Extracellular Vesicle-based Nano/Microparticles for Novel Vaccination Approaches

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“I have come that they may have life, and that they may have it more abundantly.”

- **Jesus Christ** (John 10:10)

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I. Summary

Pneumococcal infections cause many fatalities worldwide. Growing resistance to antibiotics and incomplete coverage of available vaccines against all serotypes made the search for novel vaccination approaches a global necessity. Extracellular membrane vesicles are secreted nanostructures, which are physiologically secreted from seemingly all living cells and harbor their virulence factors and immunogenic components.

In this work, several research objectives were explored: (i) **Isolation and characterization** of pneumococcal vesicles. (ii) **Biocompatibility and uptake** with cell lines and primary human cells. (iii) **Yield Enhancement** of pneumococcal vesicles. (iv) **Immunostimulation** of immune cells by pneumococcal vesicles. (v) **Formulation** of spray-dried vaccine microparticles for pulmonary immunization.

The isolated vesicles exhibited excellent biocompatibility with several cell lines and primary cells, without cytotoxic effects. Pneumococcal vesicles demonstrated rapid uptake into immune cells and stimulated the release of pro-inflammatory cytokines. We successfully formulated spray-dried vaccine microparticles with enhanced stability and increased cytokine release for pulmonary delivery.

Our findings confirm the strong potential of pneumococcal membrane vesicles as vaccine candidates, and provide a sound basis for further translation and scale-up for pulmonary delivery and immunization.

II. Zusammenfassung

Pneumokokken-Infektionen führen weltweit zu zahlreichen Todesfällen. Die zunehmende Resistenz gegen Antibiotika und die unvollständige Abdeckung durch verfügbare Impfstoffe gegen alle Serotypen machen die Suche nach neuen Impfansätzen eine globale Notwendigkeit. Extrazelluläre Membranvesikel sind sezernierte Nanostrukturen, die von ziemlich allen Zellen ausgeschieden werden und ihre Virulenzfaktoren und immunogenen Komponenten beherbergen.

In dieser Arbeit wurden mehrere Forschungsziele verfolgt: (i) **Isolierung und Charakterisierung** von Pneumokokken-Vesikeln. (ii) **Biokompatibilität und Aufnahme** gegenüber Zelllinien und primären menschlichen Zellen. (iii) **Erhöhung der Ausbeute**. (iv) **Immunstimulation** von Immunzellen durch Pneumokokken-Vesikel. (v) **Formulierung** von sprühgetrockneten Impfstoff-Mikropartikeln für die Immunisierung der Lunge.

Die isolierten Vesikel zeigten eine ausgezeichnete Biokompatibilität mit verschiedenen Zelllinien und Primärzellen. Pneumokokken-Vesikel zeigten eine schnelle Aufnahme in Immunzellen und stimulierten die Freisetzung von proinflammatorischen Zytokinen. Sprühgetrocknete Impfstoff-Mikropartikel wurden mit verbesserter Stabilität und erhöhter Zytokinfreisetzung für die pulmonale Verabreichung formuliert.

Unsere Ergebnisse bestätigen das große Potenzial von Pneumokokken-Membranvesikeln als Impfstoffkandidaten und bilden eine wichtige Grundlage für die weitere Umsetzung und das Scale-up für die pulmonale Verabreichung und Immunisierung.

III. List of Abbreviations

CLSM	Confocal laser scanning microscopy
Cryo-TEM	Cryogenic transmission electron microscopy
EVs	Extracellular vesicles
DCs	Dendritic cells
dMVs	Death-phase membrane vesicles
IL	Interleukin
LPS	Lipopolysaccharide
MDMs	Monocyte-derived macrophages
MHC	Major histocompatibility complex
MPs	Microparticles
MVs	Membrane vesicles
NPs	Nanoparticles
NTA	Nanoparticle tracking analysis
OMVs	Outer membrane vesicles
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PRRs	Pattern recognition receptors
SEC	Size exclusion chromatography
SEM	Scanning electron microscopy
sMVs	Stationary-phase membrane vesicles
TLRs	Toll-like receptors
TNF	Tumor necrosis factor

1. Abstract

Antigen delivery has always been a challenge in scientific practice of vaccine formulation. Mammalian extracellular vesicles (EVs) or bacterial membrane vesicles (MVs) provide an innovative avenue for safe and effective delivery of antigenic material. They include intrinsically loaded antigens from EV-secreting cells or extrinsically loaded antigens onto pre-formed vesicles. Interestingly, many studies shed light on potential vaccination for prophylactic applications using bacterial cell-derived MVs against infectious diseases. *Streptococcus pneumoniae* infections are a leading cause of death worldwide. Bacterial membrane vesicles (MVs) are promising vaccine candidates because of the antigenic components of their parent microorganisms. Extracellular vesicles are membranous structures shed by almost every living cell. Bacterial gram-negative outer membrane vesicles (OMVs) and gram-positive membrane vesicles (MVs) play important roles in adaptation to surrounding environment, cellular components exchange, transfer of antigens and virulence factors, and infection propagation.

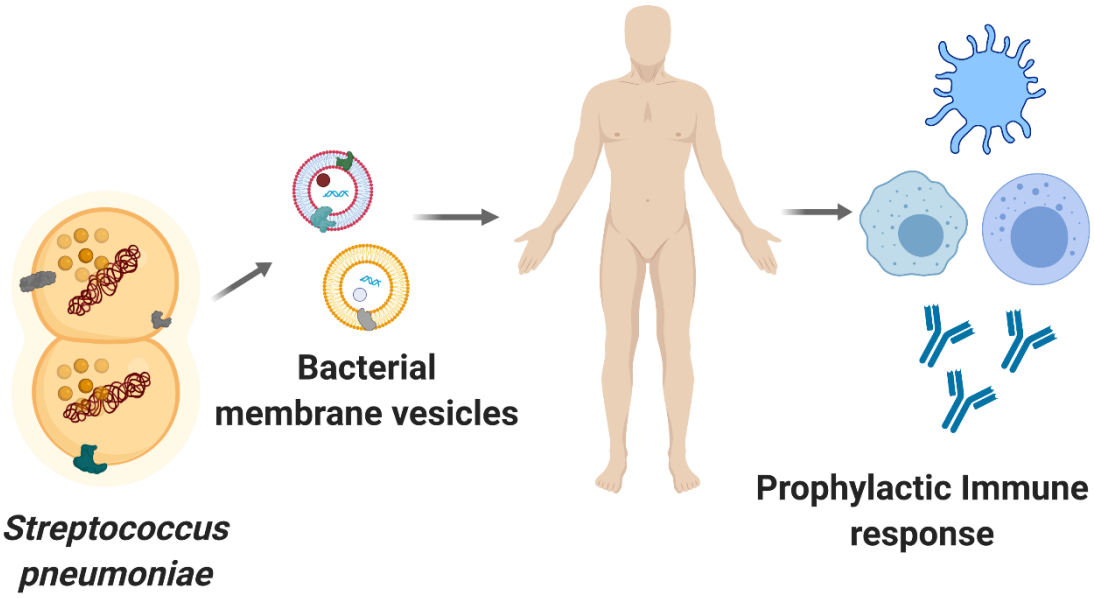
We isolated and characterized MVs produced from *Streptococcus pneumoniae* reference strain (R6). Isolated MVs showed a mean particle size range of 130-160 nm and a particle yield of around 10^{12} particle/mL. Cryogenic transmission electron microscopy (cryo-TEM) images revealed a very heterogeneous nature of isolated MVs with a broad size range. Pneumococcal MVs showed excellent biocompatibility with several somatic and immune cell lines including human lung epithelial A549 and human keratinocytes HaCaT cell lines, and immune cells including differentiated macrophage-like dTHP-1 and murine dendritic DC2.4 cells, and negligible cytotoxic effects. A rapid uptake and increased release of pro-inflammatory cytokines from DC2.4 cell line was observed.

The clinical translation of pneumococcal vesicles requires high yield and strong immunogenic effects without compromising immune cell viability. MVs were isolated during either the stationary phase (24 h) or death phase (48 h), and their yields, immunogenicity and cytotoxicity in human primary macrophages and dendritic cells were investigated. Death-phase vesicles showed higher yields, while both vesicle types displayed good compatibility, rapid uptake and stimulated release of pro-inflammatory cytokines, tumor necrosis factor and interleukin-6, with human primary immune cells. Proteomic analysis revealed similarities in vesicular immunogenic proteins such as pneumolysin, pneumococcal surface protein A, and IgA1 protease in both vesicle types, but stationary-phase MVs showed significantly lower autolysin levels than did death-phase MVs.

After confirmation of the strong immunostimulating potential of pneumococcal membrane vesicles, we explored the formulation of dry powder inhaler for the pulmonary immunization using pneumococcal MVs, to enhance their stability and application. We successfully prepared pneumococcal MVs-loaded spray-dried vaccine microparticles using lactose and leucine as the inactive carrier components. Vaccine microparticles exhibited particle size range 1-3 µm, amorphous nature, and optimal morphology and excellent aerodynamic particle size range 2.34 µm, essential for deep lung deposition. Moreover, they were readily soluble releasing the loaded pneumococcal MVs, with a yield around 81%. Macrophage-like THP-1 cells demonstrated excellent viability upon treatment with vaccine microparticles, and a rapid uptake reaching 60 % was observed after 8h of incubation. In addition, vaccine microparticles generated an increased secretion of pro-inflammatory cytokines i.e., tumor necrosis factor and interleukin-6 from human primary immune cells.

Our study provides better understanding of gram-positive pneumococcal MVs, and show their strong potential as vaccine candidates, owing to abundant antigenic proteins/lipoproteins cargo, and stimulation of primary human immune cells. Therefore, they could offer an innovative avenue for safe and effective cell-free vaccination against pneumococcal infections. Additionally, the formulated pneumococcal MVs-loaded spray-dried vaccine microparticles are promising stable candidates for pulmonary immunization.

Graphical Abstract



Keywords

Extracellular vesicles - bacterial membrane vesicles - *Streptococcus pneumoniae* - pneumococci - cytotoxicity - uptake - cytokine - vaccine – proteomics – immune cells - primary macrophages - dendritic cells - antigen - Infection - spray drying - pulmonary immunization

2. Introduction

Most of the following introduction is transferred *verbatim* from the following published review article.

Extracellular vesicles as antigen carriers for novel vaccination avenues

Mina Mehanny, Claus-Michael Lehr, Gregor Fuhrmann

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Extracellular vesicles as antigen carriers for novel vaccination avenues

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2.1. Pneumococcal Disease

Streptococcus pneumoniae (Pneumococcus) was first described in 1881 independently by both scientists Louis Pasteur and George Sternberg [1]. Pneumococcus is a gram-positive bacterium, which usually colonizes the respiratory tract and may invade through mucosal membranes causing serious diseases including otitis media, pneumonia, septicemia and meningitis [2]. The risk groups for pneumococcal invasive diseases include pediatrics, geriatrics and immune-compromised people or having underlying comorbidities, causing mortalities worldwide mainly in developing countries [3, 4]. Pneumococcal infections are commonly treated with several antibiotics including penicillins, cephalosporins, macrolides, rifampicin and vancomycin [5]. Increasing antibiotic resistance rates and higher disease burdens of pneumococci pose a global health issue. Consequently, The world health organization (WHO) declared in 2017 *Streptococcus pneumoniae* among the priority pathogens, which require prompt new anti-infective strategies [6].

Available pneumococcal vaccines include two main types, either polysaccharide vaccines or conjugate vaccines. The first developed pneumococcal vaccine were derived from their immunogenic capsular polysaccharide, which can induce a protective immune response by phagocytic immune cells and pathogenic-specific antibodies [7]. It was introduced in the 1940s [8] and the production of 14-valent vaccine was supported by the work of Robert Austrian in the 1970s [9], until it reached the 23-valent vaccine available form, known as Pneumovax 23® [10]. It is recommended for elderly, adults with serious health problems including HIV patients, and smokers to prevent exacerbations of chronic obstructive pulmonary disease. However, the WHO does not recommend the polysaccharide vaccine for routine pediatric immunization schemes [11-13]. Pneumococcal conjugate vaccine consists of purified capsular polysaccharide conjugated to a carrier protein to potentiate the antibody immune response, and longer and stronger protection. Hence, it is the recommended vaccine for children vaccination programs by WHO [14, 15]. The available brand names for the conjugate vaccine include Prevnar®, Synflorix® and recently approved Vaxneuvance®. Prevnar 13® is a 13-valent vaccine of capsular polysaccharide of 13 serotypes conjugated to diphtheria carrier protein and it was approved by FDA in 2010 to replace the former 7-valent Prevnar® [16, 17]. Recently, the FDA approved Prevnar® 20 in 2021 including 20 serotypes [18]. Synflorix® is a 10-valent vaccine containing capsular polysaccharide conjugated to mixture of carrier proteins including protein D from *Haemophilus influenza*, tetanus toxoid and diphtheria toxoid carrier proteins [19]. Vaxneuvance is a 15-valent conjugate vaccine introduced in 2021 formulated with a non-toxic mutant of diphtheria toxin called CRM_{197} [20]. Even though the incidence of invasive

pneumococcal infections declined effectively after the introduction of pneumococcal vaccine [21], nevertheless, the vaccine did not provide complete protection against all known serotypes and suffered from the rising prevalence of non-vaccine serotypes [22, 23]. Therefore, the search for novel safe and effective vaccination approaches against pneumococcal infections have never stopped.

2.2. Extracellular vesicles

The term “exosomes” was mentioned for the first time in 1981 to describe shedding vesicles with 5′ nucleotidase activity from normal and malignant cells by Trams *et al.* [24]. The family of extracellular vesicles (EVs) include exosomes, in addition to microvesicles (also denoted ectosomes or microparticles) and apoptotic bodies, identified according to their cellular origin and particle size. Exosomes (20-150 nm) are the smallest and are secreted from multivesicular bodies (MVBs) through exocytosis, while microvesicles (100-1000 nm) are directly secreted *via* budding or shedding from plasma membrane. Apoptotic bodies (50-5000 nm) are released by dying cells [25-27]. Evidence shows that almost every living cell can produce EVs, as well EVs were isolated from most biological fluids including plasma, saliva, urine and breast milk [28-30].

EVs contain seemingly the same components of their parent cells in terms of proteins, lipids and nucleic acids, and perform similar functions [31, 32]. These functions include intercellular communication, cellular homeostasis and signal transmission in physiological and pathological conditions [33]. In malignancy, EVs secreted from tumor cells and immune and non-immune host cells have a crucial impact on the tumor microenvironment (TME), they might stimulate or inhibit tumor progression, metastasis or drug resistance [34]. Since EVs play such a crucial role in TME, several studies tried to exploit them to inhibit tumor growth and activate a potent immune response against malignant cells.

As the secretion of cellular components is a vital process for all living cells [35], EVs serve as an export and/or exchange tool between cells, which is conserved in all eukaryotic and prokaryotic cells [36, 37]. Bacterial membrane vesicles (MVs) are lipid-bilayered spheres, which range from 20-500 nm [38]. Reporting on bacterial vesicles in literature dates back to 1960s, were it was shown that Escherichia coli secrete “extracellular lipoglycopeptide” when grown in lysine-limiting medium [39]. These secretions were mainly composed of cell wall components such as lipopolysaccharide. Until recently, it was thought that only gram-negative bacteria secrete MVs from their outer membranes hence denoted outer membrane vesicles (OMVs), while gram-positive bacteria were usually overlooked, owing to the inference that their thick and rigid cell wall may hinder the release of vesicles. However, many researchers in the last decade could successfully isolate and characterize vesicles from gram-positive bacteria

[40, 41]. Recent studies showed that MVs from microbiota or probiotic microorganisms help the maturation of our immune system and possess anti-inflammatory effect [42], as well as pathogenic bacterial MVs support inflammation and dissemination of infections [43]. They carry many cellular components including antigenic determinants and virulence factors, leading to immunomodulatory effects, therefore are investigated as vaccine carriers [44].

Secretion of membrane vesicles (MVs) from gram-positive bacteria had been long overlooked, owing to the inference that their thick rigid cell wall might hinder the shedding of membrane vesicular blebs [45]. Some reports of gram positive MVs might date back to the 1970s and formerly denoted mesosomes, but were considered as artifacts [46]. Recently, several studies isolated membrane vesicles from various gram-positive bacteria including Bacillus, Staphylococcus and Streptococcus species [47]. Bacterial membrane vesicles could induce immune response in host cells. They can interact with innate immune cells including macrophages, in addition to antigen-presenting cells e.g., dendritic cells (DCs) from adaptive immune system [38]. Introduction of bacterial virulence factors and/or antigens might interact with the immune system, which might respond via release of cytokines and activation of further immune cells. Thus, they might be invested to elicit a protective immune response [48]. In conclusion, the application of bacterial vesicles as vaccination approaches is well established and in clinical practice for meningococcal disease [27, 49]. In addition, bacterial MVs may constitute a good platform for preparation of cancer immunotherapy delivery systems [50]. Another important application is loading bacterial MVs with active ingredients, for drug delivery purposes, this potentially offers better targeting of specific tissues [51, 52].

2.3. Bacterial EVs for prophylactic vaccination against infectious diseases

2.3.1. Biogenesis of bacterial MVs

The exact mechanisms responsible for bacterial MVs biogenesis are not fully elucidated. Nevertheless, it is considered as a result of several processes [43], including shedding of bacterial MVs by blebbing of membranes from living bacteria or endolysin-dependent bacterial cell lysis [41].

a- Membrane blebbing

Membrane blebbing arises from disturbances in the bacterial cell envelope, due to unbalanced production of bacterial wall components or intercalation of large molecules in the outer membrane of gram-negative bacteria [53].

Various models were proposed for the bacterial MVs blebbing and shedding process including localized remodeling of cell membrane, unbalanced turgor pressure in the periplasmic space,

repulsion between anionic charges within lipopolysaccharide (LPS) molecules, and weak cross-linking between peptidoglycan and outer membranes [41, 54, 55].

b- Endolysin-dependent cell lysis

Based on enzymatic activity carried by double-stranded DNA phages, which tend to lyse their host bacterial cells in order to be released to surrounding milieu [56, 57], microbial cells liberate their contents by shedding MVs. This phenomenon is sometimes called explosive cell lysis in gram-negative bacteria and because of its milder extent in gram-positive bacteria called bubbling cell death [41].

c- Multiple stimuli impact vesiculation and contents of bacterial MVs

Several factors influence the production and release of MVs from bacterial cells. These factors are either inherent to bacteria or acquired as a response to surrounding conditions [58-60].

i. Genetic background of secreting strain

Some bacterial strains, such as genetically modified *Escherichia coli*, produce more MVs than their counterparts, owing to their genetic constitution. Therefore, their MV yield is higher, hence they are more suited for large-scale applications of vaccines or drug delivery carriers [61].

ii. Growth culture components and conditions

The availability or deprivation of some nutrients in bacterial culture medium may influence the secretion of MVs**.** A recent study demonstrated a significant difference in yield and proteome of released MVs from *Escherichia coli*, when subjected to iron-restricted or iron-supplemented conditions. Abundance of protein content of MVs was dependent on iron availability [62].

iii. Environmental factors including bacterial growth phase, temperature, and availability of oxygen

Surrounding conditions affect the release and loaded cargo into bacterial MVs. Klimentova *et al.* examined bacterial MVs from *Francisella tularensis* subsp. *holarctica*, under several stress conditions including oxidative stress, low pH values, high temperature (42 °C) or low temperature (25 °C). MVs showed many-fold increase in their vesiculation rate and/or change in their protein cargo as a response to unfavorable conditions [63]. Another study reported significant change in protein profile of *Campylobacter jejuni* OMVs, in response to exposure to low concentration of bile [64].

iv. Exposure to sublethal concentration of antibiotics

Production of MVs could be enhanced through weakening of bacterial cell wall upon exposure to sublethal concentration of antibiotics [65, 66]. It was shown that, DNA-damaging agents and antibiotics trigger SOS signals and induce bacterial MV formation, especially in lysogenic strains of *Staphylococci* [66].

For the development of MV-based vaccines, one can benefit from the accumulated knowledge about biogenesis and factors controlling bacterial vesicle secretion, to enhance their production and manipulate them to express desirable characteristics.

2.3.2. Cargo and function of bacterial OMVs/MVs

Literature provides evidence that MVs can harbor many bioactive molecules including lipopolysaccharides (LPS), periplasmic and cytosolic proteins, lipoproteins, nucleic acid material, enzymes, signaling molecules and other metabolites. For a more detailed discussion about composition and various components of bacterial MVs, the reader is referred to these recent reviews [41, 67].

Box 1. summarizes the main functions of bacterial MVs. Bacterial MVs are essential for microbial cell growth and community formation, both in planktonic and biofilm state [39]. They aid in intercellular communication and exchange between similar or different species [36]. They transport genetic material, enzymes, proteins and signal molecules [68]. Securing adaptation towards surrounding conditions and responding to stress as well as releasing stress products, decreasing levels of toxic material, and protecting microorganism against antibiotics and antimicrobial agents are amongst bacterial MVs' functions [69-71]. Microorganisms secrete MVs carrying anti-microbial molecules against competing bacteria [72-74], and control host-pathogen interaction by releasing toxins, virulence factors and antigens to spread the infection and evade the host immune system [75-78]. Bacterial MVs helps in releasing of attacking phages, through endolysin-dependent cell lysis [79].

Box 1. Key functions of bacterial membrane vesicles

- Formation of microbial communities (biofilms) [39]
- Cross species interaction [36]
- Responding to stress and release of stress products [69]
- Transport of nucleic acids [68]
- Protection against antimicrobial agents [71]
- Release of toxins and virulence factors [77, 78]
- Host-pathogen interaction and immune response [72, 75, 76]
- Release of antimicrobial peptides against competing bacteria [72]
- Aid in release of attacking phage [79]

2.3.3. Cellular interaction/uptake and Immune modulation of bacterial MVs into mammalian cells

Bacterial MVs have the ability to interact with and to be internalized inside mammalian cells, producing many effects on host cells (**Figure 1**). Hence, they are considered couriers for microbial material between bacteria and surrounding environment, including host cells. [73, 80]. This interaction is vital for the secreting microorganism, and understanding its mechanisms may provide us with valuable information to develop MVs in various applications such as vaccine delivery.

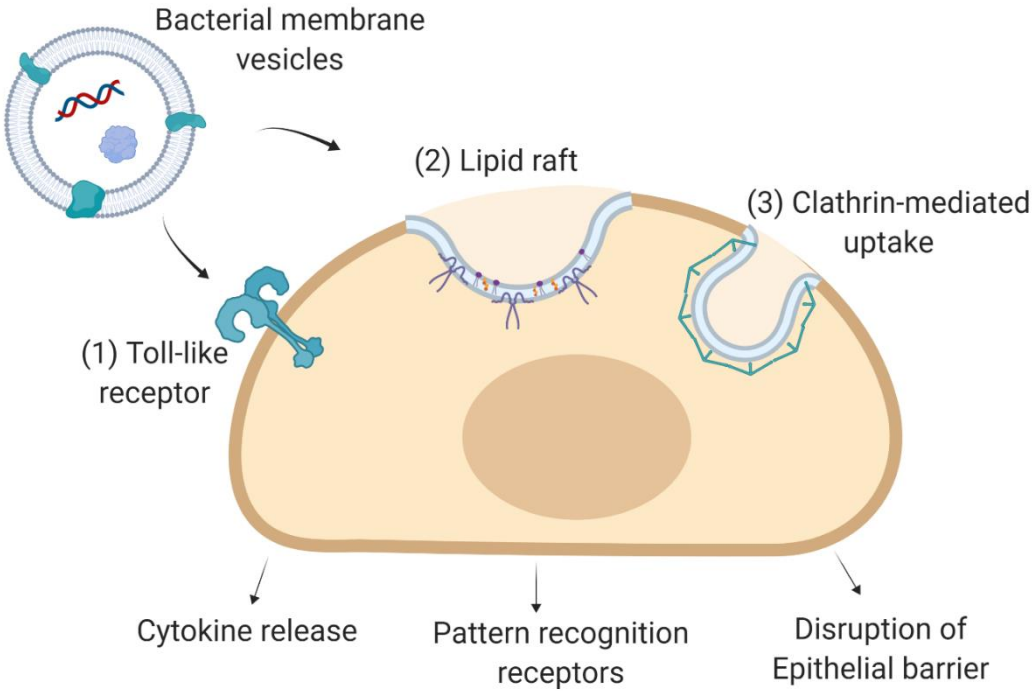

Figure 1. Various pathways for interaction/uptake of bacterial membrane vesicles (MVs) with mammalian cells and subsequent effects. Interaction pathways include immune signaling by toll-like receptors, lipid rafts, and carrier-dependent (clathrin-mediated) transport. Cytokine release, higher expression for pattern recognition receptors, and disruption of epithelial membranes are the most prominent outcomes of bacterial MVs on mammalian cells. Figure created with BioRender.com

Interaction/uptake of bacterial MVs with mammalian cells may occur through several pathways and mechanisms, including:

a. Immune signaling facilitates MV entry and uptake

Bacterial membrane pattern recognition receptors (PRRs), such as toll-like receptors (TLR), which facilitate MV entry into host cells [38]. *Moraxella catarrhalis*, which causes chronic obstructive pulmonary disease, secretes surface proteins including adhesins and virulence

factors into MVs. They interact with TLR2, causing pro-inflammatory response to evade host defense and facilitate entry into alveolar epithelial cells [81].

b. Lipid rafts

Enterotoxigenic *Escherichia coli* shows temperature-dependent uptake into adrenal and intestinal epithelial cells, which is facilitated by caveolin and cholesterol-dependent lipid rafts [82]. *Pseudomonas aeruginosa* and *Aggregatibacter actinomycetemocomitans*, common pathogens causing aggressive periodontitis, can fuse with lipid rafts to facilitate their entry into host cells [83, 84].

c. Carrier-mediated transport

Helicobacter pylori MVs can enter human gastric epithelial cells via cholesterol-independent, clathrin-mediated endocytosis [85]. Turner *et al.* demonstrated that MVs uptake is a function of their particle size. Smaller MVs (20-100 nm) showed better uptake through caveolin-mediated endocytosis, while micropinocytosis and endocytosis controlled uptake of larger MVs (90-450 nm) [86].

Bacterial MVs interact with host cells in various ways, in order to spread infection and evade the immune system. In addition, MVs could interact with immune cells (e.g. antigen-presenting cells) to produce protective immune response (**Figure 5**). The main immunomodulatory effects of bacterial MVs on mammalian cells are listed in **Table 1**.

Table 1. Main immunomodulatory effects of bacterial MVs on mammalian (epithelial and immune) cells

	Source of MVs	**Target mammalian cells**	***In vitro* effect**	**Ref.**
Epithelial cells				
Induction of cytokines	*Helicobacter pylori*	Gastric epithelial cells	Increased proliferation Dose-dependent IL-8 release	[87]
	Pseudomonas aerugionsa	Lung epithelial cells	Enhanced IL-8 secretion	[70]
	Legionella pneumoniae	Lung epithelial cells	Stimulate release of IL-6, IL-7, IL-8, IL-13, IFN-γ, G-CSF and MCP-1	[88]
	Klebsiella pneumoniae	HEp-2 cells	Increased IL-1β, IL-8	[89]

			Model: Intratracheal challenge in neutropenic mice	
	Acinetobacter baumannii	HEp-2 cells	Increased IL-1β, IL-6, Il-8, macrophage inflammatory protein-1α, monocyte chemoattractant protein-1 Model: Mice inflammation model (intradermal and intrtracheal administration)	[90]
Induction of pattern recognition receptors	*Escherichia coli*	A498 and T-24 cells	Increase Toll-like receptors (TLR-4) Increase IL-8 production	[91]
	Helicobacter pylori *Pseudomonas aeruginosa* *Neisseria gonorrhoea*	HEK 293 cells	Increased pro-inflammatory signal (NOD-1) Model: Intragastric immunization of mice	[92]
Disruption of epithelial barrier integrity	*Treponema denticala* *Porphyromonas gingivalis*	HEp-2 cells HSC-2 cells	Detachment of epithelial layers Dissemination of infection	[93, 94]
Immune cells				
Induction of cytokines and inflammatory mediators	*Neisseria meningitidis*	Neutrophils	Enhanced TNF and IL-1β	[95]
	Neisseria meningitides *Histophilus somni*	Neutrophils	Increased Neutrophil extracellular traps (NET) formulation Increased bacterial colonization	[96, 97]
	Neisseria meningitidis	Macrophages and monocytes	Increased IL-1β, IL-6, IL-8, IL-10, IL-12p40, TNF	[98]
	Salmonella	Mouse macrophages	Increased TNF and NO	[99]
	Salmonella	Dendritic cells	Increased expression of CD86 and MHC-II	[99]

			Increased release of TNF and IL-12 Model: Immunization of mice by intraperitoneal injection	
	Streptococcus	Dendritic cells	Rapid uptake of MVs into DC2.4 cell lines Increased release of TNF-α	[80]

2.3.4. Bacterial MVs as vaccine carriers against infections:

Bacterial MVs could disrupt the integrity of epithelial barriers, elicit pro-inflammatory effects and facilitate the pathogenesis of several bacterial infections and spread of bacterial cells [100, 101]. Due to the interaction of their surface ligands with membrane receptors and/or uptake into mammalian host cells, bacterial MVs deliver successfully their cargoes and express antigenic proteins and virulence factors and signaling molecules on their surface or encapsulated in their lumen [102]. Thus, they show characteristic pathogen-associated molecular patterns (PAMPs), which bind to pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) present on immune cell surface and drive inflammatory response in conjunction with complement system activation [103-106]. Consequently, they show a significant effect on both innate and adaptive immunity, which can be investigated for a protective response against pathogenic microbes. Bacterial MVs have thus sparked increasing interest to be applied for vaccine development [107]. Antigen carriers in the nanometer size range with self-adjuvant properties is an important prerequisite for vaccine development [67, 102]. Moreover, bacterial MVs are safer than live and attenuated bacteria, since they cannot replicate and have the potential to be engineered and modified [107, 108]. In addition, they can interact specifically with antigen-presenting cells e.g., dendritic cells, to stimulate antigen processing and presentation **(Figure 2)**, leading to a long-lasting protective immune response [38, 43, 109].

Nevertheless, safety of bacterial MVs needs to be considered because of the toxicity of danger signals, due to the presence of LPS, a main component of the outer membrane in gram-negative bacteria. LPS may cause a strong innate immune response because it is a potent stimulator of immune cells including monocytes and macrophages. It interacts with Toll-like receptor 4 (TLR-4)/MD-2 receptor pathway, which triggers NF-κB and interferon regulatory factor 3 (IRF-3)-dependent gene expression, leading to excessive release of cytokines [110-112]. In contrast, the resulting activation is mandatory for a subsequent adaptive immune response and protective effect, hence it is considered favorable for vaccination purpose [113]. However, LPS may trigger severe and even toxic effects including fever, inflammatory

reactions and septic shock through overshooting of innate immunity [114, 115]. Therefore, addressing toxicity problems due to bacterial MVs LPS content remains a cornerstone to develop a good vaccine candidate [116, 117]. Therefore, finding an optimal balance between the essential immune stimulation by LPS and avoiding excessive toxic or inflammatory reaction remains an important factor in successful formulation of MV-based vaccines.

In addition, the variable and small MV yield obtained from microorganisms depending on many factors and conditions is another obstacle. Lack of adequate immune response or weak immunogenic effects, owing to low expression of surface proteins and antigenic determinants, are also challenges that bacterial MVs might face [118, 119].

Hence, several studies explored possible techniques for engineering of bacterial MVs to overcome these problems, as needed for a safe and efficient vaccine. This must be free from harmful endotoxic properties, producible at sufficiently high yield, and capable to elicit adequate immune response for a protective effect.

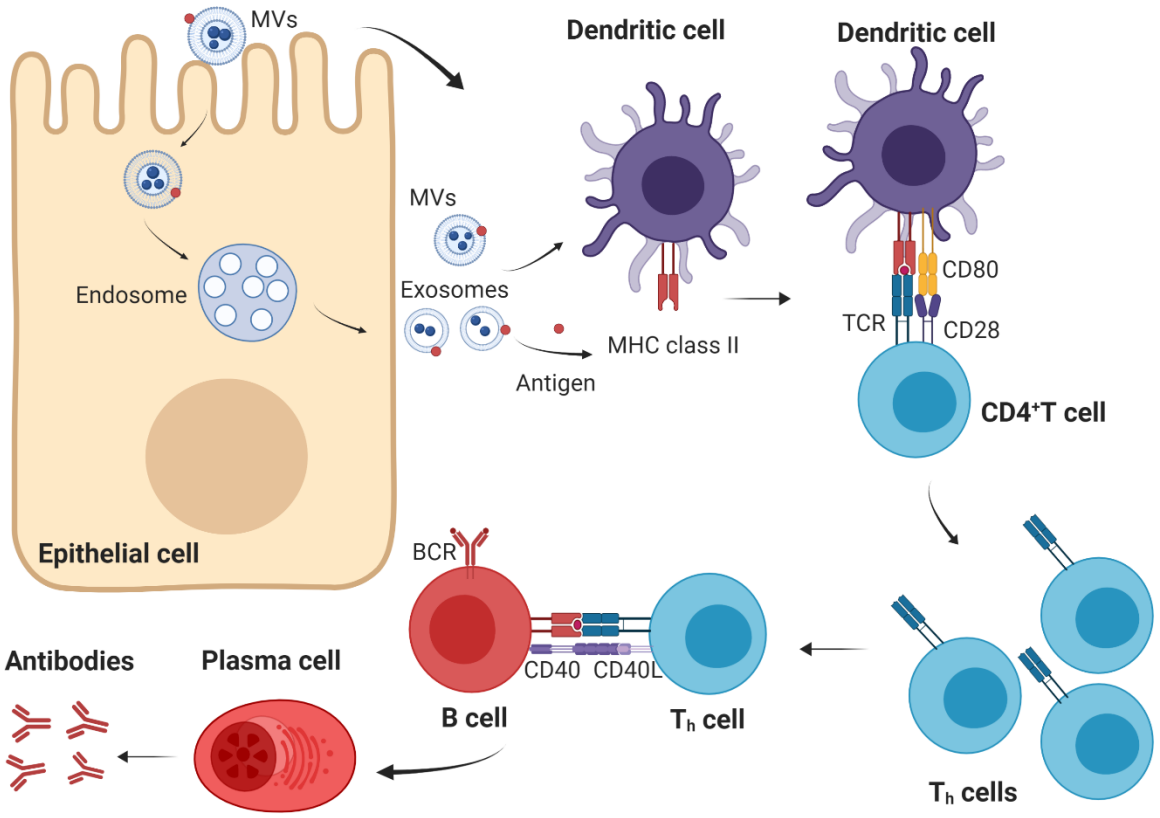

Figure 2. Interaction of bacterial membrane vesicles (MVs) with antigen-presenting cells (APCs) e.g., dendritic cells. Bacterial MVs internalize into epithelial cells and/or interact directly with APCs, leading to T cell activation, and further B cell activation and consequent protective immune response. Major histocompatibility complex (MHC), T-cell receptor (TCR), T-helper cell (T_h cells), B-cell receptor (BCR). Figure created with BioRender.com

2.3.5. Engineering of OMV vaccines

a. Surface antigen expression

Non-pathogenic bacteria were genetically engineered to display antigens from pathogenic bacteria. A common strategy is to fuse antigens to membrane proteins. Cytolysin A (ClyA) a pore-forming transmembrane protein, which is enriched on MVs, could elicit an immunological reaction [120]. Outer membrane protein (OMP) is utilized to fuse with the antigens of choice. Antigens from group A and B Streptococci fused to OmpA in *Escherichia coli* resulted in OMVs expressing streptococcal antigens onto their surface, induced higher functional antibody titers *in vivo*. Immunization with OMVs protected mice against challenge with a lethal dose of group A streptococci [121].

Surface-associated antigens on bacterial MVs are expected to cause stronger immune response than lumen-associated antigens. Nevertheless, antigens in lumen of MVs can induce adequate immune response and antibody production [107].

b. Cytotoxicity amelioration

As mentioned earlier, LPS is the main component of the gram-negative outer membrane and provokes toxic reaction of bacterial MVs in the host. Thus, removal of LPS is pivotal to prevent inflammation.

Detergent extraction is usually applied to decrease LPS content. However, they might cleave pathogen-associated molecular patterns (PAMPs) accountable for antigen recognition and presentation by immune cells, causing weaker immunogenic reaction, which is unfavorable for vaccination purposes [122]. In addition, it may cause increased MV aggregation tendency, which diminishes the product quality and results in other toxicity risks [123]. Detergent-free extraction, e.g EDTA-extraction decreases aggregation while maintaining the yield and immunogenicity of MVs [124].

Genetic engineering could be implemented to solve LPS toxicity problem. Mutational inactivation of gene msbB (known as LpxM), which encodes lipid A acyltransferase in *Escherichia coli* O157:H7, reduced endotoxicity of OMVs and produced suitable multifunctional vaccine delivery vehicles [125, 126].

MVs can be engineered with target molecules other than LPS e.g. deletion of global regulator (agr), controlling secretion of virulence factors and surface proteins, rendering MVs less toxic [127].

c. Yield enhancement

Since a variable and/or low yield of microbial secretions including MVs poses a problem for large-scale production, several methods were proposed to enhance vesicular production.

Hypervesiculating bacteria, secreting higher amounts of bacterial MVs, could be engineered via mutation in genes encoding lipoma-preferred partner (Lpp) or Tol-pal proteins, which connect membrane to peptidoglycan in gram-negative bacteria [124]. In gram-positive bacteria, degradation of peptidoglycan layer or reduction of its cross-linking enhance MV release. Treatment with sublethal concentrations of penicillin G caused weakened crosslinking of peptidoglycan layer and facilitated release of MVs, leading to increase in obtained MV yield [127].

2.3.6. Meningococcal disease

The application of MVs for the protection against meningococcal infections is one of the most elaborate examples of using MVs as vaccination avenue because it has several clinical evaluations in many countries during disease outbreaks. Since *Neisseria meningitidis* have 12 serotypes according to its capsular polysaccharide, only serotypes A, B, C, X, Y and W are able to cause human diseases. Even though serotypes A, C, Y and W have available capsular polysaccharide vaccine, yet due to antigenic structural similarity with human neural cells and meningococcal products, pose a risk for autoimmune effect [128, 129].

Several OMV-based vaccines were prepared to provide protection against meningococcal infection, such as:

a) **MenBvac®** was the earliest OMV-based vaccine, developed in Norway 1984-1986. It was isolated from wild-type strain 44/76-SL by deoxycholate extraction technique and aluminum hydroxide as adjuvant [130]. The vaccine provided promising results achieving 57.2 % protection rate against infection at the beginning, yet subsequent large-scale studies showed less protection and did not justify public vaccination [131, 132].
b) **VA-Mengoc-BC®** was introduced in Cuba, and isolated from wild-type strain CU-385. It demonstrated around 83% efficacy in clinical trials, with long-lasting and high antibody titers. It was applied to control meningococcal outbreaks in some countries such as Cuba, Brazil, Uruguay and Colombia [133-135].
c) **MeNZB®** was prepared in New Zealand, from the NZ98/554 strain, which caused an outbreak in New Zealand in 2000. It conferred significant high antibody titers in 96% of adults and around 75% in children [136]. It was discontinued in 2008, due to low levels of children protection (around 40 %) and relatively short duration (few months) of acquired immunity [137].

These OMV-based vaccines faced as a major problem, that they did not provide adequate broad protection, since the main antigen (Por A) is highly variable between different strains

[128]. Therefore, there was a need to broaden protection offered by these vaccines, aiming to cover most -if not all- meningococcal strains.

One way of addressing these challenges may be by using reverse vaccinology. Reverse vaccinology, which is a genome-based technique to determine possible antigens for vaccination, provides a promising tool to overcome this drawback and to enhance the coverage against several strains. It saves time for vaccine development, especially for pathogens, which are difficult to culture *in vitro* [138].

Therefore, the genome sequence of virulent *Neisseria meningitidis* strains was determined. Five proteins were able to induce immunity and were selected for a multicomponent vaccine. Later due to industrial scale requirement, it was developed into four of them expressed as fusion protein and available commercially as Bexsero® [139, 140].

Bexsero® is licensed in more than 35 countries, including the USA, the European union, Australia, Canada and Brazil, and sold over 1 million doses worldwide since 2013 [141]. It induced stronger response serum bactericidal antibodies, against vaccine antigens, and better strain coverage as assessed using meningococcal antigen typing system (MATS). It is recommended for high-risk patient groups, control of outbreaks and routine immunization for infants. Moreover, its safety and tolerability is comparable to other routine infant vaccines [141]. It even elicited antibodies against *Neisseria gonorrhoeae* [142]. However, little information is available about the long-term protection after infant vaccination [141].

2.3.7. Animal studies with OMV-based vaccine

Many published articles demonstrated protective immune response (**Table 2**) achieved upon application of bacterial MVs in various animal models. A study isolated OMVs from *Bordetella pertussis* and showed stronger protective effect of OMV-vaccine than acellular pertussis (aP) vaccine, and a similar effect to whole-cell pertussis (wP) vaccine, against lung infection in mice. Accumulated CD4 T cells with abundant memory cells provided long-lasting protective effect upon respiratory challenge [143].

A recent paper confirmed sufficient protection of mice after immunization using *Burkholderia pseudomallei* OMVs, against lethal pulmonary infection, in a manner similar to live attenuated vaccine **(Figure 3)**. In addition, OMV vaccination produced increased IgG, CD4+ and CD8+ T-cells, accompanied by the immune response [144].

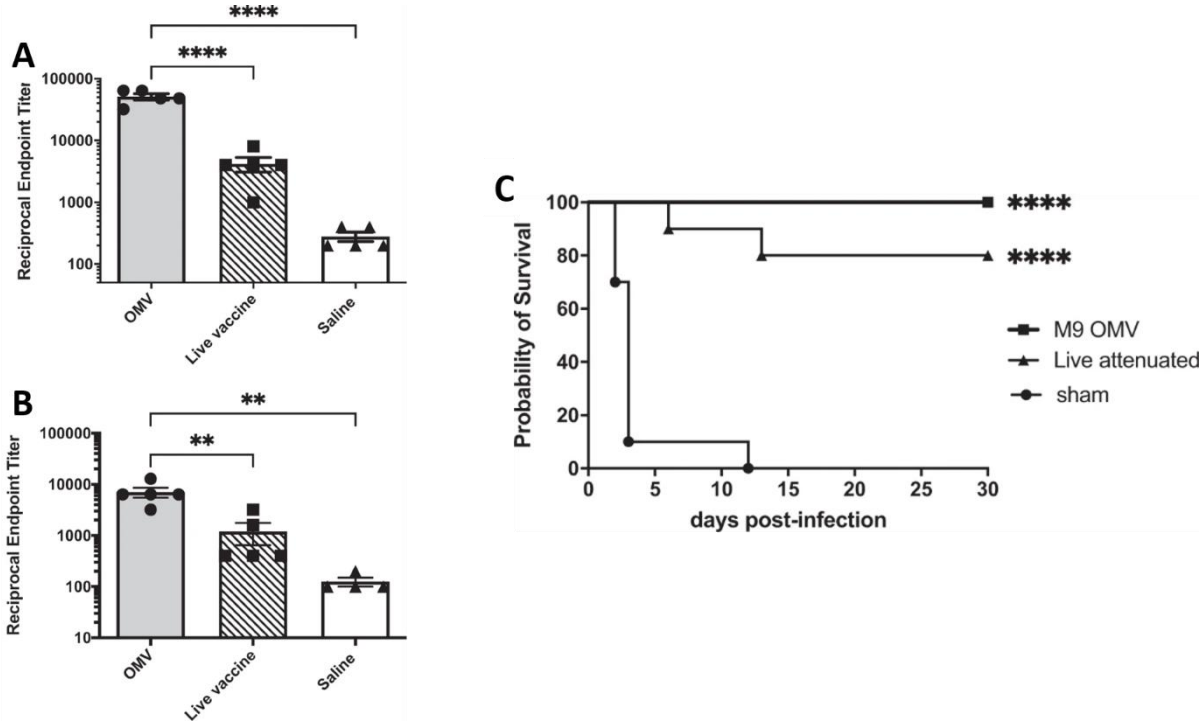

Figure 3. Protective effect of *Burkholderia pseudomallei* OMVs against pulmonary infection. Produced IgG titers upon treatment of mice with (A) OMVs and (B) heat-inactivated vaccines, showing comparable levels of immune response. (C) Similar survival rates were achieved through immunization with OMVs and live attenuated vaccines, in comparison with sham (saline) treated mice. Reprinted with permission from reference [144]

Table 2. Animal studies using bacterial membrane vesicles for vaccination and infection protection applications and their main findings

Microorganism	Main findings	Ref.
Salmonella Typhimurium	• OMVs harboring generalized modules for membrane antigens (GMMA) vaccines, induced diverse antibody profile in mice with higher bactericidal effect against nontyphoidal *Salmonella*	[145]
Francisella noatunensis subsp. *orientalis*	• OMVs contained several immunogenic factors (intracellular growth proteins, outer membrane protein (OmpA)) • Immunization protects adult zebrafish from subsequent challenge with a lethal dose of bacteria, via upregulation of acute inflammatory response and immunogenic effect	[146]
Vibrio cholerae	• OMVs vaccination to adult female mice protects infant mice against challenge with hyperinfectious bacteria. • Vaccine decreased colonization and bacterial burden and blocked transmission/infectivity of shed bacteria	[147]

Shigella boydii	• tolA-disrupted bacteria caused higher OMV yield • Oral immunization with OMV-vaccine secured 100 % protection against lethal nasal challenge with bacteria. It increased secretion of mucosal IgG and IgA, and proinflammatory cytokines (TNF-α, IL-6, IFN-γ)	[148]
Acinetobacter baumannii	• Immunization of mouse model of disseminated sepsis. • Stimulated strong antibody response against bacterial antigens and clinical isolates (including resistant strain)	[149]
Escherichia coli	• OMV vaccine protected mice against bacterial-induced lethality • OMV vaccine produced higher T-cell immunity, rather than B-cell, with increased secretion of IFN-γ and IL-17	[150]
Haemophilus parasuis	• Vaccination with OMVs provided protection of mice against lethal dose bacterial challenge	[151]
Klebsiella pneumoniae	• Intraperitoneal OMV-vaccine conferred protection against bacteria-induced lethality in mice • Enhanced innate immune response (increased proinflammatory mediators production and T-cell response)	[152]
Bacteroides thetaiotaomicron	• Genetically-engineered gut microbiota bacteria to secrete bacterial, viral or human proteins into OMVs • Bacterial proteins include (*Salmonella enterica* vaccine antigens, influenza A virus vaccine antigens) to protect against infection.	[153]

2.4. Cancer Immunotherapy

Cancer immunotherapy is therapy applied to treat tumors using the components of immune system including antibodies, cells, and proteins in addition recently EVs are promising candidates and it boosted the field of cancer therapy [154, 155]. Over the years, many scientists suggested to exploit immune system to combat cancer through activation of immune cells and creation of a potent adaptive immune response. [156, 157].

Many tumors suppress the immune response via several inhibitory pathways known as checkpoints, which are related to immune homeostasis or escape from immune detection. Two of these checkpoints, namely cytotoxic T-lymphocyte protein 4 (CTLA4) (discovered by James P. Allison) is a negative regulator of T-cell activation by competing with co-stimulatory molecule CD28 from binding to shared ligands CD80 and CD86. The other is programmed cell death

protein 1 (PD-1) (discovered by Tasuko Honjo) is expressed on T-cell surface during priming and bind to complementary ligands (PD-L1 or PD-L2) [157, 158]. Blocking these checkpoints showed good antitumor responses in animal models and several clinical trials provided evidence for the feasibility of checkpoint inhibiting antibodies and approval of these medicaments for anticancer use in market [159, 160]. Hence, both cancer immunologists received the 2018 Nobel Prize in Physiology or Medicine “for their discovery of cancer therapy by inhibition of negative immune regulation” [161].

2.4.1. Application of EVs in Cancer Immunotherapy:

Application of EVs from immune cells and/or tumor cell in anticancer immunotherapy is a promising approach. EVs may support immune regulation, matrix remodeling, signal transduction pathways, induction of angiogenesis and a pre-metastatic niche. In comparison with conventional cellular antitumor vaccines, cell-free EV-based vaccines offer many advantages including enhanced stability and ease of storage for longer periods without compromising their activity since they are non-cellular components and easier to maintain their effect unlike cell formulations [162, 163]. Our group showed that EVs could withstand several storage conditions; EVs could preserve the activity of loaded glucuronidase enzyme even after lyophilization [163]. We demonstrated that EVs exhibited minor alterations in terms of size, concentration and protein content upon exposure to physiologic temperature 37 °C. Exposure to even higher temperatures did not compromise cellular uptake of EVs into macrophage-like THP-1 cells [37].

Capturing antigens loaded onto EVs is easier than isolating soluble molecules from producing cells e.g. antigen-presenting cells [164]. As well, EVs are superior to other synthetic nanocarriers (e.g. liposomes or polymeric nanoparticles) that they are less toxic to healthy cells and environmentally friendly isolation, as they do not require much chemical material to isolate them [165]. Unlike synthetic nanocarriers, which require organic solvents and various chemicals to fabricate them. Most EVs are capable to evade phagocytosis, since they harbor CD47 on their surface that act through signal-regulatory protein alpha (SIRPα) as “don’t eat me” signal, thus prolonged circulation and decreased premature clearance [166]. However, other sources claim similar clearance profile of EVs to liposomes [167]. Some exosomes showed even rapid clearance after intravenous administration, which might be due to difference in EV source, method of isolation or presence of certain lipid/protein on their surface [168, 169]. Possession of plasma membrane-like phospholipids and proteins may decrease clearance and enhance their cellular uptake and therapeutic effect. They can permeate through biological membranes even through blood brain barrier and reach brain tumors

[170].Therefore, EVs exhibit many promising characters for cell-free antigen delivery, which can be engineered or modified to enhance their pharmacokinetic and/or pharmacodynamic properties.

The ability of DC-derived EVs to retain similar immunostimulatory properties of parent DCs made them among the most promising cell-free anticancer vaccine candidates **(Figure 4. A)** [171, 172]. Proteomic study of DC EVs demonstrated their characteristic molecular composition and immunostimulatory potential [173]. Their membrane proteins content including microdomain organizing proteins, adherins and lactadherins facilitate targeting, docking, fusion and uptake into recipient cells [174]. Another interesting aspect of DC EVs that they harbor antigen presentation machinery; peptide-MHC II complexes are present on EV membrane for antigen-specific T-cell responses [175]. DC-derived EVs express active molecules on their surfaces which interact with tumor antigens including MHC class I and II, co-stimulatory and adhesion molecules (CD40, CD80, CD86) necessary for induction of T-cell response against tumor [176] and could elicit antigen-specific tumor regression in cancer model [177]. Several studies showed that DC EVs could stimulate CD8+ T-cell [178], however some studies stated that they are more efficient in stimulating activated T-cells rather than naïve counterparts [109].

DC EVs could present antigen through MHC to T-cells either directly or indirectly. Indirect pathways include cross-dressing of APCs, where EVs are abundant on their surface and transfer peptide-MHC complex [179]. Alternatively, EVs are captured by APCs through phagocytosis, followed by reprocessing of peptide-MHC and transfer of antigens to APCs [180]. Autologous DC-derived exosomes in phase I clinical trial were well tolerated without showing any severe side effects [181]. DC-derived EVs could induce a strong T_{H1}-skewed immune response in mice [182]; while *in vivo* T- and B-cell anticancer responses are a function of whole antigen i.e. ovalbumin rather than MHC-peptide on exosome surface [183]. A study demonstrated better *in vivo* immunostimulatory effects from DC-derived smaller exosomes in comparison with larger size microvesicles. Exosomes induced stronger antigen-specific IgG response and antigen-specific CD8+ T-cell population, probably due to more amounts of loaded antigen onto exosomes [184].

2.4.2. Loading of EVs with antigens

Loading of EVs with their therapeutic payload represents a crucial step to prepare anticancer EV vaccines **(Figure 4. B)**. Cargo is loaded onto vesicles, either exogenously after their isolation from producing cells, or endogenously during their biogenesis [185]. Several techniques were employed for exogenous loading of EVs such as electroporation, sonication,

co-incubation, saponin-aided, extrusion , dialysis and freeze-thaw cycling, with variable degrees of efficiency of loading and maintaining effect [186-188]. Nevertheless, these techniques might cause aggregation of EVs or their payload, alter their physicochemical/morphological characteristics, or diminish or even destroy their effect [169]. Endogenous loading of EVs can exploit the intracellular sorting machinery for production and load certain biomolecules into vesicles before secretion. Cells might be endogenously loaded through several methods including direct transfection especially for RNA delivery [189] and co-incubation, leading to secretion of EVs loaded with these cargos.

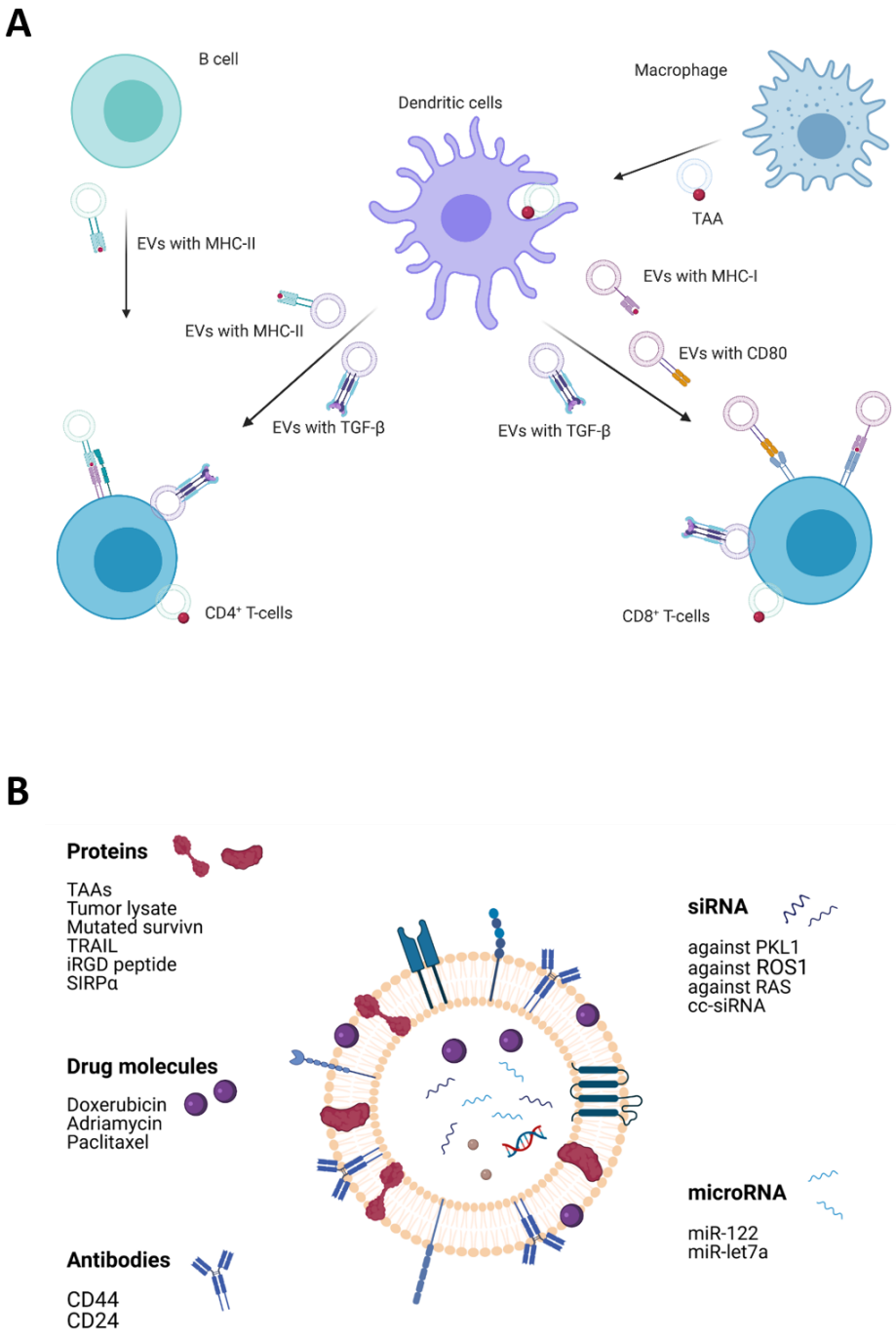

Figure 4. Role of dendritic cell extracellular vesicles and their application in cancer therapy. (A) Possible pathways of antitumor immune response depending on dendritic cells extracellular vesicles. (B) Dendritic cell extracellular vesicles harbor and/or modified with several proteins/antigens/drugs with potent anticancer effect.

2. 5. Previous studies on pneumococcal MVs in the literature

Few studies in the literature reported isolation of pneumococcal MVs and performed some characterization of the isolated nanostructures. Olaya-Abril *et al.* isolated extracellular vesicles

from pneumococci using ultracentrifugation, followed by gradient ultracentrifugation of the obtained pellet [190]. They reported a mean particle size of vesicles below 100 nm and described the process of vesicular secretion as membranous invagination from bacterial plasma membrane. Furthermore, they carried out lipidomic analysis of isolated vesicles and mentioned that both mammalian EVs and bacterial plasma membranes share similarities, yet EVs membranes were enriched in short-chain fatty acids. They described abundance of membrane-associated proteins and lipoproteins, *via* proteomic analysis. The study performed a mice survival assay after treatment with a lethal pneumococcal MVs dose, which protected the mice from death after challenge with pneumococci [190].

Another study from Choi *et al.* isolated pneumococcal vesicles using ultrafiltration followed by ultracentrifugation and the obtained pellet was purified using sucrose gradient ultracentrifugation [191]. Moreover, they described a wide range of average diameter for isolated vesicles 40 – 200 nm. The isolated vesicles from laboratory strain S. pneumoniae R6 strain BAA-255 did not harm the viability of A549 cells, while vesicles from a pathogenic strain (KCCM-41569) caused significant cytotoxic effects. In addition, they conducted a mice immunization experiment with the isolated MVs, which elicited a protective immune response and around 60% of the immunized mice survived bacterial challenge, in comparison with only 10% survival with control group [191].

Codemo *et al.* used gradient centrifugation to isolate pneumococcal vesicles and performed a proteomic analysis, which revealed the presence of several pneumococcal surface virulence proteins, as well as enrichment with the cytotoxin pneumolysin [192]. They showed that pneumococcal MVs are successfully internalized by human lung epithelial cells. Moreover, pneumococcal MVs could interact with monocyte-derived DCs and elicit release of proinflammatory cytokine response. The authors observed impairment of phagocytic activity of macrophages after addition of pneumococcal MVs to human sera [192].

A recent study by Yerneni *et al.* isolated pneumococcal vesicles in the mid-late exponential phase, using ultrafiltration followed by purification with mini-SEC columns [193]. The isolated vesicles exhibited mean size range of 27 – 400 nm, a yield of 10^9 particles from 500 mL culture supernatant and protein content around 45 µg/mL purified vesicles. The isolated MVs demonstrated successful internalization into mouse macrophages (J774A.1) and primary T-cells, in addition to induction of NF-κB in macrophages. Moreover, the authors tested systemic delivery of pneumococcal MVs by intravenous injection into mice, where they caused increased in the total macrophage count, a decrease in NK and some T-cells, and significant changes in spleen, confirming that pneumococcal vesicles triggered strong immune response in blood and spleen after systemic delivery. The proteomic analysis showed that some biological functions of vesicles are attributed to their lipoprotein contents. Further, they

reported enhanced immune cell recruitment after local application of pneumococcal MVs, as well as induction of alternatively activated macrophages. In addition, they claimed that MVs pneumolysin content aids in macrophage phagocytosis and bacterial survival [193].

Overall, this motivated us to study in further details pneumococcal membrane vesicles, isolate and purify them using optimal techniques, explore methods to increase their yields, assess their compatibility and uptake with mammalian cells, examine their interaction with primary immune cells, explore their proteomic contents, evaluate their potential immunostimulatory effects and feasibility for a cell-free vaccination avenue. Pneumococcal MVs provide a promising and innovative tool to trigger protection against pneumococcal infections and formulate vaccination avenue.

2.6. Spray drying

Vaccines save around 2-3 million lives annually, however millions of mortalities are recorded from vaccine-preventable infections [194]. Many obstacles aggravate this problem including the difficulty of vaccine distribution to remote and rural areas, where vaccines suffer from instability [195]. Temperature-dependent degradation represents a major factor in vaccine degradation, since high temperatures accelerate destabilization pathways [196]. Therefore, cold chain systems were devised to maintain vaccine stability in a frozen or refrigerated state, throughout all steps of production, transport, storage and distribution [197]. Cold chain systems are a costly challenge, especially for low-income countries and disruptions of these cold chains may lead to almost 50% wastage of vaccines worldwide [198]. Liquid vaccines are the most common dosage forms; nevertheless, liquid formulations are highly prone to degradation in aqueous environment, hence adversely affecting their shelf life [197, 199]. Many degradation pathways might destroy protein vaccines including physical pathways e.g. unfolding, denaturation, precipitation, aggregation and adsorption, while chemical degradation pathways comprise hydrolysis, oxidation and deamidation [200]. Consequently, the removal of water through drying could significantly boost the stability of vaccine components, inhibit these detrimental pathways, prolong their shelf life and eliminate the need for the costly cold chain [201, 202].

Spray drying is a single-step continuous drying process that produces dehydrated fine dispersible powder with adjustable physicochemical and morphological properties from liquid feed. Furthermore, it is a well-established technique industrially in food processing, chemical industries and generate bulk powder product that can be packaged into customizable quantities [202]. The first patent for spray drying dates back to Robert Stauf in 1901, to produce dry powder from milk, blood and other aqueous solutions [203]. Spray drying process

comprises atomization of liquid feed, droplets drying, powder collection and further processing of the obtained powder. In addition, it is relatively less expensive than lyophilization, because it does not require freezing or high vacuum and consumes less energy than lyophilization [204]. Spray drying generates fine powder, which suits unconventional drug delivery routes of vaccines including oral [205], intranasal [206] and pulmonary routes [207], without the need for reconstitution. Spray drying lacks a freezing step, which is desirable for freeze-sensitive adjuvants [204].

In spite of its various merits, spray drying may suffer from some drawbacks. The process might result in 3-7 % residual moisture content [208], while only 1-2% residual moisture contents is desirable in live attenuated vaccines [209]. Thus, additional secondary drying steps might be necessary, which expose the vaccine to shear stress of atomization and thermal stress from heated gases, leading to loss to antigenicity and higher costs [210, 211]. Nonetheless, the careful selection and optimization of spray drying process parameters can prevent many of these disadvantages [212]. Atomization could be tuned to decrease shear stress and drying air temperature and flowrate could be balanced to achieve satisfactory residual moisture content without compromising the formulated antigens with heat stress [199]. Another obstacle for spray-dried vaccines is sterilization of products. Hence, aseptic spray dryers are introduced and have applied to produce Raplixa®, the first approved spray-dried biologic [213], as well as inhalable insulin formulations [214]. Terminal sterilization could be employed, yet it might cause excessive heat stress and vaccine degradation. Irradiation terminal sterilization was successfully tested for spray-dried influenza vaccine [215].

Spray-dried vaccines have not reached the market yet [199]. However, several studies were applied to confirm their physical stability and immunogenicity *in vitro* and *in vivo* against both viral [216, 217] and bacterial [218, 219] infections. The first spray-dried pulmonary vaccine to enter a phase I clinical trial was a vaccine against measles [220]. Another clinical trial was of a subunit influenza vaccine, *via* both intramuscular and pulmonary routes, which showed a promising long-term stability of spray-dried vaccines at 20 °C for 3 years [221].

A single study in the literature prepared thermostable spray-dried bacterial OMVs from *Bordetella pertussis* for vaccination purpose. The authors reported improved stability of prepared powder in comparison with liquid formulation, without degradation of a major antigen in OMVs called Vag8. Pulmonary immunization of mice using spray-dried OMVs demonstrated similar immune response and protection against challenge with live bacteria, as liquid OMVs [222]. This study shed light on the potential of spray drying to produce safe and effective vaccine, suitable for pulmonary delivery. Thus, we wanted to explore spray drying of gram-positive vesicles and optimize the spray drying conditions to achieve highest stability without

affecting their immunogenic effect, to formulate a successful dry powder inhaler (DPI) vaccine for pulmonary immunization.

3. Aim of Work

Streptococcus pneumoniae (Pneumococcus) is a gram-positive bacterium, which normally colonizes the respiratory tract. Young children and elderly populations, in addition to immunocompromised individuals are most prone to pneumococcal-related infections [223]. These diseases account for high morbidities and mortalities worldwide, predominantly in developing countries [3, 224].

Pneumococcus, owing to its higher disease burden and increasing antibiotic resistance rates, poses a global health issue. Consequently, in 2017 the World Health Organization (WHO) announced *Streptococcus pneumoniae* in the list of priority pathogens, which require development of new antibiotic strategies [6]. Pneumococci exhibit high genetic diversity owing to variation in the capsular polysaccharide structure, which is the main virulence determinant and dominant immunogenic structure. The introduction of pneumococcal vaccines induced a significant decline in invasive pneumococcal infections and a decrease in carrier and transmission cases [225]. However, pneumococcal vaccines do not offer complete protection against all 100 known serotypes [2, 226-228]. Therefore, researchers continue to seek additional, safe and effective vaccines against pneumococci.

Bacterial membrane vesicles (MVs) are lipid-bilayer structures with particle sizes ranging from 20–500 nm [38, 229]. Gram-positive bacterial cell walls undergo local lysis and cytoplasmic membrane bulging, leading to bacterial vesicle and nanotube secretion [230]. MVs can interact with mammalian cells and successfully deliver their antigenic payload into immune cells. Consequently, they can induce considerable innate and adaptive protective immune responses against pathogenic bacteria in a safe and effective vaccine formulation [108, 229]. Nevertheless, bacterial MV production suffers from low and variable yields. Many factors influence the MV production process, yield and characteristics. Hence, the production of bacterial MVs with abundant yield is essential for vaccine development.

Many vaccine formulations are prone to physical and/or chemical degradation in aqueous environment; hence, they require cold chain to preserve their stability. Maintenance of cold chain is costly and complicates the widespread storage and distribution of vaccines worldwide, especially in low-income countries [231, 232]. Therefore, many techniques are explored to maintain the stability and efficacy of vaccines in extreme temperature in an affordable manner.

Spray drying is a single-step process of converting liquid feed into dried powder via passing an atomized spray into heated gas. It provides many merits including being an adjustable

continuous process, consistent dried powder quality, flexible dryer design and applications, suitability for both heat-labile and heat-stable, feasibility to dry various feeds such as solutions, emulsions and suspensions, and relatively affordable, scalable and consistent technique to obtain dried powder in comparison with lyophilization [195, 233]. Thus, spray drying poses a strong potential to prolong the shelf life of bacterial MVs as a dried powder formulation for vaccination application [234, 235]. Moreover, mucosal immunization with inhalable powder is potentially stronger in eliciting immune protection than parenteral injection against respirable infections, such as pneumococci and tuberculosis [236]. Therefore, spray drying might be a promising tool to maximize the stability and application of pneumococcal MVs vaccine formulation for pulmonary immunization.

Overall, these motivated the work of this PhD project to explore the following goals:

- Better understanding of pneumococcal membrane vesicles and optimization of their production conditions.
- Assessment of pneumococcal membrane vesicles safety on several epithelial and immune cell lines and measurement of cellular viability and cytotoxic effects, if any.
- Determination of uptake/interaction of pneumococcal membrane vesicles with epithelial and immune cell lines, to ensure delivery of their contents into cells.
- Verification of strong immunogenic character of pneumococcal membrane vesicles, as a promising vaccine candidate.
- Proteomic analysis of the antigenic protein and lipoprotein contents of pneumococcal vesicles, to confirm their antigenic potential
- Enhancement of yield and/or immunogenicity of pneumococcal membrane vesicles.
- Evaluation of safety, uptake and immunostimulation of pneumococcal membrane vesicles on human primary immune cells, to simulate *in vivo* environment.
- Preparation of a dry powder formulation of pneumococcal membrane vesicles, to enhance the stability of loaded vesicles.
- Characterization of the prepared microparticle formulation, as dry powder inhaler vaccine for pulmonary immunization against pneumococcal infections.
- Confirmation of immunostimulatory effect of prepared vaccine microparticles.

4. Materials and Methods

4.1. Microbial culture and MVs isolation

Streptococcus pneumoniae reference strain R6 (ATCC® BAA255™, USA), whose genome is fully sequenced and annotated was selected [237]. It lacks the capsule, as the capsular polysaccharide might diminish the yield of secreted MVs.

Bacteria were grown overnight in 1 L of Bacto™ brain heart infusion (BHI) medium (BD Biosciences) at 37 °C/5% CO_2 (**Figures 5 and 6**). We grew bacteria for 24 h to isolate stationary-phase MVs (sMVs) and 48 h to isolate death-phase MVs (dMVs) in Bacto™ brain heart infusion nutrient medium (BD Biosciences) at 37°C/5% CO_2 (**Figures 7**). The medium was then collected and centrifuged twice at 3800 × g for 15 min at 4 °C, in order to remove bacterial cells and fragmented debris. Afterwards, the collected supernatant was sterile filtered through Strericup Durapore PVDF 0.45 µm pore membrane filter (Merck Millipore, Germany). Sterility of filtrate was checked via overnight incubation of filtered supernatant and showing no bacterial growth upon inspection, as observed by absence of any turbidity and no change in optical density at 600 nm.

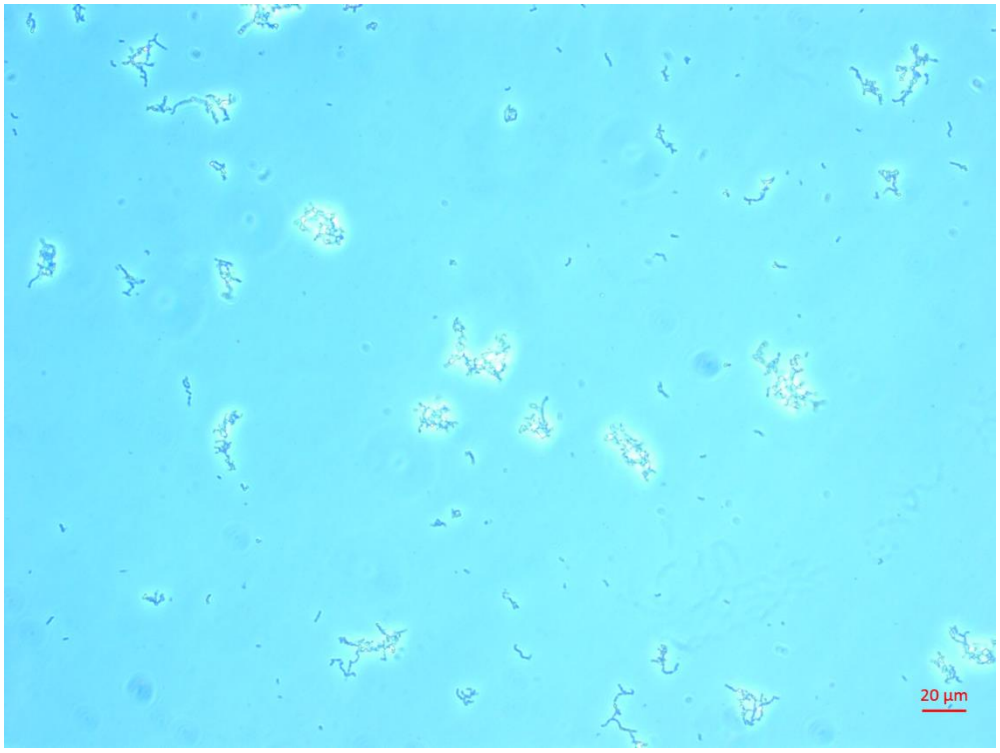

Figure 5. Morphology of *Streptococcus pneumoniae* reference strain R6, grown in brain heart infusion (BHI) medium, under light microscope (32x magnification), in stationary phase (≈ 24 h)

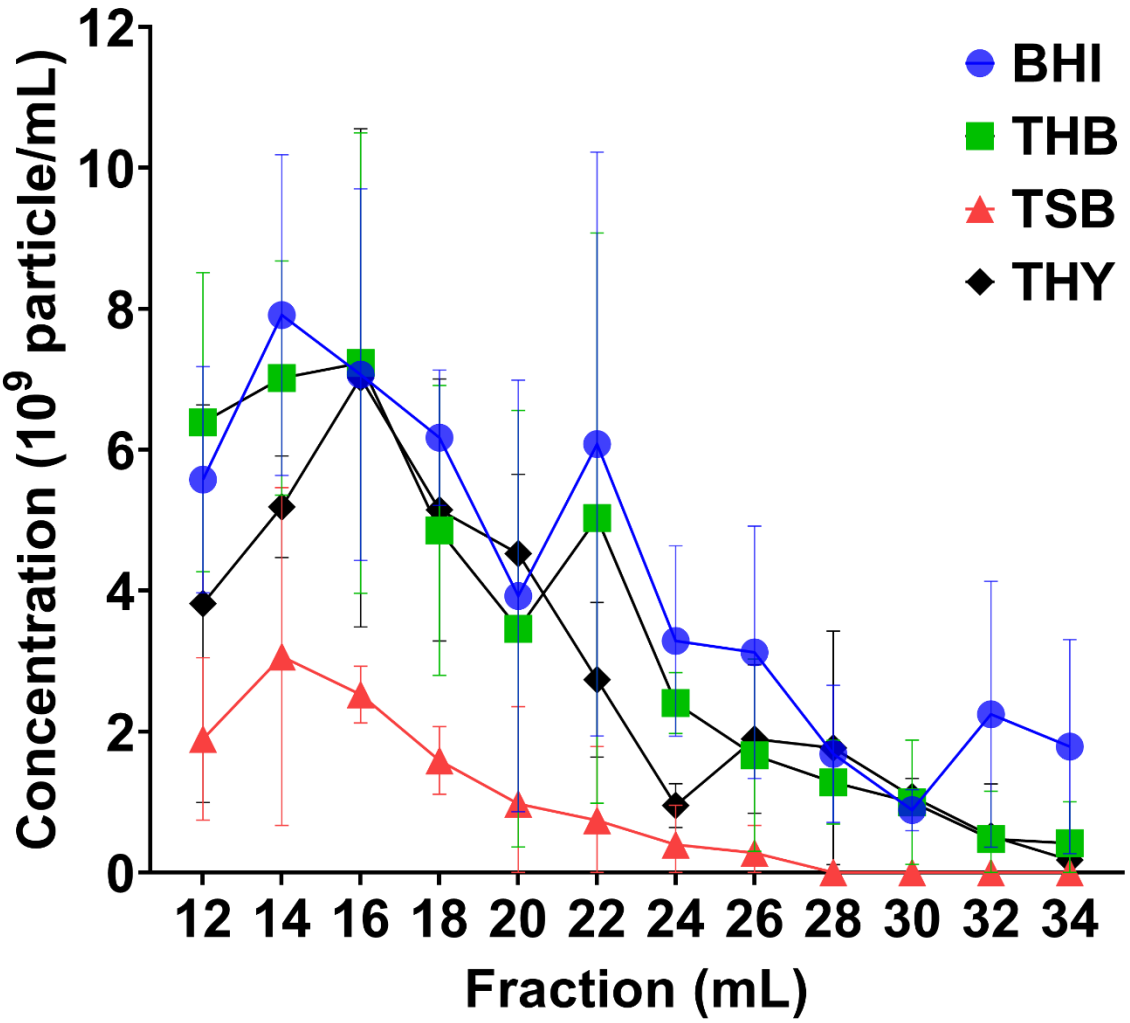

Figure 6. Preliminary study of pneumococcal membrane vesicles (MVs) production yield from four different culture media, namely brain heart infusion (BHI), tryptic soy broth (TSB), Todd Hewitt broth (THB) and Todd Hewitt broth supplemented with 0.5 % *w/v* yeast extract (TSY). A volume of 120 mL supernatant and 35-mL packed Sepharose CL-2B column for size exclusion chromatography were applied, followed by nanoparticle tracking analysis (NTA) measurement of produced MVs-rich fractions (12-34).

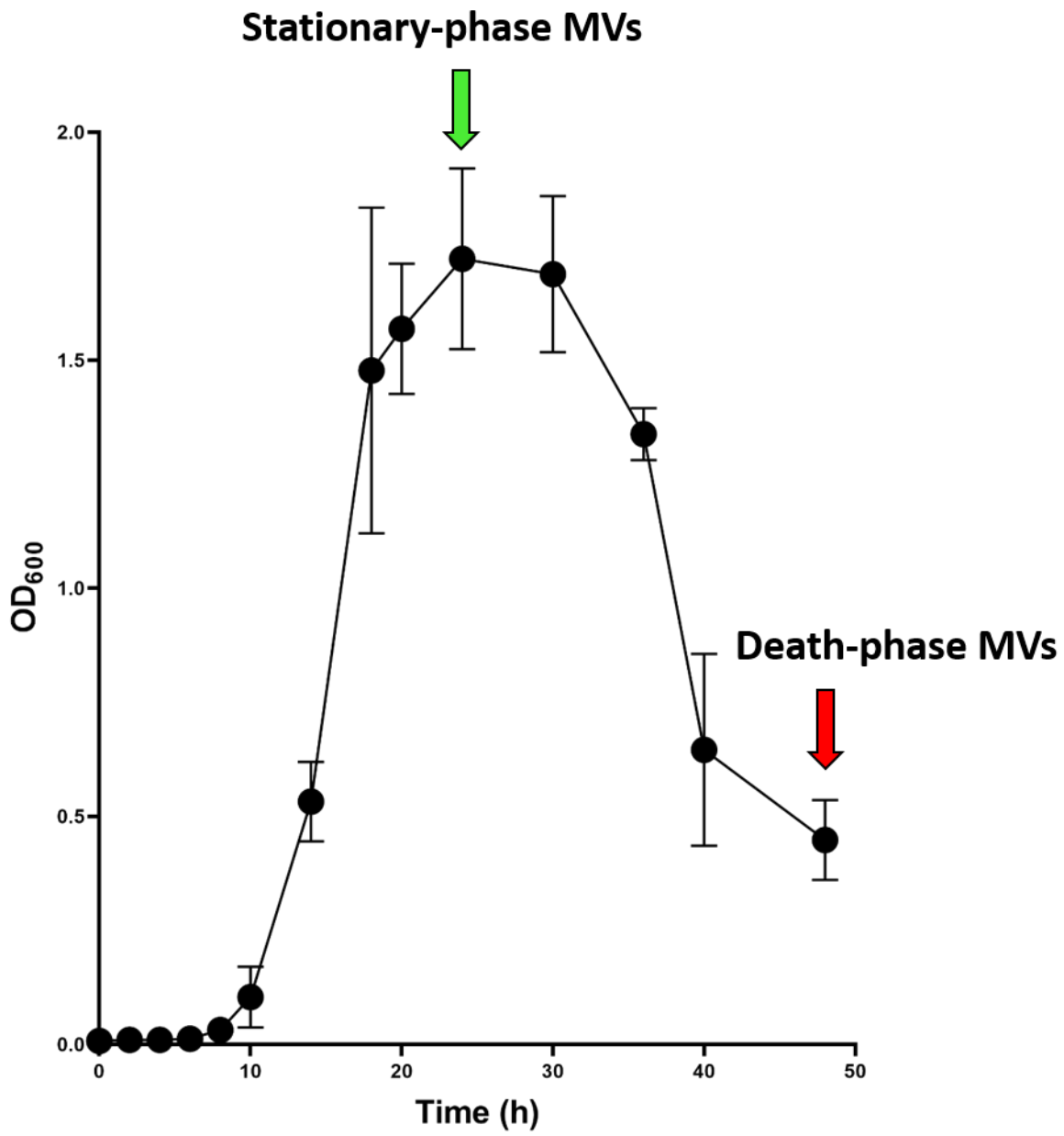

Figure 7. Growth curve of *Streptococcus pneumoniae* R6 strain (ATCC® BAA255™, USA), showing OD600 values of bacterial culture medium collected at different time points of collection. Arrows indicate the stationary-phase (after 24 h, sMVs) or death-phase (after 48 h, dMVs) of growth

Filtered supernatant was loaded in 70-mL ultracentrifuge tubes, and centrifuged at 100,000 × g for 2 h at 4 °C (rotor SW 45Ti, Optima L-90k, Beckman Coulter, Germany) to obtain vesicle-rich pellet. Then the supernatant was removed carefully, and the pellet was resuspended in minimal volume (≈ 500 µL) of filtered phosphate buffer saline (PBS) (Gibco PBS tablets without calcium, magnesium and phenol red).

4.2. Purification

The resuspended pellet was purified by size-exclusion chromatography (SEC) column loaded with Sepharose CL-2B (GE Life Science, UK) to separate MVs from soluble protein impurities. 1-mL eluted fractions were collected in polypropylene tubes (Axygen, Corning, Germany) and then, stored at 4 °C for no longer than a week until used in further experiments [238].

4.3. Bicinchoninic acid (BCA) assay

To check the efficiency of SEC purification, protein concentration in collected SEC fractions was determined through bicinchoninic assay kit (BCA) ((QuantiPro™ BCA Kit, Sigma Aldrich)), according to the manufacturer's recommendation. Analysis of samples was done in triplicates using a standard calibration curve of bovine serum albumin (BSA).

4.4. Particle size, concentration and size distribution

Particle size distribution and yield of MVs were determined using nanoparticle tracking analysis (NTA, LM-10, Malvern, UK). To achieve comparable results, samples were diluted up to 1:1000 in filtered PBS to maintain vesicle concentration within the recommended range of 20 – 120 particles/frame. Briefly, 400-µL MVs sample was introduced into a green laser-illuminated chamber, and a high-sensitivity video with camera level 13-15 was captured, three videos of 30 s length were recorded and processed by Nanosight 3.1 software [163].

4.5. Electron microscopy

Cryogenic transmission electron microscopy (cryo-TEM) was performed on MVs pelleted after ultracentrifugation. Briefly, 5 µL of sample was dropped onto a holey carbon grid (type S147-4, Plano, Wetzlar, Germany) and plotted for 2 seconds before plunging into liquid ethane at T=-165°C using a Gatan (Pleasanton, CA, USA) CP3 cryo plunger. The sample was transferred under liquid nitrogen to a Gatan model 914 cryo-TEM sample holder and investigated at T=-173°C by low-dose TEM bright-field imaging using a JEOL (Tokyo, Japan) JEM-2100 LaB6 operating at 200 kV accelerating voltage. We acquired 1024×1024-pixel images using a Gatan Orius SC1000 CCD camera with 4-s imaging time and binning 2.

Bacteria were prepared for scanning electron microscopy (SEM), by centrifugation at 1000 × g for 5 min to pellet bacterial cells. The pellet was resuspended in 4 % p-formaldehyde (PFA) to fix the cells for 1 h. Then bacterial cells were resuspended in 200 µL of hexamethyldisilazane (HMDS), and mount 2 µL suspension onto silicon wafer and dry overnight. The samples were investigated with/without gold sputter coating, using a FEI

Quanta 400 FEG (Thermo Scientific, USA) in high vacuum conditions at 5 kV accelerating voltage.

4.6. Preparation of liposome and polystyrene bead control particles

Liposomes were prepared from Dipalmitoylphosphatidylcholine (DPPC) and 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) (molar ratio 3:2 and final concentration 5 mg/mL) using thin-film hydration technique, followed by extrusion through polycarbonate filter at 40 °C. Liposomes were fluorescently labelled using the same protocol performed with MVs. Commercially available polystyrene beads (Polybead® Carboxylate 0.1 µm, Polysciences Incorporation, Germany), were used as inert nanoparticles.

4.7. Cell culture

Five cell lines were utilized in this work. The human alveolar adenocarcinoma basal epithelial cell line, A549, was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ-ACC 107, Germany) and cultured in RPMI 1640 (Life Technologies Limited, Paisley, UK) supplemented with 10% (*v/v*) fetal calf serum (FCS). The human skin keratinocyte cell line, HaCaT, was obtained from Cell Lines Service GmbH (CLS-300493, Germany) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (*v/v*) FCS. THP-1 human acute leukemia monocyte cell line (THP-1, DSMZ-ACC 16, Germany, RPMI 1640 supplemented with 10% FCS). The murine macrophage cell line, RAW264.7, was purchased from the European Collection of Authenticated Cell Cultures (Germany), and cultured in DMEM supplemented with 10% (*v/v*) FCS. DC2.4 murine dendritic cell line (DC2.4, SCC142-Merck Millipore, Germany, RPMI 1640 supplemented with 10% FCS + 1x non-essential amino acids + 1x HEPES buffer + 0.0054x β-mercaptoethanol). A549, HaCaT, DC2.4 and RAW264.7 cell lines were split once weekly, starting with 0.1×10^6 cells/13 mL for DC2.4, 0.2×10^6 cells/13 mL medium for A549 and RAW264.7 cells and 0.4×10^6 cells/13 mL medium for HaCaT and THP-1 cells. Mycoplasma tests were conducted regularly.

4.8. Cytotoxicity assessment of pneumococcal MVs with mammalian somatic and immune cells

Two epithelial cell lines were used for the determination of viability and cytotoxicity, namely A549 and HaCaT, and one immune cell line THP-1

To examine the mammalian cell viability upon treatment with several dilutions of pneumococcal MVs, cells were seeded into 96-well plates at 20,000 cells/well (A549 and RAW264.7) or 40,000 cells/well (HaCaT) [239]. All cell lines were cultured for 48 h until they reached 80%–

90% confluence. THP-1 cells were seeded into 96-well plates at a density of 100,000 cells/well, after being differentiated with phorbol 12-myristate 13-acetate (PMA) at a concentration of 30 ng/mL and let to grow for 24 h [240].

During the assays, the medium was aspirated, and cells were cultured in fresh phenol red-free RPMI 1640 medium (Life Technologies Limited, Paisley, UK), not supplemented with FCS, to prevent any false results due to traces of lactate dehydrogenase (LDH) in the medium or extracellular vesicles in the serum. Cells were incubated with 100 µL MV suspension in PBS at 10^4–10^6 purified MVs/cell and 100 µL of cell medium for 24 h. Live and dead controls were performed for each 96-well plate. A live control, in which cells were treated with PBS (100 µL PBS added to 100 µL medium), was run and showed no morphological changes. A dead control, in which cells were treated with 1% (*v/v*) Triton-X 100 (Sigma-Aldrich Co.), was run in parallel.

During the assays, the medium was aspirated, and cells were cultured in fresh phenol red-free RPMI 1640 medium (Life Technologies Limited, Paisley, UK), not supplemented with FCS, to prevent any false results due to traces of lactate dehydrogenase (LDH) in the medium or extracellular vesicles in the serum. Cells were incubated with 100 µL MV suspension in PBS at 10^4–10^6 purified MVs/cell and 100 µL of cell medium for 24 h. Live and dead controls were performed for each 96-well plate. A live control, in which cells were treated with PBS (100 µL PBS added to 100 µL medium), was run and showed no morphological changes. A dead control, in which cells were treated with 1% (*v/v*) Triton-X 100 (Sigma-Aldrich Co.), was run in parallel.

For the cytotoxicity assays, 100 µL of medium was drawn from each well to perform an LDH assay, which measures the LDH released into the medium upon cell death and membrane disruption, and mixed with 100 µL of LDH kit reagent (Roche Diagnostics GmbH, Mannheim, Germany) per the supplier's protocol. After incubating for 5 min at room temperature (RT), the absorbance was measured at 490 nm. For the viability assays, we used the PrestoBlue kit (Thermo Fisher Scientific, Waltham, MA, USA), which detects the metabolic activity in live cells, in a dilution of 1:10 with respective cell culture medium. The remaining medium was aspirated, and 100 µL of diluted PrestoBlue was added to each well, then incubated for 30 min at 37°C. The dye fluorescence was determined at a 560-nm excitation wavelength and 590-nm emission wavelength using a Tecan Infinity Pro 200 plate reader (Tecan, Männedorf, Switzerland) [74].

4.9. Cytotoxicity evaluation of pneumococcal MVs on DC2.4 cells by live-dead stain

DC2.4 cells were seeded in 24-well plates (3×10^5 cells/well), and let to grow for 24 h 37 °C/5% CO_2. The medium was aspirated, then MVs were added onto cells at concentrations of 10^2-10^5 MV/cell and incubated for 24 h. PBS-treated cells were considered as live cells (negative control with 100% viability), whereas dead cells (positive control with 0% viability) were obtained through incubation with 4% PFA for 15 min at room temperature. Afterwards, cells were washed with Hank's Balanced Salt Solution (HBSS) buffer and detached using Trypsin/EDTA and transferred to FACS tubes, then pelleted by centrifugation at 300 × g at 4 °C for 5 min. The cells were resuspended in HBSS buffer and 1 µL/tube reconstituted Live/Dead staining kit 568/583 (PromoCell, Germany) was added onto them, and incubated at 4 °C for 30 min. Then, we pelleted cells as mentioned before, washed with HBSS buffer, and centrifuged again to remove any excess stain. Eventually, the pellet is reconstituted in HBSS buffer and further analyzed using flow cytometry (LSRFortessa, BD Bioscience, USA). The dye can permeate the compromised cell membrane of dead cells, accumulate and label intracellular proteins thus become highly fluorescent, in contrast with scarce penetration through intact membrane of live cells, only labelling surface proteins, subsequently much lower fluorescence signal than dead cells.

10,000 live cells threshold was set, to be analyzed from forward versus side scatter (FSC vs. SSC) gating, where Phycoerythrin (PE-A) channel negative cells were considered as live cells, using FlowJo 10.6.1 software (FlowJo LLC, USA).

4.10. In vitro isolation of primary human monocyte-derived macrophages (MDMs) and monocyte-derived dendritic cells (DCs) from buffy coats

We isolated human monocytes from healthy adult blood donors (Blood Donation Center, Saarbrücken, Germany) and differentiated them using human macrophage colony-stimulating factor (M-CSF) (Miltenyi, 130-096-492) as reported previously [241]. Human material use and handling was reviewed and approved by the local Ethics Committees (permission no. 173/18; State Medical Board of Registration, Saarland, Germany).

We isolated peripheral blood mononuclear cells (PBMCs) using density gradient centrifugation with Lymphocyte Separation Medium 1077 (Promocell, C-44010) and Leucosep tubes (Greiner Bio-One, 227290) per the supplier's recommendations. After washing with PBS, monocytes were separated from PBMCs by magnetic cell sorting using anti-CD14 microbeads (Miltenyi, 130-050-201) per the manufacturer's instructions, except that only 10% of the recommended bead amount was used as previously described [242]. Monocytes were seeded into 24-well plates (5×10^5 cells/well) and differentiated into macrophages in RPMI 1640 supplemented with 10% FCS, 1% (*v/v*) penicillin/streptomycin (100 units penicillin and 0.01 mg/mL), 2 mM

glutamine, and 20 ng/mL M-CSF at 37 °C and 5% CO2 for 6 days. DCs were differentiated using the same procedure and culture medium supplemented with interleukin-4 at 10 ng/mL [243]. The culture medium was changed on days 4 and 6, then further treated with vesicles on day 6.

4.11. Viability assessment of human primary human MDMs and DCs *via* live-dead staining after treatment with pneumococcal MVs

Human primary MDMs and DCs were seeded at 2×10^5 cells/well into 24-well plates in 500 µL of the previously described medium. Cells were treated with pneumococcal vesicle suspensions (100 µL/well) at 5×10^3 vesicles/cell and incubated for 8 h. The MDM viability/cytotoxicity was measured using a Live/Dead staining kit (Invitrogen, ThermoFisher Scientific, L3224) via flow cytometry and confocal microscopy. In this assay, intracellular esterase activity in the live cells converts nonfluorescent cell-permeant calcein AM into intensely green-fluorescent calcein (excitation wavelength: 495 nm, emission wavelength: 515 nm). Ethidium homodimer-1 (EthD-1) accumulates in dead cells with damaged membranes by binding to their nucleic acids, producing a strong red fluorescence (excitation wavelength: 495 nm, emission wavelength: 635 nm); however, EthD-1 cannot permeate the intact plasma membranes of live cells. Cell viability/cytotoxicity was determined using flow cytometry per the manufacturer's recommendations. Briefly, cells were detached using Accutase solution (Sigma Aldrich, Germany, A6964) at 100 µL/well at 37°C for 30 min. Next, 2 µL of 50-µM calcein AM and 4 µL of 2 mM EthD-1 were added to 1 mL of cell suspension (1×10^6 cells/mL) and gently mixed. Afterwards, the cells were incubated for 15 min at RT in the dark, then analyzed using flow cytometry (LSR Fortessa, BD Bioscience, USA).

Within each experimental set, we ran live and dead cell controls. Live controls were prepared using PBS (100 µL/well), and these cells showed no changes in viability compared with that of medium-grown cells. Dead controls were prepared by heating the detached cell suspension in an 80°C water bath for 10 min before conducting flow cytometry. LPS-treated cells were prepared at 250 ng/mL as controls. Stained cells were analyzed via flow cytometry at 488 nm of excitation and set to a 10,000 cell threshold. Cells were gated after excluding debris using two channels: Phycoerythrin-Texas Red-A (PE-Texas Red-A) for red fluorescence of EthD-1 within dead cells (610/20 bandpass) and fluorescein isothiocyanate-A (FITC-A) for green fluorescence of calcein within live cells (530/30 bandpass). Flow cytometry measurements were further analyzed using FlowJo 10.6.1 software (FlowJo LLC, USA).

Cell viability/cytotoxicity was visualized using confocal microscopy per the manufacturer's manual. Briefly, 20 µL of 2 mM EthD-1 was diluted in 10 mL sterile PBS and added to 5 µL of 4 mM calcein AM, then vortexed thoroughly to achieve a working solution of 2 µM calcein AM

and 4 µM EthD-1. Next, 150 µl of working solution was added to the cells on glass slides and incubated for 45 min at RT. The cells were washed with PBS, and a drop of mounting medium (ThermoFisher, Germany) was added. The slides were then sealed with coverslips without damaging or shearing the cells. Images were captured using a Leica TCS SP8 confocal laser scanning microscope (CLSM, Leica Microsystems, Germany). Calcein was visualized using a 488-nm laser; EthD-1 was visualized using a 561-nm laser. Images were captured under a 25× water-immersion objective lens at a 1024×1024 resolution. Captured images were processed with LAS X software (LAS X 1.8.013370, Leica Microsystems).

4.12. Assessment of uptake/colocalization of streptococcal MVs within mammalian cells

Pelleted MVs after UC were fluorescently labelled by incubation with 2 µL DiI (Vybrant DiI Cell-labelling solution, Thermo Fisher, Germany) for 30 min at 37 °C (Ofir-Birin *et al.*, 2018). Non-incorporated dye was separated through SEC and the fractions with maximum fluorescence were used for further investigation.

A549 cells were seeded on 24-well plates (0.75 $\times 10^5$ cells/well), while HaCaT cells (1.25 $\times 10^5$ cells/well) and both cell lines were incubated for 48 h, until 80-90% confluence. While, differentiated THP-1 (dTHP-1) cells (4×10^5 cells/well), and DC2.4 cells (3×10^5 cells/well), were seeded in 24-well plate. Then, both cell lines were let to grow for 24 h. Further, 100 µL labelled-MVs were added onto cells, and incubated for 0.5, 2 and 4 h at 37 °C/5% CO_2. In addition, uptake of MVs into A549 and DC2.4 cells was tested after 24 h incubation at 37 °C with5% CO_2.

Then the cells were washed 3 times with PBS (for A549 and HaCaT) and HBSS buffer (for dTHP-1 and DC2.4), to remove any residual MVs. Cells were then detached using trypsin/EDTA (for A549, HaCaT and DC2.4 cells) and Accutase for (dTHP-1 cells). Then cellular suspensions were collected in 5-mL FACS polystyrene round-bottom tubes (12×75 mm style, 352054, Falcon, Corning, USA) and diluted with suitable buffers i.e., 2 % FCS in PBS (for A549 and HaCaT cells) and 2% FCS in HBSS (for dTHP-1 and DC2.4), until measurement with flow cytometry (LSRFortessa, BD Bioscience, USA) [244]. We repeated the same procedure for all cell lines incubated at 4 °C for 4 h, to determine the temperature-dependence of MV uptake and mechanisms controlling it. Liposomes were used as control for uptake study, and were fluorescently labelled using the same protocol performed with MVs.

Human primary blood-derived MDMs and DCs were seeded on 24-well plates (2×10^5 cells/well) and incubated for 6 days, then 100 µL of labelled MVs were added at 5,000 MVs/cell onto cells in 400 µL of nutrient medium and incubated for 1, 4, and 8 h at 37°C/5% CO2. Cells

were washed twice with PBS, to discard any traces of labelled MVs, and detached with 100 µL/well Accutase solution (Sigma Aldrich, Germany, A6964) at 37°C for 30 min. Afterwards, the cell suspension was collected in 5-mL polystyrene round-bottom tubes (12×75 mm style, 352054, Falcon, Corning, USA) and diluted with 2% FCS in PBS until flow cytometry analysis (LSR Fortessa, BD Bioscience, USA).

As mentioned before, 10,000 live cells threshold was set, to be analyzed from forward versus side scatter (FSC vs. SSC) gating. DiI positive cells (PE-A channel) after successful uptake of DiI-labelled MVs was determined, as compared with PBS treated cells (negative control), using FlowJo 10.6.1 software (FlowJo LLC, USA).

4.13. Confocal imaging of cells

A549 cells were seeded (0.45 $\times 10^5$ cells/well) on 8-well imaging chamber plates (SPL Life Sciences, Korea), while HaCaT cells (0.75 $\times 10^5$ cells/well), and incubated both for 48 h. While, dTHP-1 cells (3×10^5 cells/well) and DC2.4 cells (2×10^5 cells/well), were incubated and let to grow for 24 h . Then, cells were treated with 100 µL DiI-labelled MVs, and then incubated for 0.5, 2 and 4 h at 37 °C/5% CO_2. In addition, colocalization of MVs into A549 and DC2.4 cells was tested, after 24 h incubation at 37 °C with 5% CO_2.

Similarly, primary human blood-derived MDMs and DCs were seeded at 2×10^5 cells/well on 8-well imaging slides (SPL Life Sciences, Korea), incubated for 6 days, treated with 100 µL DiI-labelled MVs per well at the indicated concentration (5,000 MVs/cell), and incubated for 1, 4, and 8 h. After discarding supernatant, cells were washed twice with PBS, and incubated with fluorescein-wheat germ agglutinin (Vector Laboratories, USA) at a concentration of 10 µg/mL for 15 min at 37 °C/5% CO_2, to stain membrane glycoproteins.

Subsequently, cells were washed twice with PBS, and then fixed using 3.7 % *w/v* paraformaldehyde for 20 min at room temperature. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Life Technologies, Germany) at 1 µg/mL for 15 min at RT. The cells were washed, then a few drops of fluorescence mounting medium (Dako North America, USA) were added, and the slides were coverslipped.

Images were captured using a Leica TCS SP8 confocal laser scanning microscope (CLSM, Leica Microsystems, Germany), using a 488-nm laser to visualize fluorescein (green), a 405-nm laser to visualize DAPI (blue) and a 561-nm laser to visualize DiI (reddish-orange). We applied a 25× water-immersion objective lens with a minimum resolution of 1024×1024 and speed of 200 for image capturing. Captured images were further processed using LAS X software (LAS X 1.8.013370, Leica Microsystems).

4.14. Detection of cytokine production from DC2.4 cells treated with pneumococcal MVs using ELISA

Determination of cytokine production was done using murine Enzyme-linked immunosorbent assay (ELISA) kit (PeproTECH, USA). Briefly, special ELISA 96-well plates were coated overnight at room temperature with capture antibody. Non-specific binding was blocked with block buffer (1% w/v BSA in PBS) for 1 h. Then the plates were incubated with sample and standard dilutions for 2 h. Cytokines present in sample/standard was detected via detection antibody, incubated for 2 h. Signal amplification was employed using Avidin conjugated to horse radish peroxidase (Avidin-HRP), incubated with plates for 30 min. Eventually, incubation of plates with ABTS substrate solution, and monitoring at 5-min intervals for color development. Absorbance was measured at 405 nm, with wavelength correction at 650 nm. Controls for the assay included PBS treated cells (as negative control), and lipopolysaccharide (LPS) treated cells at a concentration of 10 µg/mL (as positive control). Two additional controls were conducted, namely liposomes and polystyrene beads, for comparison with MVs.

4.15. Determination of released cytokines from human primary MDMs and DCs upon treatment with pneumococcal MVs, using cytometric bead array (CBA)

Cytokines released from primary human blood-derived MDMs and DCs were quantified with a cytometric bead array human soluble protein master buffer kit (BD, USA, 558278) and human soluble protein flex set for cytokines: IL-6 (BD, 558276), IL-8 (BD, 558278), and tumor necrosis factor (TNF) (BD, 558273).

The assay was performed per the manufacturer's recommendations. PBS and lipopolysaccharide (LPS)-treated cells (250 ng/mL) were prepared as controls. Briefly, standards were prepared by pooling lyophilized standards together and reconstituting them in 4 mL of assay diluent, diluted from 1:2 to 1:256. Fifty microliters of standard dilutions and test samples were mixed gently with an equal volume of mixed capture beads and incubated for 1 h at RT. Next, 50 µL of mixed phycoerythrin (PE) detection reagent was added to the assay tubes and incubated for 2 h at RT. One milliliter of wash buffer was added to each tube and centrifuged at 200 g for 5 min. The supernatant was discarded, and 200 µL of wash buffer was added and vortexed to resuspend the sedimented beads, then measured via flow cytometry (LSR Fortessa, BD Bioscience, USA) after setting the software (BD FACSDiva^{TM}) as per the supplier's manual. Data were further analyzed using BD FCAP Array^{TM} software, version 3.0. Cytokine concentrations were determined and compared with the standard calibration curves.

4.16. Microparticles formulation using spray dryer

A carrier lactose (sigma-Aldrich, Taufkirchen, Germany) solution (2.5 % *w/v*) in Milli-Q water was prepared using overnight stirring, with addition of 1 % *w/v* leucine (Sigma-Aldrich, Taufkirchen, Germany) to enhance the stability and flow properties of obtained powder, until complete dissolution. Lactose was chosen, since it is the only FDA approved carrier for dry powder inhalers (DPI) [245, 246]. It is considered a safe carrier for pulmonary delivery of drug-loaded MPs, and can be applied for pulmonary immunization as well [247]. The solutions were sterile-filtered through 0.22µm Steriflip® vacuum filtration system (Merck KGaA, Darmstadt, Germany) and stored at 4 °C until further use. Two and half milliliters of pneumococcal MVs suspension at a concentration of (1×10^{12} particle/mL) were mixed with 17.5 mL of carrier solution, with or without fluorescein sodium solution, followed by gentle mixing. The solution was spray dried using a Büchi-90 Nano spray dryer (Flawil, Switzerland) at the following conditions: gas flow of 110 L/min, frequency of 140 KHz, inlet temperature at 90 °C, spraying at 40 %, pressure around 40 mbar and relative room humidity set at 20-30%. Then, vaccine microparticles (MPs) are collected with a plastic scraper, transferred to glass vials wrapped with Aluminum foil and stored in desiccator.

4.17. Physicochemical characterization of vaccine microparticles

4.17.1. Scanning Electron Microscopy (SEM)

Spray-dried vaccine MPs were mounted onto silicon wafer over sticky carbon tape fixed over a metal stage, followed by removal of excess powder with mild airflow. Afterwards, samples were sputter coated with gold (Quorum Q150R ES; Quorum Technologies Ltd., East Grinstead, UK) and examined with scanning electron microscope (Zeiss EVO MA15 LaB_6, Jena, Germany) at 10 kV and 20, 000 x magnification.

4.17.2. Static light Scattering (SLS)

The powder was dispersed in minimal volume of 1-octanol, and sonicated for 5 min to ensure proper dispersion. Further, the suspension was gradually added, with continuous magnetic stirring, to a 15-mL filled quartz cuvette in Horiba Partica LA-960 Laser Scattering Particle Size Distribution Analyzer (Darmstadt, Germany). Particle size distribution was determined based on calculated transparency reduction and light scattering.

4.17.3. X-ray Diffraction (XRD)

The spray-dried powders, in addition to inert materials including carrier solution contents i.e. lactose and leucine, and SEC eluent PBS were prepared using suitable sample holders and analyzed using a Bruker D8 X-ray diffractometer (Massachusetts, USA).

4.17.4. Aerodynamic Behavior of prepared vaccine microparticles

The aerodynamic properties of spray-dried MPs powder were determined *via* COPLEY Next Generation Impactor (NGI) (Nottingham, UK) connected to Akita airflow generating device (Bremen, Germany). Prior to the assessment, all impactor cups were coated using a mixture of 40% (v/v) from a 15% ethanolic solution of Brij®15 and 60% glycerol [248]. The pre-separator was filled with 10 mL of Milli-Q water. For each experiment, a hard gelatin capsule (size 3) was filled approximately with 10 mg of spray-dried powder formulation, and placed in a Handihaler® (Boerhringer Ingelheim, Ingelheim, Germany). After puncturing the capsule, the powder contents were aerosolized for 4 seconds at a gas flow rate of 60 L/min. Afterwards, the powders deposited in different NGI cup stages were collected by dissolving in defined volume of Milli-Q water. For quantification of collected samples, the fluorescence of MP-encapsulated fluorescein sodium was measured at λ_{ex}=460 nm and λ_{em}=515 nm, using a TECAN plate reader (Männedorf, Switzerland). For each powder formulation, an individual calibration curve was generated [249, 250].

4.18. Assessment of successful encapsulation of pneumococcal membrane vesicles within spray-dried vaccine microparticles

4.18.1. Confocal Laser Scanning Microscopy (CLSM)

Two milligrams of spray-dried powder were suspended in 200 µL of 1-octanol and sonicated for 5 min to ensure adequate dispersion. Aliquots of 2 µL were placed onto a glass slide and covered with a coverslip. Images were captured using a Leica DMi8 Confocal laser scanning microscope (Leica, Mannheim, Germany) with a 63 x water immersion objective lens (HC APO CS2 63x/1.20, Leica, Mannheim, Germany), followed by image analysis using LAS X software from Leica Application Suite X. Spray-dried vaccine MPs were stained with green fluorescence of fluorescein sodium, while pneumococcal MVs were labelled with DiI and visualized with reddish-orange fluorescence, to explore the distribution/encapsulation of MVs within MPs.

4.18.2. Nanoparticle Tracking analysis (NTA)

The spray-dried vaccine MPs powder was reconstituted in Milli-Q water and their contents of pneumococcal MVs in terms of particle size distribution and concentration were measured using nanoparticle tracking analysis (NTA, LM-10, Malvern, UK), using the earlier mentioned calculations.

4.19. Compatibility of spray-dried vaccine MPs with human THP-1 macrophage like cell line

Human macrophage-like (THP-1) cell line was selected to assess the compatibility of spray-dried vaccine MPs with human immune cells. THP-1 were seeded as described earlier into 96-well plates at density of 100,000 cells/well, after differentiation with 30 ng/mL phorbol 12-myristate 13-acetate (PMA) for 48 h. Then, the supernatant was removed and replaced with serum-free, phenol-red free RPMI 1640 medium, and treated with reconstituted vaccine MPs at a concentration of 10^4 pneumococcal MVs/single cell For 8h. Live (positive) and dead (negative) controls were prepared using PBS-treated and 1% (w/v) Triton-X-treated cells, respectively. For viability assay, cells were washed with PBS and equal volumes i.e. 100 µL of fresh medium and 10% v(v/v) PrestoBlue cell viability kit per (Thermo Fisher, Germany), were incubated at 37 °C for 30 min and fluorescence was recorded at (excitation wavelength 560 nm and emission wavelength 590 nm). One hundred microliter were transferred from the supernatant of THP-1 cells treated with vaccine MPs into 96-well plate for lactate dehydrogenase (LDH) cytotoxicity assay (Merck, Darmstadt, Germany) according to manufacturer's protocol, followed by absorbance measurement at 490 nm.

4.20. Uptake study of fluorescently labelled spray-dried pneumococcal MVs from vaccine microparticles into THP-1 cells

THP-1 cells were culture and seeded after PMA differentiation (30 ng/mL) for 48 h, as described earlier, with slight modifications, at a concentration of 200,000 cells/well in a 24-well plate for flow cytometry, as well as 8-well chamber slides (SPL Life Sciences, Korea) for confocal imaging. The cells were incubated with 100 µL reconstituted DiI fluorescently labelled spray-dried pneumococcal MVs vaccine MPs for 8 h, at a concentration of 10^4 pneumococcal MVs/single cell. Afterwards, the cells were washed twice with PBS, and detached using trypsin/EDTA, then cellular suspensions were collected in FACS tubes and diluted with appropriate buffer i.e., 2 % FCS in PBS, until flow cytometry measurement (LSRFortessa, BD Bioscience, USA). DiI positive cells (PE-A channel) were recorded as successful uptake of DiI labelled MVs, and compared to PBS and plain MPs (without MVs) as negative controls, with FlowJo 10.6.1 software (FlowJo LLC, USA).

For confocal imaging, cells were washed with PBS after removal of supernatant. Afterwards, cells were incubated with 100 µL of 1:1000 Alexa Fluor 488 Phalloidin (Thermo Fischer Scientific, Waltham, MA, USA) for 30 min to stain actin cytoskeleton, using 1% w/v bovine serum albumin and 0.1% w/v saponin in PBS for 20 min, as permeabilization and blocking solution. Subsequently, cells were washed with PBS and fixed with 4 % w/v paraformaldehyde solution in PBS for 15 min, then the nuclei were counterstained with 200 µL of 1 µg/mL 4′,6-diamidino-2-phenylindole (DAPI) (Life Technologies, Germany) for 15 min. Images were snapped *via* a Leica TCS SP8 confocal laser scanning microscope (CLSM, Leica Microsystems, Germany) using 25 x water immersion objective lens, followed by processing of images with LAS X software (LAS X 1.8.013370, Leica Microsystems, Germany).

4.21. Cytokine release determination from primary human peripheral blood mononuclear cells (PBMCs)

We assessed the immunostimulating potential of spray-dried pneumococcal MVs vaccine MPs on human primary immune cells. Determination of secreted cytokines of supernatant medium of primary human peripheral blood mononuclear cells (PBMCs) isolated from buffy coats as stated earlier. Briefly, buffy coats from healthy adult blood donors were collected (Blood Donation Center, Saarbrücken, Germany), where handling of human material was approved by local Ethics Committee (permission no. 173/18; State Medical Board of Registration, Saarland, Germany). PBMCs were isolated using density gradient centrifugation using Leucosep tubes (Greiner Bio-One, 227290) and Lymphocyte Separation Medium 1077 (Promocell, C-44010), according to manufacturer's manual. Afterwards, PBMCs were seeded at a concentration of 100,000 cells/well in 96- well plates, and incubated with 100 µL of reconstituted vaccine MPs for 8h, at a concentration of 10^4 pneumococcal MVs/single cell.

Pro-inflammatory cytokines released from treated PBMCs were recorded using cytometric bead array human soluble protein master buffer kit (BD, USA, 558278) and human soluble protein flex set: IL-6 (BD, 558276), IL-8 (BD, 558278) and tumor necrosis factor (TNF) (BD, 558273). The assay was conducted according to supplier's recommendations as previously described, in comparison with PBS and LPS-treated cells (250 ng/mL) as negative and positive controls, respectively. Samples were determined with flow cytometry (LSR Fortessa, BD Bioscience, USA) after setting its respective software (BD FACSDiva™) as per the manufacturer's manual. Furthermore, obtained data were analyzed using BD FCAP Array™ software, version 3.0, to quantify cytokine concentrations, compared with their standard calibration curves.

4.22. Statistical analysis of data

All data are shown as the means ± standard deviation, where the number *n* is independent experiments. All measurements were performed at least in triplicate in *n* independent experiments. Unpaired two-tailed t-tests were performed to compare two groups. Groups of two or more were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test.

Results and Discussion

5. Chapter One: Streptococcal extracellular membrane vesicles are rapidly internalized by immune cells and alter their cytokine release

Most of the following chapter is transferred *verbatim* from the following published original research article.

Streptococcal extracellular membrane vesicles are rapidly internalized by immune cells and alter their cytokine release

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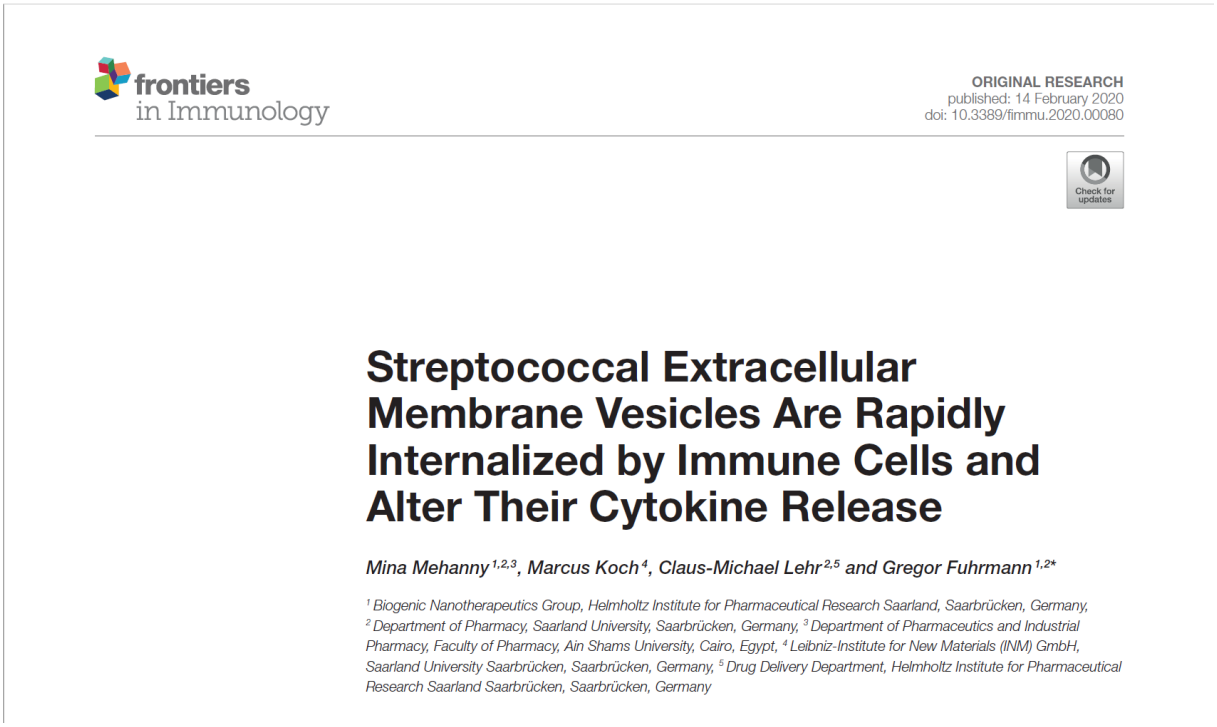

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Streptococcal Extracellular Membrane Vesicles Are Rapidly Internalized by Immune Cells and Alter Their Cytokine Release

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5. 1. SEC provides efficient purification of MVs from protein impurities

SEC was used to purify the resuspended pellet after ultracentrifugation, without affecting their integrity. After collection of eluted 1-mL fractions after SEC purification and confirming efficient separation between MV-rich fractions and unwanted soluble protein impurities using BCA assay, as demonstrated in **Figure 8. A.** Assessment of MV-containing fractions using NTA (**Figure 8. B and C**) showed the highest concentration of particles in consecutive fractions 6, 7 and 8, in the range of 10^{12} particle/mL. We designated the MV concentrations to number of particles and not protein mass per fraction, because the conformational structure of surface proteins, ligands and loaded cargo onto intact vesicles is more relevant to receptor binding, and hence their biological effect. The isolated fractions exhibited average particle size ranging 130-160 nm, upon NTA analysis (**Figure 8. C**).

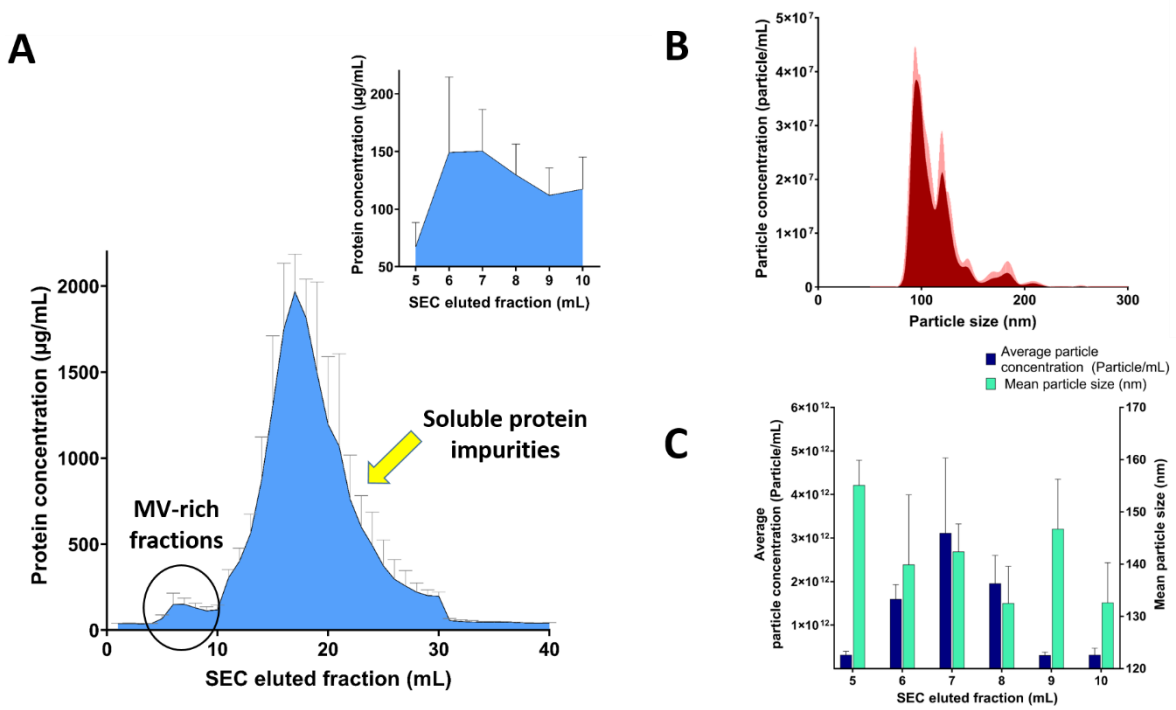


Figure 8. Analysis of streptococcal membrane vesicle (MV)-rich eluted fractions after size exclusion chromatography (SEC). (A) Bicinchoninic acid (BCA) assay of SEC eluted fractions with magnified part of MV-rich fractions in upper right corner. (B) Representative particle size-distribution curve of one of the vesicle rich fractions (fraction 7), after suitable dilution for nanoparticle tracking analysis (NTA). (C) Graph representing average particle concentration (particle/mL) and mean particle size (nm) for the MV-rich SEC eluted 1 mL fractions, after NTA measurement. n = 3, mean ± SD.

5. 2. Pneumococcal MVs display a heterogeneous nature

Visualization of streptococcus bacteria SEM **(Figure 9. A)** revealed several protruding spherical particles, which are distinct from bacterial surface texture. They may show the process of vesicular release or budding through the bacterial cell wall.

For better understanding of secreted pneumococcal MVs, cryoTEM investigation was employed (**Figure 9. B-F**). MVs exhibited a very heterogeneous nature, with respect to their morphology, particle size, arrangement and content. Various structures and shapes were observed, such as spherical, elliptical, rod-shaped and irregular forms (**Figure 9. D**). In addition, many particles were aligned in chain-like structures (**Figure 9. E and F**).

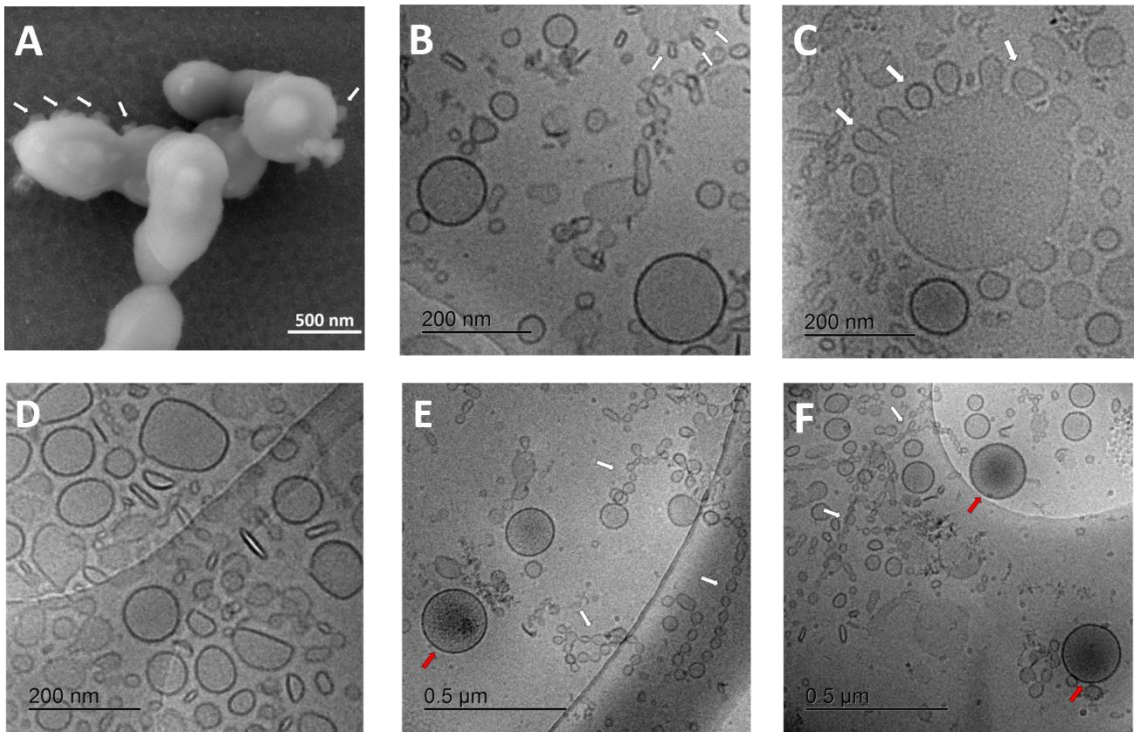


Figure 9. Characterization of streptococcal membrane vesicles (MVs) using electron microscopy. (A) Scanning electron micrograph (SEM) of *Streptococcus pneumonia* reference strain R6 cells, during shedding of vesicles from their surface. (B-F) cryogenic transmission electron micrographs (cryo-TEM) of isolated pneumococcal MVs, showing heterogeneous morphology, tiny vesicles budding from large ones (white arrows), chain-like structures (white arrows) and some vesicles with darker content (red arrows)

Interestingly, we also saw many smaller particles budding from larger structures (**Figure 9. C**). Several particle sizes were recognized; the smallest were in the range of 20-30 nm in these chain-like assemblies, another population was almost 130-160 nm mean diameter, and the largest reached around 300 nm size. Some tiny vesicles were budding from larger ones. Few MVs showed a darker color, suggesting a different content, rather than other vesicles (**Figure 9. E and F**). SEC-eluted fractions showed some dilution effect (**Figure 10**).

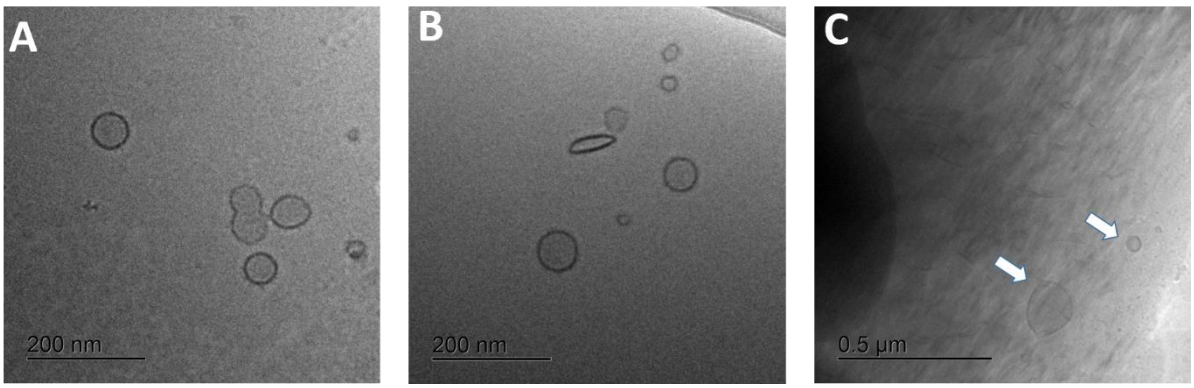


Figure 10. Cryogenic transmission electron micrographs (cryoTEM) images of (A and B) Streptococcal membrane vesicles, eluted after purification with size exclusion chromatography (SEC) with some dilution effect. (C) Streptococcal bacterial cell with some vesicles shed near its surface.

In this work, we isolated and characterized streptococcal MVs to study their structure, interaction and uptake into mammalian cells, and their potential for immunomodulatory effect. Our pneumococcal MVs showed average particle size, in the range of 130-160 nm as measured by NTA. This is comparable to what is reported by Surve *et al.* for MVs from group B Streptococci, which measured sizes of 150-300 nm using dynamic light scattering [251]. However, Olaya-Abril *et al.* described streptococcal vesicles, with particle size range 20-75 nm, based on TEM images investigation [190].

A reason for this difference could be the purification technique employed, as Olya-Abril *et al.* utilized Optiprep density gradient fractionation. They reported that their vesicles might contain some cellular fragments or debris, due to lysis of apoptotic bacteria, hence smaller-sized vesicles were observed. The difference in particle size obtained; could arise from the difference in particle size measurement techniques i.e., bulk NTA measurement versus individual electron microscopy image analysis. In contrast, we applied SEC as our purification tool, which provides milder and better recovery of pure MVs, without compromising their purity, biological activity or integrity [252, 253]. It is worth mentioning that the lower size limit for NTA detection and measurement is 50 nm [254].

The differences in bulk size measurements in comparison to literature values motivated us to study the streptococcal vesicles by electron microscopy. Protrusions of tiny vesicles from bacterial cell wall as we observed them by SEM imaging, may characterize the shedding process of pneumococcal MVs. This is in agreement with what was described earlier in literature regarding tiny blebs, appearing as rough protrusions distinct from the smooth surface texture of Streptococci [190, 251]. We furthermore detected a very broad particle size range of our isolated pneumococcal MVs, with four main populations of vesicles; i) extremely small

chain-like structures ii) tiny vesicles which bud from other vesicular forms with around 20-30 nm diameter both iii) medium-sized vesicle population of approximately 130-160 nm, also detected by NTA measurement and iv) a giant vesicular population from which the tiny ones bud, with average diameter of 300 nm. Interestingly, the budding of tiny vesicles from giant counterparts was – to the best of our knowledge – not recorded earlier in literature in gram-positive MVs. These morphologies may suggest various biogenesis mechanisms involved in pneumococcal MVs release [45]. Characteristic chain-like structures (also known as nanotubes or nanowires) are protruding blebs from gram-positive bacterial cytoplasmic membrane, a phenomenon described also in gram-negative Myxobacteria [255]. They usually decorate cellular surface, and may form membrane-bound network to connect biofilm cells through periplasmic space. They may serve as bridges for exchange of cellular components [41, 256]. In addition, a study on gram-negative Vibrio vulnificus showed that individual OMVs could detach from nanotubes, forming a unique “beads on a string” pattern. OMVs were proposed to fuse to form nanotubes and vice versa, leading to nanotubes disintegrating into OMVs [257]. This could justify the flexible nature of MV packing into chains and dismantling into separate vesicles according to the surrounding conditions as we observed, and their importance for intercellular communication and exchange.

5. 3. Streptococcal MVs demonstrate no cytotoxicity with somatic and immune cells

Assessment of cellular viability upon treatment with pneumococcal MVs and examination of whether they have any cytotoxic effects was performed on various mammalian cell lines.

Mammalian somatic cells (A549 and HaCaT) exhibited very good tolerance to pneumococcal MVs. Even upon exposure to very high concentration of 10^6 MV/cell, cellular viability was not affected (**Figure 11. A**).

Cellular viability was higher than positive control (100% viability) results. Interestingly the higher the applied MV concentration was on cells, the higher was their calculated cellular viability. Pneumococcal MVs lacked any detectable cytotoxic effects on both cell lines (**Figure 11. B**).

We examined MVs on dTHP-1 cells as a representative of innate immune cells. Concentrations (10^3-10^5 MV/cell) caused no change in viability of cells, but calculated viability values rather were slightly higher than positive control. Besides these concentrations manifested inconsiderable toxic effects (below 10%). Only the highest concentration of MVs (10^6 MV/cell), compromised dTHP-1 cellular viability.

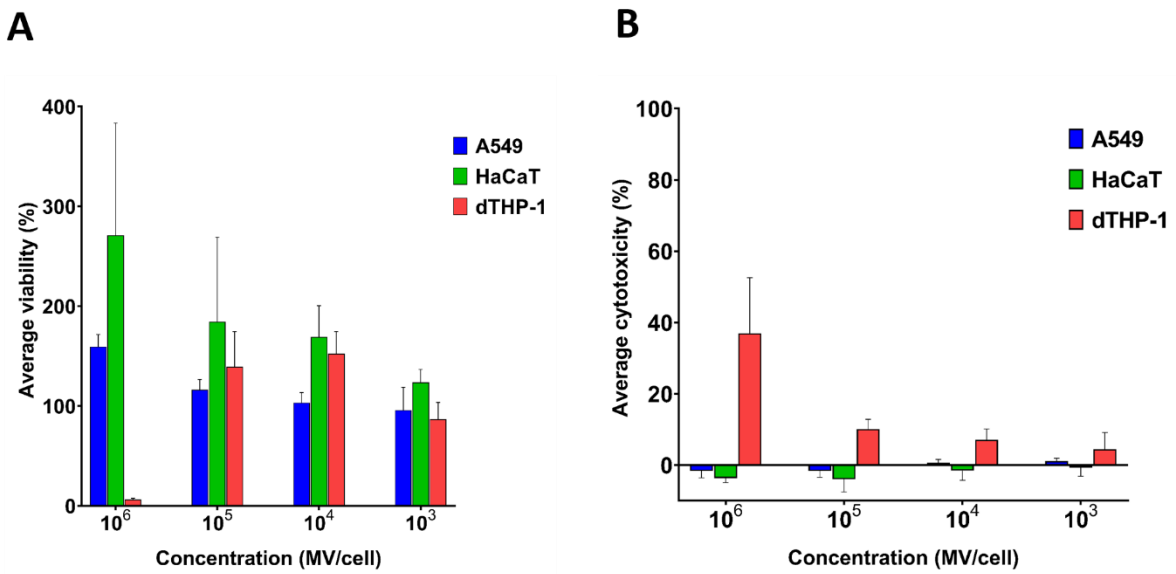


Figure 11. Streptococcal membrane vesicle (MVs) are non-cytotoxic with somatic (i.e., A549 and HaCaT), and immune (i.e., dTHP-1) cell lines. (A) Calculated percentage viability of cells determined by PrestoBlue reagent and (B) Calculated percentage cytotoxicity of cells determined by LDH reagent, in comparison with cells treated with PBS (live cell control) and 1% Triton X (dead cell control), after application of various dilutions of streptococcal MVs. n = 3, mean ± SD

Various concentrations (10^2-10^5 MV/cell) were applied on DC2.4 cells. They showed comparable viability results to positive control (PBS treated) (**Figure 12. A**), indicating their *in vitro* compatibility with adaptive immune cells and absence of any cytotoxic effects (**Figure 12. B**).

In a subsequent step, we were interested to study the impact of Streptococcal MVs on various human and murine cell lines. MVs proved to be non-toxic towards mammalian somatic cells, namely A549 and HaCaT cell lines. We used relatively high concentrations of MVs reaching 10^6 MV/cell, without observing any detrimental effects on their viability. Streptococcal MVs conferred negligible cytotoxicity on both cell lines as examined by LDH assay. Choi *et al*. reported similar results of pneumococcal MVs on A549 cells, suggesting they lack any major cytotoxic effect on mammalian somatic epithelial cells [191].

Surprisingly, cellular viability exceeded positive control (100%) the higher the applied MV concentration on cells was. MVs might be consumed by cells as a nutritional material rich in proteins and phospholipids, leading to a potential stimulatory effect on cell growth. Another possible reason, we speculate that MVs might activate cellular metabolic activity, resulting in stronger intracellular reducing environment of resazurin, the PrestoBlue reagent, to fluorescent resorufin leading to increase in overall recorded fluorescence.

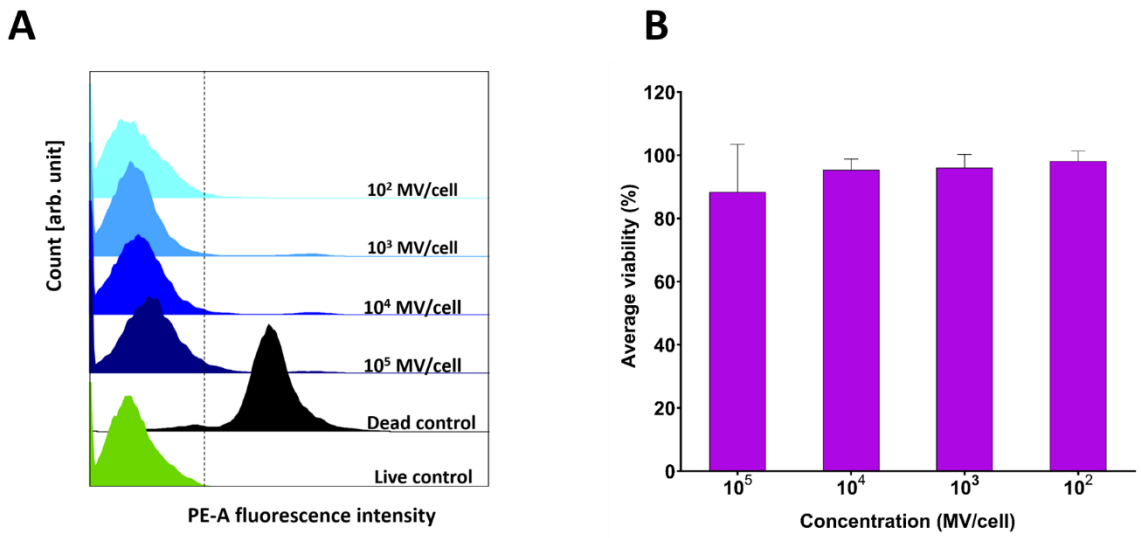


Figure 12. Viability assessment of dendritic cells (DC2.4) after exposure to pneumococcal membrane vesicles (MVs) by flow cytometry. (A) Overlay of recorded fluorescence intensity of Phycoerythrin (PE-A) channel, after exposure of DC2.4 cells to various concentrations of MVs, in addition to live and dead cell controls. (B) Calculated percentage viability of DC2.4 cells with respect to cells treated with PBS (live cells control) and 4% PFA (dead cells control), after exposure to streptococcal MVs. The dashed line separates PE-A channel negative and positive cells. n = 3, mean ± SD

Subsequently, these findings were verified in cells involved in the potential immune response to MVs, namely differentiated macrophage-like dTHP-1 cells as a representative of innate immune cells and DCs as the archetype of adaptive immune cells. Cellular viability for both cell lines (dTHP-1 and DC2.4) did not deteriorate upon treatment with concentrations (10^3-10^5 MV/cell). These concentrations did not display any negative effects on cells, proposing a potential non-cytotoxic nature of pneumococcal MVs upon incubation with immune cells. dTHP-1 cells showed very good viability profile with all applied concentrations, except for the highest concentration (10^6 MV/cell), which is an extremely high number of MVs. Nevertheless, we regarded pneumococcal MVs safe and suitable for further investigations.

5. 4. Streptococcal MVs successfully colocalize within mammalian somatic and immune cells after relatively short incubation period:

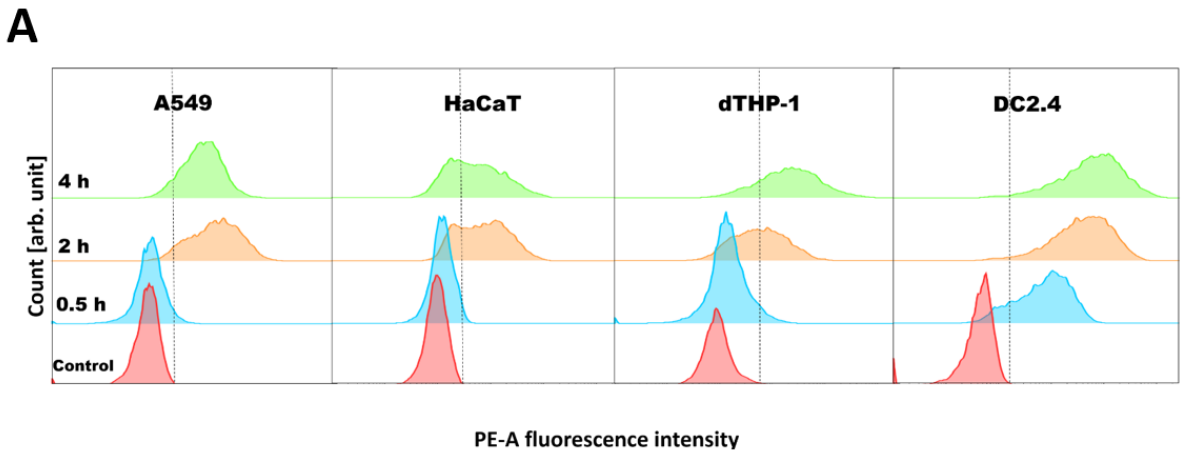
A prompt uptake of MVs by DC2.4 cells, with almost 80% of cells exhibited uptake of fluorescently labelled MVs after only 30 minutes of incubation, in comparison with minimal uptake within other examined cell lines such as A549, HaCaT and dTHP-1 (**Figure 13. A and B**).

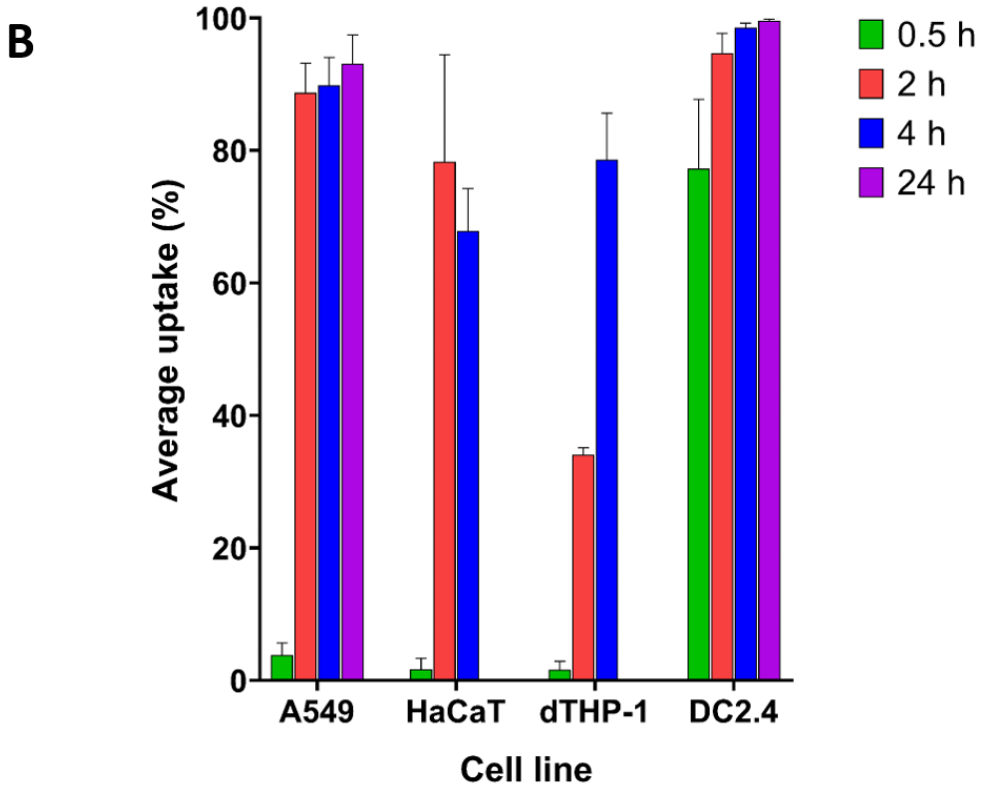
After 2 h of incubation, dTHP-1 cells showed a slow increase in uptake of MVs, reaching approximately 35%. While other cell lines demonstrated relatively, faster and higher uptake values (more than 80% for somatic A549 and HaCaT cells, and 95% for immune DC2.4 cells).

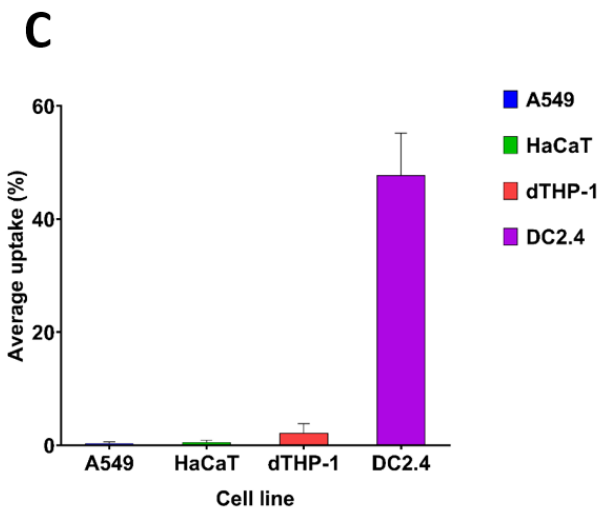
Uptake readings of MVs for somatic cells level off after 4 h and show no remarkable difference between 2 and 4 h uptake values. dTHP-1 reached almost 80% uptake after 4 h, confirming their gradual uptake of MVs. Whereas as expected, DC2.4 cells exhibited almost complete uptake of all fluorescent MVs after 4 h of incubation period. After 24 h incubation, almost complete uptake of fluorescent MVs was detected, in both A549 and DC2.4 cells, without major changes from 4 h uptake observations.

Only DC2.4 cells exhibited uptake of pneumococcal MVs after 4 h incubation at 4 °C, while no uptake was detected in other cell lines at this temperature (**Figure 13. C**)

Liposomal controls exhibited negligible cellular uptake into somatic (A549) or immune (DC2.4) cells within 30 min, followed by gradual increase reaching around 45% fluorescent cells after 4 h of incubation and almost complete uptake after 24 h (**Figure 13. D**).


A
A549
HaCaT
dTHP-1
DC2.4
Count [arb. unit]
4 h
2 h
0.5 h
Control
PE-A fluorescence intensity


B
Average uptake (%)
0
20
40
60
80
100
A549
HaCaT
dTHP-1
DC2.4
Cell line
0.5 h
2 h
4 h
24 h


C
Average uptake (%)
0
20
40
60
A549
HaCaT
dTHP-1
DC2.4
Cell line

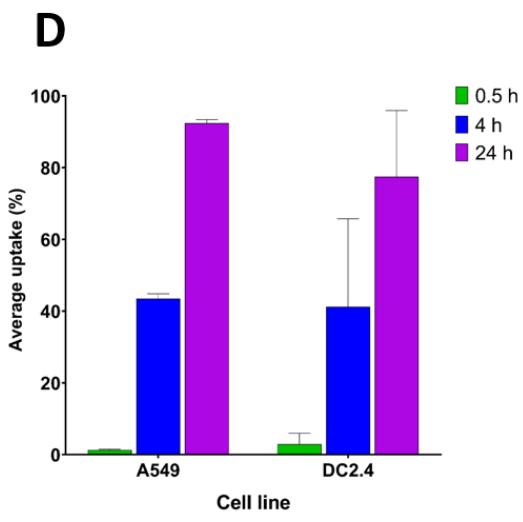

D
Average uptake (%)
0
20
40
60
80
100
A549
DC2.4
Cell line
0.5 h
4 h
24 h

Figure 13. Streptococcal membrane vesicles (MVs) show successful uptake within mammalian cells shown by colocalization during flow cytometry measurement. (A) Phycoerythrin (PE-A) channel fluorescence intensity after incubation of cells with DiI-labelled MVs, with respect to control (PBS-treated) cells. The dashed line separates PE-A channel positive and negative cells. (B) Average percentage uptake of fluorescently labelled MVs inside four mammalian cell lines (A549, HaCaT, dTHP-1 and DC2.4) after different incubation periods (0.5, 2 and 4 h) and uptake of MVs into (A549 and DC2.4) cells after 24 h incubation. (C) Average percentage uptake of fluorescently labelled MVs into four mammalian cell lines (A549, HaCaT, dTHP-1 and DC2.4) after 4 h incubation period at 4 °C. (D) Average percentage uptake of fluorescently labelled liposomes into (A549 and DC2.4) cells after various incubation periods (0.5, 4, 24 h). n = 3, mean ± SD

Confocal laser scanning microscopy images (**Figure 14**) confirmed flow cytometry results for uptake of MVs into cells. The images showed minimal intracellular internalization of MVs after 30 min incubation period, except for DC2.4 cells, which presented high and rapid uptake of MVs, as detected by appearance of numerous orange-red dots of DiI fluorescently labelled MVs within the green-stained cellular perimeter (**Figure 14**).

After 2 h of incubation, gradual increase in MVs taken up by cells. In correlation with flow cytometry observations, dTHP-1 cells exhibited fewer red dots, in comparison with other cells. As anticipated, DC2.4 cells displayed more intracellular MVs indicating the highest internalization of them into adaptive immune cells. All cell lines revealed abundant amounts of fluorescent-MVs internalized especially after 4 h, confirming successful uptake of streptococcal MVs into mammalian cells (complete channels and overlay confocal images, in (**Figures 15-17**). No major change of MVs colocalization into A549 and DC2.4 cells was detected, after 24 h incubation (**Figure 18**).

We first studied the interaction and/or internalization of MVs within A549, HaCaT, dTHP-1 and DC2.4 cell lines. Pneumococcal MVs were successfully taken up, and colocalized within different mammalian cells. DC2.4 cells could promptly internalize streptococcal MVs, reaching around 80 % of positive cells after an incubation period of 30 minutes. This may suggest a specific receptor-mediated and/or endocytosis uptake mechanism for antigen-carrying MVs into antigen presenting cells as it has been reported in literature [258]. Negligible uptake of liposomes by DC2.4 cells after 30 min, suggesting a specific interaction between DC2.4 cells and streptococcal MVs.

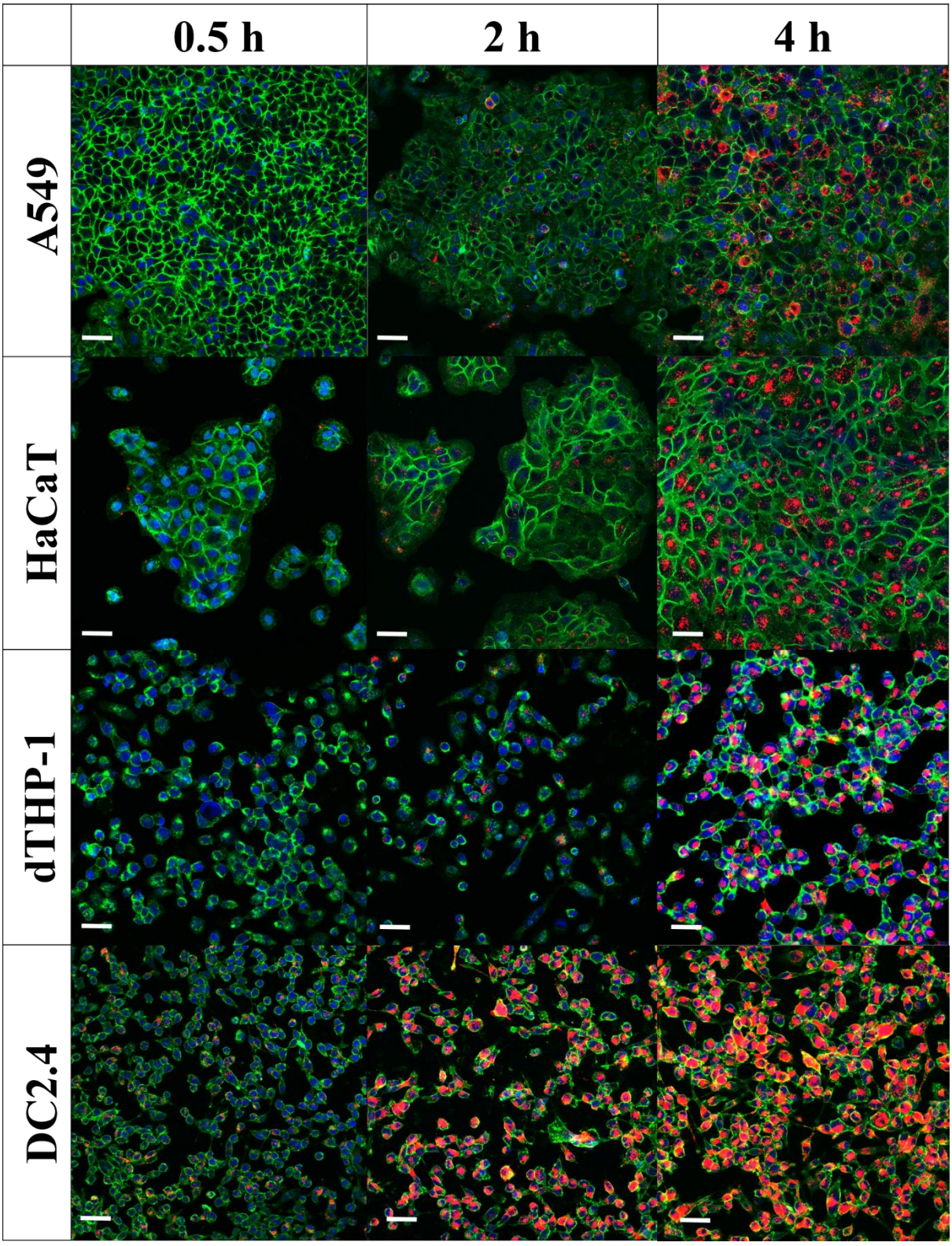


Figure 14. Internalization of pneumococcal membrane vesicles (MVs) into various somatic (A549 and HaCaT) and immune cell lines (dTHP-1 and DC2.4), as confirmed by confocal microscopy imaging, after various incubation periods (0.5, 2, and 4 h). Overlay images are shown, where nuclei are blue-stained (DAPI), cellular membranes are green-stained (Fluorescein-WGA), while MVs are orange-red stained (DiI). Scale bar represents 50 μm.

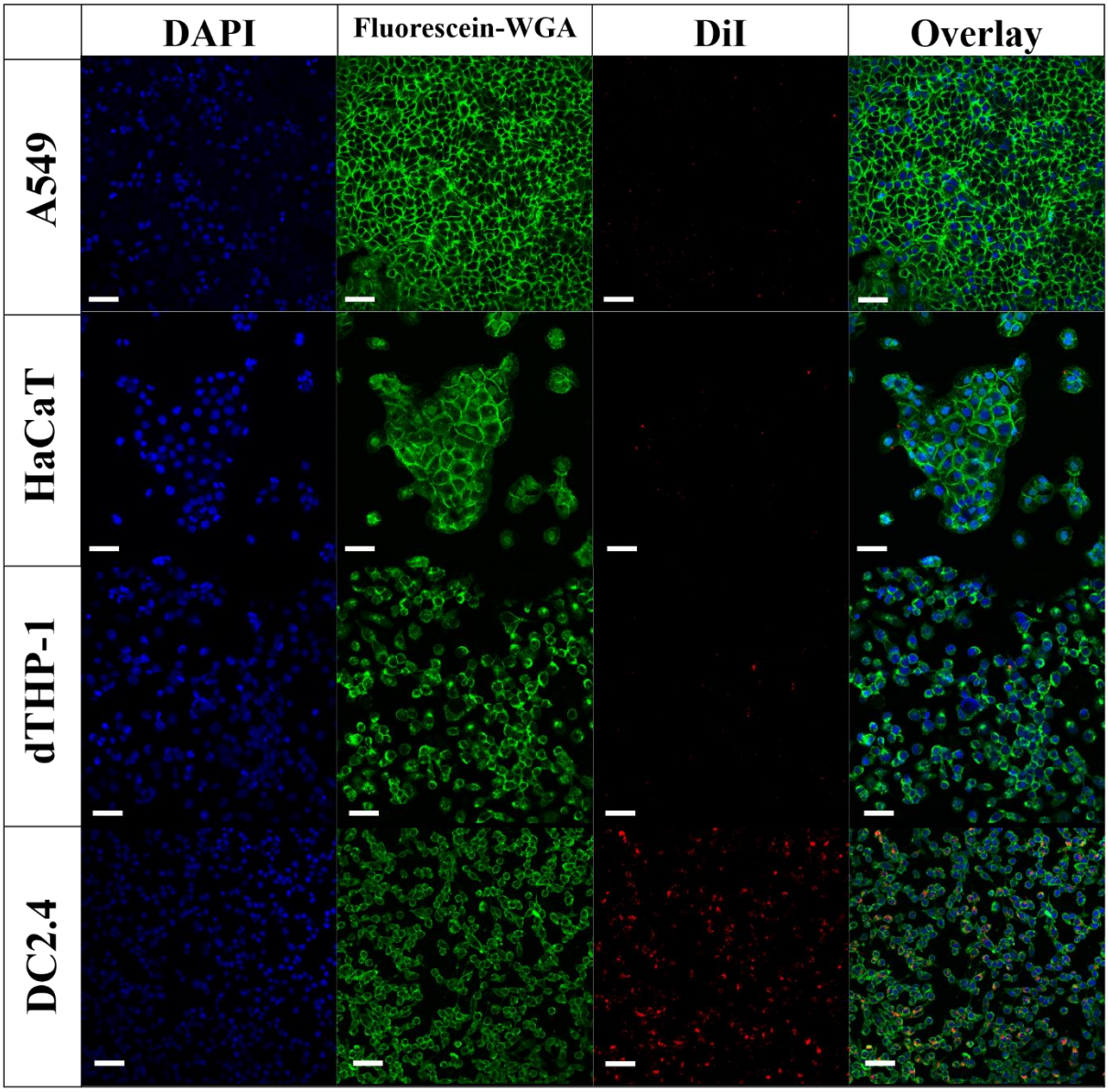


Figure 15. Confocal laser scanning microscopy images of various somatic (A549 and HaCaT) and immune cell lines (dTHP-1 and DC2.4), showing internalization of DiI-fluorescently labelled streptococcal membrane vesicles (MVs) after 0.5 h incubation period. Scale bar represents 50 µm.

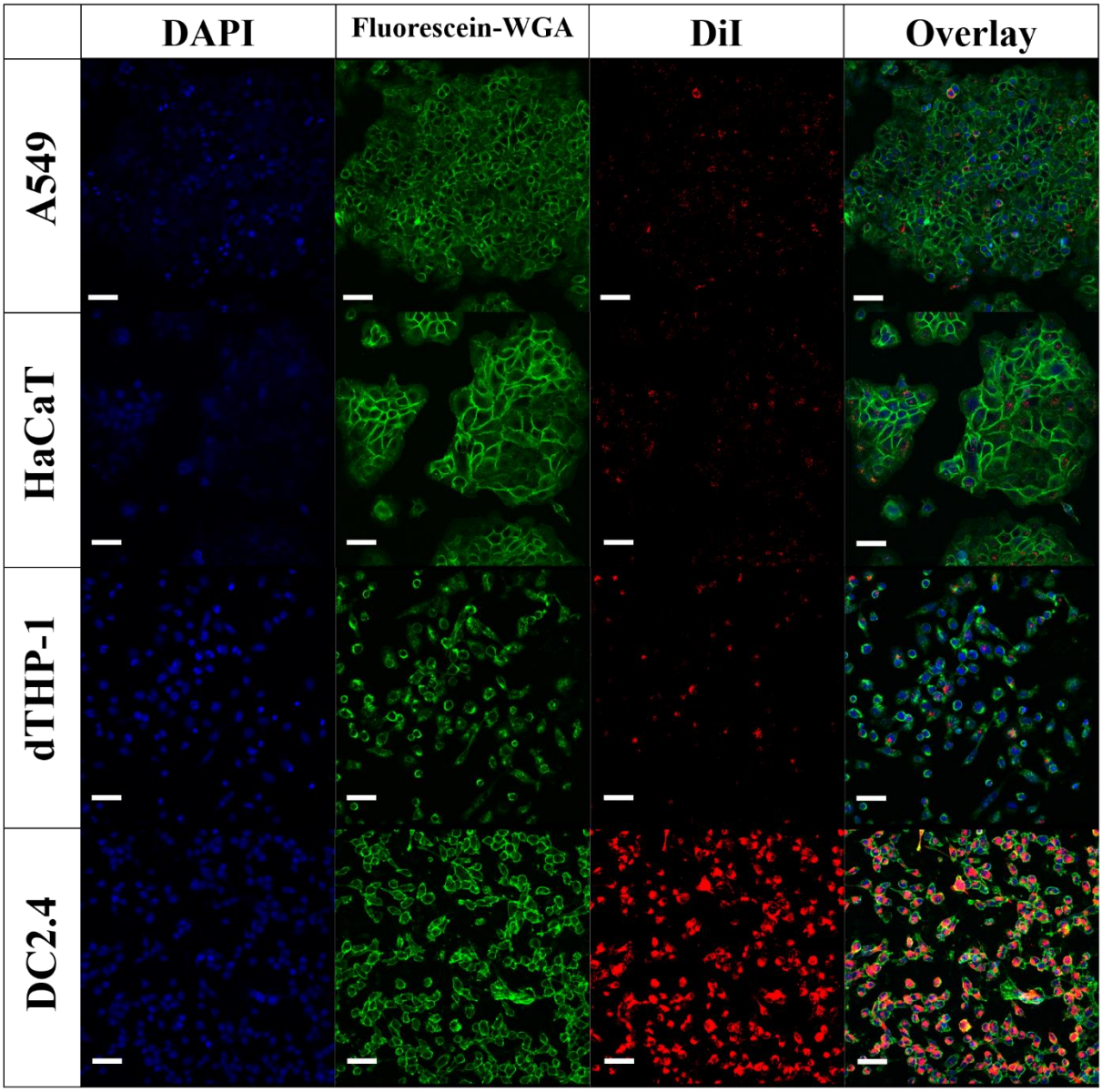


Figure 16. Confocal laser scanning microscopy images of various somatic (A549 and HaCaT) and immune cell lines (dTHP-1 and DC2.4), showing internalization of DiI-fluorescently labelled streptococcal membrane vesicles (MVs) after 2 h incubation period. Scale bar represents 50 µm.

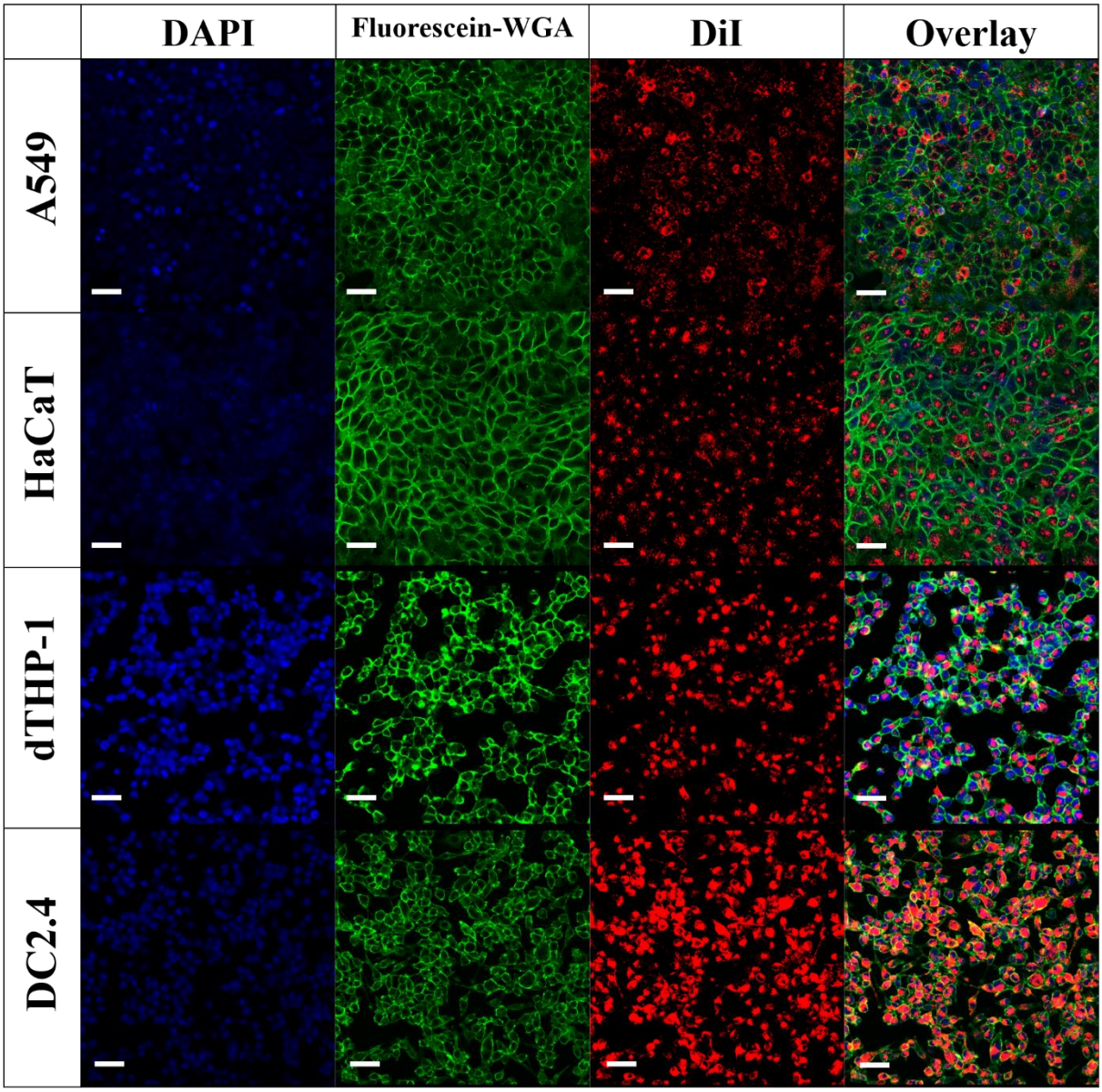


Figure 17. Confocal laser scanning microscopy images of various somatic (A549 and HaCaT) and immune cell lines (dTHP-1 and DC2.4), showing internalization of DiI-fluorescently labelled streptococcal membrane vesicles (MVs) after 4 h incubation period. Scale bar represents 50 µm.

Uptake of vesicles into mammalian cells might be a function of their particle size [259], as illustrated also by Turner *et al.* [86]. They examined the mechanisms responsible for cellular entry of *Helicobacter pylori* OMVs. Smaller OMVs (20-100 nm) preferentially entered the cells through caveolin-mediated endocytosis, while larger OMVs (90-450 nm) transferred through micropinocytosis and endocytosis. Taking into account the mixed populations of MVs with larger and smaller sizes, the rapid uptake of streptococcal MVs into DCs after incubation for only 30 min is presumably due to mixed mechanisms. In literature, extracellular vesicle uptake relies mainly on endocytosis, phagocytosis, micropinocytosis, lipid raft internalization and cell surface membrane fusion [244, 260, 261]. Almost complete uptake of pneumococcal MVs in 4

h for DC2.4 cell line supports our hypothesis that MVs tend to be preferably uptaken up by DC2.4, owing to their role as antigen presenting cells, in order to be processed and presented for further adaptive immune pathways. Interestingly, solely only DC2.4 cells demonstrated uptake of pneumococcal MVs upon incubation at 4 °C, which might be ascribed to passive processes including lipid raft uptake through seemingly dynamic membrane areas enriched with sterols and sphingolipids, and membrane fusion of vesicles with cell surface [260, 261]. In addition, we speculate that quick uptake by DCs might be beneficial, since they are the cells, we want to target, for antigen processing and potential selective adaptive immune response.

These mixed uptake processes could explain the rapid uptake of MVs into DC2.4 cells after only 30 min incubation at 37 °C. Further investigations will allow us to study the uptake kinetics of various MV populations and mechanisms controlling them.

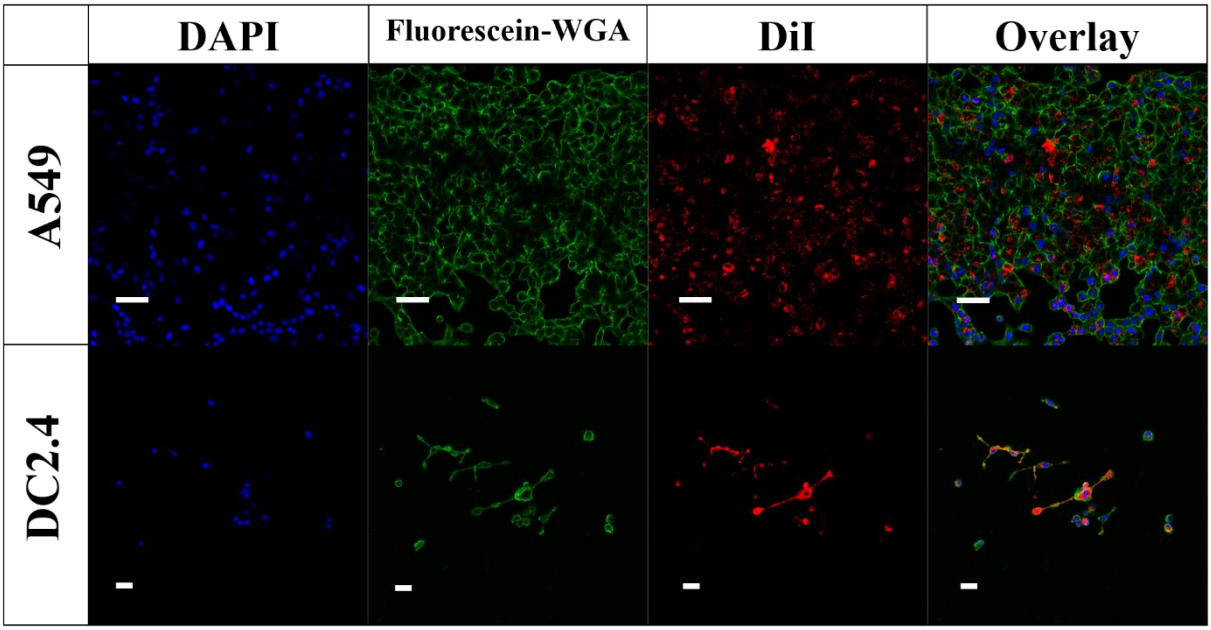


Figure 18. Confocal laser scanning microscopy images of somatic (A549) and immune (DC2.4) cell lines, showing internalization of DiI-fluorescently labelled streptococcal membrane vesicles (MVs) after 24 h incubation period. Scale bar represents 50 µm.

Codemo *et al.* examined internalization of pneumococcal in A549 after 24 h incubation and reported a concentration-dependent increase in intracellular localization of vesicles [192]. 24 h uptake results of streptococcal MVs into (A549 and DC2.4), exhibited no major changes from 4 h uptake, suggesting that MVs accumulate inside cells. This might be beneficial for sufficient processing of streptococcal antigens within immune APCs e.g. DCs.

To further validate flow cytometry experiments, we performed additional confocal microscopy visualization of cellular internalization of DiI-labelled MVs. We found that approximately all DC2.4 cells have taken up fluorescent MVs, assuming that APCs have the highest rate in a

very short incubation time (30 min), and extent (4 h) that almost all cells showed MV uptake, in comparison with other examined cell lines. This suggests the ability of immune system especially APCs (e.g. DC2.4) to recognize microbial MVs, in order to process them and elicit a protective immune response.

5. 5. Streptococcal MVs modulate cytokine production from dendritic cells

Streptococcal MVs, after 24 h incubation period with DC2.4 cells, could enhance the release of TNF-α (**Figure 19. A**), small increase in the levels of IL-10 (**Figure 19. B**) and no detected effect on IL-12 release (Figure 7. C) was observed. Increased secretion of TNF-α demonstrated an inflammatory effect, and suggested a potential immunostimulatory effect upon application of MVs on DC2.4 cells.

In a final step, the ability of MVs to modulate the interleukin release pattern of DCs was assessed. We observed increased secretion of TNF-α upon exposure of DC2.4 cells to streptococcal MVs. TNF-α is an endogenous alarm signal, which coordinates gene expression, cellular activity and drives inflammatory responses in injury or infection [262]. It has a prominent role in regulation of immune cells and creates an inflammatory signal to recruit other immune cells to evoke an immunostimulatory cascade [263]. A previous study used several pneumococcal strains (virulent serotype 4, TIGR4 and its isogenic mutants). They reported increased TNF-α production from human monocyte-derived DCs, after exposure to pneumococcal MVs [192]. Their observations support our findings that pneumococcal vesicles display an immunomodulatory effect.

We noticed slight increase in production of IL-10 from DC2.4 cells treated with pneumococcal MVs. IL-10 possesses a broad anti-inflammatory activity, chiefly on macrophages and DCs. It inhibits antigen presentation, decreases major histocompatibility complex class II expression, hinders DC differentiation from precursor monocytes and DC maturation, and decreases secretion of pro-inflammatory cytokines from immune cells [264]. This observation might support our hypothesis that streptococcal MVs could modulate antigen presentation and immune response. Our results are similar to what was described by Codemo *et al*. They reported an increased release of IL-10 after pneumococcal MV exposure [192]. We believe that enhanced TNF-α secretion is necessary for an adequate inflammatory response from immune cells, which elicits an immune response afterwards [265].

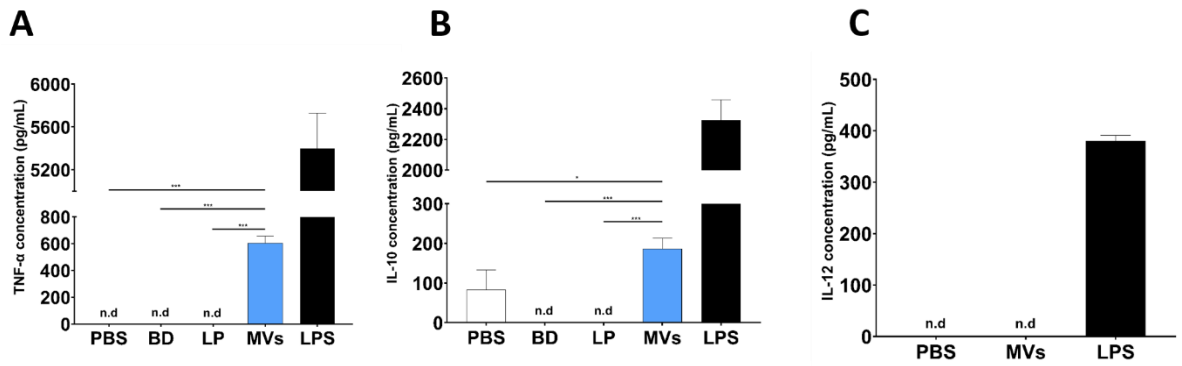


Figure 19. Pneumococcal membrane vesicles (MVs) possess immunomodulatory effect on antigen-presenting cells (APCs), e.g., dendritic cells (DC2.4). Phosphate buffer saline (PBS), polystyrene beads (BDs), liposomes (Lipo), membrane vesicles (MVs) and lipopolysaccharide (LPS). Negative values are denoted not detected (n.d), and considered zero value in statistical analysis. (A) Induction of TNF release, upon treatment of DC2.4 cells with pneumococcal MVs for 24 h incubation period. (B) Slight increase of IL-10 release, upon treatment of DC2.4 cells with pneumococcal MVs for 24 h incubation period. (C) Absence of any detected effect on IL-12 release, upon treatment of DC2.4 cell with pneumococcal MVs for 24 h incubation period. Differences between groups were determined using one-way analysis of variance (ANOVA) followed by (Sidak) *post hoc* test for multiple comparisons. n = 6, mean ± SD, where *P≤0.05, **P≤ 0.001, ***P≤ 0.0001

We observed no change in secretion of IL-12 from MV-treated DC2.4 cells. This cytokine is secreted mainly by activated APCs during antigen presentation. It functions as a bridge between innate and adaptive immune systems, as it aids in differentiation of naïve T cells into memory cell and cytokine-producing T-helpers. It regulates several cellular pathways necessary for proper functioning of immune system, as it provides protection against infections [266]. This result might be due to difference in activation of DCs upon exposure to live bacteria and their vesicles. This observation is in line with previously reported results, as they reported inability of pneumococcal MVs to induce secretion of IL-12 from DCs [192].

5. 6. Conclusion

Our study provides better understanding of MVs secreted from gram-positive Streptococci. We isolated and purified streptococcal MVs and characterized them physico-chemically. We exhibited a heterogeneous nature of streptococcal MVs and presence of distinct populations, with respect to morphology, constitution, particle size and content. Our findings confirmed rapid internalization of pneumococcal MVs within adaptive immune cells and rather slower uptake into somatic cells after relatively short incubation periods. This uptake pattern is optimal for interaction with immune cells. MVs altered cytokine release pattern from dendritic cells, indicating a possible immune interaction/response. Thus, streptococcal MVs could be investigateded to develop new avenue of safe and effective, cell-free vaccines against pneumococcal infections.

6. Chapter Two: Yields and immunomodulatory effects of pneumococcal membrane vesicles differ with the bacterial growth phase

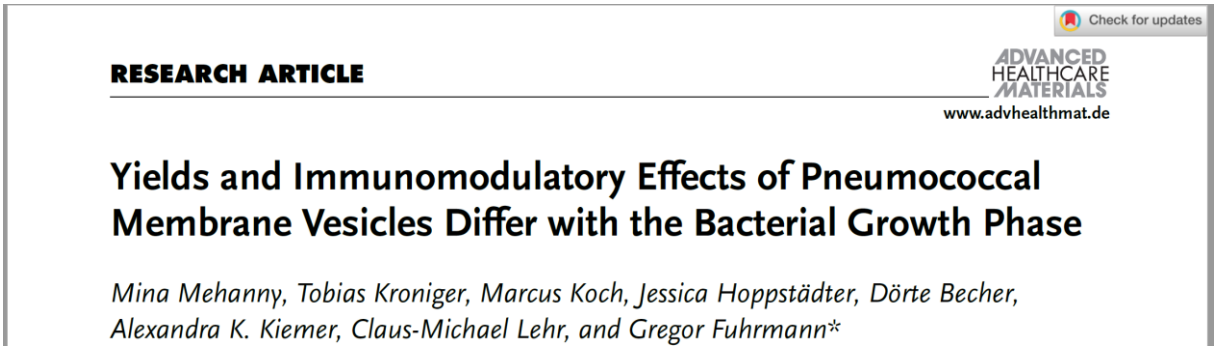
Most of the following chapter is transferred *verbatim* from the following published original research article.

Yields and immunomodulatory effects of pneumococcal membrane vesicles differ with the bacterial growth phase

Mina Mehanny, Tobias Kroniger, Marcus Koch, Jessica Hoppstädter, Dörte Becher, Alexandra K. Kiemer, Claus-Michael Lehr, Gregor Fuhrmann

Advanced Healthcare Materials, 2021, 2101151

DOI: 10.1002/adhm.202101151



Bacterial MVs are promising candidates as vaccination tools because they carry many microbial contents including lipopolysaccharides, cytosolic and membrane proteins, lipoproteins, and nucleic materials, which can interact with immune cells and initiate a protective immune response [267]. Pneumococcal MVs are considered miniature structures, with contents similar to those of their parent microorganisms [229]. We explored whether isolation during the death phase or the stationary phase, would affect vesicular yields, composition and immunogenicity to achieve better vaccine applicability.

6. 1. Death-phase MVs showed higher yields and a slightly higher size range to those of stationary-phase MVs

At 48 h, pneumococcal death-phase (dMVs) yields were several folds higher (~10^{12} particles/mL) than the stationary-phase (sMVs) yields, as determined via a nanoparticle-tracking assay. However, at 24 h, sMVs yielded approximately 10^{11} particles/mL (**Figure 20. A**). The yield was almost 10-fold higher, upon doubling the harvest time. We previously reported that fractions 6, 7, and 8 had the highest vesicle concentrations [80]. The dMVs exhibited a protein concentration of 404.33 µg/mL, which is approximately 4×10^{-10} µg/vesicle, while the sMV protein concentration was 218.33 µg/mL, which is approximately 2×10^{-9} µg/vesicle. We determined the vesicle concentration in terms of the number of particles per unit volume rather than the protein mass per fraction because proteins can originate from both vesicle membrane proteins and traces of nutrient medium as soluble protein impurities. Vesicular surface proteins, ligands, and/or antigens are biologically active, with suitable conformational structures and are located only on intact vesicles for relevant receptor binding. This is in contrast to soluble proteins, which can be ineffective or inactive [117].

dMVs had a mean particle size of 150–200 nm, while sMVs ranged from 120–180 nm **(Figure 20. B)**. SEC enables adequately purifying vesicles while maintaining their biological activity, as we previously reported [163]. We successfully purified the resuspended pellet obtained after ultracentrifugation, as BCA confirmed adequate separation of MVs from soluble protein impurities. Visualizing dMVs via cryo-TEM enabled studying their morphology in detail (**Figure 20. C–H**). Pneumococcal dMVs exhibited patterns similar to those of sMVs. Filtration allowed removing most of the protein impurities; thus, the pellet exhibited a clean background. Both vesicles showed very heterogeneous morphologies, with several arrangements and structures that included rounded, rod-shaped, irregular and chain-like vesicular forms. Additionally, some MVs showed darker contrast, suggesting different contents from those of other structures (**Figure 20. F**). Many vesicles were detected within other vesicles; some smaller vesicles were budding from larger vesicles. Cryo-TEM investigation showed various particle sizes from 20–30 nm to approximately 200 nm in diameter. Interestingly, some vesicles had double

membranes, and others had darker contents, indicating that they may harbor different cargo than do other vesicular forms. **Figures 21 and 22** show more cryo-TEM images of sMVs and dMVs.

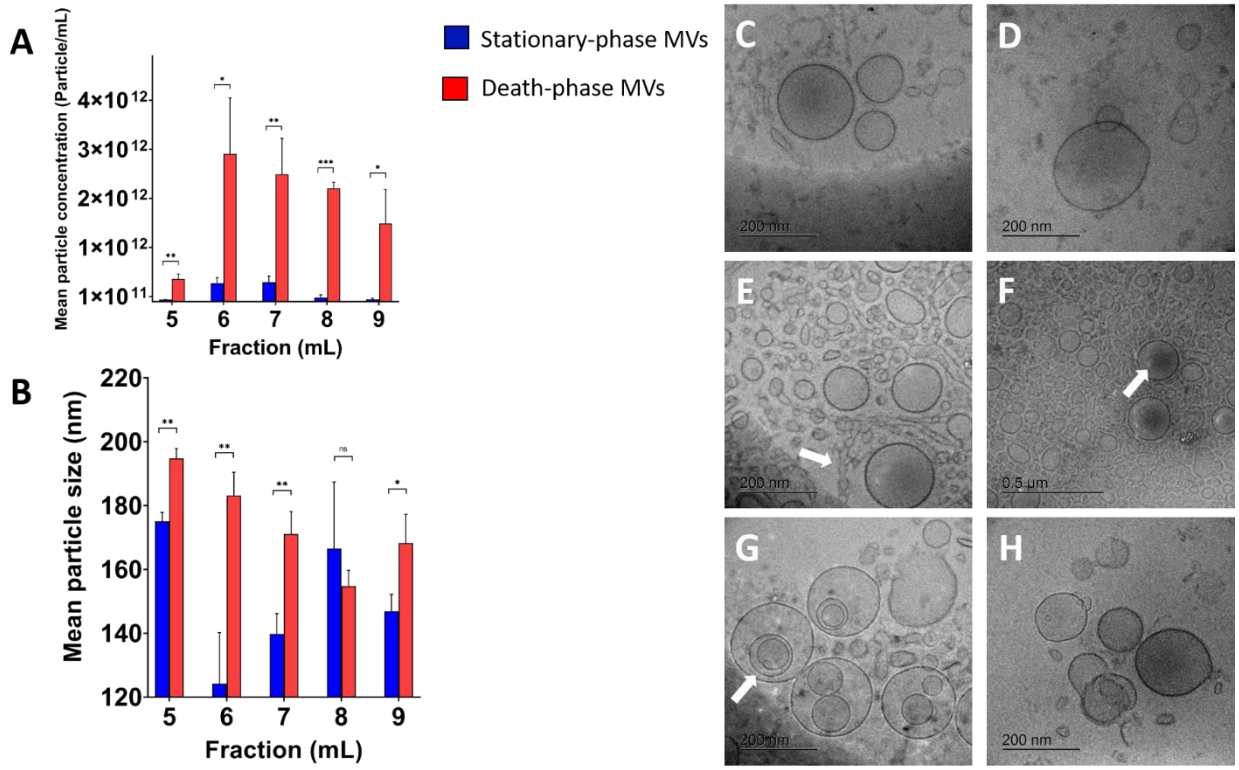


Figure 20. Nanoparticle tracking analysis results for stationary-phase membrane vesicles (sMVs) and death-phase membrane vesicles (dMVs). (A) Average particle concentration of the collected size-exclusion chromatography vesicle-rich fractions, where all dMV-isolated fractions showed statistically significantly higher concentrations than did the sMV fractions as per unpaired two-tailed t-tests comparing individual fractions. (B) Average particle size of collected vesicle-rich fractions, where most dMV fractions exhibited significantly increased particle sizes compared with those of sMVs as per unpaired two-tailed t-tests for individual fractions. The average number of independent experiments was n=3, where each sample was recorded in triplicate; *p<0.05, **p<0.01, ***p<0.001, and n: non-significant (C–H). Cryogenic transmission electron microscopy investigation of isolated pneumococcal sMVs and dMVs. (C, D) Isolated dMV pellet after ultracentrifugation; the white arrows show darker content structures (E) and chain-like vesicle assembly (F). Purified dMVs after size-exclusion chromatography, where the white arrows show superimposed (G) and one-inside-another (H) vesicle arrangement. Some vesicles were seen within each other (smaller vesicles were budding from larger vesicles). Particle size varied greatly, ranging from 20–30 nm to ~200 nm in diameter.

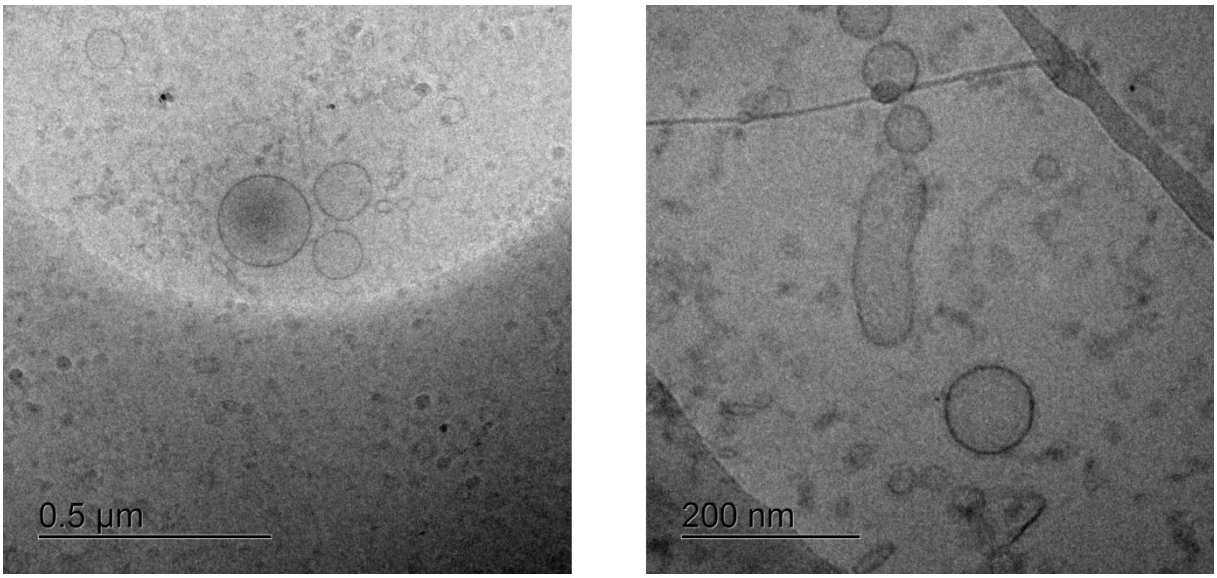


Figure 21. Cryogenic-transmission electron microscopy (cyro-TEM) investigation of isolated pneumococcal stationary-phase membrane vesicles (sMVs) pellet after ultracentrifugation

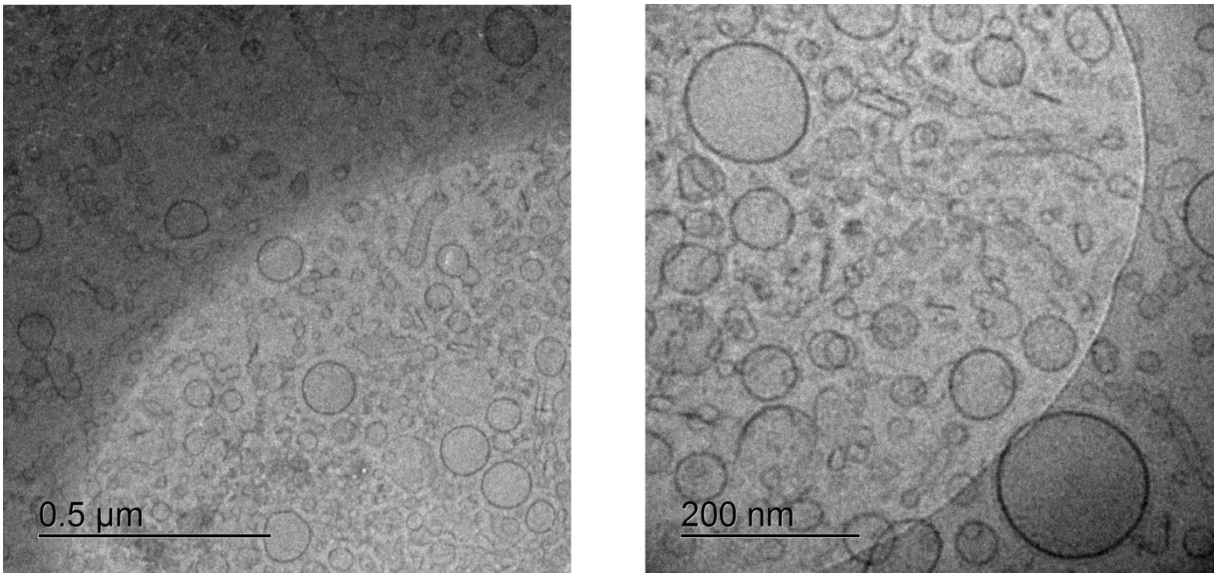


Figure 22. Cryogenic-transmission electron microscopy (cyro-TEM) investigation of isolated pneumococcal death-phase membrane vesicles (dMVs) pellet after ultracentrifugation

Nanoparticle tracking analysis data showed a relatively larger particle size range for dMVs than sMVs, while both having a wide size range as determined by cryo-TEM images. The mechanism of vesicular biogenesis in gram-positive bacteria remains poorly understood; however, a complex global gene network is likely to be involved in MV production [268]. Heat-inactivated bacteria reportedly cannot produce MVs in their supernatants; only metabolically active microorganisms can undergo vesiculogenesis [269]. In both the stationary and death phases, viable and active bacterial cells can secrete their vesicles into the surrounding environment. This process starts with budding of the cytoplasmic membrane and depends on

the turgor pressure of the membrane. Some lipidomic studies found similar fatty acid and phospholipid contents [270], whereas other studies found differences in the phospholipid contents between membrane vesicles and cell membranes [271]. dMVs showed significantly higher yields (i.e., more particles detected in the field), possibly because of collapsing cells or bubbling cell death via autolysin (Q7ZAK4) [41]; our proteomic analysis data confirmed an abundance of autolysin in both vesicle types, especially in dMVs.

The higher yield might arise from increased MV release due to loss of membrane integrity or fragmented parts of the dying microorganism. Toyofuku *et al.* reported an increased release of MVs during lysis of *Bacillus subtilis*. Expression of prophage-encoded endolysin in bacteria produced holes in the peptidoglycan wall, allowing MVs to protrude through it [57]. Nevertheless, a former study reported lower yield from dying pneumococcal cells, in contrast to our observation [190]. The bubbling mechanism from dying cells could explain the heterogeneous nature of pneumococcal vesicles isolated under both conditions, where dMVs are more abundant in terms of particle numbers. This is consistent with the findings of Resch *et al.* of heterogeneously sized circular structures, presumably for extracellular MVs from group A *Streptococci* [269]. Pneumolysin, a pore-forming toxin harbored on vesicles, can insert pores into lipid bilayers with protein-membrane aggregation of synthetic liposomes, causing membrane deformation and wrinkling and a layer-by-layer peeling of giant vesicles [272]. Pneumolysin (Q7ZAK5) was detected in our proteomic study of pneumococcal vesicles under both conditions. Therefore, pneumolysin may play a role in the heterogeneous morphology of pneumococcal vesicles.

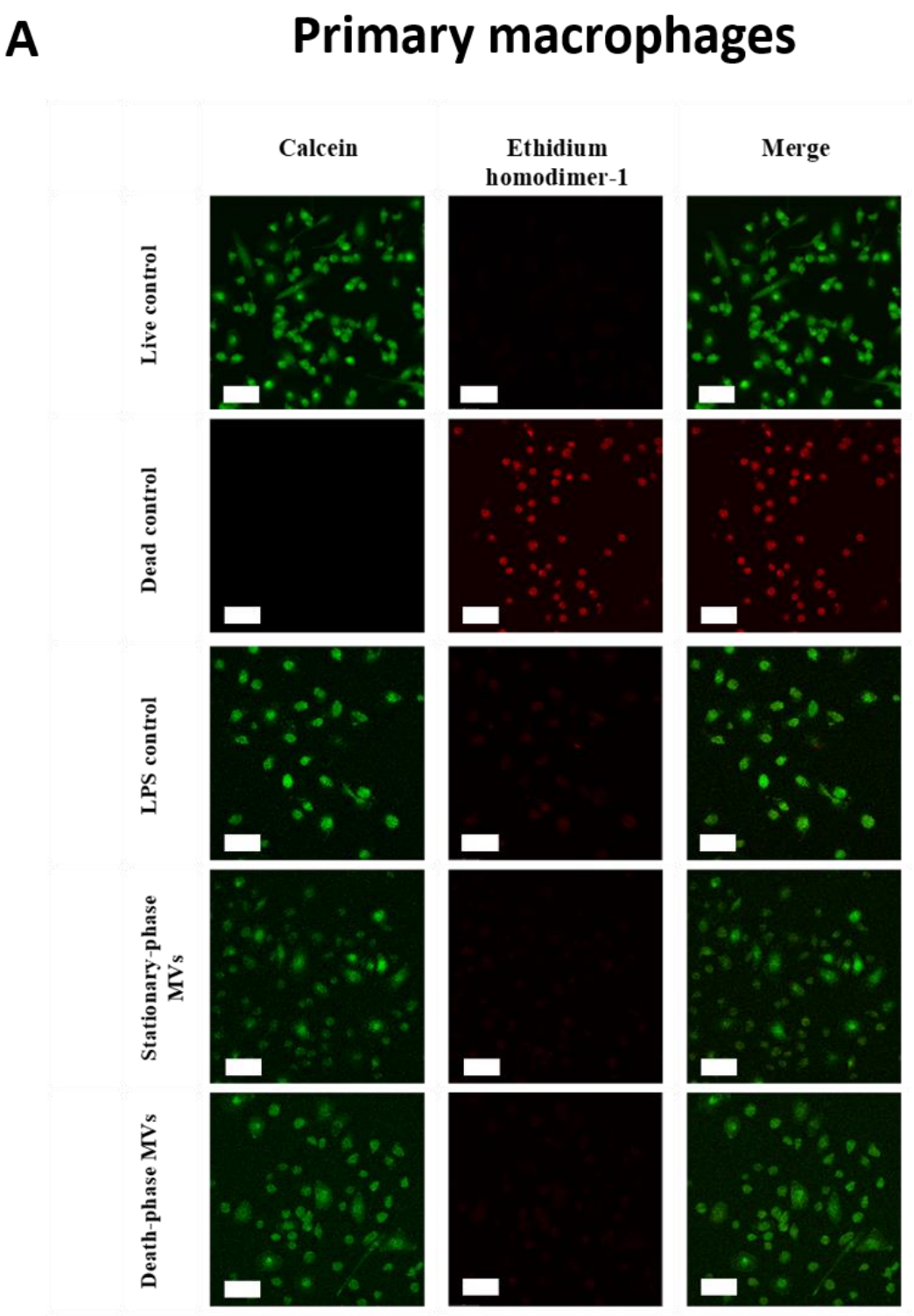
6. 2. Primary human immune cells

6.2.1. Pneumococcal MVs induced no severe cytotoxicity in primary human immune cells

To better understand the behavior and interactions of pneumococcal MVs inside the human body and confirm the suitability of applying bacterial vesicles for vaccinations, we examined the compatibility with various epithelial and immune cells. We previously confirmed the excellent compatibility of pneumococcal MVs with cell lines as an initial indication of their safety [80]; thus, we assessed them with primary human immune cells to ensure their applicability for a safe and effective human vaccine.

Primary human blood-derived MDMs and DCs exhibited good viability upon incubation with pneumococcal vesicles for 8 h, at concentrations reaching 5000 MVs/cell, showing minimal changes from the positive live controls (**Figures 23-25**). Whereas, dMVs showed very faint reddish staining in the confocal images (**Figure 23**) due to the presence of few dead cells in

MDMs and DCs. Concentrations up to 5000 vesicles/cell did not produce severe cytotoxicity, and caused no major changes in cellular morphology or membrane integrity **(Figure 26)**. Green fluorescence accumulated in the living cells, suggesting good tolerance with primary human cells, but further *in vivo* animal experiments are needed to confirm this finding. Both pneumococcal vesicles scored around 85% viability with MDMs and 75% viability with DCs at a concentration of 5000 MVs/cell (**Figure 24**). No significant difference was observed between pneumococcal MVs and LPS-treated immune cells.


A
Primary macrophages
Calcein
Ethidium homodimer-1
Merge
Live control
Dead control
LPS control
Stationary-phase MVs
Death-phase MVs

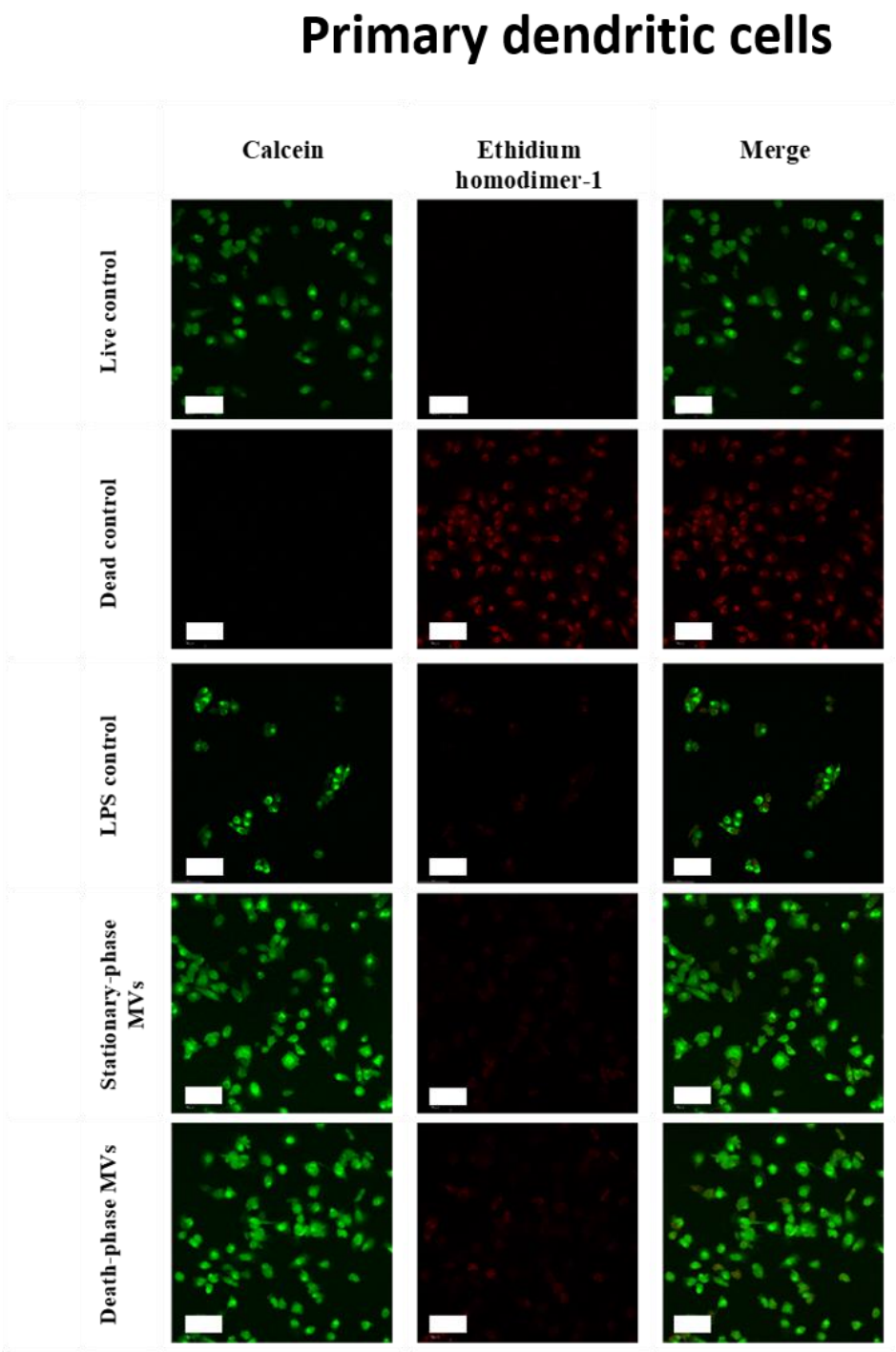


Figure 23. Confocal laser scanning microscopy images of primary human (A) monocyte-derived macrophages (MDMs) and (B) monocyte-derived dendritic cells (DCs) live-dead staining after treatment with stationary-phase membrane vesicles (sMVs) and death-phase membrane vesicles (dMVs) compared with live and dead controls and LPS-treated cells (250 ng/mL for 8h), where calcein (green) indicates live cells, and ethidium homodimer-1 (red) indicates dead cells (Scale bar = 50 µm)

However, some studies reported cytotoxic effects from other streptococcal strain vesicles. A study that isolated MVs from group B *Streptococci* showed hemolytic effects and induced cellular death of primary human cells after incubating neutrophils for 3 h and B and T cells for 1 h with 10 µL of vesicles at 2 × 10^{-4} µg vesicle protein/cell using an LDH assay [273]. Group B streptococcal MVs disrupted the feto-maternal barrier in lab mice after intra-amniotic injection due to their matrix-degrading proteases and pore-forming toxins, causing preterm birth [251]. Our study showed acceptable compatibility and relative safety of pneumococcal vesicles, possibly owing to more efficient purification using SEC, and suggesting the vesicles' promising applicability for vaccines.

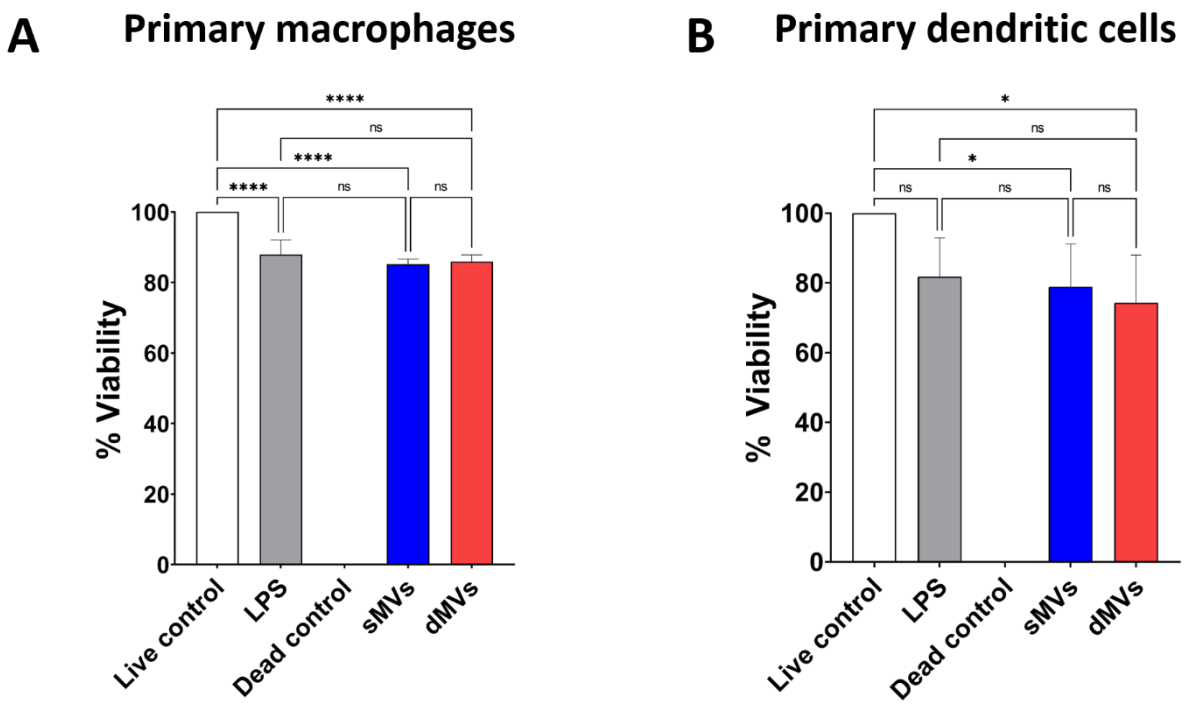


Figure 24. Quantitative determination of viability of primary monocyte-derived macrophages (MDMs) and monocyte-derived dendritic cells (DCs) *via* flow cytometry, using a live-dead kit. Cell viability percentage of stationary-phase membrane vesicles (sMVs) and death-phase membrane vesicles (dMVs) compared with live controls for (A) MDMs and (B) DCs. Average number of independent samples (n=4), where each sample was recorded in duplicate, using cells from four donors. LPS treatment was at concentration of (250 ng/mL) for 8h. One-way ANOVA was applied, followed by a Tukey post-hoc test, where *= p≤0.05, ****= p≤0.0001 and ns = non-significant.

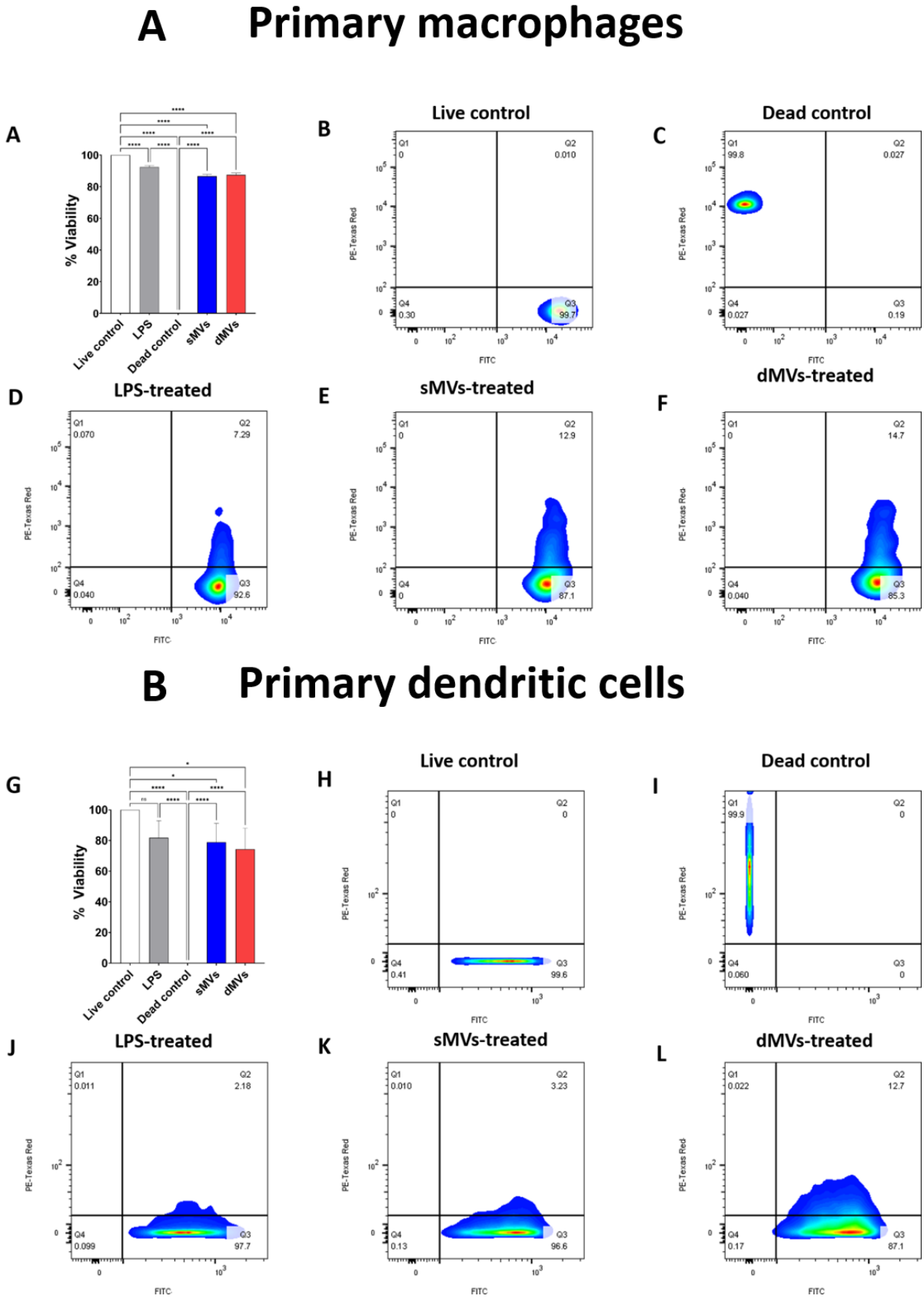


Figure 25. Quantitative determination of the viability of primary monocyte-derived macrophages (MDMs) and monocyte-derived dendritic cells (DCs) via flow cytometry using

the Live-dead stain kit. (A and G) Cell viability was expressed as a percentage compared to Live control cells after treatment with stationary-phase (sMVs) and death-phase (dMVs) membrane vesicles at a concentration of 5000 MVs/cell. Average number of independent samples n = 4, where each sample was recorded in duplicates, using cells from 4 different donors. One-way ANOVA was applied, followed by a Tukey post-hoc test, where *= p≤0.05, ****= p≤0.0001 and ns = non-significant. (B-F and H-L) FlowJo® analysis of viability of MDMs and DCs of live control, dead control, lipopolysaccharide, sMVs and dMVs respectively, showing gating of both Phycoerythin-Texas Red A (PE-A) and FITC-A channels. Recorded cells were gated for singlets, followed by biexponential transformation to facilitate visualization of cell populations.

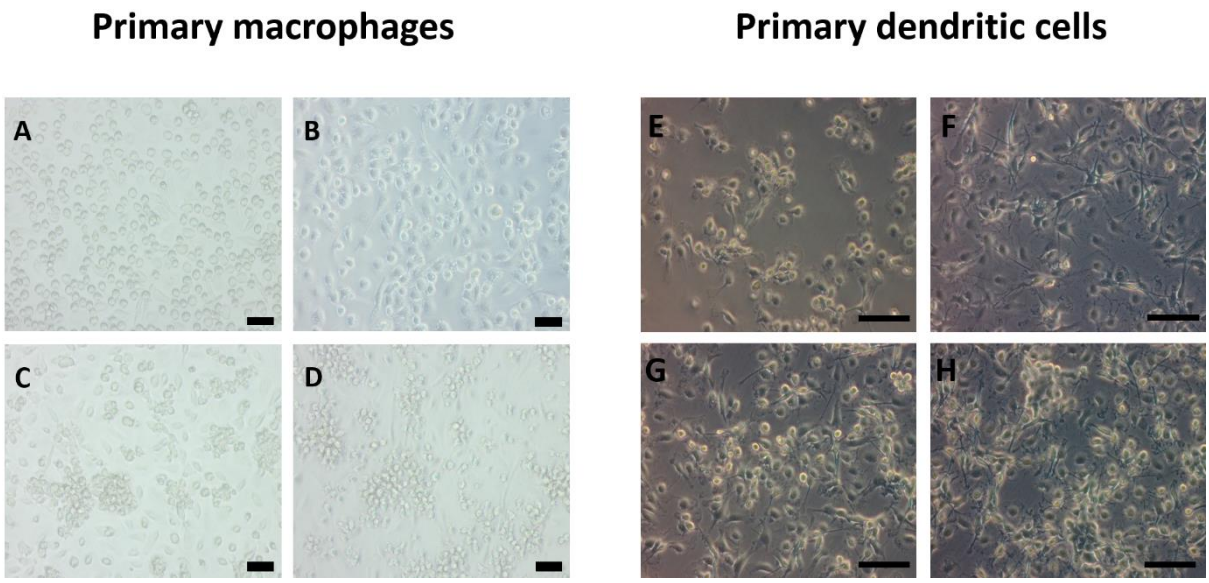


Figure 26. Light microscopy images of human primary blood monocyte-derived macrophages (MDMs) and dendritic cells (DCs) after 8 h treatment with pneumococcal membrane vesicles (MVs) at a concentration of 5000 MVs/cell. (A and E) PBS-live controls, (B and F) LPS-treated cells, (C and G) sMVs-treated cells and (D and H) dMVs-treated cells. The images show intact cell walls of the human primary immune cells upon incubation with pneumococcal MVs, as well as their tendency to accumulate and migrate when activated in presence of vesicles. (Scale bar = 50 µm)

6. 2. 2. Pneumococcal MVs showed excellent viability with cell lines

Cellular viability was excellent upon treatment with several dMV and sMV concentrations for all examined cell lines, and no cytotoxic effects were observed (**Figure 27**). Epithelial cells, including pulmonary cells (A549) and skin cells (HaCaT), were used to assess the effects of dMVs and sMVs on these cells as potential routes for vaccine administration. The pulmonary route is an important infection pathway for pneumococci. Murine macrophages (RAW264.7) were used as examples of immune cells to test their potential safety for vaccination interactions. These cells were applied to confirm pneumococcal MV suitability before further primary human immune cell testing.

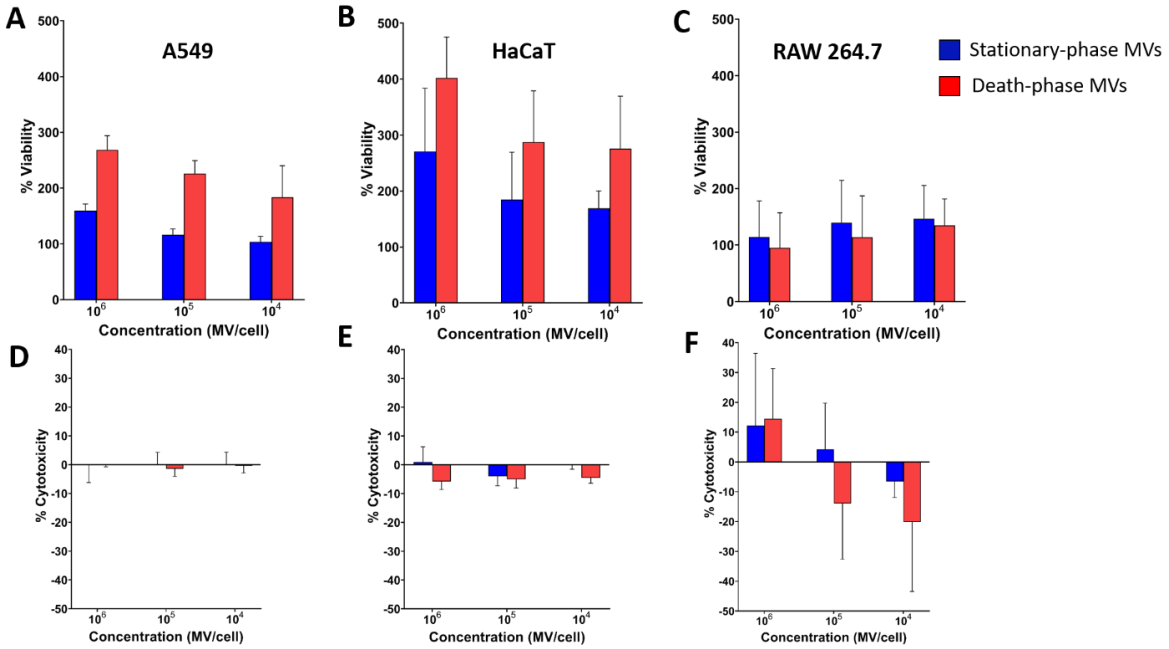


Figure 27. Assessment of compatibility of pneumococcal stationary-phase (sMVs) and death-phase (dMVs) membrane vesicles, with several mammalian cell lines. (A-C) Viability assay was determined using the PrestoBlue kit for A549 (p-value = 0.0289), HaCaT (p-value = 0.091) and RAW 264.7 (p-value = 0.2771) cell lines. (D-F) Cytotoxicity was assessed using lactate dehydrogenase kit for A549 (p-value = 0.1322), HaCaT (p-value = 0.0565) and RAW 264.7 (p-value = 0.456) cell lines. Average number of independent samples n = 3, where each sample was recorded in duplicates, using unpaired two-tailed t-test.

All cell lines showed very good tolerance to MVs upon 24 h incubation with concentrations reaching 10^6 MV/cell. Cellular viability even exceeded that of the positive live controls (PBS-treated cells with 100% calculated viability), as reported previously [80]. Higher vesicle concentrations yielded higher recorded viability values for A549 and HaCaT cell lines, while RAW264.7 cells demonstrated similar viability values for all applied vesicle concentrations. dMVs tended to have higher calculated viability than did sMVs for A549 and HaCaT cells, while RAW264.7 cells demonstrated almost same values for both vesicles, possibly due to consumption of these vesicles for cellular nutrition [42]. MVs might also stimulate the metabolic activity in the cells, leading to a stronger intracellular environment to reduce the resazurin PrestoBlue reagent to the fluorescent resourfin, leading to increased overall recorded fluorescence and hence, viability [80].

Pneumococcal MVs caused negligible, if any, cytotoxic effects on all cell lines and at all concentrations applied. All cell lines tolerated high concentrations of pneumococcal vesicles up to 10^6 MVs/cell, without compromising their viability. RAW264.7 cells showed some

cytotoxic effects at the highest concentration (10^6 MV/cell), with around 10%–20% cell cytotoxicity.

Consistent with our observations, a study reported that pneumococcal MVs lack cytotoxic effects on A549 cells for both sMVs and dMVs [191]. Codemo *et al.* similarly found that pneumococcal MVs could successfully internalize into A549 cells without causing cytotoxic effects after incubation for 24 h. These authors observed that pneumolysin-deficient mutant MVs produced no cell death in monocyte-derived DCs [192]. Although pneumolysin (Q7ZAK5) was identified in our vesicles, no cytotoxic effects were recorded in the cell lines or primary human immune cells.

6. 2. 3. Pneumococcal MVs were taken up successfully by primary human immune cells

For successful vaccine development, vesicles should interact and colocalize and be successfully taken up by antigen-presenting cells. Therefore, we assessed the interaction and uptake of pneumococcal vesicles into primary human cells by analyzing DiI fluorescence-labelled vesicles via flow cytometry and confocal microscopy.

Both pneumococcal dMVs and sMVs showed gradual time-dependent uptake into primary human blood-derived MDMs and DCs. No significant difference in uptake into MDMs was observed except in 4h uptake samples, however sMVs displayed slightly higher tendency to interact with human blood-derived primary MDMs than did dMVs at different time points, while both MVs exhibited similar uptake with DCs (**Figure 28**). Flow cytometry assays for uptake/interaction of fluorescent DiI-labelled vesicles into primary MDMs showed a fast interaction after 1 h of incubation of up to 40% for sMVs and 25% for dMVs. For sMVs, uptake reached 67% after 4 h and 75% after 8 h of incubation, whereas dMVs displayed 34% uptake after 4 h and 49% after 8 h of incubation. Faster uptake was recorded for primary DCs, reaching around 60% and 70% after 1 h of uptake for sMVs and dMVs, respectively. Near complete uptake was achieved after 4 h of incubation for both vesicle types, supporting our previous observation that pneumococcal MVs are taken up rapidly by antigen-presenting DCs [80].

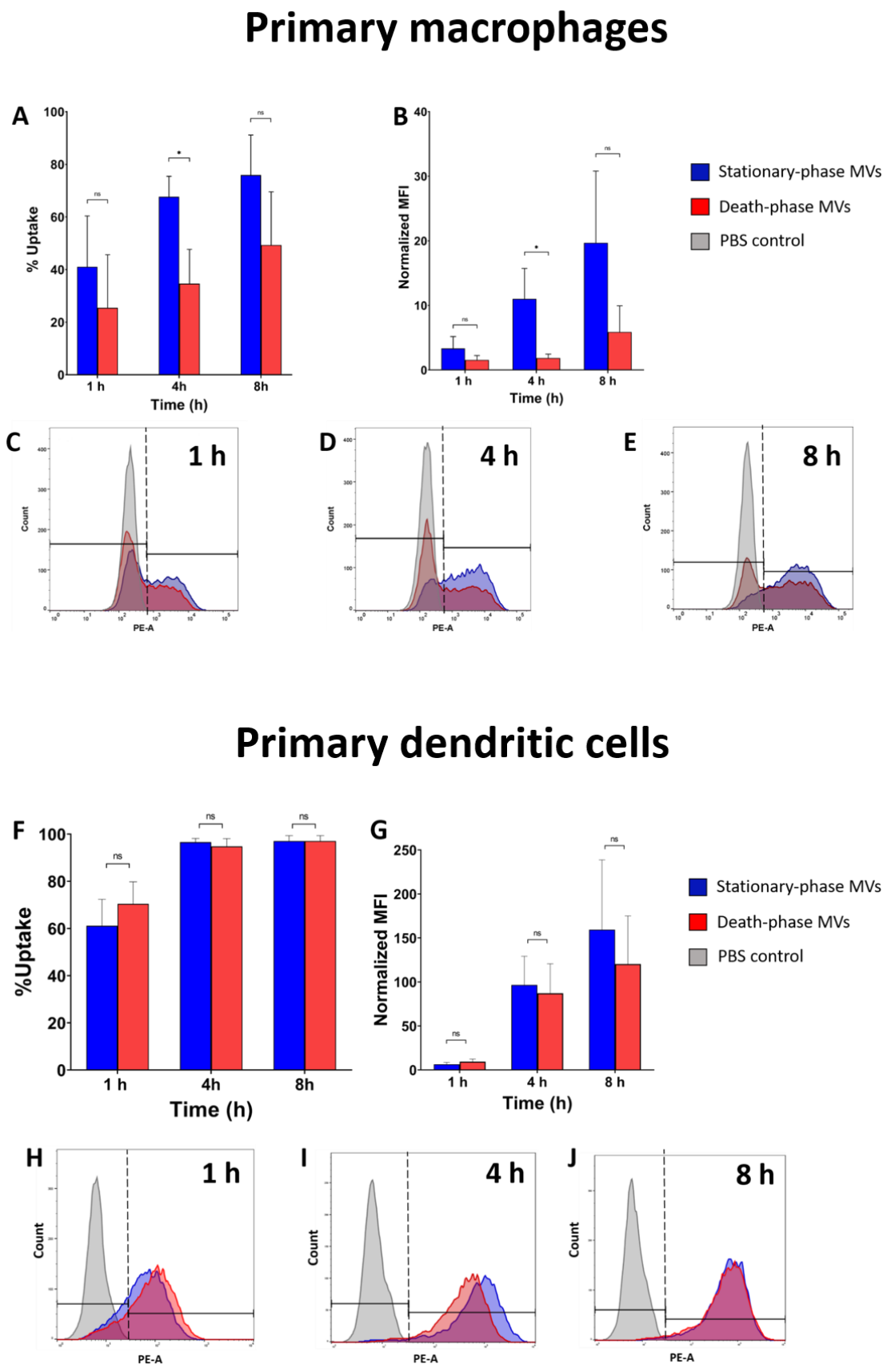
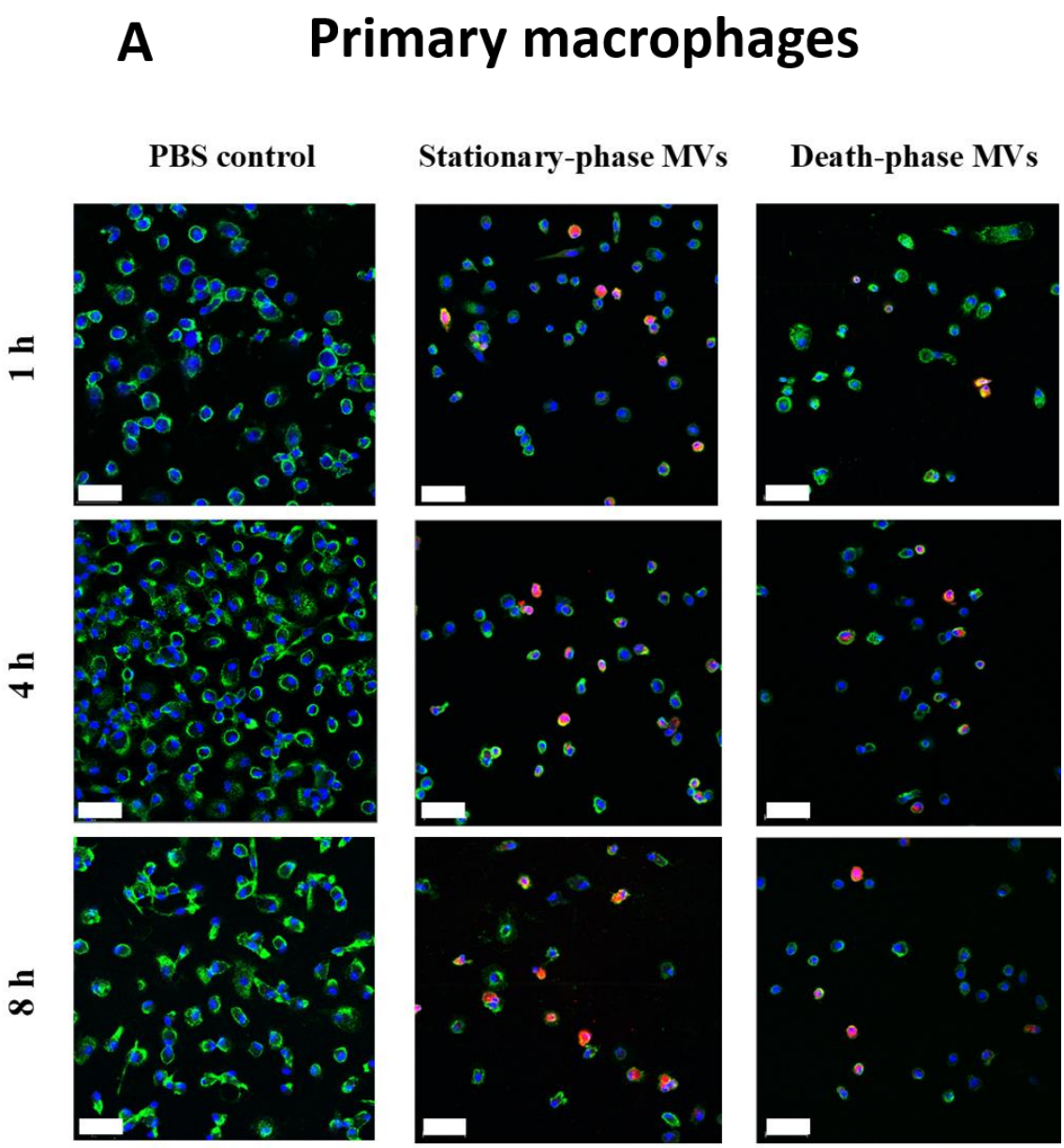


Figure 28. Uptake of DiI fluorescence-labelled stationary-phase membrane vesicles (sMVs) and death-phase membrane vesicles (dMVs) into human primary monocyte-derived macrophages (MDMs) and monocyte-derived dendritic cells (DCs) at 1, 4 and 8 h as per flow

cytometry. Percentage uptake calculated as percentage of positive phycoerythrin (PE) cells at 1, 4 and 8 h compared with PBS-treated negative controls for MDMs (A) and DCs (F). Normalized median fluorescence intensity of MDMs (B) and DCs (G) after uptake of DiI fluorescence-labelled sMVs and dMVs compared with PBS-treated cells at 1, 4 and 8 h. Average number of independent experiments (n=3), where each sample was recorded in triplicate and compared using unpaired two-tailed t-tests between individual time intervals; *p<0.05 and ns: non-significant (C–E and H–J) FlowJo® analysis plots of PE-positive cells compared with PBS-treated cells at 1, 4 and 8 h, respectively. Average number of independent samples (n=3), where each sample was recorded in duplicate, using cells from four donors.

Confocal laser scanning microscopy images showed successful internalization of pneumococcal vesicles into primary MDMs and DCs (**Figure 29**). After 1 h of incubation, minimal intracellular localization occurred in the cells because few red DiI-labelled MVs were observed within or attached to the green-stained cytoskeleton for both vesicle types. Internalization of vesicles inside macrophages increased gradually after 4 and 8 h, where sMVs exhibited slightly higher uptake than did dMVs. DCs demonstrated faster uptake, and nearly all cells internalized the vesicles after only 4 h of incubation. This supports the flow cytometry results that pneumococcal vesicles can successfully interact and colocalize inside immune cells and suggests successful delivery of loaded antigens for antigen presentation and immune response elicitation. Another study reported successful uptake of pneumococcal vesicles into monocyte-derived DCs after 2 h of incubation, supporting our results [192].

In summary, both sMVs and dMVs demonstrated gradual time-dependent uptake into primary MDMs. Internalization of pneumococcal MVs into immune cells might play a role in antigenic payload delivery and interaction with cells to elicit an immune response. No statistically significant differences were observed, although sMVs tended to have higher uptake into cells than did dMVs. This suggests that some dMVs are merely bacterial cell fragments and might possess a vesicular structure or morphology but lack functionality or ability to traverse biological membranes.


A
Primary macrophages
PBS control
Stationary-phase MVs
Death-phase MVs
1 h
4 h
8 h

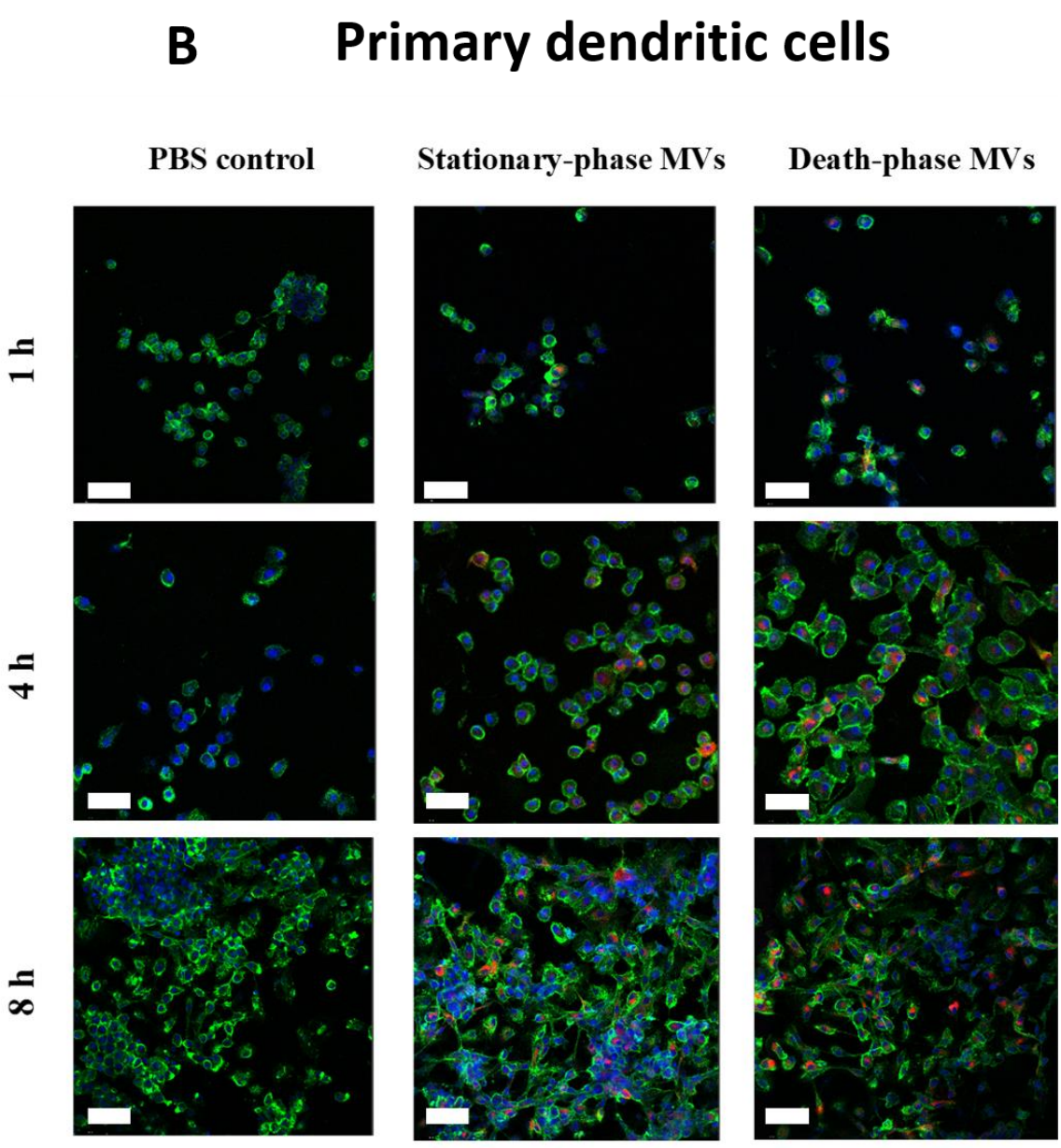


Figure 29. Confocal laser scanning microscopy of uptake of DiI (red) fluorescence-labelled stationary-phase membrane vesicles (sMVs) and death-phase membrane vesicles (dMVs) into human primary monocyte-derived macrophages (MDMs) at 1, 4 and 8 h (Scale bar = 50 µm). Cell membrane glycoproteins were stained with fluorescein-wheat germ agglutinin (green) at 10 µg/mL for 15 min, fixed in 3.7 % *w/v* paraformaldehyde, and incubated with 1 µg/mL DAPI (blue) for 15 min to stain the nuclei. Representative image for 3 independent experiments.

6. 2. 4. Pneumococcal MVs stimulated the release of inflammatory cytokines from primary human immune cells

We tested the ability of pneumococcal vesicles to stimulate primary MDMs and DCs to elicit a protective immune response for vaccination purposes. Measurement of released cytokines into the supernatant medium of primary MDMs and DCs upon 8-h incubation with pneumococcal

MVs showed stimulated release of TNF and IL-6 (**Figure 30**). Both sMVs and dMVs exhibited similar effects on cytokine release, and no significant differences were observed. IL-8 could not be quantified because its concentrations exceeded the detection limit in both untreated and treated samples. However, four cytokines (IL-1β, IL-2, IL-12p70 and IFN-γ) were undetected in the collected supernatant, possibly because they are not secreted in large amounts from macrophages or only secreted after longer times [274]. IL-8 is a major chemokine that enhances neutrophil and monocyte migration to inflammation sites. Monocytes and macrophages secrete IL-6 upon stimulation of their pattern recognition receptors including toll-like receptors with pathogen-associated molecular patterns. IL-6 plays a crucial role in host defenses against infection and causes acute-phase response and fever [275]. TNF is necessary in protecting against pathogens and is produced from effector T cells and innate immune cells to initiate killing infected cells [276]. TNF and IL-6 levels were elevated upon treatment with pneumococcal vesicles, thus confirming the ability of pneumococcal MVs to initiate the inflammatory reaction necessary for a protective immune response. This indicates the potential of pneumococcal vesicles as vaccination tools.

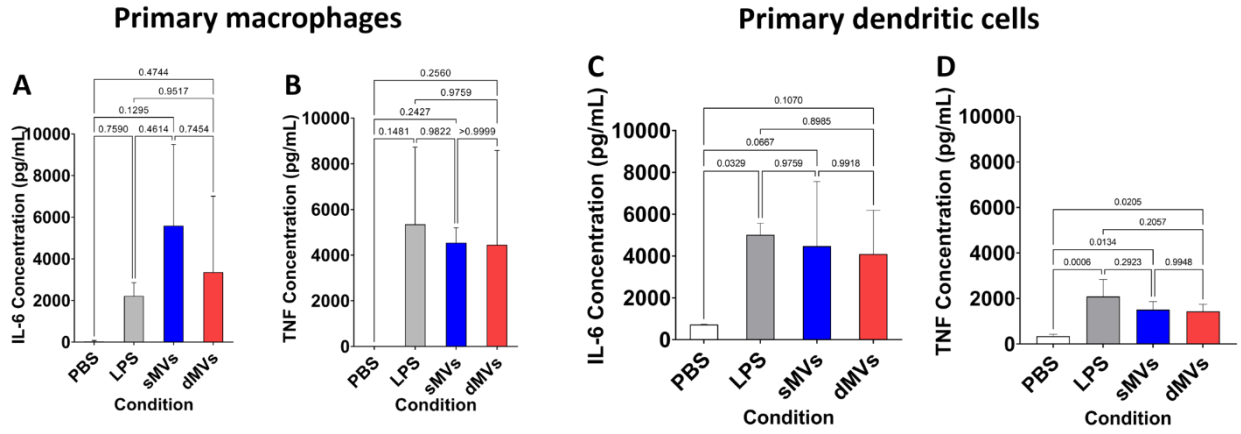


Figure 30. Determination of immunostimulatory cytokines released after incubation of pneumococcal stationary-phase membrane vesicles (sMVs) and death-phase membrane vesicles (dMVs) with human primary monocyte-derived macrophages (MDMs) and monocyte-derived dendritic cells (DCs) at concentration of 5000 MVs/cell, for 8 h compared with lipopolysaccharide (LPS)-treated controls at 250 ng/mL Concentrations of released interleukin-6 (IL-6) from MDMs (A) and DCs (C). Concentrations of released tumor necrosis factor (TNF) from MDMs (B) and DCs (D). Average number of independent samples (n=4), where each sample was recorded in triplicate, using cells from four donors. One-way ANOVA was applied, followed by the Tukey post-hoc test. P-values are plotted between various samples.

6. 3. Proteomic analysis of isolated pneumococcal MVs

In the MV proteomic analysis, we detected 586 proteins in isolated pneumococcal sMVs (24 h) and dMVs (48 h). We used the prediction tools, PSORTb and CELLO, to analyze the theoretical protein subcellular localization.

We used PSORTb, version 3.0.2 (https://www.psort.org/psortb/), an online bacterial peptide subcellular localization prediction database hosted by the Brinkman laboratory, Simon Fraser University, British Columbia, Canada [277, 278]. Most proteins in the sample (357 proteins) were assigned as cytoplasmic, followed by 125 cytoplasmic membrane proteins, thus showing a possible abundance of pneumococcal integral and transmembrane peptide proteins with probable antigenic characteristics. The program predicted that 16 proteins originated from the cell wall, and 11 were extracellular and might play roles as antigens and elicit an immune response. Seventy-seven proteins were considered to be of unknown origin (**Figure 31. A**).

We applied another online tool, CELLO v.2.5, a subcellular localization prediction tool (http://cello.life.nctu.edu.tw/), ran by the Molecular Bioinformatics Center, National Chiao Tung University, Taiwan [279]. The platform predicted 405 proteins to be cytoplasmic, while 121 and 60 proteins were considered membrane and extracellular localized proteins, respectively (**Figure 31. B**).

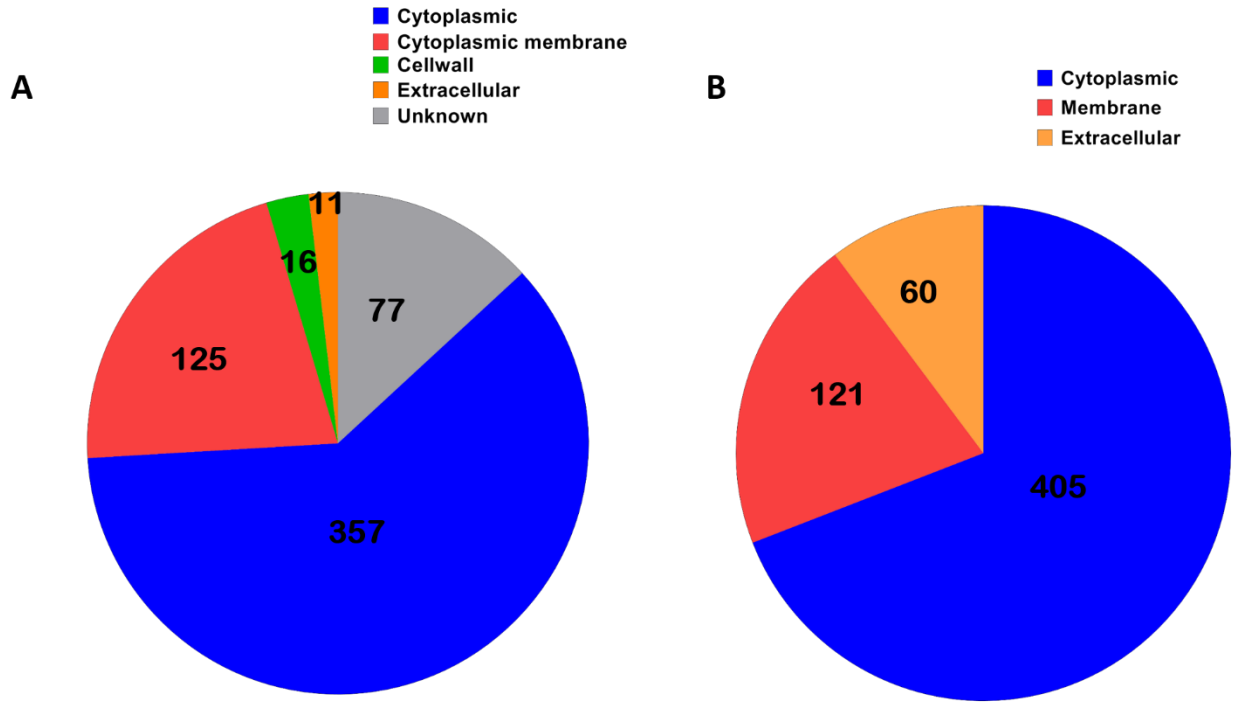


Figure 31. Subcellular localization prediction of shared proteins from the proteomic analysis in both pneumococcal stationary-phase membrane vesicles and death-phase membrane vesicles, using (A) PSORTb and (B) CELLO online databases.

Autolysin (Q7ZAK4) was detected in both sMVs and dMVs. Interestingly, autolysin was significantly more abundant in the dMVs than in the sMVs (**Figure 32**). Autolysin is considered essential for pneumococcal virulence and for an immune response against pneumococci. Eleven proteins were detected exclusively in sMVs, and 30 proteins were exclusive for dMVs; none of these carried specific antigenic importance for vaccine development. Further, we studied how SEC purification influenced both the sMV and dMV proteomes. Only five proteins were found exclusively in the isolated sMV pellet, whereas many proteins were exclusive to SEC-purified sMVs (**Figure 33**). SEC-purified dMVs exhibited many exclusive proteins, whereas the pellet showed only sialidase A, as a single exclusive protein (**Figure 34**). The low number of exclusive proteins detected in the pellets after ultracentrifugation confirmed the robust nature of pneumococcal vesicles to ultracentrifugation and the absence of vesicular lysis during this process. Previous studies on pneumococcal vesicles reported that MVs are enriched with transmembrane proteins and lipoproteins [190]. Another study identified 61 putative antigenic proteins in pneumococcal vesicles, which might induce adaptive immunity [191]. Codemo *et al.* speculated that pneumococcal MVs are derived from the plasma membrane and carry some cytoplasmic proteins as well as surface proteins docked to the membrane [192].

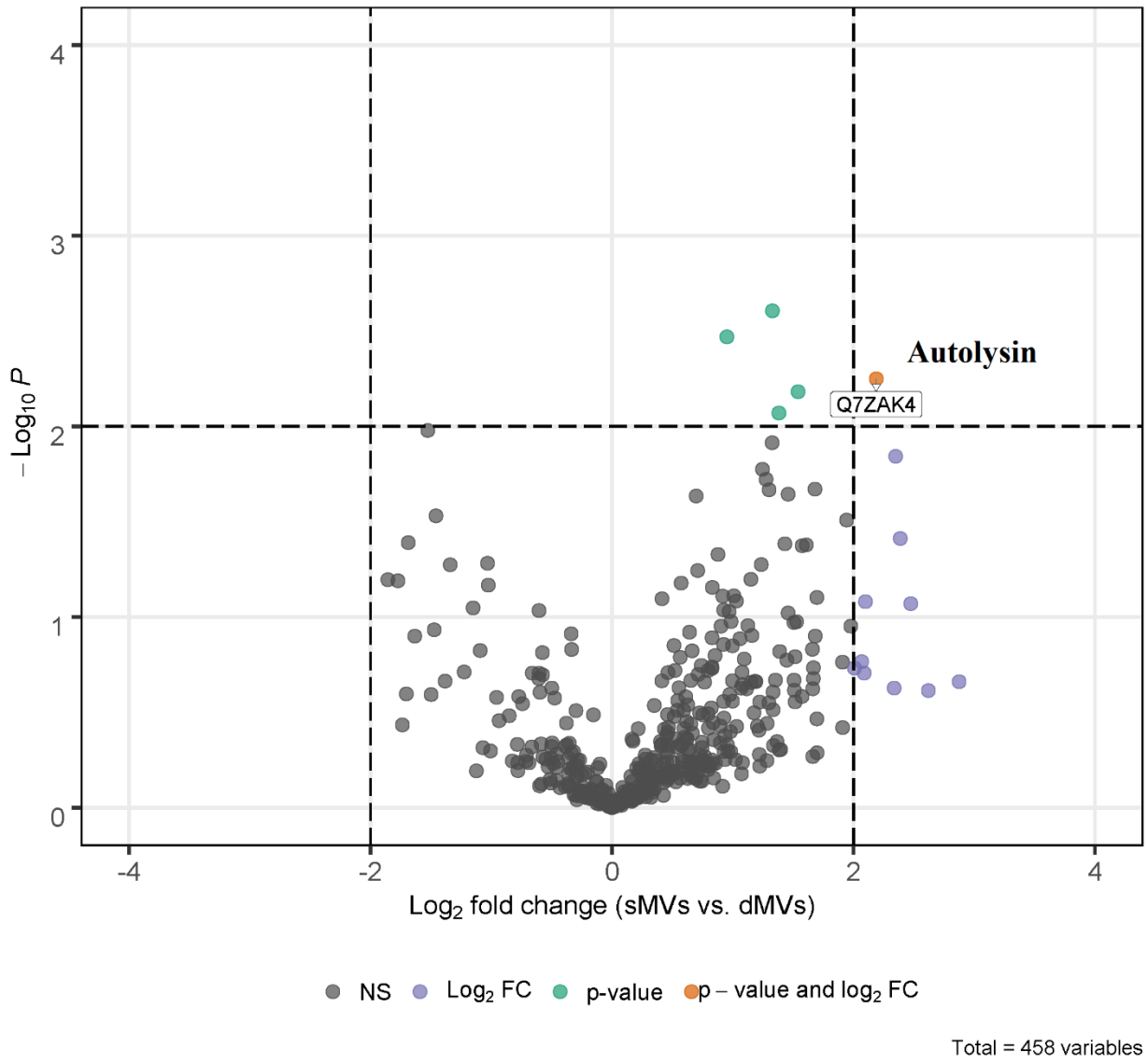


Figure 32. Volcano plot of potential differences between measured proteins in both size-exclusion chromatography-purified stationary-phase membrane vesicles (sMVs) and death-phase membrane vesicles (dMVs). Significantly changed proteins are in orange ($p<0.01$, $\log_2$ fold-change >2); proteins above the p-value threshold are in green; proteins above the fold-change threshold are in purple. The only significantly different protein between them was autolysin, which was significantly more abundant in dMVs.

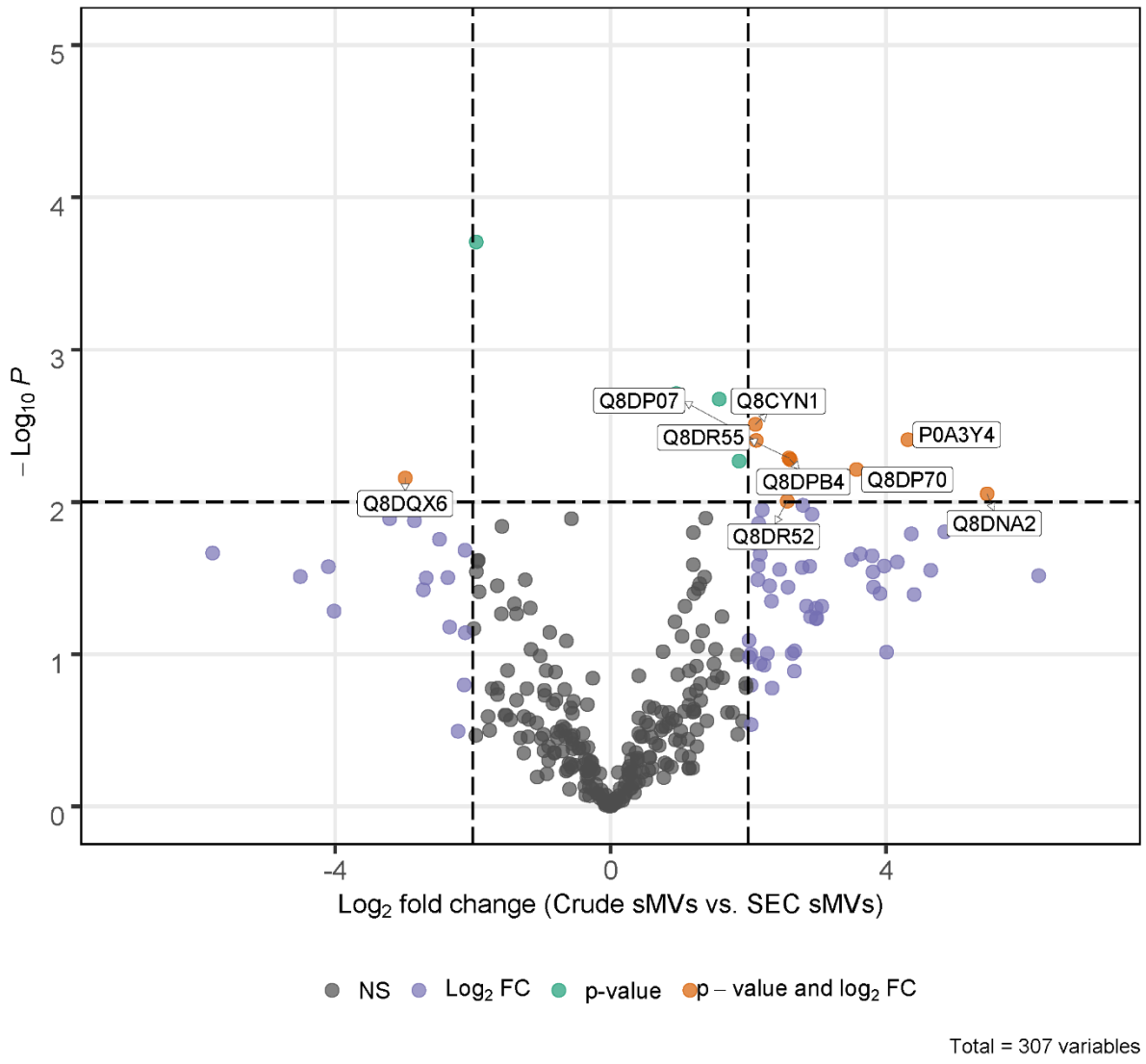


Figure 33. Volcano plot of potential differences between measured proteins in both size exclusion chromatography (SEC) purified stationary-phase (sMVs) membrane vesicles and crude pellet after ultracentrifugation without purification, both collected after 24 h, showing significantly different proteins. Significantly changed proteins are indicated in orange (p-value < 0.01, log_2 fold-change > 2), proteins above the p-value threshold are indicated in green, and proteins above the fold-change threshold are indicated in purple.

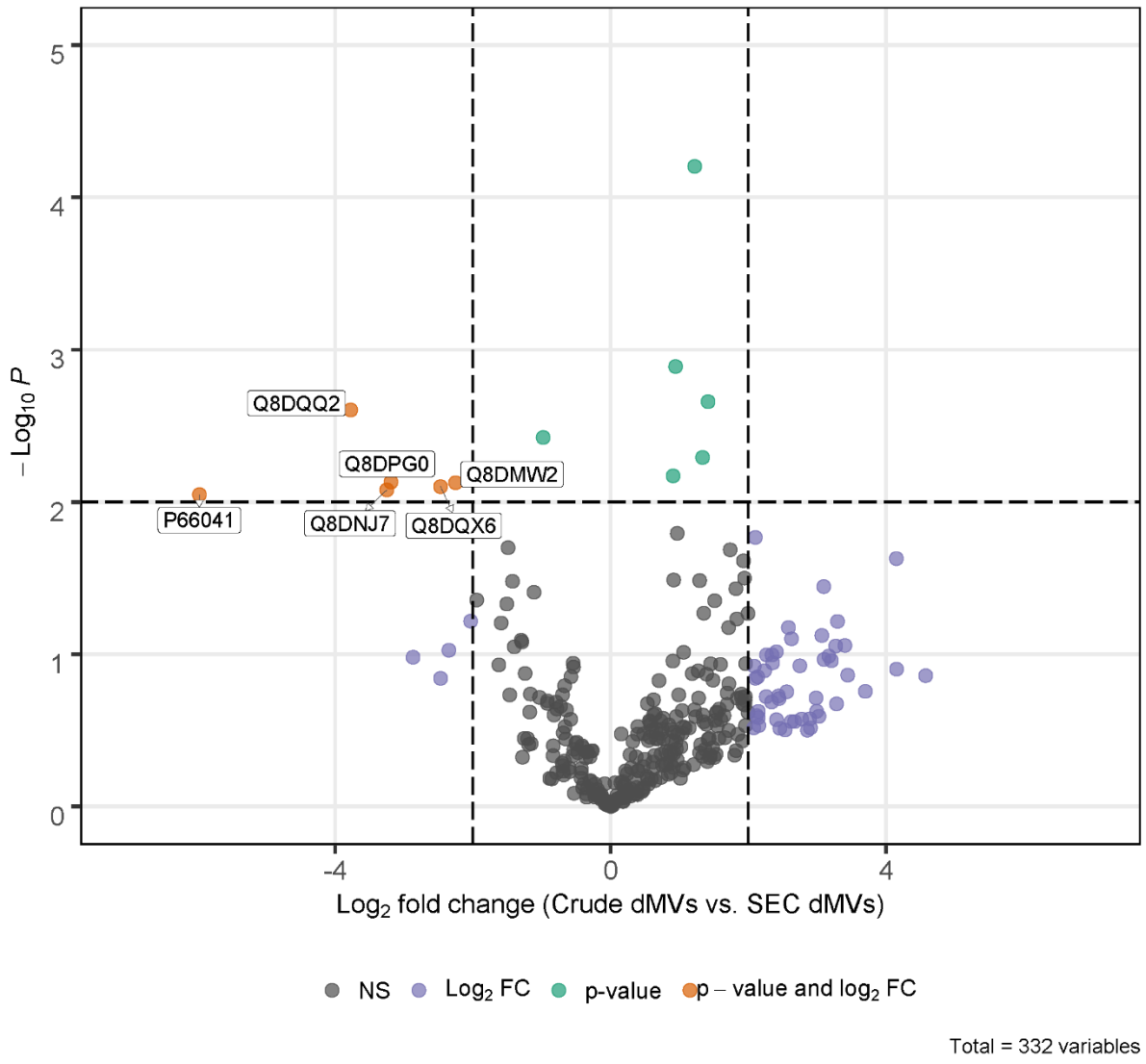


Figure 34. Volcano plot of potential differences between measured proteins in both size exclusion chromatography (SEC) purified death-phase (dMVs) membrane vesicles and crude pellet after ultracentrifugation without purification, both collected after 48 h, showing differences in their proteomic contents. Significantly changed proteins are indicated in orange (p-value < 0.01, log_2 fold-change > 2), proteins above the p-value threshold are indicated in green, and proteins above the fold-change threshold are indicated in purple.

Proteomic analysis of pneumococcal MVs revealed additional information about their contents and potential functions. Several putative antigenic proteins and lipoproteins were abundant and identified in the isolated pneumococcal vesicles (**Box. 2**). Pneumococcal lipoproteins play crucial roles for bacteria, including nutrient acquisition, resistance of stress conditions, enzymatic activities, and pathogenic processes such as adhesion, invasion and immune evasion. Additionally, lipoproteins trigger host innate immunity by activating toll-like receptor-

2, which elicits strong cellular and humoral adaptive immune responses. Hence, lipoproteins are considered promising vaccine candidates [280, 281]. Several lipoproteins were abundant in the analyzed MVs, including phosphatidylglycerol-prolipoprotein diacylglyceryl transferase (EC 2.5.1.145, Lgt) (Q8DPA3). Lgt is considered a crucial integral membrane enzyme involved in lipoprotein generation and its presence is essential for bacterial virulence and hence, immunostimulating effects [282, 283]. Manganese ABC transporter substrate-binding lipoprotein (pneumococcal surface adhesin A, PsaA) (P0A4G3) was detected in both vesicle types, which is a surface lipoprotein abundant in all known pneumococcal serotypes and is responsible for manganese transport and acts as an adhesive for host cell attachment. PsaA was the third most abundant protein in our analysis. It is considered a strong virulence factor and highly immunogenic, leading to antibody formation against it, and is being evaluated for vaccine development [284]. In addition, other lipoproteins were present in both MVs including Zinc-binding lipoprotein AdcA (Q8CWN2), lipoproteins (Q8DQ09) and (Q8DRG1) confirming their good immunogenic potential.

Pneumococcal vesicles harbored oligopeptide-binding protein (AliB) (P0A4G1), an ATP-binding cassette transporter involved in nutrient uptake and an important virulence factor that aids in nasopharyngeal colonization [285]. Autolysin (Q7ZAK4) is an important virulence factor and immunogenic protein loaded onto the vesicles, which causes release of pneumococcal factors, inflammatory macrophage reactions and oxidative stress in pneumococcus-infected lung epithelial cells [286, 287]. Pneumolysin (Q7ZAK5) is a key virulence determinant that induces pore formation, cellular damage, host tissue injury, and host immunity escape. It causes inflammatory responses and aids in transmission, colonization and cell death [288]. Importantly, it exhibits limited variation between different pneumococcal strains; thus, several studies demonstrated its protective effect as an immunogenic agent [289-291]. Alkaline amylopullulanase (ApuA; Q8DRA6) is a cell-wall-anchored enzyme that hydrolyzes pullulan and glycogen in the nasopharyngeal and oral cavities, which might enhance mucosal adhesion and could be a virulence factor [292]. Another cell-wall-associated protein is serine protease (PrtA) (Q8DQP7), which is highly conserved among many pneumococcal strains. PrtA is a good candidate for vaccine development because it has strong immunogenicity, limited genetic variation and importance for pneumococcal virulence [293, 294]. Another highly conserved pneumococcal protein is pneumococcal histidine triad protein D (Q8DQ08), which has been used in many studies of pneumococcal antigens for vaccination purposes. This protein induced functional antibodies and demonstrated a good safety profile, and expanded protection against pneumococci in animal studies and clinical trials [295-297].

Pneumococcal surface protein A (Q8DRI0) is a highly immunogenic protein, highly abundant on vesicles, and is a common vaccine candidate. It is expressed on the surface of all

pneumococci, which confers promising potential for a protective immune response against pneumococcal infection [298]. It is reported to induce both systemic and mucosal immune responses, causing long-term T- and B-cell induction [299-301]. Immunoglobulin A1 protease (Q59947) is a proteolytic enzyme responsible for cleaving specific peptide bonds in the host's immunoglobulin A1 (IgA1) hinge region sequence. It is a crucial virulence factor in bacterial infection and colonization [302]. Monoclonal antibodies can inhibit its binding and block infection at the host interface [303]. Immunization using recombinant IgA1 protease protected lab mice against lethal meningococcal and pneumococcal infections [304]. Choline-binding protein A (Q8DN05) is a member of a polypeptide family that anchors proteins to choline residues in the cell wall. It elicits release of chemokines from activated endothelial cells and alveolar macrophages, leading to recruitment of neutrophils into the alveolar space in early pneumonia [305, 306].

Olaya-Abril *et al.* performed lipidomic analysis of pneumococcal vesicles and confirmed that they are enriched in lipoproteins with slightly different fatty acid compositions from those of the bacterial cell membrane. Short-chain saturated fatty acids, especially lauric, myristic and palmitic acids, were enriched [190].

These proteomic analysis data confirm the high potential of pneumococcal MVs as carriers for several highly immunogenic proteins and lipoproteins, which are considered promising multiple-antigen hybrid vaccine candidates against pneumococcal infections as a novel strategy for next-generation vaccines [307, 308]. Our data indicate that dMVs offer no advantage over sMVs in terms of loaded antigenic proteins and lipoproteins, except for autolysin, which is significantly more abundant in dMVs.

Box 2. Immunogenic proteins/lipoproteins detected in both stationary-phase and death-phase pneumococcal membrane vesicles during proteomic analysis

Box 2. Abundant immunogenic proteins/lipoproteins in pneumococcal vesicles

- Oligopeptide-binding protein (AliB)
- Pneumococcal surface protein A (PspA)
- Autolysin
- Pneumolysin
- Immunoglobulin A1 protease (IgA1 protease)
- Serine protease (PrtA)
- Pneumococcal surface adhesin A (PsaA)
- Choline-binding protein A
- Pneumococcal histidine triad protein D

6. 4. Conclusion

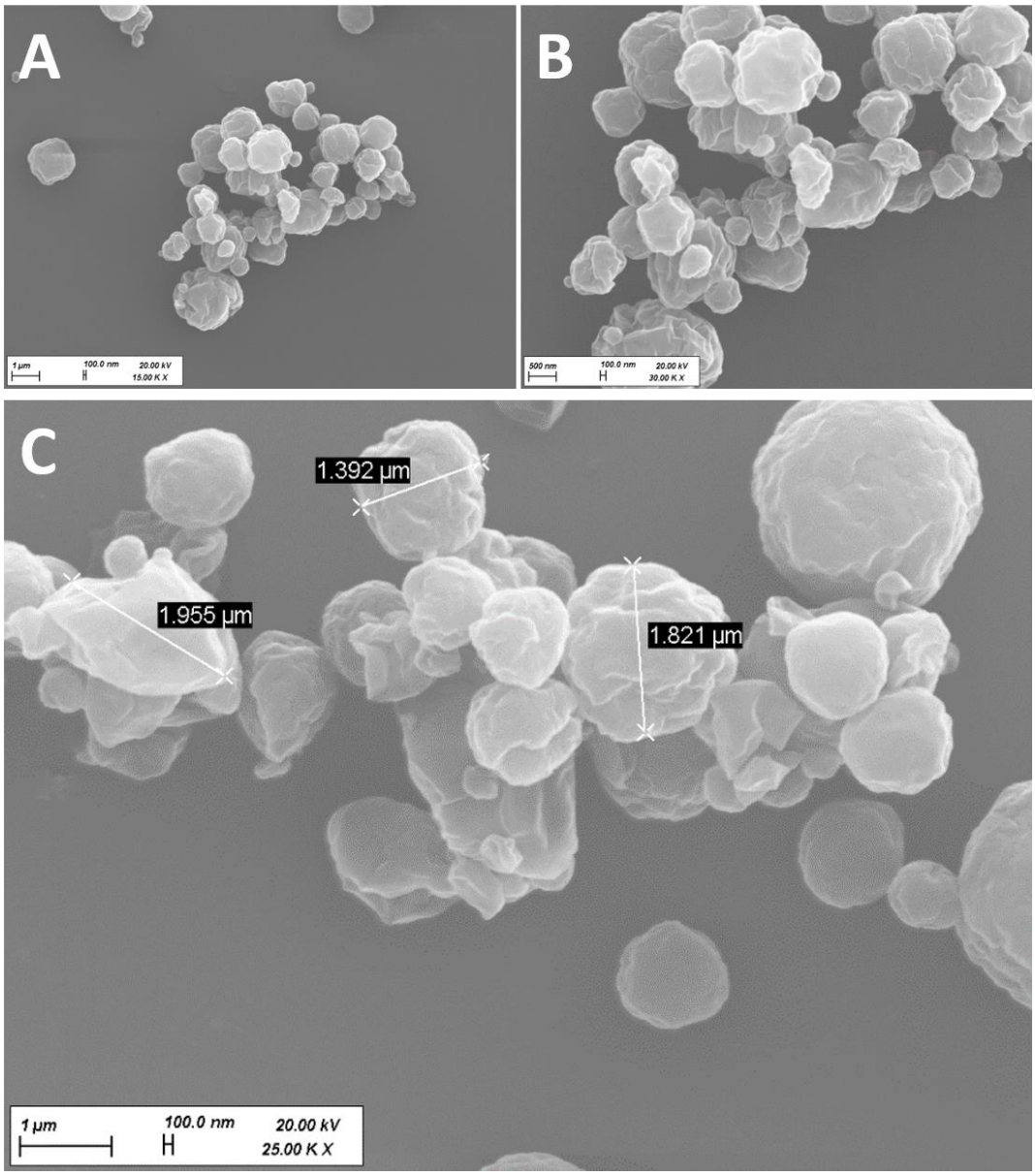
Our study revealed that pneumococcal dMVs achieved a higher vesicular yield than did sMVs. dMVs showed slightly larger particle size range, while both vesicle types exhibited excellent tolerability with several mammalian somatic and immune cell lines. Primary human immune cells showed acceptable compatibility with pneumococcal vesicles. Pneumococcal MVs displayed a gradual uptake into primary MDMs, with slightly higher trend of uptake values for sMVs than for dMVs. DCs showed comparable uptake for both vesicle types. The vesicles activated both primary MDMs and DCs to release the inflammatory cytokines, TNF and IL-6. Additionally, several strong antigenic proteins and lipoproteins were detected on isolated MVs, confirming their strong potential for protective immunostimulation and as promising vaccine candidates. Although dMVs achieved higher yields, they did not show better cellular uptake or stronger immune responses. More research should be implemented to test the feasibility of dMVs as models for prokaryote membranes [309]. In spite of minimal cytotoxicity observed with primary human immune cells, pneumococcal MVs confer a strong technological advantage being easily produced and more accessible, as well as harboring several strong immunogenic proteins and lipoproteins. This study thus supports applying pneumococcal sMVs as promising vaccine candidates.

7. Chapter Three: Spray-dried Pneumococcal Membrane Vesicles Microparticles are Promising Vaccine Candidates for Pulmonary Immunization

7.1. Successful Spray drying of pneumococcal MVs into vaccine microparticles

Spray drying is an affordable single-step and continuous process to achieve dry powder, without affecting the loaded antigens of vaccine microparticles (MPs). Scanning electron micrographs confirmed the successful formulation of vaccine MPs, where (**Figure 35. A-C**) shows the plain MPs without loaded vesicles, having an average diameter of 1-2 µm, while their pneumococcal MV-loaded vaccine MPs counterparts (**Figure 35. D-F**) exhibited similar surface morphology and dimensions. Moreover, vaccine MPs topography demonstrated a rough corrugated surface, possibly due to presence of lactose and addition of leucine to the carrier solution, which improves their flow properties and mitigates their tendency to agglomerate [310]. In addition, leucine offers several privileges as a carrier including increased dispersibility of the generated powder and improved stability during spraying and storage [311, 312]. Furthermore, it possesses surface-active properties and enriches the surface during spraying process, creating a hydrophobic surface and reducing their hygroscopic nature and moisture uptake properties of the formed vaccine dried MPs [313, 314]. Reports mentioned enhancement of aerosolization behavior for DPIs containing leucine [315]. Thus, leucine is considered a very important excipient in the formulation of DPI, imparting a myriad of desirable properties and enhances their scale-up feasibility and clinical application. Similarly, Lactose, as mentioned earlier, is considered a generally regarded as safe (GRAS) carrier material by health authorities including the US FDA and the European Pharmaceutical Union (EPU). Hence, it is approved in DPI formulations as the main excipient [246, 316]. Therefore, our formulation could successfully produce pneumococcal MVs-loaded vaccine MPs using approved GRAS excipients, which make them amenable to further scale-up, animal testing and clinical trials.

Plain Microparticles



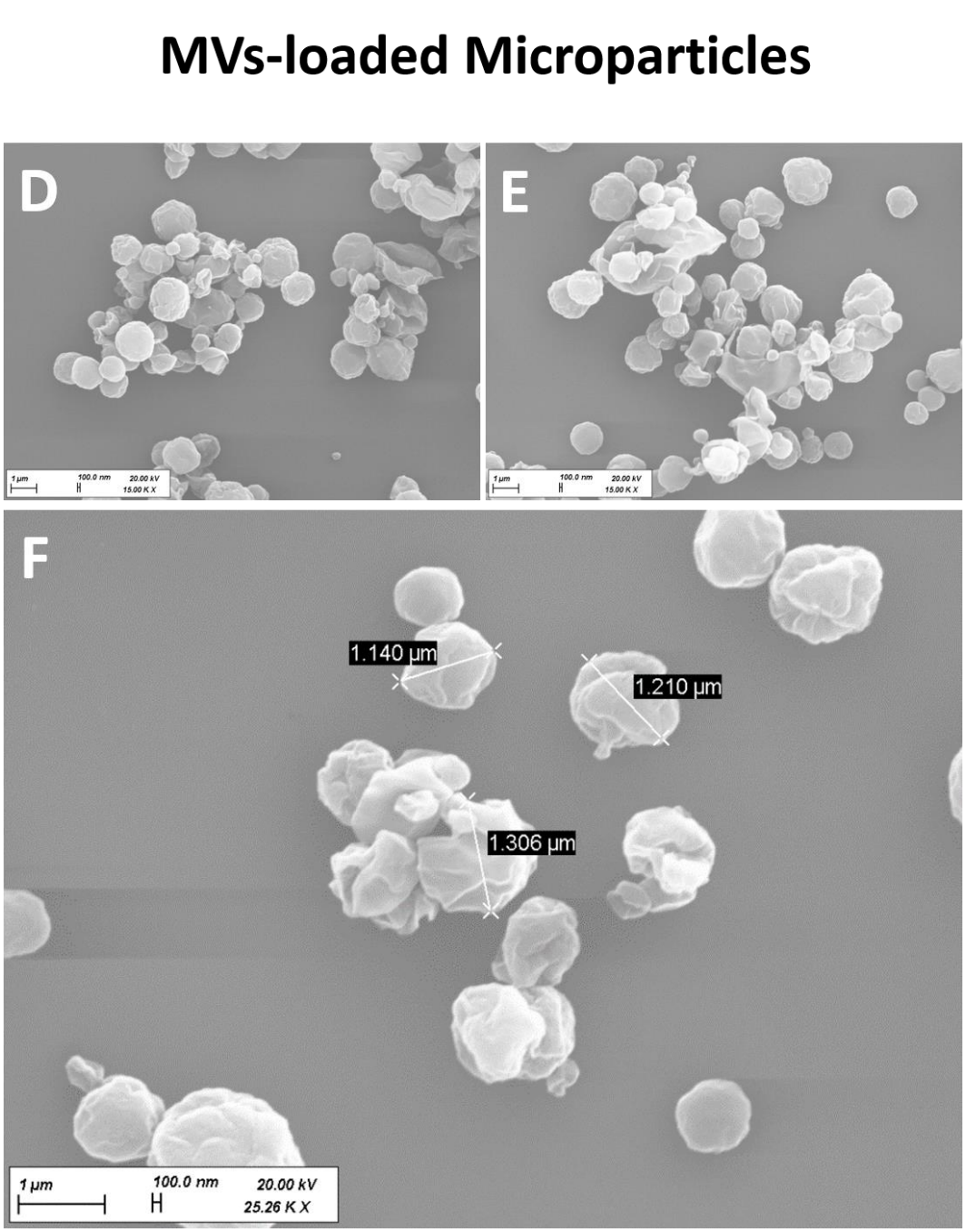


Figure 35. Scanning electron micrographs (SEM) of prepared microparticles. (A-C) Multiple images of plain microparticles consisting of lactose 2.5 % (*w/v*) and leucine 1% (*w/v*) in Milli-Q water. (D-F) Multiple images of pneumococcal MVs-loaded vaccine microparticles of the same formulation. Both microparticles manifested an average particle size within the range of 1-3 µm.

Static light scattering (**Figure 36**) showed particle size distribution of plain MPs (A and B) and pneumococcal MVs-loaded vaccine MPs (C and D), where plain powder MPs had a median particle size of 1.77 µm and MPs-loaded vaccine MPs exhibited a comparable median particle size of 2.06 µm. Both formulations demonstrated a uniform particle size distribution, which supported the obtained SEM images of prepared powder vaccine MPs [317]. Therefore, our

formulated vaccine MPs possessed optimal dimensions for DPI application for pulmonary immunization.

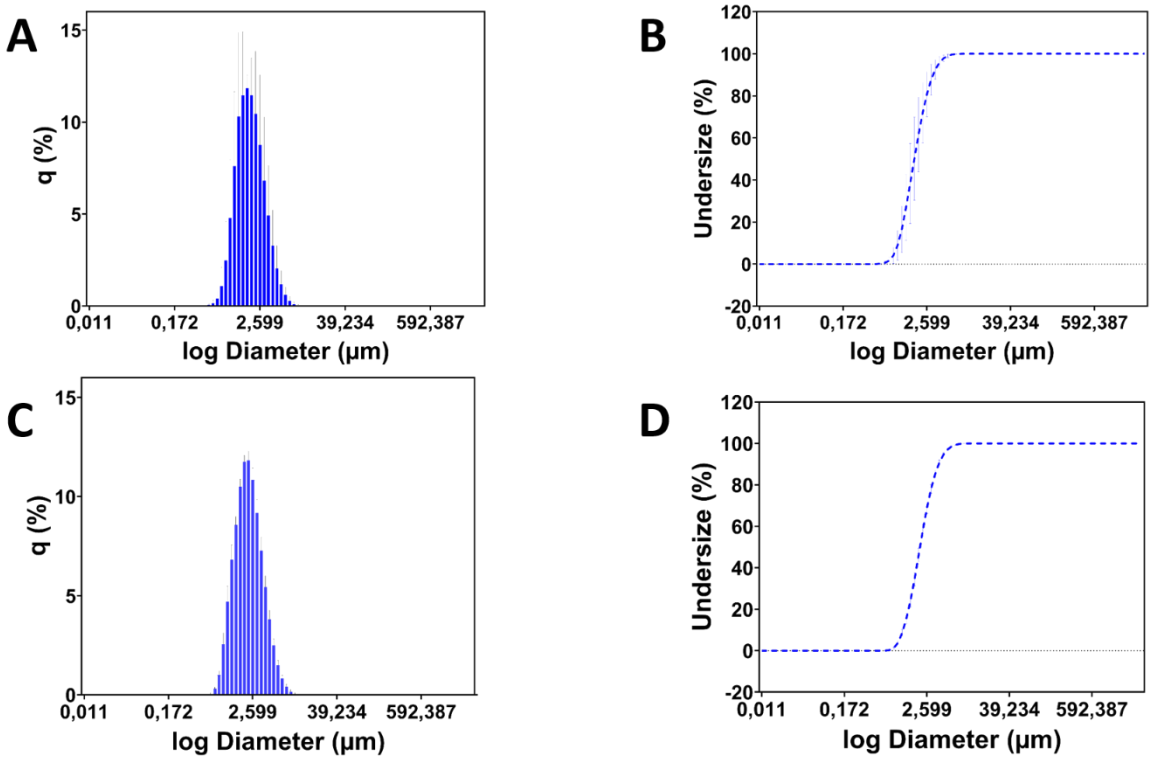


Figure 36. Particle size distribution of prepared microparticles as determined using static light scattering. (A-B) Plain microparticles. (C-D) Pneumococcal MVs-loaded microparticles. Where both particles exhibited median particle size 1.77 and 2.06 µm, respectively.

Additionally, we wanted to assess the nature of prepared vaccine MPs and we applied x-ray diffraction analysis to judge whether they exist in crystalline or amorphous state. Diffractograms (**Figure 37**) emphasized the crystalline nature of carrier ingredients i.e., lactose, leucine and PBS tablets, which is characterized by sharp diffraction peaks. Whereas spray-dried plain and pneumococcal MVs-loaded powders exhibited broad and diffuse peaks, typical for long-range disorder, hence confirming their amorphous nature [317, 318]. The amorphous nature of produced powder provide the advantage of being readily soluble to release their pneumococcal MVs payload, directly available for interaction with alveolar immune cells, to elicit a local protective response. Meanwhile, the amorphous powder suffers from potential instability during manufacture and/or aerosolization. Consequently, the application of a hydrophobic excipient such as leucine provides a protective role against instability, while maintaining good flow properties and therapeutic effects [319-321].

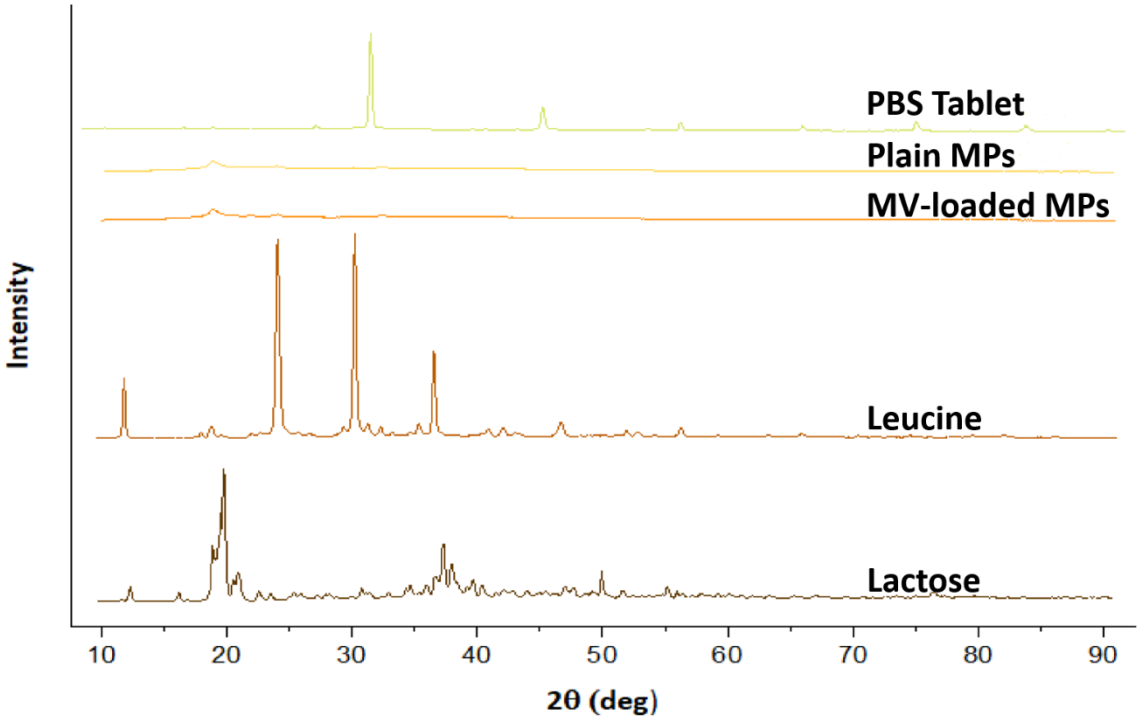


Figure 37. X-ray diffractograms of individual components of microparticle formulation including carrier solution (lactose and leucine), PBS tablet powder and formulated dry powder of plain microparticles and pneumococcal MV-loaded microparticles. Lactose, leucine and PBS demonstrated sharp peaks due to their crystalline nature, while both microparticle powders manifested diffuse peak confirming their amorphous nature.

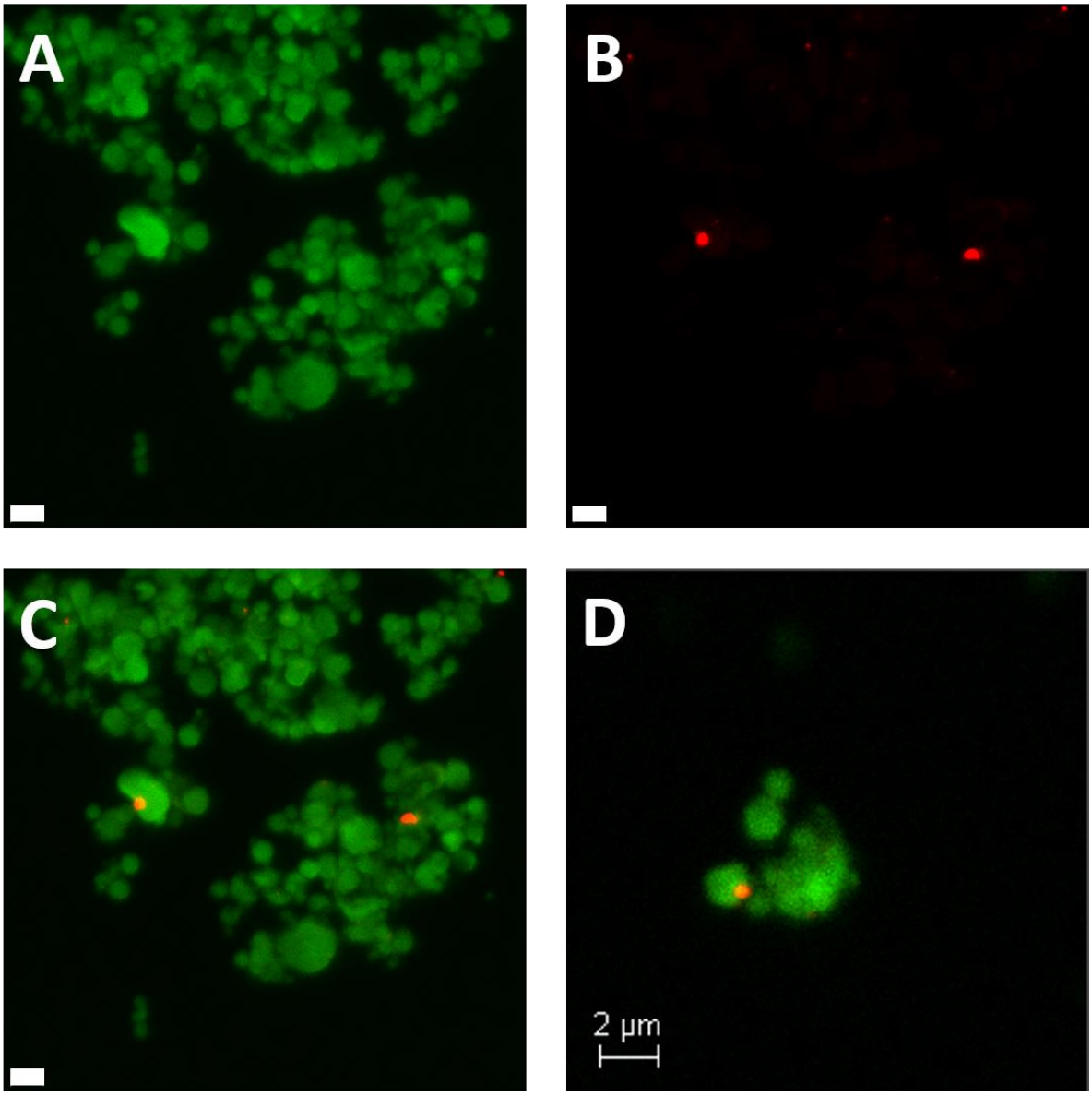
The aerodynamic properties of dry powder intended for inhalation are crucially important for pulmonary delivery, since they control their fate within the respiratory tract and hence, their ability to achieve their desirable effects. Large microparticles with aerodynamic diameter exceeding 5 µm suffer from deposition in oropharynx and large conducting airways, whereas particles in the range 1 to 5 µm predominantly achieve small airways and alveoli deposition [322, 323]. Therefore, *in vitro* investigation of aerodynamic properties of the prepared vaccine MPs was conducted using NGI. The prepared pneumococcal MVs-loaded vaccine MPs demonstrated a mass median aerodynamic diameter (MMAD) of 2.34 ± 0.09 µm and a geometric standard deviation (GSD) of 4.20 ± 1.38 µm, thus they are capable of deep lung alveolar deposition. They can reach the alveolar macrophages, the main phagocytic immune cells in deep lungs, and hence, can elicit strong protective local and systemic immune responses [324, 325].

The average percentage emitted dose from the capsule was 88.84 ± 3.17 %, showing that the powder exhibit good flow properties, to be released from the capsule. In addition, the calculated fine powder fraction (FPF) was 79.68 ± 5.37 %, showing that most of vaccine MPs could be successfully delivered from the capsule and travel through the respiratory tree to deep lung,

due to their small cut off value below 5 µm and excellent flow properties, which is very promising for the pulmonary immunization purpose [326, 327]. Accordingly, the formulated vaccine MPs manifested favorable *in vitro* aerodynamic powder properties, which make them promising candidates as DPI for deep pulmonary delivery.

7.2. Encapsulation of pneumococcal MVs within spray-dried vaccine microparticles

Even though spray drying is a non-stressful technique for dry MP production, it might cause loss of the loaded nanoparticles or vesicles, if the process conditions are not optimized to maintain their stability within the produced MPs. We examined the stability and successful encapsulation of fluorescently DiI labelled pneumococcal MVs (orange-red) inside the produced vaccine fluorescein-labelled MPs (green), using CLSM images. **Figure 38** confirmed the encapsulation of orange-red vesicles inside the formulated dry powder vaccine MPs [249]. In addition, z-stack (D-F) images indicated the presence of pneumococcal MVs in all powder layers and their homogeneous distribution throughout vaccine MPs, which is a crucial factor in the dose uniformity for pulmonary delivery of dry powder inhaler.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B
C
D
2 µm

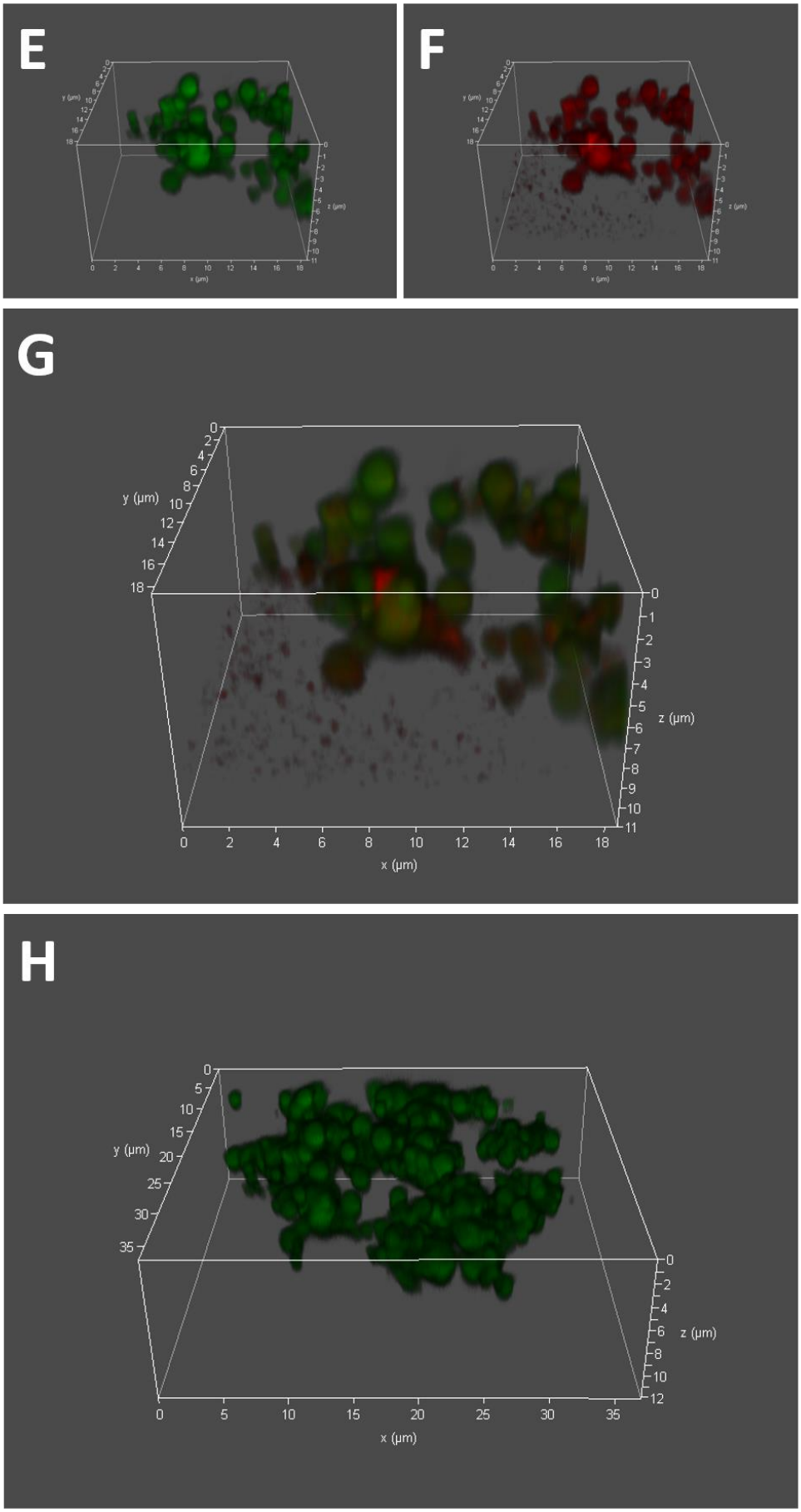

E
F
G
y (µm)
z (µm)
x (µm)
H
y (µm)
z (µm)
x (µm)

Figure 38. Confocal laser scanning micrographs (CLSM) images of (A) fluorescein sodium stained microparticles (green), (B) fluorescently DiI-labelled pneumococcal MVs (orange-red), (C) the merge of both channels and (D) magnified vaccine MPs encapsulated pneumococcal vesicles, manifesting successful encapsulation of pneumococcal MVs within produced vaccine microparticles. (Scale bar = 2 µm) (E-G) CLSM Z-stack of fluorescein sodium stained microparticles, DiI stained pneumococcal MVs and merge of both channels, confirming successful encapsulation and well dispersed nature of loaded MVs within all vaccine MPs, in comparison with (H) the plain microparticles with pneumococcal MVs, having only green fluorescent appearance.

As a complementary step, we wanted to evaluate the integrity of encapsulated pneumococcal MVs within MPs, and release after reconstitution of vaccine MPs in contact with water or *in vivo* pulmonary fluids. NTA results confirmed that spray-drying process did not compromise the integrity of loaded pneumococcal MVs, hence successful encapsulation within formulated vaccine MPs. The average particle size of pneumococcal MVs did not exhibit a change after spray drying, where before spraying 149.2 ± 8.5 nm and after spray drying 144.8 ± 2.6 nm (**Figure 39. A**). Furthermore, The average particle concentration of dispersed pneumococcal MVs after reconstitution of vaccine MPs was 1.23×10^{11} particles/mL, in comparison with 1.51×10^{11} particles/mL for pneumococcal MVs before drying (**Figure 39. B**). Therefore, the yield of pneumococcal MVs after reconstitution of powder vaccine MPs was around 81.5 %, which confirms the suitability of optimized drying protocol to maintain the integrity of pneumococcal vesicles and hence, their stability during the process. Some studies explored spray drying of liposomes and vesicular nanoparticles and reported successful drying of these nanostructures [328, 329]. Thus, the applied spray drying conditions were optimized and milder temperature (90 °C) than the temperature (135 °C) reported by Kanojia *et al.* for OMVs, which implies less stress on the pneumococcal MVs and hence, better stability for their antigenic protein and lipoprotein contents [222].

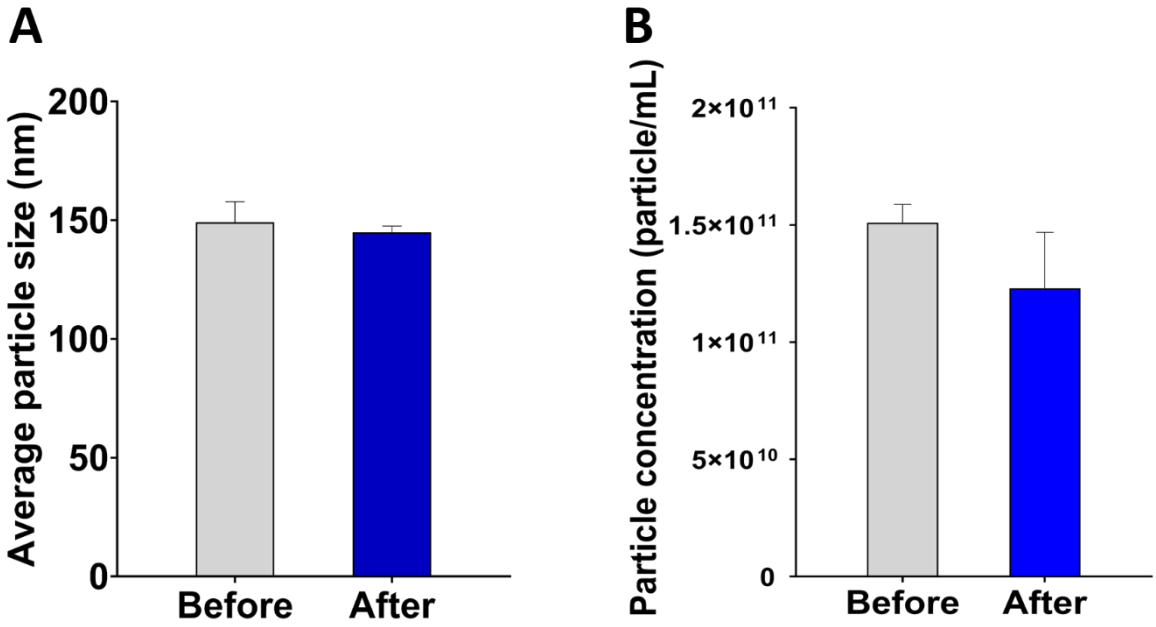


Figure 39. Nanoparticle tracking analysis (NTA) determination of (A) Average particle size (nm) and (B) Average particle concentration (particles/mL) of pneumococcal MVs before (gray) and after (blue) spray drying process.

On the other hand, plain MPs, free from loaded MVs, were investigated using NTA, and did not show any vesicles or particles, since the microparticle contents of lactose and leucine are completely soluble and no nanoparticulate structures were present. These observations confirmed successful spray drying of pneumococcal MVs within vaccine MPs, without adversely affecting their stability in terms of their particle concentration and average particle size. Our observations demonstrate the strong potential of spray drying to prolong stability of extracellular vesicles and produce dry powders suitable for scale-up in pharmaceutical industry.

7.3. Compatibility of spray-dried vaccine MPs with human THP-1 macrophage like cell line

We explored the compatibility of our spray-dried vaccine MPs with human macrophage-like THP1 cell line, after 8h of incubation. PrestoBlue viability assay (**Figure 40. A**) manifested higher calculated percentage viability values above 100 % of PBS-treated live controls for both plain MPs unloaded with vesicles and MV-loaded vaccine MPs. This expected finding agrees with earlier findings, upon treatment of cells with pneumococcal MVs, higher calculated viability results were observed [80, 330]. We speculate that the presence of sugar-containing carrier solution of vaccine MPs enhance the metabolic activity of THP cells, as the cells consume them as nutritious material, in addition to MVs rich in lipids and proteins. LDH assay (**Figure**

40. B) exhibited almost no signs of cellular cytotoxicity and did not exceed 10% calculated cytotoxicity for both plain and MV-loaded MPs, supporting PrestoBlue viability test results. Furthermore, live dead stain (**Figure 40. C-E**) confirmed absence of any cytotoxicity of formulated MPs on cellular morphology and/or viability [331], where cells pneumococcal-MVs vaccine MPs showed a bright green staining indicating live cells and absence of red discoloration due to dead cells. Therefore, spray-dried vaccine MPs are safe and compatible with macrophage-like THP1 immune cell lines.

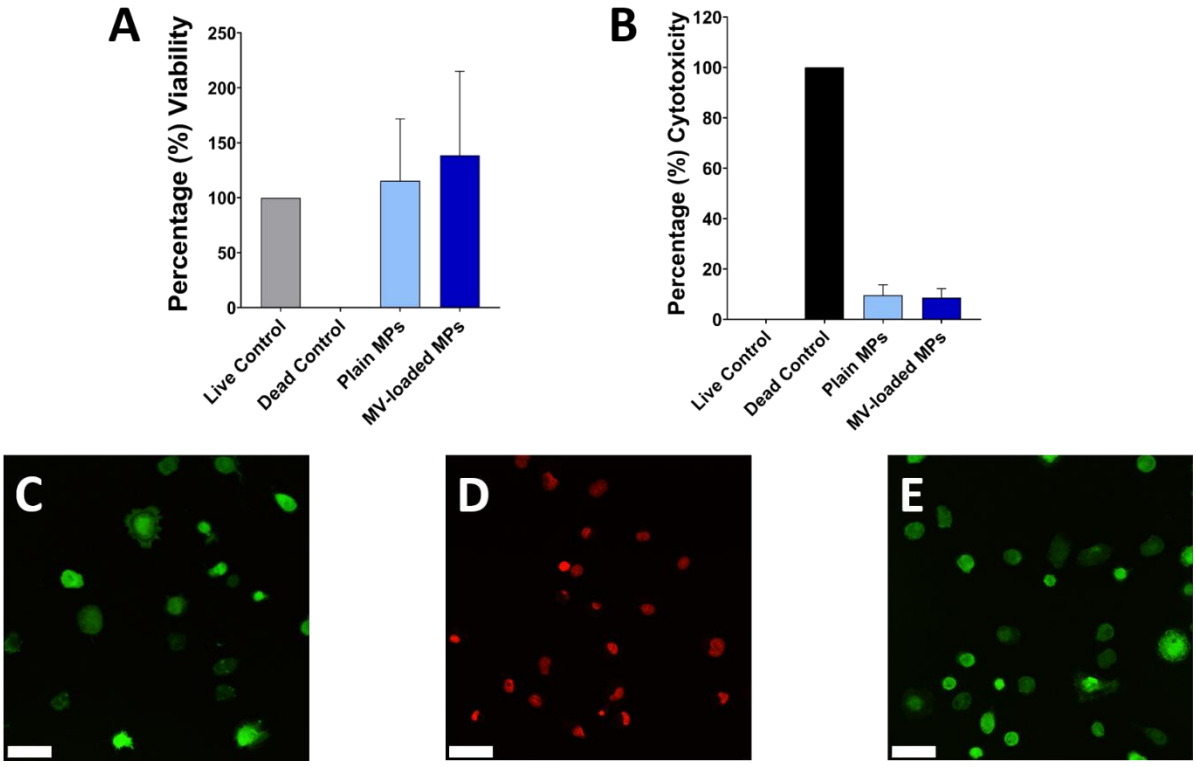
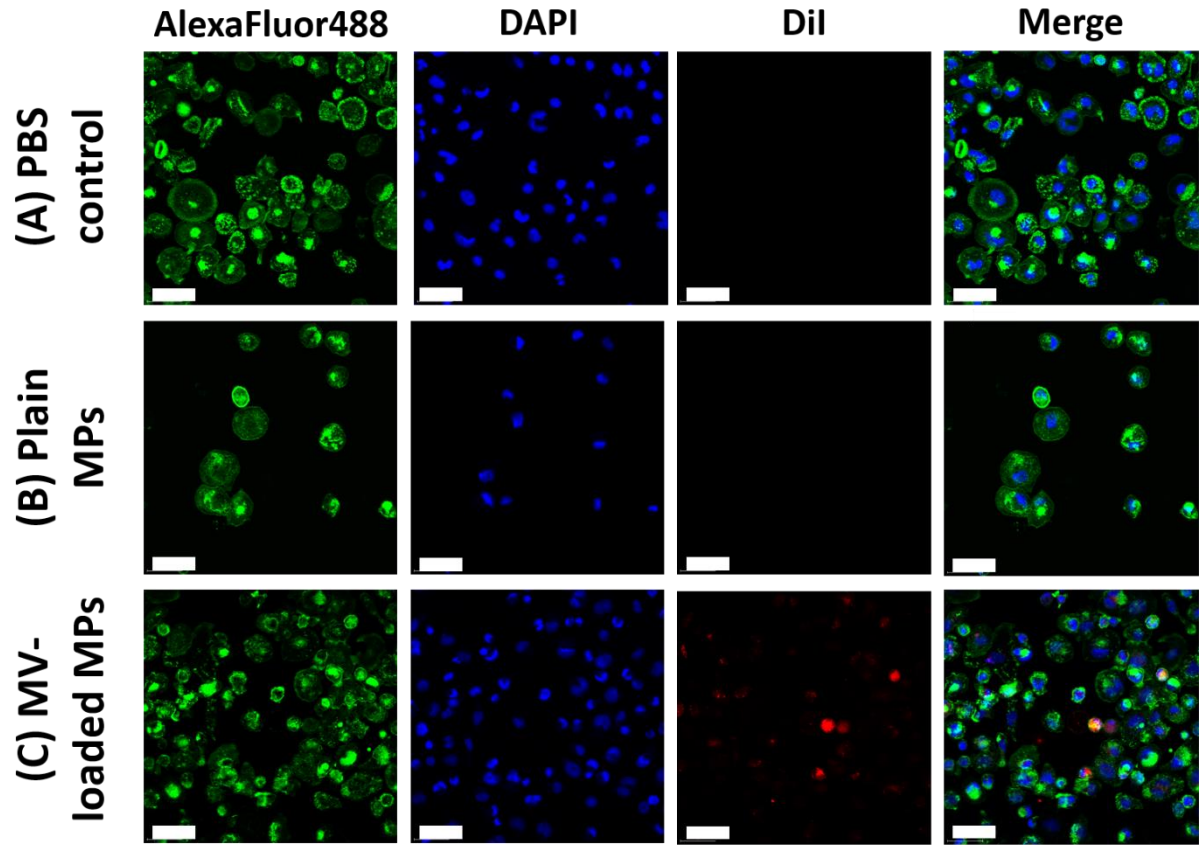


Figure 40. Viability study of human THP-1 macrophage-like immune cell lines after 8 h incubation with vaccine microparticles. (A) PrestoBlue viability assay (B) LDH cytotoxicity assay, confirming the compatibility of pneumococcal MVs-loaded vaccine microparticles with THP-1 cells. (C-E) Live dead stain of THP-1 cells after 8h incubation with pneumococcal MVs-loaded vaccine microparticles, confirming the viability of immune cells after 8h treatment. (Scale bar = 50 µm)

7.4. Spray-dried vaccine microparticles exhibit rapid uptake of encapsulated pneumococcal MVs into THP-1 macrophage-like cell lines

The ability of vaccine MPs to release loaded antigenic vesicles and successful interaction and/or uptake of these nanostructures into immune cells, is a crucial step to initiate a protective immune response and hence, potential as immunization delivery system. **Figures 41 and 42** indicated the successful internalization of fluorescently DiI-labelled pneumococcal MVs into human THP-1 macrophage-like cell lines after 8 h of incubation, as observed by significant red

dots (DiI-labelled pneumococcal MVs) within THP-1 cells. Z-stacks confirmed this finding by the diffuse appearance of red-stain DiI labelled pneumococcal MVs throughout all THP cell layers and hence, successful uptake and delivery of loaded antigens into immune cells [229]. Flow cytometry was utilized for quantitative determination of cellular interaction and uptake. The results demonstrated that uptake reached almost 60% of encapsulated vesicles in vaccine into THP-1 cells. Accordingly, we conclude that pneumococcal MVs within spray-dried vaccine MPs could internalize into immune cells, deliver their antigen payload, and expected to initiate an immune response [331, 332].



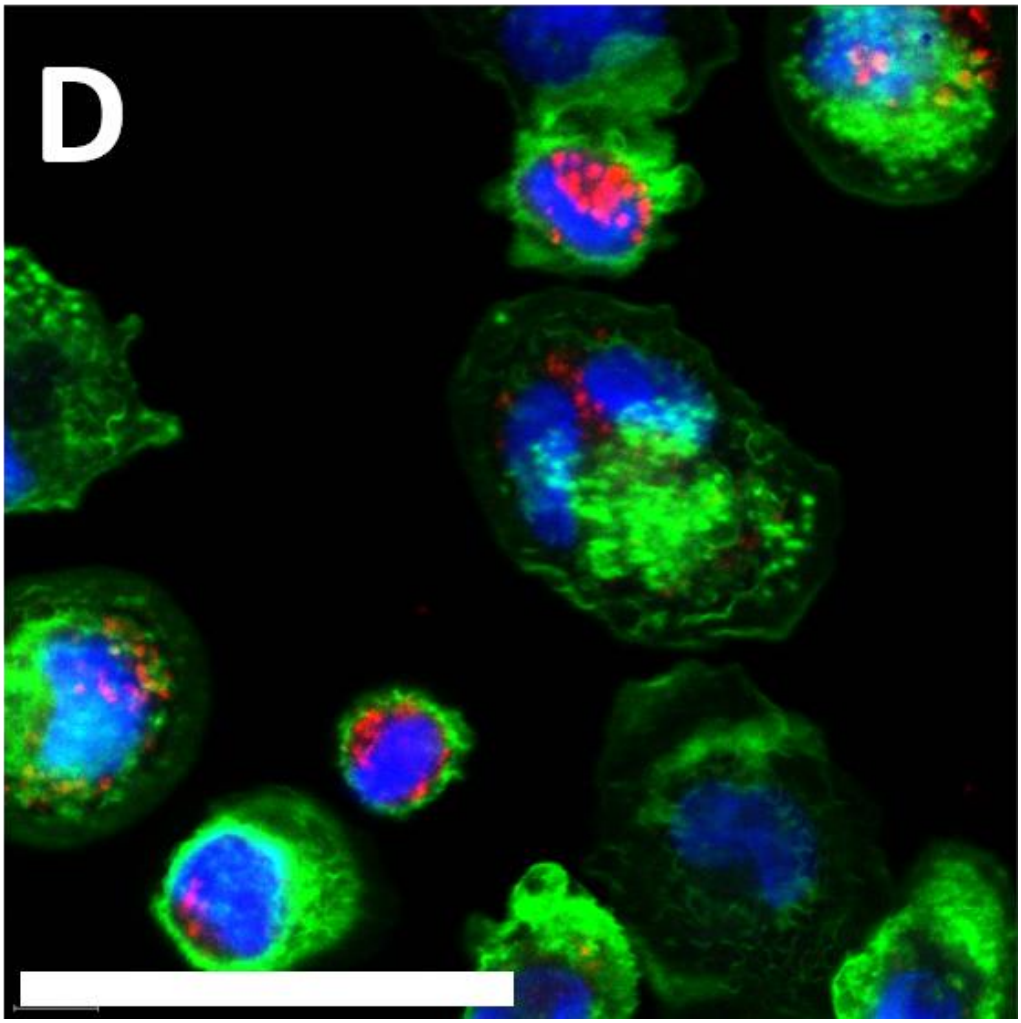
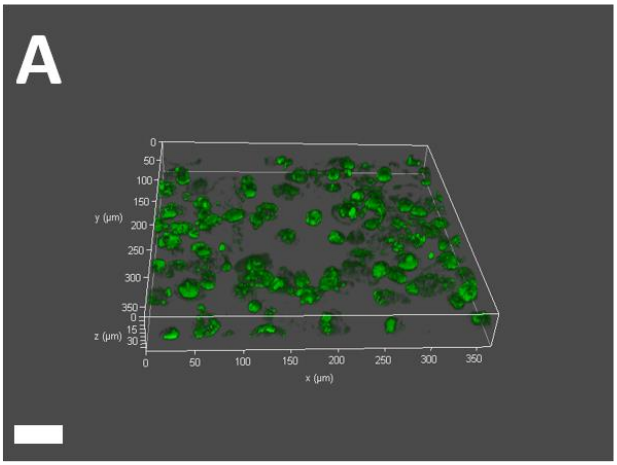
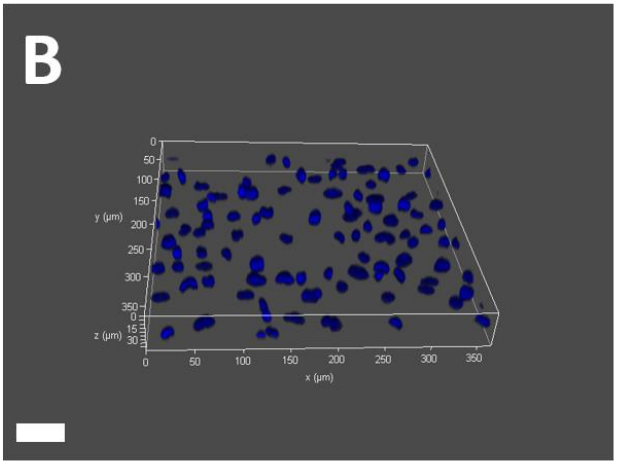
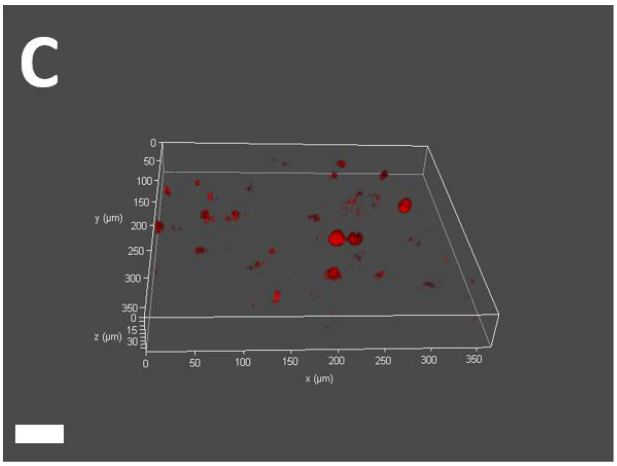


Figure 41. Confocal Laser scanning micrographs of fluorescently DiI labelled pneumococcal MVs (red) in vaccine microparticles uptake into human THP-1 macrophage cells after 8h of incubation (C), in comparison with PBS control (A) and Plain microparticles control (B), where cytoskeleton was stained with AlexaFluor488 (green) and nuclei with DAPI (blue). (D) Magnified image of DiI labelled pneumococcal MVs vaccine MPs uptake. (Scale bar = 50 µm)


A


B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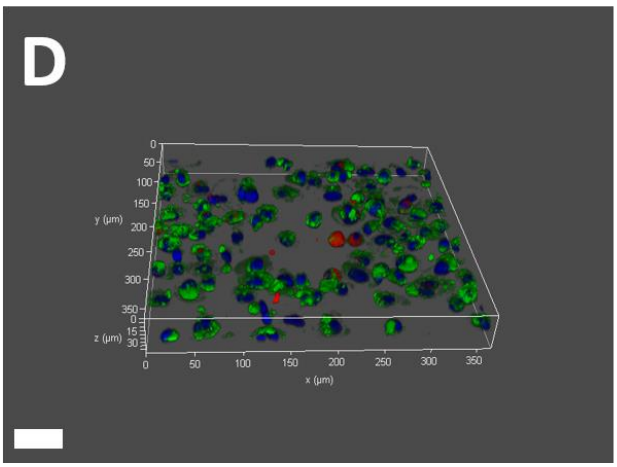
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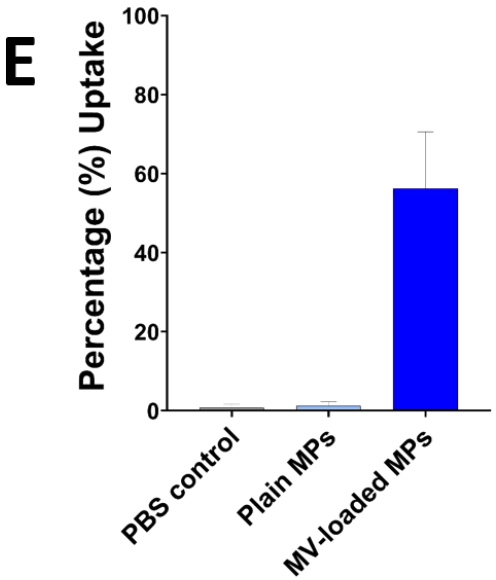
E
Percentage (%) Uptake
100
80
60
40
20
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PBS control
Plain MPs
MV-loaded MPs

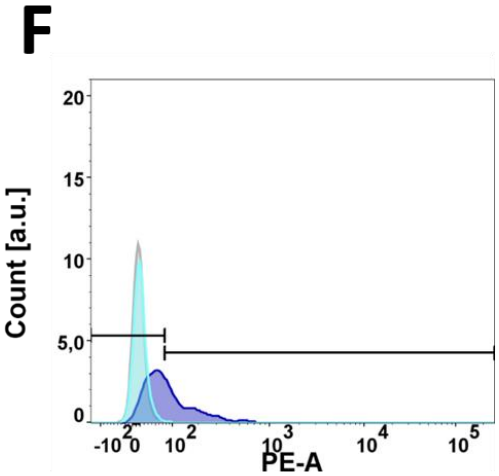
F
Count [a.u.]
20
15
10
5,0
0
PE-A

Figure 42. Confocal Laser scanning Z-stack micrographs (A-D) of fluorescently DiI labelled pneumococcal MVs (red) in vaccine microparticles uptake into human THP-1 macrophage cells after 8h of incubation, where cytoskeleton was stained with AlexaFluor488 (green) and nuclei with DAPI (blue). Magnified image of DiI labelled pneumococcal MVs vaccine MPs uptake is in the right side of image. (Scale bar = 50µm) (E and F) Quantitative assessment of interaction/uptake of fluorescently DiI labelled pneumococcal MVs in vaccine microparticles into human THP-1 macrophage cells after 8h of incubation, as determined via flow cytometry.

7.5. Spray-dried vaccine microparticles enhance the release of pro-inflammatory cytokines from human primary peripheral blood mononuclear cell (PBMCs)

As a follow-up, we wanted to explore the efficacy of spray-dried vaccine microparticles, whether they maintained the ability of encapsulated pneumococcal MVs to stimulate immune cells to release pro-inflammatory cytokines, essential to initiate a protective immune response.

The cytokine analysis (**Figure 43)** revealed a strong increase in the secretion of interleukin-6 (IL-6) from human primary peripheral blood mononuclear cell (PBMCs), for both free pneumococcal MVs suspension and MVs-loaded spray-dried vaccine MPs, in comparison with negative controls including PBS-treated cells and plain carrier MPs. Even though, the mean value for IL-6 production was higher for the free suspension than spray-dried vaccine MPs, yet the range of secreted cytokine was comparable.

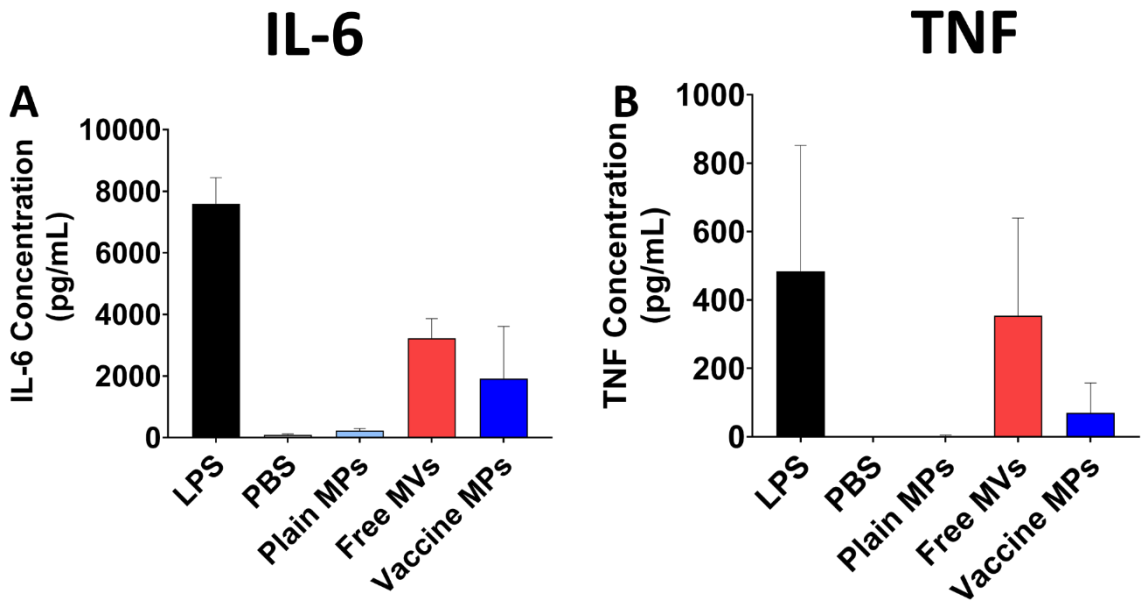

Figure 43. Cytokine analysis of the pro-inflammatory cytokines (A) Interleukin-6 and (B) tumor necrosis factor (TNF), secreted from human primary peripheral blood mononuclear cells (PBMCs) after 8h incubation with free pneumococcal membrane vesicles (free MVs) suspension, pneumococcal MVs-loaded spray-dried vaccine microparticles (vaccine MPs). Controls included lipopolysaccharide (LPS) at a concentration of 250 ng/mL as positive control and, PBS and plain unloaded microparticles (Plain MPs) as negative controls

Tumor necrosis factor (TNF) exhibited a similar behavior, since vaccine MPs and pneumococcal MVs suspension showed higher release of the pro-inflammatory cytokine than negative controls. However, vaccine MPs showed slightly less activation of TNF, in comparison with free pneumococcal MVs suspension. We speculate this observation due to the limited solubility of spray dried carriers (lactose and leucine) in cell culture medium, which might be hindering the fast release of encapsulated pneumococcal vesicles to be freely available for cells, and thus decrease their immunostimulating effect on PBMCs [333, 334]. However, more optimization is required for the formulation of the dry powder carrier, testing new carrier sugars, and dose adjustment of loaded MVs to potentiate their immunostimulatory effect.

Overall, spray-dried vaccine MPs generated an immunostimulatory effect on human primary PBMCs, and increased the release of pro-inflammatory cytokines. Therefore, spray-dried vaccine MPs are promising candidates as dry powder inhalers for immune cell activation and further investigation should follow to explore their potential as anti-pneumococcal pulmonary immunization.

7.6. Conclusion

Pneumococcal MVs showed a strong potential as vaccination candidates against pneumococcal infections, since they harbor many antigenic contents as determined from proteomic analysis. Moreover, they stimulate the release of pro-inflammatory cytokines from human primary immune cells. We explored the feasibility to prolong their stability and shelf life using spray drying and to formulate dry powder for DPI application as pulmonary immunization. We successfully prepared spray-dried pneumococcal MVs vaccine MPs composed of FDA-approved and GRAS materials i.e., lactose and leucine as inactive excipients, boosting their spraying, stability, flow properties and *in vitro/vivo* aerosolization behavior. Assessment of encapsulation confirmed homogeneous distribution of pneumococcal MVs throughout the prepared vaccine MP powder and readily release of intact vesicles after reconstitution in water, without large loss of MV feed. The formulated MPs were applied on macrophage-like THP-1 cell line without compromising their viability or manifesting any severe cytotoxic effects on cells. Moreover, confocal microscopy images demonstrated rapid uptake of spray-dried pneumococcal MVs from vaccine MPs into THP-1 cells, confirming successful intact nature of spray-dried MVs, keeping their rapid uptake into immune cells and delivery of their antigenic component payload. The prepared vaccine MPs increased the release of pro-inflammatory cytokines TNF and IL-6 from human primary PBMCs, thus they possess an immunostimulatory effect and are promising vaccine candidates.

8. General Conclusion and Outlook

Pneumococci can cause severe invasive infections and account for high morbidities and mortalities in young children and elderly population. Our study explored the application of pneumococcal membrane vesicles and their potential as promising vaccine candidates. We isolated successfully pneumococcal membrane vesicles and characterized their physical and chemical properties. Moreover, excellent viability and rapid uptake into several cell lines and human primary immune cells was observed, upon treatment with pneumococcal vesicles.

We investigated to achieve higher yields of vesicles, by harvesting during bacterial death-phase. Death-phase vesicles displayed a higher yield, while possessed comparable uptake, cellular compatibility as their counterparts, isolated during stationary-phase. Proteomic analysis revealed abundance of many strong immunogenic proteins and lipoproteins onto vesicles. Pneumococcal vesicles increased the release of pro-inflammatory cytokines including interleukin-6 and tumor necrosis factor from human primary immune cells, confirming their strong potential as vaccine candidates. We formulated spray-dried pneumococcal vesicles into dry powder vaccine microparticles for pulmonary immunization, to prolong their shelf-life stability. The prepared vaccine microparticles showed successful encapsulation of membrane vesicles, optimal physical and aerodynamic behavior, excellent compatibility with immune cells, and enhanced release of pro-inflammatory cytokines.

Hence, our study confirmed that pneumococcal membrane vesicles are promising vaccine candidates and amenable for formulation into delivery dosage forms for further investigations. Therefore, the outlook for future follow-up include:

- Optimization of membrane vesicle production from other wild-type pneumococcal strains. We used in our project the avirulent R6 lab strain; therefore, the isolation of pneumococcal membrane vesicles from non-encapsulated wild-type strains such as D39 strain should follow to mimic the realistic situation with microorganism. Moreover, wild-type strains may generate membrane vesicles with expected stronger immune response.

- Assessment of standardization and scale-up of membrane vesicles production. Since the pharmaceutical industry requires well-defined and known compounds or contents, hence standardization is a pivotal step towards approval from regulatory authorities. Identification of the most important immunogenic proteins/lipoproteins and

determination of the concentration accurately is essential for scale-up of extracellular vesicle products.

- *In vivo* immunological animal study to verify adequate protective immune response of pneumococcal membrane vesicles. A detailed immunological animal study using appropriate model will offer helpful information about the safety and efficiency of pneumococcal vesicles as vaccine candidates. First, the study should determine the recommended dose range for application. Second, the extent of mucosal protection should be explored using non-invasive model such as the nasopharyngeal colonization animal model, to keep the welfare of lab animal and decrease the pain and distress to minimum. Further, an invasive pneumonia model may be applied, if the primary steps show promising results, to confirm their protective potential against severe infection.

- Improvement of formulation of spray-dried/lyophilized dosage forms for vaccine delivery. The search for other safe carriers and optimized production conditions should follow to formulate vaccine microparticles loaded with pneumococcal membrane vesicles with better technical properties for scale-up, preferable aerodynamic behavior for deep lung delivery and enhanced immune response.

- Application for clinical trials using the most promising candidates from animal models. Once the animal study offers promising candidates, application for clinical trial should follow to explore their safety and efficacy in human. Therefore, the potential and feasibility of introduction of a commercially available extracellular membrane vesicle-based vaccine against *Streptococcus pneumoniae* should continue.

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10. Scientific Output

Publications:

1- Original research article entitled “Streptococcal extracellular membrane vesicles are rapidly internalized by immune cells and alter their cytokine release” in ***Frontiers In Immunology*** journal, 2020, 11:80.
DOI: 10.3389/fimmu.2020.00080

2- Review article entitled “Extracellular vesicles as antigen carriers for novel vaccination avenues” in ***Advanced Drug Delivery Reviews*** journal, 2021, 173:164-180.
DOI: 10.1016/j.addr.2021.03.016

3- Original research article entitled “Yields and immunomodulatory effects of pneumococcal membrane vesicles differ with the bacterial growth phase” in ***Advanced Healthcare Materials*** journal, 2021, 2101151
DOI: 10.1002/adhm.202101151

4- Original research article entitled “Spray-dried Pneumococcal Membrane Vesicles Vaccine for Pulmonary Immunization” under preparation.

Selected Podium presentations:

1- Selected Podium presentation entitled “Characterization of Pneumococcal extracellular membrane vesicles” at ***Helmholtz Institute for Pharmaceutical Research Saarland (HIPS) Symposium***
Place: Saarland University, Saarbrücken, Germany
Date: June 2019

2- Selected Podium presentation entitled “Extracellular membrane vesicles from Streptococcus alter the cytokine release of immune cells” at ***Autumn meeting of German Society for Extracellular Vesicles (GSEV) and Austrian Society for Extracellular Vesicles (ASEV)*.**
Place: Technical University of Munich, Freising, Germany
Date: November 2019

3- Selected Podium presentation entitled “Pneumococcal membrane vesicles are promising vaccine candidates” at the ***Ist Drug Delivery in Microbial Cells (DDMiC) Conference*.**
Place: Porto, Portugal.
Date: September 2021.

4- Selected Podium presentation entitled “Spray-dried Pneumococcal Membrane Vesicles Vaccine for Pulmonary Immunization” at the ***13th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology***
Place: Rotterdam, Netherlands.
Date: March 2022.

Selected Poster presentations:

1- Selected Poster presentation entitled “Streptococcal membrane vesicles are not cytotoxic to mammalian somatic and immune cells” at ***PhD day for Faculty of Natural Science – Saarland University***
Place: Saarland University, Saarbrücken, Germany
Date: November 2019.
2- Selected Poster presentation entitled “Advanced Vaccine Carriers – from biogenic extracellular vesicles to polymeric nucleic acid nanocarriers” at ***Virtual Helmholtz Institute for Pharmaceutical Research Saarland (HIPS) Symposiu****m*
Place: Saarland University, Saarbrücken, Germany
Date: May 2021.
3- Selected Poster presentation entitled “Pneumococcal death-phase membrane vesicles achieve higher yield, while being poor vaccination candidates” at ***Controlled Release Society (CRS) 2021 Virtual Annual Meeting***
Date: July 2021.

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