*175*)
22	*N. meningitidis*	Protein D	*Haemophilus influenzae*	Mixed	Antibody production	(*176*)
23	*E. coli* Nissle 1917	ClyA-Apxr	*Actinobacillus pleuropneumoniae*	Engineered: external (0.6%)	Antibody production	(*177*, *178*)
24	*N. meningitidis*	RBD	SARS-CoV-2 virus	Mixed	Antibody production	(*178*)
25	*S. parathyphi GMMA*	Vi, 0:2 O	*Salmonella serovars*	Engineered: external (9,6%)	Antibody production	(*179*)

26	*E. coli*	EsxA, Sbi, SpAKKAA	*Staphylococcus aureus*	Mixed	Antibody production	(*180*)
27	*Escherichia coli* W3110 ΔmsbB/ΔpagP	Influenza antigens (A/California/7/2009 (H1N1), A/Victoria/361/2011 (H3N2), and B/Massachusetts/2/2012)	Influenza A virus	Mixed	Antibody production	(*118*)
28	*S. typhimurium*	RBD	SARS-CoV-2 virus	Mixed	Antibody production	(*181*)
	OMVs producing strain	**Heterologous epitope**	**Tumor**	**Type of OMV-antigen association**	**Type of immune response analyzed**	**Ref**
29	*E. coli* DH5α	BFGF	TC-1 skin tumor model	Engineered: luminal, mixed	Autoantibodies production	(*182*)
30	*E. coli* Nissle, *E. coli* BL21(DE3)Δ*omp A*	SV40 epitope (VVYDFLKL), OVA (SIINFEKL)	B16F10 melanoma model	Engineered: external	CD8+ T cells	(*149*)
31	*E. coli* BL21(DE3)ΔompA	EGFRvIII, M30	B16F10 melanoma model	Engineered: external	CD4+ and CD8+ T cells	(*95*)
32	*E. coli* DH5α	HPV16E7	TC-1 skin tumor model	Engineered, mixed	CD4+ and CD8+ T cells	(*183*)
33	*E. coli*	OVA, Adpgk	B16F10 melanoma model, MC38 CRC model	Conjugated, mixed	CD4+ and CD8+ T cells	(*184*)
34	*E. coli*	OVA, Adpgk	B16F10 melanoma model	Engineered: external, mixed	CD4+ and CD8+ T cells	(*132*)

Table 5. State of the Art Research on OMV-based vaccines

6 Materials and methods

6.1 Animals

Female BALB/C, CD1 and C57BL/6 mice between 6–8 weeks of age were purchased from Charles River laboratories. All Mice were treated in accordance with the Animal Ethical Committee of The University of Trento and the Italian Ministry of Health. Mice were monitored once a day in order to evaluate any signs of stress such as respiration rate, posture, and loss of weight (more than 20%) according to humane endpoints. Mice showing such conditions were anesthetized and subsequently sacrificed in accordance with experimental protocols.

6.2 Mice immunization

CD1 mice were intraperitoneally immunized three times (0-14-28) with different concentrations of $OMVs_{\Delta OmpA}$ and $OMVs_{\Delta 60}$ empty or engineered with FhuD2, HlA or SpA_{KKAA} antigens formulated alone or in presence of 2 mg/ml Alum hydroxide as adjuvant in a final 200µl solution containing phosphate-buffered saline (PBS)(Gibco) to arrive at volume. Mouse sera were collected after 10 days of the last immunization. Instead for T cell generation, BALB/C or C57BL/6 mice were immunized two or three times per intraperitoneal, subcutaneous or intramuscular routes with different peptide and OMV, derived from *E. coli* BL21(DE3)ΔOmpA or Δ60 strains, concentrations using different schedule of immunizations as described previously (0-7-12; 0-7-14-19). Mice immunized intraperitoneally received a final volume of vaccine of 200ul, instead mice immunized subcutaneously 100 µl and mice immunized intramuscularly 50 µl of vaccine. Mice immunized per subcutaneous and intramuscular routes were sedated.

6.3 Bacterial strains and culture conditions

In this work were used four *E. coli* strains: *E. coli* BL21(DE3)ΔOmpA, *E. coli* BL21(DE3)Δ60, *E. coli* HK100 and *E. coli* BL21(DE3). These *E. coli* strains were grown in Luria-Bertani (LB) broth medium

(Sigma 25g/L) at two different temperatures at 37°C or 30°C with shaking at 200 RPM, adding Ampicillin when required at the final concentration of 100 μg/mL. Depending on the need the *E. coli* strains were grown in different LB volumes, 50 ml, 500ml or in 2L in fermenter. For fermentation processes, bacteria grew at temperature 30°C until optical density at 600nm (OD_{600}) 0.5, then growth continued at 25°C; pH 6.8 (±0.2), dO2 > 30%; rpm 280-500 rpm; antigen induction at OD600 1.0 by adding 0.1 mM IPTG, 1 mL ampicillin (100 mg/mL).

6.4 Chemically competent cells preparation

E. coli BL21(DE3)ΔOmpA, *E. coli* BL21(DE3)Δ60, *E. coli* HK100 and *E. coli* BL21(DE3) stocked in glycerol were inoculated in 5 mL of sterile LB medium and incubated overnight at 37°C while shaking 200rpm. The precultures were diluted to OD_{600} 0.1 in 100mL medium until they reach an OD_{600nm} of 0.5-0.7. The pellet was collected with a centrifugation of the culture at 4 °C, 2500 x g for 20 minutes. The supernatant was discarded and the pellet was gently resuspended in 10 mL of cold sterile 100mM MgCL2 on ice for 30 minutes. After another centrifugation at 2500xg for 20 minutes at 4 °C, discarded the supernatant, the pellet was resuspended in 2mL of a cold sterile solution of 100mM CaCl2 in 15% glycerol. The aliquots were rapidly frozen at -80°C.

6.5 Plasmids construction using the Polymerase Incomplete Primer Extension (PIPE)

The Polymerase Incomplete Primer Extension (PIPE) method was used to insert all *E. coli* BL21(DE3) genes described in this thesis project in pET15. The PIPE method is based on the observation that normal PCR amplifications result in mixtures of products which are not fully double stranded (Table 6). This technique combines cloning and mutagenesis. In fact DNA polymerase, once the primer is recognized, duplicate sequences starting 15-20 nucleotides further downstream from the start site. 5’ ends segment on the synthetic amplification of the primers overlap with the unpaired 5’ ends on the PCR products. In fact the primers that recognize the insert PCR (I-PIPE) are designed with the first 19 bases on the 5’ ends that overlap with 5’ ends to the vector PCR (V-PIPE). In this way it is possible to introduce sequences in the terminal portion of these incomplete fragments to build an hybrid consisting of vector and insert. At this point,

enzyme manipulation is not needed. V-PIPE and I-PIPE products are transformed in HK100 *E.coli* recombinase chemical competent strain and plated overnight on LB-Agar.

To eliminate DNA not duplicated with PCR cycle, it is necessary to add 1µl of DpnI (Thermofisher scientific) a restriction enzyme.

In Table 7 are represented the list of primers designed and applied for the construction of plasmids, in table 8 the list of plasmids used in this work and in table 9 the recombinant proteins features exploited in this work

PCR Mix	
Q5 polymerase	0,25µl
5x buffer	5µl
forward primer (10mM)	1,25µl
Reverse primer (10mM)	1,25µl
DNA	1ng
dNTPs	0,5µl
H2O	up to 25µl

PCR program		
Initial denaturation	98°C x 30 sec	
Denaturation	98°C x 10 sec	35x
Annealing	*°C x 30 sec	
Extension	72°C x *sec	
Final extension	NO EXTENSION	

Table 6. PCR protocol

Code	Description	Sequence (5'-3')
Primers for Vector (V) PIPE PCR		
EK-1084	Primer forward V-PIPE pET15b	GCCCTGGAAGTACAGGTTTTC
EK-1085	Primer reverse V-PIPE pET15b	CGCGACTTAATTCTAGCATAAC
Primers for Insert (I) PIPE-PCR ("flap"- primer)		
EK-1486	Primer forward I-PIPE BamA	ctgtacttccagggc GCTGAAGGGTTCGTAGTGAA
LC-1498	Primer reverse I-PIPE BamA	tagaattaagtcgcg TTACCAGGTTTTACCGATGTTAAAC
i-f-cpoB	Primer forward I-PIPE CpoB	ctgtacttccagggc CAGGCACCAATCAGTA
i-r-cpoB	Primer reverse I-PIPE CpoB	tagaattaagtcgcg TTACATCGCGTTCAGACGT
EK-1486	Primer forward I-PIPE FhuE	ctgtacttccagggc GCACCAGCCACTGAAGAAAC
LC-1499	Primer reverse I-PIPE FhuE	tagaattaagtcgcg TCAGAATTGATACGTGCCGGTAA
i-f-fkpA	Primer forward I-PIPE FkpA	ctgtacttccagggc ATGAAATCACTGTTTAAAGTAACGC

i-r-fkpA	Primer reverse I-PIPE FkpA	tagaattaagtcgcg TTATTTTTTAGCAGAATCTGCGGC
i-f-glpQ	Primer forward I-PIPE GlpQ	ctgtacttccagggc ATGAAATTGACGCTGAAAAACC
i-r-glpQ	Primer reverse I-PIPE GlpQ	tagaattaagtcgcg TTACTCTTTATTAAGGAATTTTACTGCC
EK-1482	Primer forward I-PIPE HisJ	ctgtacttccagggc GCGATTCCGCAAAACATCCG
LC-1494	Primer reverse I-PIPE HisJ	tagaattaagtcgcg TTAGCCACCATAAACATCAAAATCGAAG
EK-1484	Primer forward I-PIPE LpoA	ctgtacttccagggc TGTGGCACCCATACTCCCGA
LC-1496	Primer reverse I-PIPE LpoA	tagaattaagtcgcg TTAACTGACGGGGACTACCTGACC
i-f-malE	Primer forward I-PIPE MalE	ctgtacttccagggc ATGAAAATAAAAACAGGTGCACGC
i-r-malE	Primer reverse I-PIPE MalE	tagaattaagtcgcg TTACTTGGTGATACGAGTCTGC
EK-1479	Primer forward I-PIPE OmpF	ctgtacttccagggc GCAGAAATCTATAACAAAGATGGCA
LC-1491	Primer reverse I-PIPE OmpF	tagaattaagtcgcg TTAGAACTGGTAAACGATACCCAC
i-f-oppA	Primer forward I-PIPE OppA	ctgtacttccagggc ATGACCAACATCACCAAGAGAAG
i-r-oppA	Primer reverse I-PIPE OppA	tagaattaagtcgcg TTAGTGCTTCACAATGTACATATTCCG
i-f-pal	Primer forward I-PIPE PaL	ctgtacttccagggc TGTTCTTCCAACAAGAA
i-r-pal	Primer reverse I-PIPE PaL	tagaattaagtcgcg TTAGTAAACCAGTACCGCA
EK-1485	Primer forward I-PIPE RlpA	ctgtacttccagggc TGTACAAGCGATGATGGTCAG
LC-1497	Primer reverse I-PIPE RlpA	tagaattaagtcgcg CTACTGCGCGGTAGTAATAAATGA
EK-1489	Primer forward I-PIPE SurA	ctgtacttccagggc GCCCCCCAGGTAGTCGATAA
LC-1501	Primer reverse I-PIPE SurA	tagaattaagtcgcg TTAGTTGCTCAGGATTTTAACGTAGGC
EK-1483	Primer forward I-PIPE YbiS	ctgtacttccagggc GTAACTTATCCTCTGCCAACCG
LC-1495	Primer reverse I-PIPE YbiS	tagaattaagtcgcg TTAATTCAGACGAACCGGCATC
i-f-ydcL	Primer forward I-PIPE YdcL	ctgtacttccagggc ATGCGTACCACATCATTTGC
i-r-ydcL	Primer reverse I-PIPE YdcL	tagaattaagtcgcg CTACTTTTTGTTAACGTCAAACATGG
EK-1490	Primer forward I-PIPE YncE	ctgtacttccagggc GCAGAAGAAATGCTGCGTAAAG
LC-1502	Primer reverse I-PIPE YncE	tagaattaagtcgcg TTACAGCGCAATACGAATCACAT
Primers for screenings		
LC-1507	Primer reverse colony PCR	CTGAATACTGATTTTCTGTG

CI-1004	(T7P) Primer forward sequencing	CCCTATAGTGAGTCGTATTA
CI-1005	(T7T) Primer reverse sequencing	GCTAGTTATTGCTCAGCGG

Table 7. List of primers designed and applied for the construction of plasmids through V-PIPE and I-PIPE, for the expression of N-terminal His-tagged recombinant proteins*. Primers used to perform colony PCR screenings and next-generation sequencing are indicated at the bottom of the table.*

Plasmid name	**Source**
Plasmids for production of engineered OMVs	
pET21b(+)_lpp_FhuD2	In-house (*123*)
pET21b(+)_lpp_Hla_{H35L}	In-house (*123*)
pET21b(+)_lpp_SpA_{KKAA}	In-house (*123*)
Plasmids for expression of E. *coli* recombinant proteins	
pET15b_His_6_TEV_BamA	Cloned
pET15b_His_6_TEV_CpoB	Cloned
pET15b_His_6_TEV_FhuE	Cloned
pET15b_His_6_TEV_FkpA	Cloned
pET15b_His_6_TEV_GlpQ	Cloned
pET15b_His_6_TEV_HisJ	Cloned
pET15b_His_6_TEV_LpoA	Cloned
pET15b_His_6_TEV_MalE	Cloned
pET15b_His_6_TEV_OmpF	Cloned
pET15b_His_6_TEV_OppA	Cloned
pET15b_His_6_TEV_PaL	Cloned
pET15b_His_6_TEV_RlpA	Cloned
pET15b_His_6_TEV_SurA	Cloned
pET15b_His_6_TEV_YbiS	Cloned
pET15b_His_6_TEV_YdcL	Cloned
pET15b_His_6_TEV_YncE	Cloned

Table 8. List of plasmids used in this work*. Plasmids for production of strains producing engineered OMVs were cloned as described in Irene et al. 2019* (*123*).

Protein	Compartment	Solubility	MW [kDa]	pI	Molar extinction coefficient (ε)	Buffer A and B pH
BamA*	OM	insoluble	88,4	4.9	140165	7.6
CpoB*	PP	soluble	28.2 (25.4)	8	21890	7.6
FhuE*	OM	insoluble	77,4	4.5	143590	7.6
FkpA	PP	soluble	26,2	6.7	15930	7.6
GlpQ	PP	soluble	40,8 (38.2)	5.4 (5.2)	51800	7.2
HisJ	PP	soluble	26,2	5.2	17545	7.6
LpoA*	LP	soluble	70	5.1	67505	7.6
MalE	PP	soluble	40,7	5.6 (5.2)	66350	7.2
OmpF*	OM	insoluble	37	4.6	54210	7.6
OppA	PP	soluble (partially)	58,4	6 (5.85)	113345	7.2
Pal*	LP	soluble	18.8 (16.6)	5.6 (6.1)	11920	7.6
RlpA*	LP	soluble	35,7	5.1	16055	7.0
SurA*	PP	soluble	45	6.1	29450	7.6
YbiS	PP	soluble (partially)	30,8	5.6	27390	7.6
YdcL	LP	soluble	22,4	6.8	22920	7.6
YncE*	PP	soluble	35,3	8.8	28420	7.0

Protein	Solubility	MW [kDa]	pI	Molar extinction coefficient (ε)	Buffer A and B pH
Hla_{H35L} (Hla)	Soluble	30	8.2	64860	6.3
FhuD2	Soluble	34	9.7	55350	7.6
SpA_{KKAA} (SpA)	Soluble	28.3	4.6	5960	7.6

The table 9 summarizes the recombinant protein features exploited in this work*. Proteins specified with the asterisk are present in the original strain E. coli BL21(DE3) ΔompA and not deleted in the E. coli BL21(DE3) Δ60 strain. Information on protein compartment was retrieved from UniProt (The UniProt Consortium UniProt: the universal protein knowledgebase in 2021). Protein solubility was evaluated with the B-PER® protein solubility test. Molar extinction coefficient, isoelectric point (pI) and molecular weight were predicted with the EMBOSS-Pepstats tool (EMBL-EBI).*

6.6 Colony PCR and sequencing

Colony PCR allows checking the insertion of I-PIPE in the plasmid. Two primers were drawn, T7 promoter and another specific primer on the insert gene sequence. The reaction mixture is

performed by Taq DNA polymerase, dNTPs and primers, 1x buffer. The presence of a band to the correct molecular weight in the agarose gel, confirms the good success of cloning (agarose gel (1,5%) in TAE 1X buffer at 120 Volts). The positive colonies were incubated ON in agarose gel and plasmids were extracted with QIAprep Spin Miniprep Kit (QIAGEN). All plasmids were sequenced by Eurofins genomics using mix2seq kit. The results of sequencing were analyzed using the benchling software. Finally, *E. coli* BL21 (DE3) were transformed with correct pET15-HIS6x-gene plasmids.

6.7 Purification of recombinant *E. coli* BL21 (DE3) Proteins

Recombinant *E. coli* BL21 (DE3) Proteins were purified using the TEV protease purification strategy (cesaratto et al.,2016) . As described before, the synthetic *E. coli* BL21 (DE3) genes, amplified by I-PIPE, were fused at their 5' to the sequence coding for His 6 -tag and the TEV cleavage site and regulated by T7 inducible promoter. 0,8-1,6 g of bacterial biomass were resuspended in 10 ml of lysis buffer A (500 mM NaCl, 10 mM NaH_2PO_4, variable pH 6-8) with the addition of 0.2 mM phenylmethansulfonyl fluoride solution protease inhibitor (PMSF) (Sigma life science), sonicated 5 time for 30" at 4°C and the total cell lysate was finally centrifuged (15.000 x g, 30 min, 4°C). The supernatant was accurately filtered (0.22 µm) using an ÄKTA purifier System (GE) and was applied to Ni-affinity chromatography (IMAC) and a 5 ml HiTrap IMAC column (GE) monitoring absorbance at 280 nm. The column washing was performed at concentration of 50 mM imidazole (10%). Proteins bound to column were eluted using a linear gradient by increasing buffer B (500 mM NaCl, 10 mM NaH_2PO_4, 500 mM imidazole, pH 6-8) from 10% to 100% (the only concentration that changes is imidazole). Several fractions, containing the His-tagged recombinant proteins, were produced and were dialyzed at 4°C against buffer A and then digested with Tobacco Etch Virus Protease (TEV) (1 mg per 100 mg protein) with the addition of 5 mM β-mercaptoethanol. TEV-digested proteins pool were applied to Ni-affinity chromatography and the flow-through, containing the untagged recombinant proteins, were applied to a final polishing step by size-exclusion chromatography using a HiLoad 16/600 Superdex 75 pg column.

6.8 Western blot

1µg or 1.5 µg $OMVs_{\Delta OmpA}$ and decreasing protein concentrations were resuspended in Laemmli buffer and were boiled at 95 °C. Than samples were run in (SDS-PAGE) and transferred on nitrocellulose membrane (iBlot™ Transfer Stack, nitrocellulose, Thermo Fisher Scientific) with iBlot® Dry Blotting System (Thermo Fisher Scientific). Blocking was done at RT for 30 minutes by shaking using 10% skimmed milk (Serva) dissolved in TPBS. Membranes were treated with serum of mice immunized with 10µg of $OMVs_{\Delta OmpA}$ serum (in black in the image) or serum of mice immunized with 10µg recombinant protein + alum (in blue in the image) resuspended in TPBS 3% skimmed milk at final concentration of 1:1000, ON at 4°C. After three washing steps of 5' in PBST, polyclonal rabbit anti-mouse immunoglobulins (Dako) were resuspended in TPBS 3% skimmed milk at the final concentration of 1:2000 and used to treat membranes for 45 minutes. Subsequently, 2 washes were done of 5' in PBST and 1 wash of 5' in PBS, antibody binding was detected by using the Amersham™ detection Kit (Cytiva) at ChemiDOC (BioRAS) instrument. To allow some bands to develop, many membranes were clipped. In this way, the intensity of the protein bands adjacent to the one of interest did not disturb its proper development.

Protein name	Loaded protein quantity
FkpA	65 ng
GlpQ	6 µg
MalE	350 ng
OppA	70 ng
YbiS	4,5 µg
HisJ	5 µg
YdcL	5 µg
CpoB	4µg
LpoA	300 ng
PaL	6µg
RlpA	800 ng
SurA	400 ng
YncE	150 ng
BamA	70 ng
OmpF	6 µg
FhuE	1,5 µg

Table 9 shows protein and quantity loaded on western blot

6.9 pET-FhuD2-OVA generation

50 µl of chemically competent cells were mixed with 100 ng of purified pET21B plasmid containing the gene of interest. Vials were incubated on ice for 30 minutes. Subsequently, cells were heat-shocked at 42°C for 45 seconds and immediately transferred on ice for 2 minutes. The cells were

moved in a volume of 950 µL of LB broth at 37°C and shaking at 200 rpm and subsequently 800 µl and 150 µl were plated overnight on LB-Agar (Luria/Miller) plate (Carl Roth), containing Ampicillin at final concentration of 100 µg/mL.

pET-Sta6-OVA3x generation plasmid assembly was made by the polymerase incomplete primer extension (PIPE) method. Full sequence of FhuD2 was previously cloned in our laboratory in the pET21b+ plasmid. The synthetic gene encoding for 3 copies of $OVA_{257-264}$ epitope with its flanking sequences separated by a glycine-glycine flexible spacer was purchased from GeneArt® Gene Synthesis (LifeTechnologies). The sequence of the construct is as follows: 5'-CAGCTGGAAAGCATTATTAACTTTGAAAAACTGACCGAAGGTGGTCAGCTGGAAAGCATTATTAACTTTG AAAAACTGACCGAAGGTGGTCAGCTGGAAAGCATCATCAACTTCGAAAAACTGACCGAA-3'. To clone OVA to pET-FhuD2 and obtain the fusion protein FhuD2-OVA, pET-FhuD2 was linearized, OVA construct was amplified with OVA-F and OVA-R primers. Subsequently, PCR products were mixed together and plasmids were used to transform *E. coli* HK100 competent cells, obtaining pET-FhuD2 -OVA.

6.10 OMVs purification and SDS-PAGE

E. coli BL21(DE3)ΔompA and *E. coli* BL21(DE3)Δ60 strains were engineered with pET21b+ plasmid encoding the genes of interest. Recombinant clones were grown at 37°C and 200 rpm in LB medium starting OD_{600} = 0.1 and, when the strains reached an OD_{600} of 0,5-0,7 protein expression was induced by addition of 0.1 mM IPTG (Sigma-Aldrich). Bacterial growth was stopped after 4 hours of culture by a centrifugation at 5,000 x g for 20 minutes at 4 °C. Supernatants were filtered through a 0.22 µm pore size filter (Millipore). Medium containing OMVs was centrifugated in high-speed centrifugation (200,000 x g for 2 hours). After being pelleted, OMVs were resuspended in variable concentration of PBS (1x) 50ul or 500ul and were filtered through a 0.22 µm pore size filter (Millex, syringe-driven filter unit). Absorbance of OMV proteins was measured through nanodrop spectrophotometer (280nm). Absorbance value was used to determine OMV protein concentration using DC protein assay (Bio-Rad). To separate OMV proteins, 10 µg of OMVs (protein content) and Laemmli buffer 1x were heated at 95°C for 10' and were resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using Any kD™ Criterion™ TGX Stain-Free™ Protein Gel (BioRad) in TrisGlicyne buffer (BioRad). Finally proteins contained in SDS-PAGE were revealed by Coomassie Blue staining.

6.11 ELISA

Purified proteins were plated over-night at 4 °C on 96-well plates in 1x PBS (200ng/well). The plates were blocked with a solution 1% BSA in 1X PBS (100 µl/well) for 45 minutes at room temperature. Sera were diluted 1:100 in a Eppendorf tube and subsequently diluted serially 1:3 on the 96-well plates with the immobilized proteins for 1 hour at RT. The wells were washed for 5 times with 1x PBS-0,5% Tween20 (TPBS) and then anti-protein specific IgG were detected with a goat anti-mouse IgG secondary antibodies (Sigma-Aldrich) diluted 1 to 2000 for 45 minutes at RT. The secondary antibodies are conjugated to the alkaline phosphatase enzyme. After triple TPBD wash, Alkaline phosphatase conjugated antibody was detected by adding 100 µl/well of 3 mg/L of para-nitrophenyl-phosphate disodium hexaidrated in 1M diethanolamine buffer (pH 9.8). After 20 minutes at room temperature in the dark, plates were analyzed at 405 nm spectrophotometer by Varioskan plate reader.

6.12 Splenocytes and lymph node cells

Spleens and lymph nodes were homogenized and splenocytes and lymph node cells filtered using a 70 µm cell Strainer (BD). Splenocytes and lymph node cells were resuspended at a concentration of $2.5x10^6$ cells per ml in Roswell Park Memorial Institute (RPMI) medium (Gibco)+ 10% heat inactivate fetal bovine serum (FBS) (Gibco) and 2mM L-glutamine (Fisher scientific) and 1X Penicillin/Streptomycin (EuroClone). As positive and negative controls, cells were stimulated with phorbol 12-myristate 13-acetate (PMA, 0.5 mg/ml) and Ionomycin (1 mg/ml) or with 5 mg/ml of an unrelated peptide, respectively.

6.13 PBMC

Blood was collected by cardiac puncture. About 500µl of blood was obtained from each BALB/C mouse to which was added 20µl heparin (2,6 mg/ml) (Sigma-Aldrich). The blood sample was diluted to a 1:1 volume ratio with PBS. It was added 5ml of Ficoll-Paque PLUS (GE Healthcare) to a fresh tube. After centrifugation at 400x g for 30 minutes, PBMC were isolated with a pipette

directly through the upper plasma layer at the interface. PBMC were washed twice in the PBS (400x g).

6.14 IFN-γ ELISpot

ELISpot plates (Mouse IFN-γ ELISpot^{PLUS} kit (ALP), Mabtech) were washed 4 times with sterile PBS (200µl/well). Subsequently the plates were conditioned with medium (200 µl/well), containing 10% of the same serum used for the cell suspension and they were incubated 30 minutes at room temperature. Removed the medium, 2.5x 10^5 cells resuspended in 100µl of medium were added followed by peptides diluted in 100µl of medium(5µg/ml). As positive and negative controls, cells were stimulated with phorbol 12-myristate 13-acetate (PMA, 0.5 µg/ml) and Ionomycin (1 µg/ml) or with 5 µg/ml of an unrelated peptide, respectively. The plates were putted in a 37°C humidified incubator with 5% CO_2 and incubated for 12-18 hours. The cells were removed by the plates, washing the wells 5 times with PBS (200µl/well). The detection antibody (R4-6A2-biotin) was diluted to 1µg/ml in PBS containing 0.5% fetal calf serum (PBS-0.5% FCS). 100µl/well were added and the plates were incubated for 2 hours at room temperature. After this step, the plates were washed 5 times with sterile PBS (200µl/well). To detect primary antibodies were added 100µl/well of Streptavidin-ALP (1:1000) in PBS-0,5% FCS for 1 hour at room temperature. The plates were washed as the previous steps. The ready-to-use substrate solution (BCIP/NBT-plus) was filtered through 0.5 µm filter and 100µl/well were added. The color was stopped by washing with water. The spots were counted by stereomicroscope. Graphs were processed using GraphPad Prism 5.03 software.

6.15 Synthetic peptides

All synthetic peptides used in this work were purchased from GenScript.

7 Literature

1. J. Varadé, S. Magadán, Á. González-Fernández, Human immunology and immunotherapy: main achievements and challenges. *Cellular & Molecular Immunology 2020 18:4*. **18**, 805–828 (2020).

2. L. S. Taams, Neuroimmune interactions: how the nervous and immune systems influence each other. *Clin Exp Immunol*. **197** (2019), pp. 276–277.

3. J. S. Marshall, R. Warrington, W. Watson, H. L. Kim, An introduction to immunology and immunopathology. *Allergy, Asthma and Clinical Immunology*. **14** (2018), , doi:10.1186/s13223-018-0278-1.

4. S. E. Turvey, D. H. Broide, Innate immunity. *Journal of Allergy and Clinical Immunology*. **125** (2010), doi:10.1016/j.jaci.2009.07.016.

5. N. S. Merle, S. E. Church, V. Fremeaux-Bacchi, L. T. Roumenina, Complement system part I - molecular mechanisms of activation and regulation. *Front Immunol*. **6** (2015), , doi:10.3389/fimmu.2015.00262.

6. R. Medzhitov, P. Preston-Hurlburt, C. A. Janeway, “A human homologue of the Drosophila Toll protein signals activation of adaptive immunity” (1997).

7. M.-A. Newman, T. Sundelin, J. T. Nielsen, G. Erbs, M. Tör, MAMP (microbe-associated molecular pattern) triggered immunity in plants (2013), doi:10.3389/fpls.2013.00139.

8. A. Gulati, D. Kaur, G. V. R. K. Prasad, A. Mukhopadhaya, PRR Function of Innate Immune Receptors in Recognition of Bacteria or Bacterial Ligands 18, doi:10.1007/978-981-13-3065-0_18.

9. K. Newton, V. M. Dixit, Signaling in innate immunity and inflammation. *Cold Spring Harb Perspect Biol*. **4** (2012), doi:10.1101/cshperspect.a006049.

10. S. E. Turvey, D. H. Broide, Innate immunity. *Journal of Allergy and Clinical Immunology*. **125** (2010), doi:10.1016/j.jaci.2009.07.016.

11. T. H. Mogensen, Pathogen recognition and inflammatory signaling in innate immune defenses. *Clin Microbiol Rev*. **22** (2009), pp. 240–273.

12. S. E. Turvey, D. H. Broide, Innate immunity. *Journal of Allergy and Clinical Immunology*. **125** (2010), doi:10.1016/j.jaci.2009.07.016.

13. K. D. Stone, C. Prussin, D. D. Metcalfe, IgE, mast cells, basophils, and eosinophils. *Journal of Allergy and Clinical Immunology*. **125** (2010), doi:10.1016/j.jaci.2009.11.017.

14. M. G. Netea, J. Domínguez-Andrés, L. B. Barreiro, T. Chavakis, M. Divangahi, E. Fuchs, L. A. B Joosten, J. W. M Meer, M. M. Mhlanga, W. J. M Mulder, N. P. Riksen, A. Schlitzer, J. L. Schultze, C. Stabell Benn, J. C. Sun, R. J. Xavier, E. Latz, Defining trained immunity and its role in health and disease, doi:10.1038/s41577-020-0285-6.

15. B. Novakovic, E. Habibi, S. Y. Wang, R. J. W. Arts, R. Davar, W. Megchelenbrink, B. Kim, T. Kuznetsova, M. Kox, J. Zwaag, F. Matarese, S. J. van Heeringen, E. M. Janssen-Megens, N.

Sharifi, C. Wang, F. Keramati, V. Schoonenberg, P. Flicek, L. Clarke, P. Pickkers, S. Heath, I. Gut, M. G. Netea, J. H. A. Martens, C. Logie, H. G. Stunnenberg, β-Glucan Reverses the Epigenetic State of LPS-Induced Immunological Tolerance. *Cell*. **167**, 1354-1368.e14 (2016).

16. L. C. J. de Bree, V. A. C. M. Koeken, L. A. B. Joosten, P. Aaby, C. S. Benn, R. van Crevel, M. G. Netea, Non-specific effects of vaccines: Current evidence and potential implications. *Semin Immunol*. **39** (2018), pp. 35–43.

17. Alberts B, Johnson A, Lewis J, et al., Molecular Biology of the Cell. 4th edition. *New York: Garland Science; 2002*.

18. F. A. Bonilla, H. C. Oettgen, Adaptive immunity. *Journal of Allergy and Clinical Immunology*. **125** (2010), doi:10.1016/j.jaci.2009.09.017.

19. T. Kurosaki, K. Kometani, W. Ise, Memory B cells. *Nat Rev Immunol*. **15**, 149 (2015).

20. T. Yoshlmoto, A. Bendelac, C. Watson, J. Hu-LI, W. E. Paul, C. H. Barton, J. K. Whte, T. I. Roach, J. M. Blackwell, R. Ahmed, D. Gray, “994) 53 L. C. C. Afonso et a.”

21. H. W. Schroeder, L. Cavacini, Structure and function of immunoglobulins. *Journal of Allergy and Clinical Immunology*. **125** (2010), doi:10.1016/j.jaci.2009.09.046.

22. P. Shen, S. Fillatreau, Antibody-independent functions of B cells: a focus on cytokines (2015), doi:10.1038/nri3857.

23. B. León, A. Ballesteros-Tato, J. L. Browning, R. Dunn, T. D. Randall, F. E. Lund, Regulation of T H 2 development by CXCR5 + dendritic cells and lymphotoxin-expressing B cells. *Nat Immunol*. **13** (2012), doi:10.1038/ni.2309.

24. A. Achour, "Major Histocompatibility Complex: Interaction with Peptides" in *eLS* (Wiley, 2001).

25. K. Murphy, C. & Weaver, Janeway’s, Immunobiology. 9th Edition. . *(Garland Science, 2016).*

26. P. J. & Delves, I. M. Roitt, The Immune System: Firts of Two Parts. *N. Engl. J. Med. 343, 37–49 (2000)*.

27. P. A. Roche, K. Furuta, Central tolerance The ins and outs of MHC class II-mediated antigen processing and presentation. *Nat Rev Immunol*. **15** (2015), doi:10.1038/nri3818.

28. Abul K. Abbas, Andrew H. Lichtman, Shiv Pillai, *Cellular and Molecular Immunology*, vol. 6.

29. H. Harjunpää, M. L. Asens, C. Guenther, S. C. Fagerholm, Cell adhesion molecules and their roles and regulation in the immune and tumor microenvironment. *Front Immunol*. **10** (2019), , doi:10.3389/fimmu.2019.01078.

30. O. S. Burren, A. Rubio García, B. M. Javierre, D. B. Rainbow, J. Cairns, N. J. Cooper, J. J. Lambourne, E. Schofield, X. Castro Dopico, R. C. Ferreira, R. Coulson, F. Burden, S. P. Rowlston, K. Downes, S. W. Wingett, M. Frontini, W. H. Ouwehand, P. Fraser, M. Spivakov, J. A. Todd, L. S. Wicker, A. J. Cutler, C. Wallace, Chromosome contacts in activated T cells identify autoimmune disease candidate genes. *Genome Biol*. **18** (2017), doi:10.1186/s13059-017-1285-0.

31. T. R. Mosmann, S. Sad, “The expanding universe subsets: Thl, Th2 and.”

32. N. Schmitt, H. Ueno, Regulation of human helper T cell subset differentiation by cytokines. *Curr Opin Immunol*. **34** (2015), pp. 130–136.

33. G. Trinchieri, S. Pflanz, R. A. Kastelein, “Minireview The IL-12 Family of Heterodimeric Cytokines: New Players in the Regulation of T Cell Responses the heterodimer when p40 is also produced by the same cells. IL-12 is a potent inducer of interferon-(IFN-) production from T, NK, and other cell types, and it has been shown to be a potent inducer of differentiation of T helper 1 (Th1) cells. A very large number of studies in” (2003).

34. R. M. Steinman, D. Hawiger, M. C. Nussenzweig, Tolerogenic dendritic cells. *Annu Rev Immunol*. **21** (2003), pp. 685–711.

35. PIIS0092867400807023.

36. Y. Simoni, M. Fehlings, H. N. Kløverpris, N. McGovern, S. L. Koo, C. Y. Loh, S. Lim, A. Kurioka, J. R. Fergusson, C. L. Tang, M. H. Kam, K. Dennis, T. K. H. Lim, A. C. Y. Fui, C. W. Hoong, J. K. Y. Chan, M. Curotto de Lafaille, S. Narayanan, S. Baig, M. Shabeer, S. A. E. S. Toh, H. K. K. Tan, R. Anicete, E. H. Tan, A. Takano, P. Klenerman, A. Leslie, D. S. W. Tan, I. B. Tan, F. Ginhoux, E. W. Newell, Human Innate Lymphoid Cell Subsets Possess Tissue-Type Based Heterogeneity in Phenotype and Frequency. *Immunity*. **46**, 148–161 (2017).

37. E. Lantelme, { S Mantovani, B. Palermo, R. Campanelli, F. Sallusto{, C. Giachino, “Kinetics of GATA-3 gene expression in early polarizing and committed human T cells.”

38. J. Zhu, L. Guo, C. J. Watson, J. Hu-Li, W. E. Paul, Stat6 Is Necessary and Sufficient for IL-4’s Role in Th2 Differentiation and Cell Expansion. *The Journal of Immunology*. **166**, 7276–7281 (2001).

39. N. J. Wilson, K. Boniface, J. R. Chan, B. S. McKenzie, W. M. Blumenschein, J. D. Mattson, B. Basham, K. Smith, T. Chen, F. Morel, J. C. Lecron, R. A. Kastelein, D. J. Cua, T. K. McClanahan, E. P. Bowman, R. de W. Malefyt, Development, cytokine profile and function of human interleukin 17-producing helper T cells. *Nat Immunol*. **8**, 950–957 (2007).

40. E. v. Acosta-Rodriguez, L. Rivino, J. Geginat, D. Jarrossay, M. Gattorno, A. Lanzavecchia, F. Sallusto, G. Napolitani, Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat Immunol*. **8**, 639–646 (2007).

41. S. Trifari, C. D. Kaplan, E. H. Tran, N. K. Crellin, H. Spits, Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from TH-17, TH1 and TH2 cells. *Nat Immunol*. **10**, 864–871 (2009).

42. M. Veldhoen, C. Uyttenhove, J. van Snick, H. Helmby, A. Westendorf, J. Buer, B. Martin, C. Wilhelm, B. Stockinger, Transforming growth factor-β “reprograms” the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nat Immunol*. **9**, 1341–1346 (2008).

43. I. R. Cohen, I. N. S. A. Lajtha, R. Paoletti, J. D. Lambris, “Volume 841 Series editors,” (available at http://www.springer.com/series/5584).

44. K. N. Couper, D. G. Blount, E. M. Riley, IL-10: The Master Regulator of Immunity to Infection. *The Journal of Immunology*. **180**, 5771–5777 (2008).

45. J. Brummelman, K. Pilipow, E. Lugli, The Single-Cell Phenotypic Identity of Human CD8+ and CD4+ T Cells. *Int Rev Cell Mol Biol*. **341**, 63–124 (2018).

46. D. L. Woodland, R. W. Dutton, Heterogeneity of CD4+ and CD8+ T cells. *Curr Opin Immunol*. **15** (2003), pp. 336–342.

47. V. Kalia, S. Sarkar, S. Subramaniam, W. N. Haining, K. A. Smith, R. Ahmed, Prolonged Interleukin-2Rα Expression on Virus-Specific CD8+ T Cells Favors Terminal-Effector Differentiation In Vivo. *Immunity*. **32**, 91–103 (2010).

48. S. Solouki, W. Huang, J. Elmore, C. Limper, F. Huang, A. August, TCR Signal Strength and Antigen Affinity Regulate CD8 + Memory T Cells . *The Journal of Immunology*. **205**, 1217–1227 (2020).

49. C. Lahariya, Vaccine epidemiology: A review. *J Family Med Prim Care*. **5**, 7 (2016).

50. M. R. Hilleman, "A simplified vaccinologists' vaccinology and the pursuit of a vaccine against AIDS" (1998).

51. C. Lahariya, Vaccine epidemiology: A review. *J Family Med Prim Care*. **5**, 7 (2016).

52. A. J. Pollard, E. M. Bijker, A guide to vaccinology: from basic principles to new developments. *Nat Rev Immunol*. **21** (2021), pp. 83–100.

53. E. de Gregorio, E. Tritto, R. Rappuoli, Alum adjuvanticity: Unraveling a century old mystery. *Eur J Immunol*. **38** (2008), pp. 2068–2071.

54. A. Grandi, M. Tomasi, G. Grandi, Vaccinology: The art of putting together the right ingredients. *Hum Vaccin Immunother*. **12**, 1311–1317 (2016).

55. Office of Infectious Disease and HIV/AIDS Policy, "Vaccine types U.S. Department of Health & Human Services."

56. N. I. Ho, L. G. M. Huis in 't Veld, T. K. Raaijmakers, G. J. Adema, Adjuvants Enhancing Cross-Presentation by Dendritic Cells: The Key to More Effective Vaccines? *Front Immunol*. **9** (2018), , doi:10.3389/fimmu.2018.02874.

57. W. Burny, A. Callegaro, V. Bechtold, F. Clement, S. Delhaye, L. Fissette, M. Janssens, G. Leroux-Roels, A. Marchant, R. A. van den Berg, N. Garçon, R. van der Most, A. M. Didierlaurent, I. Carletti, M. Esen, J. Gabor, E. Haelterman, C. Hervé, Y. Horsmans, M. Janssens, P. Kremsner, P. Moris, T. F. Schwarz, F. T. da Silva, P. van Belle, P. van Damme, D. Zuchner, Different adjuvants induce common innate pathways that are associated with enhanced adaptive responses against a model antigen in humans. *Front Immunol*. **8** (2017), doi:10.3389/fimmu.2017.00943.

58. E. H. Nardin, G. A. Oliveira, J. M. Calvo-Calle, K. Wetzel, C. Maier, A. J. Birkett, P. Sarpotdar, M. L. Corado, G. B. Thornton, A. Schmidt, Phase I testing of a malaria vaccine composed of hepatitis B virus core particles expressing Plasmodium falciparum circumsporozoite epitopes. *Infect Immun*. **72**, 6519–6527 (2004).

59. S. C. Eisenbarth, O. R. Colegio, W. O'connor, F. S. Sutterwala, R. A. Flavell, LETTERS Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants, doi:10.1038/nature06939.

60. D. T. O'Hagan, G. S. Ott, E. de Gregorio, A. Seubert, The mechanism of action of MF59 - An innately attractive adjuvant formulation. *Vaccine*. **30** (2012), pp. 4341–4348.

61. C. Maisonneuve, S. Bertholet, D. J. Philpott, E. de Gregorio, Unleashing the potential of NOD- and toll-like agonists as vaccine adjuvants. *Proc Natl Acad Sci U S A*. **111** (2014), pp. 12294–12299.

62. G. del Giudice, R. Rappuoli, A. M. Didierlaurent, Correlates of adjuvanticity: A review on adjuvants in licensed vaccines. *Semin Immunol*. **39** (2018), pp. 14–21.

63. N. Garc¸on, S. Morel, A. Didierlaurent, D. Descamps, M. Wettendorff, M. van Mechelen, "Development of an AS04-Adjuvanted HPV Vaccine with the Adjuvant System Approach."

64. A. Olotu, G. Fegan, J. Wambua, G. Nyangweso, A. Leach, M. Lievens, D. C. Kaslow, P. Njuguna, K. Marsh, P. Bejon, Seven-Year Efficacy of RTS,S/AS01 Malaria Vaccine among Young African Children. *New England Journal of Medicine*. **374**, 2519–2529 (2016).

65. S. Detienne, I. Welsby, C. Collignon, S. Wouters, M. Coccia, S. Delhaye, L. van Maele, S. Thomas, M. Swertvaegher, A. Detavernier, A. Elouahabi, S. Goriely, A. M. Didierlaurent, Central role of CD169+ lymph node resident macrophages in the adjuvanticity of the QS-21 component of AS01. *Sci Rep*. **6** (2016), doi:10.1038/srep39475.

66. J. D. Campbell, "Development of the CpG adjuvant 1018: A case study" in *Methods in Molecular Biology* (Humana Press Inc., 2017), vol. 1494, pp. 15–27.

67. A. Medical Association, "A Two-Dose Hepatitis B Vaccine for Adults (Heplisav-B)" (2018), (available at www.medicalletter.org/tmlj.).

68. H. Sultan, J. Wu, V. I. Fesenkova, A. E. Fan, D. Addis, A. M. Salazar, E. Celis, Poly-IC enhances the effectiveness of cancer immunotherapy by promoting T cell tumor infiltration. *J Immunother Cancer*. **8** (2020), doi:10.1136/jitc-2020-001224.

69. T. W. Dubensky, D. B. Kanne, M. L. Leong, Rationale, progress and development of vaccines utilizing STING-activating cyclic dinucleotide adjuvants. *Ther Adv Vaccines*. **1**, 131–143 (2013).

70. R. L. Coffman, A. Sher, R. A. Seder, Vaccine adjuvants: Putting innate immunity to work. *Immunity*. **33** (2010), pp. 492–503.

71. C. Schwechheimer, M. J. Kuehn, Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions (2015), doi:10.1038/nrmicro3525.

72. A. Kulp, M. J. Kuehn, Biological Functions and Biogenesis of Secreted Bacterial Outer Membrane Vesicles. *Annu. Rev. Microbiol*. **64**, 163–184 (2010).

73. S. N. Chatterjee A N, "Electron Microscopic Observations on the Excretion of Cell-wall Material by Vibriu chulerae."

74. Electron Microscopic Observations on the Excretion of Cell-wall Material by Vibrio cholerae S. N. Chatterjee1, J. Das1 Published: 01 October 1967 https://doi.org/10.1099/00221287-49-1-1.

75. E. L. Johnston, B. Heras, T. A. Kufer, M. Kaparakis-Liaskos, Detection of bacterial membrane vesicles by NOD-like receptors. *Int J Mol Sci*. **22** (2021), pp. 1–14.

76. A. T. Jan, Outer Membrane Vesicles (OMVs) of gram-negative bacteria: A perspective update. *Front Microbiol*. **8** (2017), doi:10.3389/fmicb.2017.01053.

77. A. J. Manning, M. J. Kuehn, "Contribution of bacterial outer membrane vesicles to innate bacterial defense" (2011), , doi:10.1186/1471-2180-11-258.

78. C. Schwechheimer, C. J. Sullivan, M. J. Kuehn, Envelope Control of Outer Membrane Vesicle Production in Gram-Negative Bacteria (2013), doi:10.1021/bi400164t.

79. K. J. McMahon, M. E. Castelli, E. G. Vescovi, M. F. Feldman, Biogenesis of outer membrane vesicles in Serratia marcescens is thermoregulated and can be induced by activation of the Rcs phosphorelay system. *J Bacteriol*. **194**, 3241–3249 (2012).

80. A. J. Mcbroom, M. J. Kuehn, Release of outer membrane vesicles by Gram-negative bacteria is a novel envelope stress response (2006), doi:10.1111/j.1365-2958.2006.05522.x.

81. C. Schwechheimer, M. J. Kuehn, Synthetic Effect between Envelope Stress and Lack of Outer Membrane Vesicle Production in Escherichia coli (2013), doi:10.1128/JB.02192-12.

82. A. J. Mcbroom, M. J. Kuehn, Release of outer membrane vesicles by Gram-negative bacteria is a novel envelope stress response (2006), doi:10.1111/j.1365-2958.2006.05522.x.

83. Y. C. Yeh, L. R. Comolli, K. H. Downing, L. Shapiro, H. H. McAdams, The Caulobacter Tol-Pal complex is essential for outer membrane integrity and the positioning of a polar localization factor. *J Bacteriol*. **192**, 4847–4858 (2010).

84. E. Cascales, A. Bernadac, M. Gavioli, J. C. Lazzaroni, R. Lloubes, Pal lipoprotein of Escherichia coli plays a major role in outer membrane integrity. *J Bacteriol*. **184**, 754–759 (2002).

85. B. L. Deatherage, J. C. Lara, T. Bergsbaken, S. L. R. Barrett, S. Lara, B. T. Cookson, Biogenesis of bacterial membrane vesicles. *Mol Microbiol*. **72**, 1395–1407 (2009).

86. M. Kaparakis-Liaskos, R. L. Ferrero, Immune modulation by bacterial outer membrane vesicles (2015), doi:10.1038/nri3837.

87. S. Ismail, M. B. Hampton, J. I. Keenan, Helicobacter pylori outer membrane vesicles modulate proliferation and interleukin-8 production by gastric epithelial cells. *Infect Immun*. **71**, 5670–5675 (2003).

88. F. Galka, N. W. Sun, H. Kusch, S. Engelmann, M. Hecker, B. Schmeck, S. Hippenstiel, B. E. Uhlin, M. Steinert, Proteomic characterization of the whole secretome of Legionella pneumophila and functional analysis of outer membrane vesicles. *Infect Immun*. **76**, 1825–1836 (2008).

89. R. Tavano, S. Franzoso, P. Cecchini, E. Cartocci, F. Oriente, B. Aricò, E. Papini, The membrane expression of Neisseria meningitidis adhesin A (NadA) increases the proimmune effects of MenB OMVs on human macrophages, compared with NadA-OMVs, without further stimulating their proinflammatory activity on circulating monocytes. *J. Leukoc. Biol*. **86**, 143–153 (2009).

90. B. T. Cookson, R. C. Alaniz, B. L. Deatherage, J. C. Lara, Protective Immunity In Vivo and T Cell Responses, and Stimulate Potently Activate Dendritic Cells, Prime B That Salmonella typhimurium Facsimiles of Membrane Vesicles Are Immunogenic. *J Immunol References*. **179**, 7692–7701 (2022).

91. B. T. Cookson, R. C. Alaniz, B. L. Deatherage, J. C. Lara, Protective Immunity In Vivo and T Cell Responses, and Stimulate Potently Activate Dendritic Cells, Prime B That Salmonella typhimurium Facsimiles of Membrane Vesicles Are Immunogenic. *J Immunol References*. **179**, 7692–7701 (2022).

92. N. C. Kesty, K. M. Mason, M. Reedy, S. E. Miller, M. J. Kuehn, Enterotoxigenic Escherichia coli vesicles target toxin delivery into mammalian cells. *EMBO Journal*. **23**, 4538–4549 (2004).

93. M. Bielaszewska, C. * Rü Ter, L. Kunsmann, L. Greune, A. Bauwens, Enterohemorrhagic Escherichia coli Hemolysin Employs Outer Membrane (2013), doi:10.1371/journal.ppat.1003797.

94. O. Y. Kim, B. S. Hong, K.-S. Park, Y. J. Yoon, S. J. Choi, W. H. Lee, T.-Y. Roh, J. Lötvall, Y.-K. Kim, Y. S. Gho, Immunization with Escherichia coli Outer Membrane Vesicles Protects Bacteria - Induced Lethality via Th1 and Th17 Cell Responses . *The Journal of Immunology*. **190**, 4092–4102 (2013).

95. A. Grandi, L. Fantappiè, C. Irene, S. Valensin, M. Tomasi, S. Stupia, R. Corbellari, E. Caproni, I. Zanella, S. J. Isaac, L. Ganfini, L. Frattini, E. König, A. Gagliardi, S. Tavarini, C. Sammicheli, M. Parri, G. Grandi, Vaccination With a FAT1-Derived B Cell Epitope Combined With Tumor-Specific B and T Cell Epitopes Elicits Additive Protection in Cancer Mouse Models. *Front Oncol*. **8** (2018), doi:10.3389/FONC.2018.00481.

96. L. Fantappiè, M. de Santis, E. Chiarot, F. Carboni, G. Bensi, O. Jousson, I. Margarit, G. Grandi, Antibody-mediated immunity induced by engineered Escherichia coli OMVs carrying heterologous antigens in their lumen. *J Extracell Vesicles*. **3** (2014), doi:10.3402/jev.v3.24015.

97. D. Serruto, M. J. Bottomley, S. Ram, M. M. Giuliani, R. Rappuoli, The new multicomponent vaccine against meningococcal serogroup B, 4CMenB: Immunological, functional and structural characterization of the antigens. *Vaccine*. **30** (2012), doi:10.1016/j.vaccine.2012.01.033.

98. N. C. Kesty, M. J. Kuehn, Incorporation of Heterologous Outer Membrane and Periplasmic Proteins into Escherichia coli Outer Membrane Vesicles* (2004), doi:10.1074/jbc.M307628200.

99. O. Youn Kim, H. Taek Park, N. Thi Hong Dinh, S. Jin Choi, J. Lee, J. Hyun Kim, S.-W. Lee, Y. Song Gho, N. Thi, Bacterial outer membrane vesicles suppress tumor by interferon-γ-mediated antitumor response, doi:10.1038/s41467-017-00729-8.

100. D. H. Lee, S. H. Kim, W. Kang, Y. S. Choi, S. H. Lee, S. R. Lee, S. You, H. K. Lee, K. T. Chang, E. C. Shin, Adjuvant effect of bacterial outer membrane vesicles with penta-acylated lipopolysaccharide on antigen-specific T cell priming. *Vaccine*. **29**, 8293–8301 (2011).

101. I. Zanella, E. König, M. Tomasi, A. Gagliardi, L. Frattini, L. Fantappiè, C. Irene, F. Zerbini, E. Caproni, S. J. Isaac, M. Grigolato, R. Corbellari, S. Valensin, I. Ferlenghi, F. Giusti, L. Bini, Y.

Ashhab, A. Grandi, G. Grandi, A. Zeneca, Proteome-minimized outer membrane vesicles from Escherichia coli as a generalized vaccine platform (2021), doi:10.1002/jev2.12066.

102. R. C. Alaniz, B. L. Deatherage, J. C. Lara, B. T. Cookson, Membrane Vesicles Are Immunogenic Facsimiles of Salmonella typhimurium That Potently Activate Dendritic Cells, Prime B and T Cell Responses, and Stimulate Protective Immunity In Vivo . *The Journal of Immunology*. **179**, 7692–7701 (2007).

103. B. B. Scorza, F. M. Colucci, A. M. Maggiore, L. Sanzone, S. Rossi, High Yield Production Process for Shigella Outer Membrane Particles. *PLoS One*. **7**, 35616 (2012).

104. A. L. Carvalho, S. Fonseca, A. Miquel-Clopés, K. Cross, K. S. Kok, U. Wegmann, K. Gil-Cordoso, E. G. Bentley, S. H. M. al Katy, J. L. Coombes, A. Kipar, R. Stentz, J. P. Stewart, S. R. Carding, Bioengineering commensal bacteria-derived outer membrane vesicles for delivery of biologics to the gastrointestinal and respiratory tract. *J Extracell Vesicles*. **8** (2019), doi:10.1080/20013078.2019.1632100.

105. R. Adriani, S. L. Mousavi Gargari, S. Nazarian, S. Sarvary, N. Noroozi, Immunogenicity of Vibrio cholerae outer membrane vesicles secreted at various environmental conditions. *Vaccine*. **36**, 322–330 (2018).

106. M. Qasim, M. Wrage, B. Nüse, J. Mattner, Shigella Outer Membrane Vesicles as Promising Targets for Vaccination (2022), doi:10.3390/ijms23020994.

107. R. Acevedo, S. Fernández, C. Zayas, A. Acosta, M. E. Sarmiento, V. A. Ferro, E. Rosenqvist, C. Campa, D. Cardoso, L. Garcia, J. Luis Perez, S. D. Gray-Owen, Bacterial outer membrane vesicles and vaccine applications (2014), doi:10.3389/fimmu.2014.00121.

108. D. Boutriau, J. Poolman, R. Borrow, J. Findlow, J. D. Domingo, J. Puig-Barbera, J. M. Baldó, V. Planelles, A. Jubert, J. Colomer, A. Gil, K. Levie, A. D. Kervyn, V. Weynants, F. Dominguez, R. Barberá, F. Sotolongo, Immunogenicity and safety of three doses of a bivalent (B:4:P1.19,15 and B:4:P1.7-2,4) meningococcal outer membrane vesicle vaccine in healthy adolescents. *Clinical and Vaccine Immunology*. **14**, 65–73 (2007).

109. E. Rosenqvist, E. A. Høiby, E. Wedege, K. Bryn, † Jan Kolberg, A. Klem, E. Rønnild, G. Bjune, H. Nøkleby, “Human Antibody Responses to Meningococcal Outer Membrane Antigens after Three Doses of the Norwegian Group B Meningococcal Vaccine” (1995).

110. Loring BJ, Turner N, Petousis-Harris H, (November 2008), MeNZB vaccine and epidemic control: when do you stop vaccinating. *vaccine PMID: 18804134 DOI: 10.1016/j.vaccine.2008.08.062*.

111. R. Arnold, Y. Galloway, A. McNicholas, J. O’Hallahan, Effectiveness of a vaccination programme for an epidemic of meningococcal B in New Zealand. *Vaccine*. **29**, 7100–7106 (2011).

112. X. Bai, J. Findlow, R. Borrow, Recombinant protein meningococcal serogroup B vaccine combined with outer membrane vesicles. *Expert Opin Biol Ther*. **11**, 969–985 (2011).

113. C. A. MacLennan, S. Grow, L. F. Ma, A. D. Steele, The Shigella Vaccines Pipeline. *Vaccines (Basel)*. **10** (2022), , doi:10.3390/vaccines10091376.

114. Q. Liu, Q. Liu, J. Yi, K. Liang, B. Hu, X. Zhang, R. Curtiss Iii, Q. Kong, Outer membrane vesicles from flagellin-deficient Salmonella enterica serovar Typhimurium induce cross-

reactive immunity and provide cross-protection against heterologous Salmonella challenge OPEN (2016), doi:10.1038/srep34776.

115. S. Fernández, E. M. Fajardo, A. Mandiarote, G. Año, M. A. Padrón, M. Acosta, R. A. Cabrera, L. A. Riverón, M. Álvarez, K. Blaín, M. Fariñas, D. Cardoso, L. G. García, C. Campa, J. L. Pérez, "A proteoliposome formulation derived from Bordetella pertussis induces protection in two murine challenge models" (2011), , doi:10.1186/1471-2172-14-S1-S8.

116. C. J. A. Asensio, M. E. Gaillard, G. Moreno, D. Bottero, E. Zurita, M. Rumbo, P. van der Ley, A. van der Ark, D. Hozbor, Outer membrane vesicles obtained from Bordetella pertussis Tohama expressing the lipid a deacylase PagL as a novel acellular vaccine candidate. *Vaccine*. **29**, 1649–1656 (2011).

117. S. Schild, E. J. Nelson, A. L. Bishop, A. Camilli, Characterization of Vibrio cholerae outer membrane vesicles as a candidate vaccine for cholera. *Infect Immun*. **77**, 472–484 (2009).

118. O. Y. Kim, B. S. Hong, K.-S. Park, Y. J. Yoon, S. J. Choi, W. H. Lee, T.-Y. Roh, J. Lötvall, Y.-K. Kim, Y. S. Gho, Immunization with Escherichia coli Outer Membrane Vesicles Protects Bacteria - Induced Lethality via Th1 and Th17 Cell Responses . *The Journal of Immunology*. **190**, 4092–4102 (2013).

119. M. J. H. Gerritzen, D. E. Martens, R. H. Wijffels, L. van der Pol, M. Stork, Bioengineering bacterial outer membrane vesicles as vaccine platform. *Biotechnol Adv*. **35** (2017), pp. 565–574.

120. L. Fantappiè, M. de Santis, E. Chiarot, F. Carboni, G. Bensi, O. Jousson, I. Margarit, G. Grandi, M. de Santis, Journal of Extracellular Vesicles Antibody-mediated immunity induced by engineered Escherichia coli OMVs carrying heterologous antigens in their lumen Antibody-mediated immunity induced by engineered Escherichia coli OMVs carrying heterologous antigens in their lumen. *J Extracell Vesicles*. **3** (2401), doi:10.3402/jev.v3.24015.

121. C. Irene, L. Fantappiè, E. Caproni, F. Zerbini, A. Anesi, M. Tomasi, I. Zanella, S. Stupia, S. Prete, S. Valensin, E. König, L. Frattini, A. Gagliardi, S. J. Isaac, A. Grandi, G. Guella, G. Grandi, E. Research Council, " Omv, Bacterial outer membrane vesicles engineered with lipidated antigens as a platform for Staphylococcus aureus vaccine, doi:10.1073/pnas.1905112116.

122. R. Li, Q. Liu, Engineered Bacterial Outer Membrane Vesicles as Multifunctional Delivery Platforms. *Front Mater*. **7** (2020), , doi:10.3389/fmats.2020.00202.

123. C. Irene, L. Fantappiè, E. Caproni, F. Zerbini, A. Anesi, M. Tomasi, I. Zanella, S. Stupia, S. Prete, S. Valensin, E. König, L. Frattini, A. Gagliardi, S. J. Isaac, A. Grandi, G. Guella, G. Grandi, E. Research Council, " Omv, Bacterial outer membrane vesicles engineered with lipidated antigens as a platform for Staphylococcus aureus vaccine, doi:10.1073/pnas.1905112116.

124. S. G. Maher, G. Multhoff, G. Grandi, A. Grandi, M. Tomasi, I. Zanella, L. Ganfini, E. Caproni, L. Fantappiè, C. Irene, L. Frattini, S. J. Isaac, E. König, F. Zerbini, S. Tavarini, C. Sammicheli, F. Giusti, I. Ferlenghi, M. Parri, synergistic Protective activity of Tumor-specific epitopes engineered in Bacterial Outer Membrane Vesicles. **7**, 7 (2017).

125. D. J. Chen, N. Osterrieder, S. M. Metzger, E. Buckles, A. M. Doody, M. P. DeLisa, D. Putnam, Delivery of foreign antigens by engineered outer membrane vesicle vaccines. *Proc Natl Acad Sci U S A*. **107**, 3099–3104 (2010).

126. E. B. 1#, E. I. 1#, E. Frigimelica, R. Petracca, G. Galli, F. B. Scorza, N. Norais, D. Laera, F. Giusti, A. Pierleoni, M. Donati, R. Cevenini, O. Finco, G. Grandi, R. Grifantini, Recombinant outer membrane vesicles carrying Chlamydia muridarum HtrA induce antibodies that neutralize chlamydial infection in vitro, doi:10.3402/jev.v2i0.20181.

127. C. G. Rappazzo, H. C. Watkins, C. M. Guarino, A. Chau, J. L. Lopez, M. P. DeLisa, C. A. Leifer, G. R. Whittaker, D. Putnam, Recombinant M2e outer membrane vesicle vaccines protect against lethal influenza A challenge in BALB/c mice. *Vaccine*. **34**, 1252–1258 (2016).

128. S. T. T. Schetters, W. S. P. Jong, S. K. Horrevorts, L. J. W. Kruijssen, S. Engels, D. Stolk, M. H. Daleke-Schermerhorn, J. Garcia-Vallejo, D. Houben, W. W. J. Unger, J. M. M. den Haan, J. Luirink, Y. van Kooyk, Outer membrane vesicles engineered to express membrane-bound antigen program dendritic cells for cross-presentation to CD8+ T cells. *Acta Biomater*. **91**, 248–257 (2019).

129. M. Muralinath, M. J. Kuehn, K. L. Roland, R. Curtiss, Immunization with Salmonella enterica serovar typhimurium-derived outer membrane vesicles delivering the pneumococcal protein PspA confers protection against challenge with Streptococcus pneumoniae. *Infect Immun*. **79**, 887–894 (2011).

130. W. Huang, S. Wang, Y. Yao, Y. Xia, X. Yang, K. Li, P. Sun, C. Liu, W. Sun, H. Bai, X. Chu, Y. Li, Y. Ma, Employing Escherichia coli-derived outer membrane vesicles as an antigen delivery platform elicits protective immunity against Acinetobacter baumannii infection. *Nature Publishing Group* (2016), doi:10.1038/srep37242.

131. K. Kuipers, M. H. Daleke-Schermerhorn, W. S. P. Jong, C. M. ten Hagen-Jongman, F. van Opzeeland, E. Simonetti, J. Luirink, M. I. de Jonge, Salmonella outer membrane vesicles displaying high densities of pneumococcal antigen at the surface offer protection against colonization. *Vaccine*. **33**, 2022–2029 (2015).

132. K. Cheng, R. Zhao, Y. Li, Y. Qi, Y. Wang, Y. Zhang, H. Qin, Y. Qin, L. Chen, C. Li, J. Liang, Y. Li, J. Xu, X. Han, G. J. Anderson, J. Shi, L. Ren, X. Zhao, G. Nie, Bioengineered bacteria-derived outer membrane vesicles as a versatile antigen display platform for tumor vaccination via Plug-and-Display technology, doi:10.1038/s41467-021-22308-8.

133. A. L. Bishop, S. Schild, B. Patimalla, B. Klein, A. Camilli, Mucosal immunization with Vibrio cholerae outer membrane vesicles provides maternal protection mediated by antilipopolysaccharide antibodies that inhibit bacterial motility. *Infect Immun*. **78**, 4402–4420 (2010).

134. J. Zhang, L. de Masi, B. John, W. Chen, D. M. Schifferli, Improved delivery of the OVA-CD4 peptide to T helper cells by polymeric surface display on Salmonella. *Microb Cell Fact*. **13** (2014), doi:10.1186/1475-2859-13-80.

135. J. Liang, K. Cheng, Y. Li, J. Xu, Y. Chen, N. Ma, Q. Feng, F. Zhu, X. Ma, T. Zhang, Y. Yue, G. Liu, X. Guo, Z. Chen, X. Wang, R. Zhao, Y. Zhao, J. Shi, X. Zhao, G. Nie, Personalized cancer vaccines from bacteria-derived outer membrane vesicles with antibody-mediated persistent uptake by dendritic cells. *Fundamental Research*. **2**, 23–36 (2022).

136. V. Durand, J. MacKenzie, J. de Leon, C. Mesa, V. Quesniaux, M. Montoya, A. le Bon, S. Y. C. Wong, Role of lipopolysaccharide in the induction of type I interferon-dependent cross-priming and IL-10 production in mice by meningococcal outer membrane vesicles. *Vaccine*. **27**, 1912–1922 (2009).

137. S. Kreiter, M. Vormehr, N. van de Roemer, M. Diken, M. Löwer, J. Diekmann, S. Boegel, B. Schrörs, F. Vascotto, J. C. Castle, A. D. Tadmor, S. P. Schoenberger, C. Huber, O. ¨ Zlem Türeci, U. Sahin, Mutant MHC class II epitopes drive therapeutic immune responses to cancer, doi:10.1038/nature14426.

138. J. Thoma, S. Manioglu, D. Kalbermatter, P. D. Bosshart, D. Fotiadis, D. J. Müller, Protein-enriched outer membrane vesicles as a native platform for outer membrane protein studies. *Commun Biol*. **1** (2018), doi:10.1038/s42003-018-0027-5.

139. G. Zhang, B. M. Ueberheide, S. Waldemarson, S. Myung, K. Molloy, J. Eriksson, B. T. Chait, T. A. Neubert, D. Fenyö, Protein quantitation using mass spectrometry. *Methods Mol Biol*. **673**, 211–222 (2010).

140. S. Bhakdil, J. Tranum-Jensen2, “Alpha-Toxin of Staphylococcus aureus” (1991).

141. P. Mariotti, E. Malito, M. Biancucci, P. lo Surdo, R. P. N. Mishra, V. Nardi-Dei, S. Savino, M. Nissum, G. Spraggon, G. Grandi, F. Bagnoli, M. Bottomley, Structural and functional characterization of the Staphylococcus aureus virulence factor and vaccine candidate FhuD2. *Biochemical Journal*. **449**, 683–693 (2013).

142. F. Falugi, H. K. Kim, D. M. Missiakas, O. Schneewind, Role of protein a in the evasion of host adaptive immune responses by Staphylococcus aureus. *mBio*. **4** (2013), doi:10.1128/mBio.00575-13.

143. U. Weller, L. Muller, M. Messner, M. Palmer, A. Valeva, J. Tranum-Jensen, P. Agrawal, C. Biermann, A. Dobereiner, M. A. Kehoe, S. Bhakdi, Expression of active streptolysin O in Escherichia coli as a maltose-binding-protein-streptolysin-O fusion protein. The N-terminal 70 amino acids are not required for hemolytic activity. *Eur J Biochem*. **236**, 34–39 (1996).

144. C. Zingaretti, F. Falugi, V. Nardi-Dei, G. Pietrocola, M. Mariani, S. Liberatori1 Marilena Gallotta, M. Tontini, C. Tani, P. Speziale, G. Grandi, I. Margarit, Streptococcus pyogenes SpyCEP: a chemokine-inactivating protease with unique structural and biochemical features . *The FASEB Journal*. **24**, 2839–2848 (2010).

145. Y. Gu, J. Huang, X. Wang, L. Wang, J. Yang, B. Zhan, X. Zhu, Identification and characterization of CD4+ T cell epitopes present in Trichinella spiralis paramyosin. *Vet Parasitol*. **231**, 59–62 (2016).

146. S. Yu, H. Zhang, D. Yao, W. Liu, X. Wang, X. Chen, Y. Wei, Z. Zhang, J. Wang, L. Yu, H. Sun, Z. Wu, Y. Yu, B. Song, J. Ma, C. Tong, Y. Cui, Identification of CD4+ T-cell epitopes on iron-regulated surface determinant B of Staphylococcus aureus. *Microb Pathog*. **89**, 108–113 (2015).

147. U. Sahin, E. Derhovanessian, M. Miller, B. P. Kloke, P. Simon, M. Löwer, V. Bukur, A. D. Tadmor, U. Luxemburger, B. Schrörs, T. Omokoko, M. Vormehr, C. Albrecht, A. Paruzynski, A. N. Kuhn, J. Buck, S. Heesch, K. H. Schreeb, F. Müller, I. Ortseifer, I. Vogler, E. Godehardt, S. Attig, R. Rae, A. Breitkreuz, C. Tolliver, M. Suchan, G. Martic, A.

Hohberger, P. Sorn, J. Diekmann, J. Ciesla, O. Waksmann, A. K. Brück, M. Witt, M. Zillgen, A. Rothermel, B. Kasemann, D. Langer, S. Bolte, M. Diken, S. Kreiter, R. Nemecek, C. Gebhardt, S. Grabbe, C. Höller, J. Utikal, C. Huber, C. Loquai, Ö. Türeci, Personalized RNA mutanome vaccines mobilize poly-specific therapeutic immunity against cancer. *Nature*. **547**, 222–226 (2017).

148. P. A. Ott, Z. Hu, D. B. Keskin, S. A. Shukla, J. Sun, D. J. Bozym, W. Zhang, A. Luoma, A. Giobbie-Hurder, L. Peter, C. Chen, O. Olive, T. A. Carter, S. Li, D. J. Lieb, T. Eisenhaure, E. Gjini, J. Stevens, W. J. Lane 10, I. Javeri 11, K. Nellaiappan, A. M. Salazar, H. Daley, M. Seaman, E. I. Buchbinder, C. H. Yoon, M. Harden, N. Lennon, S. Gabriel, S. J. Rodig, D. H. Barouch, C. Aster, G. Getz, K. Wucherpfennig, D. Neuberg, J. Ritz, E. S. Lander, E. F. Fritsch, N. Hacohen, C. J. Wu, An immunogenic personal neoantigen vaccine for patients with melanoma. *Nature Publishing Group* (2017), doi:10.1038/nature22991.

149. M. Tomasi, E. Caproni, M. Benedet, I. Zanella, S. Giorgetta, M. Dalsass, E. König, A. Gagliardi, L. Fantappiè, A. Berti, S. Tamburini, L. Croia, G. Di Lascio, E. Bellini, S. Valensin, G. Licata, G. Sebastiani, F. Dotta, F. Armanini, F. Cumbo, F. Asnicar, A. Blanco-Míguez, E. Ruggiero, N. Segata, G. Grandi, A. Grandi, Outer Membrane Vesicles From The Gut Microbiome Contribute to Tumor Immunity by Eliciting Cross-Reactive T Cells. *Front Oncol*. **12** (2022), doi:10.3389/fonc.2022.912639.

150. M. Tomasi, M. Dalsass, F. Beghini, I. Zanella, E. Caproni, L. Fantappiè, A. Gagliardi, C. Irene, E. König, L. Frattini, G. Masetti, S. J. Isaac, F. Armanini, F. Cumbo, A. Blanco-míguez, A. Grandi, N. Segata, G. Grandi, Commensal bifidobacterium strains enhance the efficacy of neo-epitope based cancer vaccines. *Vaccines (Basel)*. **9** (2021), doi:10.3390/vaccines9111356.

151. Q. Feng, X. Ma, K. Cheng, G. Liu, Y. Li, Y. Yue, J. Liang, L. Zhang, T. Zhang, X. Wang, X. Gao, G. Nie, X. Zhao, Engineered Bacterial Outer Membrane Vesicles as Controllable Two-Way Adaptors to Activate Macrophage Phagocytosis for Improved Tumor Immunotherapy. *Advanced Materials*. **34** (2022), doi:10.1002/adma.202206200.

152. Old Rotzschkeo, Kirsten Falko, Stefan St evanovif +, Giinther Jug+, Peter WaldenO and, Hans-Georg RammenseeO, Exact prediction of a natural T cell epitope. *Eur. J. Immunol. 1991. 21: 2891-2894* .

153. D. Assudani, H. il Cho, N. DeVito, N. Bradley, E. Celis, In vivo expansion, persistence, and function of peptide vaccine-induced CD8 T cells occur independently of CD4 T cells. *Cancer Res*. **68**, 9892–9899 (2008).

154. M. Ghosh, L. H. Shapiro, “In vitro Ag Cross-presentation and in vivo Ag Cross-presentation by Dendritic Cells in the Mouse Materials and Reagents.”

155. S. Rm. K. Clarke, M. Barnden, C. Kurts, F. R. Carbone, J. F. Miller, W. R. Heath, Characterization of the ovalbumin-specific TCR transgenic line OT-I: MHC elements for positive and negative selection. *Immunol Cell Biol*. **78**, 110–117 (2000).

156. D. Dersh, J. W. Yewdell, J. Wei, "A SIINFEKL-based system to measure MHC class I antigen presentation efficiency and kinetics" in *Methods in Molecular Biology* (Humana Press Inc., 2019), vol. 1988, pp. 109–122.

157. Y. Tanaka, S. S. Tevethia, In vitro selection of SV40 T antigen epitope loss variants by site-specific cytotoxic T lymphocyte clones. *The Journal of Immunology*. **140**, 4348–4354 (1988).

158. L. M. Mylin, T. D. Schell, D. Roberts, M. Epler, A. Boesteanu, E. J. Collins, J. A. Frelinger, S. Joyce, § And, S. S. Tevethia, "Quantitation of CD8 T-Lymphocyte Responses to Multiple Epitopes from Simian Virus 40 (SV40) Large T Antigen in C57BL/6 Mice Immunized with SV40, SV40 T-Antigen-Transformed Cells, or Vaccinia Virus Recombinants Expressing Full-Length T Antigen or Epitope Minigenes" (2000).

159. Q. Ni, F. Zhang, Y. Liu, Z. Wang, G. Yu, B. Liang, G. Niu, T. Su, G. Zhu, G. Lu, L. Zhang, X. Chen, "A bi-adjuvant nanovaccine that potentiates immunogenicity of neoantigen for combination immunotherapy of colorectal cancer" (2020).

160. C. H. Huang, C. Y. Huang, M. H. Huang, Impact of antigen-adjuvant associations on antigen uptake and antigen-specific humoral immunity in mice following intramuscular injection. *Biomedicine and Pharmacotherapy*. **118** (2019), doi:10.1016/j.biopha.2019.109373.

161. N. O. Fischer, A. Rasley, M. Corzett, M. H. Hwang, P. D. Hoeprich, C. D. Blanchette, Colocalized delivery of adjuvant and antigen using nanolipoprotein particles enhances the immune response to recombinant antigens. *J Am Chem Soc*. **135**, 2044–2047 (2013).

162. J. Yang, S. Arya, P. Lung, Q. Lin, J. Huang, Q. Li, Hybrid nanovaccine for the co-delivery of the mRNA antigen and adjuvant. *Nanoscale*. **11**, 21782–21789 (2019).

163. F. Necchi, G. Stefanetti, R. Alfini, E. Palmieri, M. Carducci, R. Di Benedetto, F. Schiavo, M. G. Aruta, F. Giusti, I. Ferlenghi, Y. S. Goh, S. Rondini, F. Micoli, Neisseria meningitidis factor h binding protein surface exposure on salmonella typhimurium gmma is critical to induce an effective immune response against both diseases. *Pathogens*. **10** (2021), doi:10.3390/pathogens10060726.

164. A. L. Carvalho, A. Miquel-Clopés, U. Wegmann, E. Jones, R. Stentz, A. Telatin, N. J. Walker, W. A. Butcher, P. J. Brown, S. Holmes, M. J. Dennis, E. D. Williamson, S. G. P. Funnell, M. Stock, S. R. Carding, Use of bioengineered human commensal gut bacteria-derived microvesicles for mucosal plague vaccine delivery and immunization. *Clin Exp Immunol*. **196**, 287–304 (2019).

165. K. Hu, E. Palmieri, K. Samnuan, B. Ricchetti, D. Oldrini, P. F. McKay, G. Wu, L. Thorne, A. R. Fooks, L. M. McElhinney, H. Goharriz, M. Golding, R. J. Shattock, F. Micoli, Generalized Modules for Membrane Antigens (GMMA), an outer membrane vesicle-based vaccine platform, for efficient viral antigen delivery. *J Extracell Vesicles*. **11** (2022), doi:10.1002/jev2.12247.

166. P. A. van der Ley, A. Zariri, E. van Riet, D. Oosterhoff, C. P. Kruiswijk, An Intranasal OMV-Based Vaccine Induces High Mucosal and Systemic Protecting Immunity Against a SARS-CoV-2 Infection. *Front Immunol*. **12** (2021), doi:10.3389/fimmu.2021.781280.

167. T. Y. Lee, C. U. Kim, E. H. Bae, S. H. Seo, D. G. Jeong, S. W. Yoon, K. T. Chang, Y. S. Kim, S. H. Kim, D. J. Kim, Outer membrane vesicles harboring modified lipid A moiety augment the efficacy of an influenza vaccine exhibiting reduced endotoxicity in a mouse model. *Vaccine*. **35**, 586–595 (2017).

168. B.-J. Lee, H. Kwon, E.-H. Kim, S.-J. Park, S.-H. Lee, Y. K. Choi, S.-H. Kim, Assessment of mOMV adjuvant efficacy in the pathogenic H1N1 influenza virus vaccine. *Clin Exp Vaccine Res*. **3**, 194 (2014).

169. M. Malekan, S. D. Siadat, M. Aghasadeghi, N. Shahrokhi, P. Afrough, A. Behrouzi, K. Ahmadi, S. F. Mousavi, Evaluation of protective immunity responses against pneumococcal PhtD and its C-terminal in combination with outer-membrane vesicles as adjuvants. *J Med Microbiol*. **69**, 465–477 (2020).

170. H. C. Watkins, C. G. Rappazzo, J. S. Higgins, X. Sun, N. Brock, A. Chau, A. Misra, J. P. B. Cannizzo, M. R. King, T. R. Maines, C. A. Leifer, G. R. Whittaker, M. P. DeLisa, D. Putnam, Safe Recombinant Outer Membrane Vesicles that Display M2e Elicit Heterologous Influenza Protection. *Molecular Therapy*. **25**, 989–1002 (2017).

171. Q. Li, G. Zhou, X. Fei, Y. Tian, S. Wang, H. Shi, Engineered Bacterial Outer Membrane Vesicles with Lipidated Heterologous Antigen as an Adjuvant-Free Vaccine Platform for Streptococcus suis. *Appl Environ Microbiol* (2023), doi:10.1128/aem.02047-22.

172. R. Nakao, H. Kobayashi, Y. Iwabuchi, K. Kawahara, S. Hirayama, M. Ramstedt, Y. Sasaki, M. Kataoka, Y. Akeda, M. Ohnishi, A highly immunogenic vaccine platform against encapsulated pathogens using chimeric probiotic Escherichia coli membrane vesicles. *NPJ Vaccines*. **7** (2022), doi:10.1038/s41541-022-00572-z.

173. D. Santana-Mederos, R. Perez-Nicado, Y. Climent, L. Rodriguez, B. S. Ramirez, S. Perez-Rodriguez, M. Rodriguez, C. Labrada, T. Hernandez, M. Diaz, I. Orosa, U. Ramirez, R. Oliva, R. Garrido, F. Cardoso, M. Landys, R. Martinez, H. Gonzalez, T. Hernandez, R. Ochoa-Azze, J. L. Perez, J. Enriquez, N. Gonzalez, Y. Infante, L. A. Espinosa, Y. Ramos, L. J. González, C. Valenzuela, A. V. Casadesus, B. Fernandez, G. Rojas, B. Pérez-Massón, Y. Tundidor, E. Bermudez, C. A. Plasencia, T. Boggiano, E. Ojito, F. Chiodo, S. Fernandez, D. Garcia-Rivera, C. Fang, G. W. Chen, D. G. Rivera, Y. Valdes-Balbin, D. Garcia-Rivera, V. Verez Bencomo, A COVID-19 vaccine candidate composed of the SARS-CoV-2 RBD dimer and: Neisseria meningitidis outer membrane vesicles. *RSC Chem Biol*. **3**, 242–249 (2022).

174. J. Wo, Z. Y. Lv, J. N. Sun, H. Tang, N. Qi, B. C. Ye, Engineering probiotic-derived outer membrane vesicles as functional vaccine carriers to enhance immunity against SARS-CoV-2. *iScience*. **26** (2023), doi:10.1016/j.isci.2022.105772.

175. I. Valdés, L. Hermida, J. Martín, T. Menéndez, L. Gil, L. Lazo, J. Castro, O. Niebla, C. López, L. Bernardo, J. Sánchez, Y. Romero, R. Martínez, M. G. Guzmán, G. Guillén, Immunological evaluation in nonhuman primates of formulations based on the chimeric protein P64k-domain III of dengue 2 and two components of Neisseria meningitidis. *Vaccine*. **27**, 995–1001 (2009).

176. A. Behrouzi, S. Bouzari, S. D. Siadat, M. Oloomi, M. Davari, H. Mazaheri, Evaluation of the immunogenic property of NT H. Influenzae protein D with neisseria meningitidis OMV in BALB/c. *J Infect Dev Ctries*. **10**, 1345–1351 (2016).

177. K. Xu, Q. Zhao, X. Wen, R. Wu, Y. Wen, X. Huang, Y. Huang, Q. Yan, X. Han, X. Ma, Y. F. Chang, S. Cao, A trivalent Apx-fusion protein delivered by E. coli outer membrane vesicles induce protection against Actinobacillus pleuropneumoniae of serotype 1 and 7 challenge in a murine model. *PLoS One*. **13** (2018), doi:10.1371/journal.pone.0191286.

178. E. B. Gaspar, C. R. Prudencio, E. De Gaspari, Experimental studies using OMV in a new platform of SARS-CoV-2 vaccines. *Hum Vaccin Immunother*. **17** (2021), pp. 2965–2968.

179. G. Gasperini, R. Alfini, V. Arato, F. Mancini, M. G. Aruta, P. Kanvatirth, D. Pickard, F. Necchi, A. Saul, O. Rossi, F. Micoli, P. Mastroeni, Salmonella paratyphi a outer membrane vesicles displaying Vi polysaccharide as a multivalent vaccine against enteric fever. *Infect Immun*. **89** (2021), doi:10.1128/IAI.00699-20.

180. J. Sun, X. Lin, Y. He, B. Zhang, N. Zhou, J. Huang, A bacterial outer membrane vesicle-based click vaccine elicits potent immune response against Staphylococcus aureus in mice. *Front Immunol*. **14** (2023), doi:10.3389/fimmu.2023.1088501.

181. L. Jiang, T. A. P. Driedonks, W. S. P. Jong, S. Dhakal, H. Bart van den Berg van Saparoea, I. Sitaras, R. Zhou, C. Caputo, K. Littlefield, M. Lowman, M. Chen, G. Lima, O. Gololobova, B. Smith, V. Mahairaki, M. Riley Richardson, K. R. Mulka, A. P. Lane, S. L. Klein, A. Pekosz, C. Brayton, J. L. Mankowski, J. Luirink, J. S. Villano, K. W. Witwer, A bacterial extracellular vesicle-based intranasal vaccine against SARS-CoV-2 protects against disease and elicits neutralizing antibodies to wild-type and Delta variants. *J Extracell Vesicles*. **11** (2022), doi:10.1002/jev2.12192.

182. W. Huang, C. Shu, L. Hua, Y. Zhao, H. Xie, J. Qi, F. Gao, R. Gao, Y. Chen, Q. Zhang, W. Li, M. Yuan, C. Ye, Y. Ma, Modified bacterial outer membrane vesicles induce autoantibodies for tumor therapy. *Acta Biomater*. **108**, 300–312 (2020).

183. S. Wang, W. Huang, K. Li, Y. Yao, X. Yang, H. Bai, W. Sun, C. Liu, Y. Ma, Engineered outer membrane vesicle is potent to elicit HPV16E7-specific cellular immunity in a mouse model of TC-1 graft tumor. *Int J Nanomedicine*. **12**, 6813–6825 (2017).

184. Y. Li, X. Ma, Y. Yue, K. Zhang, K. Cheng, Q. Feng, N. Ma, J. Liang, T. Zhang, L. Zhang, Z. Chen, X. Wang, L. Ren, X. Zhao, G. Nie, Rapid Surface Display of mRNA Antigens by Bacteria-Derived Outer Membrane Vesicles for a Personalized Tumor Vaccine. *Advanced Materials*. **34** (2022), doi:10.1002/adma.202109984.

8 Acknowledgement