



**University of
Nottingham**

UK | CHINA | MALAYSIA

Role of extracellular vesicles in allergic sensitization

Thesis submitted to the University of Nottingham for the degree of
Doctor Philosophy

Davis Tucis

Student ID: 20205324

School of Life Sciences, The University of Nottingham, Nottingham NG7
2UH, UK

List of contents

List of contents

[Acknowledgments](#)

[Publications](#)

[Covid-19 impact statement](#)

[Abstract](#)

[Main hypothesis](#)

[Objectives](#)

[Abbreviations](#)

[Chapter 1: General introduction](#)

[1.1. Allergy](#)

- [1.1.1 What is allergy?](#)
- [1.1.2 Prevalence of Type I hypersensitivity](#)
- [1.1.3 Predisposition of Type I hypersensitivity](#)
- [1.1.4 Mechanisms of Type I hypersensitivity](#)
- [1.1.5 Treatment of Type I hypersensitivity](#)

[1.2. Immune cells in allergic sensitization](#)

- [1.2.1. Epithelial cells](#)
- [1.2.2. Dendritic cells](#)
- [1.2.3. Naïve T cells](#)
- [1.2.4. CD4+ T cells](#)
- [1.2.5. Regulatory T cells](#)
- [1.2.6. B cells](#)
- [1.2.7. Innate Lymphoid Cells](#)

[1.3. Extracellular vesicles](#)

- [1.3.1. EV subtypes](#)
- [1.3.2. Biogenesis and release of exosomes](#)
- [1.3.3. Biogenesis and release of ectosomes](#)
- [1.3.4. Tetraspanins associated with EVs](#)

[1.4. EVs in allergies](#)

- [1.4.1. Cargo of EVs in allergies](#)
- [1.4.2. DNA in EVs in allergies](#)
- [1.4.3. RNA in EVs in allergies](#)
- [1.4.4. Proteins in EVs in allergies](#)

- 1.4.5. [EVs as biomarkers in allergies](#)
- 1.4.6. [EVs as therapeutics in allergies](#)
- 1.5. [Hypothesis and Aims of the study](#)

Chapter 2: Materials and methods

2.1 Materials

- 2.1.1 [Culture media](#)
- 2.1.2 [Human blood samples](#)
- 2.1.3 [Immunofluorescent staining for flow cytometry](#)
- 2.1.4 [Immunomagnetic Cell Separation](#)
- 2.1.5 [Allergen](#)
- 2.1.6 [T cell stimulation](#)
- 2.1.7 [Extracellular vesicle isolation and concentration](#)
- 2.1.8 [Nano-tracking analysis](#)
- 2.1.9 [Super-resolution microscopy](#)
- 2.1.10 [Enzyme-Linked Immunosorbent Assay](#)

2.2 Methods

- 2.2.1 [PBMC isolation](#)
- 2.2.2 [Monocyte isolation](#)
- 2.2.3 [Dendritic cell culture](#)
- 2.2.4 [Cell count](#)
- 2.2.5 [Immunofluorescent staining for flow cytometry](#)
 - 2.2.5.1 [Extracellular staining](#)
 - 2.2.5.2 [Intracellular staining](#)
- 2.2.6 [Annexin and Propidium Iodide staining](#)
- 2.2.7 [T cell activation](#)
- 2.2.8 [Extracellular vesicle isolation](#)
- 2.2.9 [Enzyme-Linked Immunosorbent Assay](#)
- 2.2.10 [TEM and confocal microscope preparation](#)
- 2.2.11 [Nano-tracking analysis](#)
- 2.2.12 [Extracellular vesicle staining](#)
- 2.2.13 [Super-resolution microscopy](#)
- 2.2.14 [Statistical analysis](#)

Chapter 3: Systematic review ‘The role of extracellular vesicles in allergic sensitization’

3.1. Introduction

3.2. Methods

3.2.1. Search strategy

3.2.2. Selection of studies

3.2.3. Study quality assessment

3.3. Results

3.3.1. PRISMA search and selection

3.3.2. Host-derived extracellular vesicles

3.3.2.1. Promotion of allergic sensitization

3.3.2.2. Promotion of immune tolerance

3.3.3. Exogenous extracellular vesicles

3.3.3.1. Promotion of allergic sensitization via the skin epithelium

3.3.3.2. Promotion of immune tolerance

3.4 Discussion

Chapter 4: Dendritic cell culture conditions

4.1. Introduction

4.1.1. Dendritic cells as immune response modulators

4.1.2. Chapter objectives

4.2. Methods

4.2.1. PBMC isolation

4.2.2. CD14+ monocyte isolation

4.2.3. DC generation

4.2.4. Flow cytometry

4.2.5. Extracellular vesicle staining

4.3. Results

4.3.1. Gating strategies

4.3.2. Optimal culture media for DCs

4.3.3. Cell seeding density and platelet counts

4.3.4. DC seeding density and optimal culture media

4.3.5. DC maturation rate using different OVA concentrations

4.3.6. Final DC assay

4.4. Discussion

Chapter 5: Optimization of EV techniques

5.1. [Introduction](#)

5.1.1. [Current techniques for EV research](#)

5.1.2. [Chapter objectives](#)

5.2. [Methods](#)

5.2.1. [PBMC isolation](#)

5.2.2. [T cell activation](#)

5.2.3. [CD14+ monocyte isolation](#)

5.2.4. [Dendritic cell culture](#)

5.2.5. [Primary human bronchial epithelium cell culture](#)

5.2.6. [Size exclusion chromatography](#)

5.2.7. [Transmission electron microscopy](#)

5.2.8. [Super-resolution microscopy](#)

5.2.9. [Nanoparticle tracking analysis](#)

5.2.10. [Sizing of EVs](#)

5.2.11. [Tetraspanin staining of EVs](#)

5.3. [Results](#)

5.3.1. [T cell optimizations](#)

5.3.2. [Imaging flow cytometry optimizations](#)

5.3.3. [Confirmation of EV presence](#)

5.3.4. [EV sizing](#)

5.3.5. [Optimal calcein-AM concentrations](#)

5.3.6. [Detergent optimization](#)

5.3.7. [Cytokine removal](#)

5.3.8. [EV tetraspanin profile](#)

5.4. [Discussion](#)

[Chapter 6: DC-derived EVs influence on naïve T cells](#)

6.1. [Introduction](#)

6.1.1. [Dendritic cell communication as APCs for naïve T cells](#)

6.1.2. [EVs as intracellular communicators](#)

6.1.3. [Chapter objectives](#)

6.2. [Methods](#)

6.2.1. [PBMC isolation](#)

6.2.2. [CD14+ monocyte isolation](#)

6.2.3. [Dendritic cell culture](#)

6.2.4. [Naïve T cell isolation and culture](#)

6.2.5. [EV isolation](#)

- 6.2.6. [DC-derived EVs and T cell assay](#)
- 6.2.7. [T cell staining](#)
- 6.2.8. [Supervised analysis](#)
- 6.2.9. [Unsupervised analysis](#)
- 6.3. [Results](#)
 - 6.3.1. [Naïve T cell gating strategy](#)
 - 6.3.2. [The effect of DC-derived EVs alone on naïve T cell responses](#)
 - 6.3.2.1. [Effect of DC-derived EVs on naïve T cell viability between healthy and allergic individuals](#)
 - 6.3.2.2. [Effect of DC-derived EVs on naïve T cell activation levels between healthy and allergic individuals](#)
 - 6.3.2.3. [Effect of DC-derived EVs on naïve T cell IFN- \$\gamma\$ production between healthy and allergic individuals](#)
 - 6.3.2.4. [Effect of DC-derived EVs on naïve T cell TNF- \$\alpha\$ production between healthy and allergic individuals](#)
 - 6.3.2.5. [Effect of DC-derived EVs on naïve T cell IL-4 production between healthy and allergic individuals](#)
 - 6.3.2.6. [Effect of DC-derived EVs on naïve T cell IL-2 production between healthy and allergic individuals](#)
 - 6.3.3. [The effect of DC-derived EVs, along with anti-CD3 and anti-CD28, on naïve T cell responses](#)
 - 6.3.3.1. [Effect of co-stimulation with DC-EVs and CD3/CD28 on naïve T cell viability between healthy and allergic individuals](#)
 - 6.3.3.2. [Effect of co-stimulation with DC-EVs and CD3/CD28 on naïve T cell activation levels between healthy and allergic individuals](#)
 - 6.3.3.3. [Effect of co-stimulation with DC-EVs and CD3/CD28 on naïve T cell IFN- \$\gamma\$ production between healthy and allergic individuals](#)
 - 6.3.3.4. [Effect of co-stimulation with DC-EVs and CD3/CD28 on naïve T cell TNF- \$\alpha\$ production between healthy and allergic individuals](#)
 - 6.3.3.5. [Effect of co-stimulation with DC-EVs and CD3/CD28 on naïve T cell IL-4 production between healthy and allergic individuals](#)
 - 6.3.3.6. [Effect of co-stimulation with DC-EVs and CD3/CD28 on naïve T cell IL-2 production between healthy and allergic individuals](#)
 - 6.3.4. [Unsupervised clustering analysis of DC-EVs following co-stimulation with anti-CD3/anti-CD28 between healthy and allergic individuals](#)

6.3.4.1 [Unsupervised clustering analysis of 2nd dose of LPS-stimulated DC-EVs co-stimulated with anti-CD3/anti-CD28 between healthy and allergic individuals](#)

6.3.4.2 [Unsupervised clustering analysis of 2nd dose of OVA-stimulated DC-EVs following co-stimulation between healthy and allergic individuals](#)

6.3.4.3 [Unsupervised clustering analysis of 2nd dose of OVA/LPS stimulated DC-EVs following co-stimulation between healthy and allergic individuals](#)

6.3.4.4 [Unsupervised clustering analysis of 2nd dose of unstimulated DC-EVs following co-stimulation between healthy and allergic individuals](#)

6.3.4.5 [Unsupervised clustering analysis of 2nd dose of PBS following co-stimulation between healthy and allergic individuals](#)

6.3.5. [The effect of co-stimulation with DC-EVs and anti-CD3/anti-CD28 on naïve T cells across conditions in healthy and allergic individuals](#)

6.3.5.1 [Effect of co-stimulation with DC-EVs and anti-CD3/anti-CD28 on naïve T cell viability in healthy and allergic individuals across conditions](#)

6.3.5.2 [Effect of co-stimulation with DC-EVs and CD3/CD28 on naïve T cell activation levels in healthy and allergic individuals across conditions](#)

6.3.5.3 [Effect of co-stimulation with DC-EVs and CD3/CD28 on naïve T cell IFN- \$\gamma\$ production in healthy and allergic individuals across conditions](#)

6.3.5.4 [Effect of co-stimulation with DC-EVs and CD3/CD28 on naïve T cell TNF- \$\alpha\$ production in healthy and allergic individuals across conditions](#)

6.3.5.5 [Effect of co-stimulation with DC-EVs and CD3/CD28 on naïve T cell IL-2 production in healthy and allergic individuals across conditions](#)

6.3.5.6 [Effect of co-stimulation with DC-EVs and CD3/CD28 on naïve T cell IL-4 production in healthy and allergic individuals across conditions](#)

6.4. [Discussion](#)

[Chapter 7: General discussion](#)

7.1. [Differences in T cells between healthy and allergic individuals](#)

7.2. [EVs influence on naïve T cell activation in healthy and allergic individuals](#)

7.3. [General limitations](#)

7.4. [Applications](#)

7.5. [Concluding remarks](#)

[References](#)

Acknowledgments

I would like to extend my heartfelt gratitude to my supervisors, Professor Lucy Fairclough, Dr. David Onion, and Dr. Victoria James, for their unwavering support, insightful guidance, and warm companionship throughout the past four and a half years. I consider myself incredibly fortunate to have had the privilege of working with three exceptional mentors who consistently offered their expertise and assistance at every phase of my PhD journey. A special thank you to Lucy for choosing me for this project and providing numerous inspiring opportunities along the way, and above all, for motivating me to strive for excellence in all my endeavours—your encouragement is deeply valued.

I wish to express my deep appreciation to my family, and friends for their steadfast love and encouragement throughout my PhD journey. Additionally, I am immensely grateful to my parents, who have always been my greatest inspirations; their unwavering support and patience during my upbringing have been instrumental in enabling me to accomplish this PhD, for which I am profoundly thankful.

My experience in the Fairclough lab was enriched by the vibrant community of students and staff, and I am deeply grateful for the friendships, encouragement, and joyful laughter they brought to my journey over the past few years.

Publications

Gumber L, Jackson H, Gomez N, Hopkins G, **Tucis D**, Chakravorty M, Tighe P, Grainge MJ, Rutter M, Ferraro A, Power S, Pradère MJ, Lanyon PC, Pearce FA, Fairclough L (2023). Antibody response to four doses of SARS-CoV-2 vaccine in rare autoimmune rheumatic diseases: an observational study. *Rheumatol Adv Pract.* (<https://doi.org/10.1093/rap/rkad097>).

Gumber L, Gomez N, Hopkins G, **Tucis D**, Bartlett L, Ayling K, Vedhara K, Steers G, Chakravorty M, Rutter M, Jackson H, Tighe P, Ferraro A, Power S, Pradère M, Onion D, Lanyon P, Pearce F, Fairclough L (2023). Humoral and cellular immunity in patients with rare autoimmune rheumatic diseases following SARS-CoV-2 vaccination, *Rheumatology*. (<https://doi.org/10.1093/rheumatology/keac574>)

Hopkins G, Gomez N, **Tucis D**, Bartlett L, Steers G, Burns E, Brown M, Harvey-Cowlishaw T, Santos R, Lauder SN, Scurr M, Capitani L, Burnell S, Rees T, Smart K, Somerville M, Gallimore A, Perera M, Potts M, Metaxaki M, Krishna B, Jackson H, Tighe P, Onion D, Godkin A, Wills M, Fairclough L (2024). Lower Humoral and Cellular Immunity Following Asymptomatic SARS-CoV-2 Infection Compared to Symptomatic Infection in Education (The ACE Cohort). *J Clin Immunol.* (<https://doi.org/10.1007/s10875-024-01739-0>)

Tucis, D.; Hopkins, G.; Browne, W.; James, V.; Onion, D.; Fairclough, L.C (2024). The Role of Extracellular Vesicles in Allergic Sensitization: A Systematic Review. *Int. J. Mol. Sci.* (<https://doi.org/10.3390/ijms25084492>)

COVID-19 impact statement

During the COVID-19 pandemic, our lab was closed for 6 months, which significantly impacted the progression of my PhD, due to the requirement of a tissue-culture facility to conduct my experiments. Upon re-opening of the lab, the pandemic continued to affect our research for months due to the delay of deliveries for essential materials. In addition to these experimental delays, being an Immunology lab, we were heavily involved in COVID-19 research throughout the pandemic. Since August 2020, I was involved in several COVID19 research projects, with many different roles including the recruitment of participants across The University of Nottingham, completing a phlebotomy training course to aid research nurses in collecting blood samples from the large cohort, as well as conducting and analysing data from cell-based assays in the lab. I progressed with these COVID-19-related roles alongside my PhD studies, until the funding for the project was done. Our team was awarded a Vice-Chancellor's medal for these efforts during the pandemic.

Abstract

Introduction

The escalating global prevalence of IgE-mediated allergic diseases, including food allergies such as egg allergy, represents a significant public health challenge, with implications for quality of life and healthcare systems worldwide. Affecting approximately 1.5% of children in Western countries and often persisting into adulthood, egg allergy exemplifies the growing burden of allergic conditions, characterized by severe symptoms that can escalate to life-threatening anaphylaxis. This rising incidence underscores an urgent need to unravel the complex mechanisms driving allergic sensitization—the initial immunological event triggered by first-time allergen exposure—to inform the development of effective therapeutic and preventive strategies for type 1 hypersensitivity. Despite advances in understanding allergen recognition and immune activation, the precise processes determining why certain individuals become sensitized remain incompletely elucidated, particularly the role of intercellular communication mediators. This PhD thesis investigates the immunomodulatory role of dendritic cell-derived extracellular vesicles (DC-EVs) in allergic sensitization, with a specific focus on their influence on naïve T-cell responses, using egg allergy as a model. Emerging evidence suggests that DC-EVs, potentially carrying allergens like ovalbumin (OVA), may serve as critical vectors in transmitting immunological signals, thereby shaping the trajectory of allergic immune responses. By exploring this hypothesis, the study addresses a critical knowledge gap, aiming to enhance our understanding of how these nanoscale vesicles contribute to the initiation and progression of allergic sensitization.

Methods

The research employed a novel human *in vitro* assay, meticulously developed and optimized across multiple chapters to investigate the role of DC-EVs in allergic sensitization. Methods included the isolation of peripheral blood mononuclear cells (PBMCs) from healthy and egg-allergic individuals, followed by CD14⁺ monocyte isolation and differentiation into DCs under optimized conditions (RPMI + 10% FBS for 5 days, then X-VIVO serum-free media, with 24-hour stimulation using 100 ng/ml LPS and/or OVA at 10

µg/ml). DCs were stimulated to generate EVs, which were isolated using size-exclusion chromatography and exposed to naïve T cells in three sequential doses. Flow cytometry was utilized to phenotype T cells and monitor cytokine production, including CD69, IFN- γ , IL-2, TNF- α , and IL-4.

Results

Chapter 4 established optimal DC culture conditions to ensure high viability and maturation, while Chapter 5 optimized EV isolation and characterization methods, adhering to MISEV 2024 guidelines. Key results, detailed in Chapter 6, revealed that DC-EVs do not act as the sole signal for naïve T-cell activation but function effectively as signal 3 when supported by anti-CD3 and anti-CD28 co-stimulation. Significant differences emerged between healthy and allergic groups, with healthy naïve T cells exhibiting a Th1-biased profile (higher IFN- γ and IL-2 expression) in control and unstimulated EV conditions, while allergic individuals showed a Th2 tendency. Exposure to LPS/OVA-stimulated DC-EVs in allergic individuals led to increased CD69 expression, indicating T-cell activation, alongside a marked reduction in IFN- γ and a rise in IL-4 production. This Th2 shift suggests that OVA-carrying DC-EVs may exacerbate allergic sensitization by promoting naïve T-cell differentiation toward a Th2 phenotype. Clustering analysis further confirmed inherent immunological differences between groups, which were modulated by EV source, highlighting the role of stimulatory context (e.g., LPS, OVA) in shaping EV immunogenicity.

Conclusion

In conclusion, this thesis demonstrates that DC-EVs play a pivotal role in allergic sensitization, particularly through their capacity to modulate naïve T-cell responses in an allergen-specific manner. The developed human assay provides a robust platform for future research, with implications for EV-based diagnostics and therapies targeting Th2-driven allergies. The findings underscore the importance of EV cargo and source in immune polarization, suggesting potential interventions to restore Th1/Th2 balance. However, limitations such as single-time-point cytokine measurements and unanalysed EV cargo necessitate further studies with time-course analyses and advanced profiling techniques. Continued research into EV diversity and their

longitudinal effects remains essential to harness their full therapeutic potential, advancing the management of allergic diseases.

Main hypothesis

The proposed hypothesis of my PhD was that allergen-matured dendritic cell EVs could influence the shift of naïve T cells into Th2 cells.

Objectives

- To explore the role of extracellular vesicles in allergic sensitization by developing a human, in-vitro, cell-based assay.
- To investigate T cell population differences between healthy and allergic individuals.

Abbreviations

AIT - Allergen Immunotherapy
APC - Antigen-Presenting Cell
BAL - Bronchoalveolar Lavage
BMMC - Bone Marrow-Derived Mast Cell
CD - Cluster of Differentiation (e.g., CD3, CD9, CD11a, CD14, CD28, CD40, CD45, CD51, CD61, CD63, CD69, CD80, CD81, CD82, CD83, CD86, CD90, CD209, HLA-DR)
DAMPs – Damage-associated molecular patterns
DC - Dendritic Cell
DLS - Dynamic Light Scattering
EM - Electron Microscopy
ELISA - Enzyme-Linked Immunosorbent Assay
ESCRT - Endosomal Sorting Complexes Required for Transport
EV - Extracellular Vesicle
FCM - Flow Cytometry
FC - Flow Cytometry
FcεRI - High-Affinity IgE Receptor
FOV - Field of View
GATA3 - GATA Binding Protein 3
GM-CSF - Granulocyte-Macrophage Colony-Stimulating Factor
HLA - Human Leukocyte Antigen
ICAM - Intercellular Adhesion Molecule
iDC – Immature DC
IFC - Imaging Flow Cytometry
IgE - Immunoglobulin E
IgG - Immunoglobulin G (e.g., IgG1, IgG2a, IgG3)
IL - Interleukin (e.g., IL-1b, IL-2, IL-4, IL-5, IL-6, IL-8, IL-12, IL-13, IL-17, IL-17F, IL-27, CXCL8)
ILCs – Innate Lymphoid Cells
ISEV - International Society for Extracellular Vesicles
ISX – ImageStreamX
IFN-γ – Interferon gamma

LPS - Lipopolysaccharide
MHC - Major Histocompatibility Complex
MISEV - Minimal Information for Studies of Extracellular Vesicles
moDC - Monocyte-Derived Dendritic Cell
MSC - Mesenchymal Stem Cell
MUC - Mucin (e.g., MUC5AC)
NALF - Nasal Lavage Fluid
NKT – Natural Killer T cells
nFCM - Nanoflow Cytometry
NTA - Nanoparticle Tracking Analysis
OVA – Ovalbumin
PBA – Phosphate buffer albumin
PBMC - Peripheral Blood Mononuclear Cell
PBS - Phosphate-Buffered Saline
PI - Propidium Iodide
PRR – Pattern recognition receptor
PRISMA - Preferred Reporting Items for Systematic Reviews and Meta-Analyses
rMFI - Relative Mean Fluorescence Intensity
SCIT - Subcutaneous Immunotherapy
SEC - Size-Exclusion Chromatography
SOCS - Suppressor of Cytokine Signaling (e.g., SOCS3)
STAT - Signal Transducer and Activator of Transcription (e.g., STAT3, STAT6)
TEM - Transmission Electron Microscopy
TGF - Transforming Growth Factor (e.g., TGF- β)
Th - T Helper (e.g., Th1, Th2)
TNF - Tumor Necrosis Factor (e.g., TNF- α)
Treg - Regulatory T Cell
TSG - Tumor Susceptibility Gene (e.g., TSG101)
TSLP - Thymic Stromal Lymphopoietin
TCR - T-Cell Receptor

Chapter 1: General introduction

1.0. Introduction

1.1. Allergy

1.1.1 What is allergy?

The concept of ‘allergy’ was first introduced by Austrian paediatrician Clemens von Pirquet in 1906, following his observations of unusual immune responses to cowpox vaccinations. He noted that some patients, after an initial vaccination, developed skin reactions, and subsequent doses triggered symptoms like hives or asthma [1]. This led to the term ‘allergy’, describing an exaggerated immune reaction to typically harmless substances, known as allergens. Allergies, also termed hypersensitivity, involve the immune system mistakenly targeting non-threatening substances, such as pollen, pet dander, or certain foods. These reactions differ from autoimmune diseases, where the immune system attacks the body’s own tissues, mistaking ‘self’ for ‘foreign’. Hypersensitivity reactions are classified into four types (I-IV), with type I being the most common form of allergy. It involves Immunoglobulin E (IgE) antibodies and rapid responses, leading to symptoms such as asthma or urticaria. Type I hypersensitivity is characterised by the binding of allergen-specific IgE molecules to effector cells, such as mast cells and basophils, via a high-affinity FC receptor (FcεR1). These allergies could be caused by aeroallergens – airborne allergens (e.g., pollen causing allergic rhinitis or asthma), food allergens (e.g., shellfish leading to anaphylaxis), and topical allergens (e.g., dust mites contributing to atopic dermatitis). Symptoms can range from mild, such as sneezing, to severe, like airway constriction and anaphylaxis. Types II-IV hypersensitivities are less frequent and involve different immune mechanisms. Type II hypersensitivity relies on IgG or IgM antibodies, which activate antibody-dependent cellular cytotoxicity. This can result in conditions like transfusion-related allergic haemolytic anaemia, where immune cells target red blood cells. Type III hypersensitivity arises from IgG or IgM antigen-antibody complexes depositing in tissues or blood vessels, causing inflammation and diseases like hypersensitivity pneumonitis, often linked to inhaled organic dust. Type IV, or delayed-type hypersensitivity, is mediated by T cells and macrophages, leading to inflammatory responses over

hours or days. A common example is contact dermatitis, triggered by substances like nickel or poison ivy ([2]; [3]). Recent studies highlight how environmental factors, such as pollution, can exacerbate these responses, amplifying allergic disease prevalence.

1.1.2. Prevalence of Type I hypersensitivity

Over the past few decades, Type I hypersensitivity reactions, driven by IgE antibodies, have surged globally, marking a significant public health challenge. The initial phase of this ‘allergy epidemic’, emerging over 60 years ago, was characterised by a sharp rise in aeroallergen-related conditions, such as allergic rhinitis and asthma, affecting 20-40% of populations in various countries ([4]; [5]). This was followed by a ‘second wave’ starting roughly 20 years ago, with a notable increase in IgE-mediated food allergies, particularly in industrialized nations ([6]). Recent evidence also points to a growing prevalence in rapidly urbanizing regions like China, driven by environmental and lifestyle changes ([7]). The most recent data from 2023-2025 indicate that IgE-mediated food allergies affect approximately 8-10% of children and 6-7% of adults worldwide, with regional variations ([8]; [9]). In the UK, a 2024 study reported a prevalence of 4% for challenge-proven food allergies in children under 5, consistent with earlier findings ([10]). Notably, about 40% of affected children experience severe reactions, and roughly one-third are allergic to multiple foods, with peanuts, cow’s milk, and shellfish being the most common triggers ([11]). The ASSESS FA study (2023) highlights higher prevalence in countries like China (8.7%) and Canada (7.45%), compared to lower rates in Germany (2.35%) and Italy (3.04%), emphasizing the role of genetic and environmental factors. This escalating burden underscores the urgent need to deepen our understanding of the immune mechanisms and to develop targeted therapies, such as oral immunotherapy or biologics, to mitigate the impact of these allergies.

1.1.3 Predisposition to Type I hypersensitivity

Type I hypersensitivity reactions, which can range from mild irritation to severe, life-threatening anaphylaxis, highlight the critical need for accurate prediction of both the likelihood and intensity of allergic responses ([12]). While the precise mechanisms driving the onset of Type I hypersensitivity remain elusive, research points to a complex interplay of factors including

genetics, environmental exposures, gut microbiome composition, and the mode of allergen contact (e.g., ingestion, inhalation, or skin exposure) as key contributors to allergic disease development. These factors likely interact in dynamic ways, influencing immune dysregulation and contributing to the global rise in allergic conditions, such as asthma, eczema, and food allergies. Emerging studies also suggest that modern lifestyle changes, like reduced microbial diversity due to urbanization and increased exposure to pollutants, may further amplify the incidence of these disorders ([13]; [14]).

A strong familial background of allergic conditions serves as a critical indicator for the likelihood of developing IgE-driven allergic responses. Pioneering research from the early 20th century revealed that nearly half of those with allergies had close relatives with comparable conditions, in stark contrast to just 15% of individuals without allergies ([15]). Genetic influence on allergic disorders varies widely, with estimates suggesting up to 95% heritability for asthma and 84% for circulating IgE levels ([16]). The advent of genome-wide association studies (GWAS) has revolutionized the field, uncovering specific genetic markers linked to complex diseases like allergies ([17]). Most genes identified through these studies are predominantly active in the skin, hinting that compromised skin barriers may play a pivotal role in initiating allergic sensitization. Although the catalogue of relevant genes remains incomplete, recent analyses have spotlighted a set of key candidates, including C11ORF30, STAT6, SLC25A46, HLA-DQB1/DQA1, IL1RL1/IL18R1, TLR1/6/10, LPP, MYC/PVT1, IL2/ADAD1, and HLA-B ([18]). These findings are complemented by emerging evidence from epigenetic studies, which suggest that environmental triggers, such as early exposure to pollutants or microbial shifts, may modify gene expression, further amplifying allergy risk. Ongoing research continues to refine this genetic landscape, promising deeper insights into personalized prevention and treatment strategies.

Beyond genetic predisposition, environmental influences play a significant role in driving allergic conditions in susceptible individuals. One prominent theory, known as the hygiene hypothesis, posits that early exposure to a diverse microbial environment during infancy can shape immune development and reduce allergy risk. Key areas like the gastrointestinal tract

and respiratory system serve as critical zones where microbial interactions occur in early life. Recent epidemiological data indicate that greater microbial diversity in childhood is linked to lower rates of allergic diseases, such as asthma and rhinitis, in later years ([19]), suggesting that heightened sanitation in industrialized nations may inadvertently foster allergic sensitization. The escalation of public health initiatives – such as widespread vaccinations programs, water purification, and the widespread use of pasteurized and sterilized dairy products – has successfully curbed infectious diseases but may have diminished essential microbial encounters ([20]). This shift is thought to skew immune maturation, favouring a T helper 2 (Th2)-dominant response, which is associated with allergies, over the protective Th1 response typically bolstered by pathogen exposure ([21]). Moreover, cutting-edge studies have raised concerns about vaccine adjuvants- substances, such as aluminium, used to enhance immune response in inactivated vaccines – which may contribute to rising allergy rates. A 2020 investigation found that increased use of these adjuvants in infant vaccinations correlates with higher incidences of conditions like eczema and asthma. While the association was statistically significant, the authors noted that the effect size was modest and that further research is needed to clarify whether aluminium itself, the timing of exposure, or other factors (such as the overall vaccination schedule) are responsible. ([22]). This line of inquiry remains controversial: large epidemiological reviews have concluded that there is currently no clear evidence of a causal link between aluminium adjuvants and allergic diseases, but they also highlight the need for more high-quality, long-term studies that specifically examine sensitisation outcomes in children. The debate continues because the hygiene hypothesis and changes in early-life microbial exposure already explain a large part of the allergy rise, making it difficult to isolate the contribution of adjuvants ([23]). The cumulative impact of such exposures could amplify the likelihood of allergic disorders, highlighting the need of for a balanced approach to immunization and microbial exposure in early development.

The rise in allergic conditions has been increasingly tied to the adoption of a modern, urbanized lifestyle prevalent in Western societies. Elements such as excessive weight gain, sedentary habits, consumption of heavily processed foods, limited social interaction during childhood (e.g., growing up as an only

child), and frequent antibiotic use have been linked to a decline in environmental microbial diversity and imbalances in the microbiota of protective tissues like the skin, lungs, and gut. Emerging economies experiencing rapid industrialization, such as parts of Southeast Asia and Africa, are also witnessing a surge in allergic disorders, underscoring the connection between economic progress and shifts in dietary patterns and living conditions ([24]). These changes appear to lower the innate immune system's activation threshold and weaken immune tolerance, paving the way for allergic sensitization in adulthood. Conversely, a rural upbringing surrounded by farm animals, a diet abundant in whole grains and vegetables, and regular early contact with peers have demonstrated protective effects against allergy development in later life. Such environments foster a rich microbial ecosystem across the skin, respiratory tract, and digestive system, bolstering the body's first line of defence. Early exposure to beneficial microbes is thought to calibrate pattern recognition receptors (PRRs) and their downstream signalling cascades, shaping long-term immune balance and reducing hypersensitivity ([25]). A modern lifestyle, however, disrupts intestinal epithelial function, creating conditions that favour pathogenic bacteria like *Escherichia coli* and *Clostridium difficile* while diminishing beneficial species such as *Bifidobacterium*, *Akkermansia*, and *Faecalibacterium* ([26]). This microbial imbalance can lead to increased gut permeability – often referred to as 'leaky gut' – where damaged epithelial layers allow allergens to penetrate and trigger immune responses ([27]). This interplay highlights the critical role of early microbial environments in modulating allergy risk.

The pathway through which an individual encounters an allergen significantly influences whether they develop allergic sensitization or immune tolerance. Historically, it was thought that allergen entry via the digestive system primarily triggered food-related allergies. However, recent findings indicate that skin exposure through damaged epithelium can initiate sensitization, while oral ingestion may instead promote tolerance, challenging earlier assumptions. This shift is supported by evidence showing that environmental stressors can compromise the skin barrier, leading to immune dysregulation and heightened allergic risk ([28]). Genome-wide association studies have further pinpointed genes linked to skin integrity as key players in

allergic conditions, reinforcing the importance of cutaneous exposure ([29]). This concept is encapsulated in the ‘dual exposure paradigm’, which posits that oral consumption of food allergens fosters tolerance, while exposure through impaired skin – such as in cases of eczema – drives allergic responses ([30]). Oral tolerance, mediated by gut-associated lymphoid tissues, enables the immune system to distinguish harmless food proteins from threatening pathogens, establishing a natural state of unresponsiveness that shields healthy individuals from unwarranted allergic reactions ([31]). Research highlights that children with atopic dermatitis, a condition characterized by compromised skin barrier, are at an elevated risk of developing milk allergies, while those introduced to milk orally from an early age exhibit a significant decrease in allergy prevalence ([32]). Studies also show that individuals with atopic dermatitis have diminished microbial diversity in both skin and gut microbiomes, a factor that often precedes sensitization to food allergens ([33]). Emerging evidence points to the respiratory tract as a potential sensitization for both food and airborne allergens, with increased IL-33 levels playing a key role in triggering allergic reactions in the airways and skin ([34]). Notably, intact milk proteins found in household dust, when inhaled alongside dust mite allergens acting as adjuvants, can sensitize infants, especially those with pre-existing barrier dysfunction ([35]). These findings underscore that the initial route of allergen exposure is crucial in determining whether immune tolerance or sensitization develops.

1.1.4 Mechanisms of Type I hypersensitivity

The process of Type I hypersensitivity allergic reactions is now recognized as involving two distinct stages: the initial sensitization phase, where the immune system first encounters and responds to an allergen, and the subsequent effector phase, where symptom activation occurs upon re-exposure. Recent research has expanded this framework, highlighting the role of early immune priming in shaping long-term allergic potential and the complex interplay of inflammatory mediators during symptom manifestation ([36]). This dual-phase model provides a foundation for understanding how environmental and genetic factors converge to drive allergic diseases, offering new avenues for targeted interventions ([37]). Figure 1.1 illustrates the contemporary model of Type I hypersensitivity allergic responses, detailing the

process from initial allergen encounter to symptom onset. The immune reaction begins with sensitization, triggered when an allergen – such as house dust mite (HDM), tree pollen, or milk – first interacts with epithelial tissues in the respiratory tract, gastrointestinal system, nasal passages, or skin, which serve as the body's primary defensive layer. In food allergy, the most common allergens are water-soluble glycoproteins that resist digestion and can cross the intestinal epithelial barrier in an immunogenic form. Cow's milk allergy serves as a classic example, with the major allergens being casein (α s1-, α s2-, β -, κ -casein) and whey proteins (β -lactoglobulin, α -lactalbumin), which are highly resistant to gastric acid and pepsin cleavage, allowing significant amounts to reach the lamina propria and Peyer's patches intact ([38]). These epithelial cells not only provide a physical shield but also express pattern recognition receptors (PRRs) that detect allergens and release signaling molecules, such as cytokines, to activate antigen-presenting cells (APCs). For example, the activation of Toll-like receptor 4 (TLR4) on nasal epithelial cells by pollen allergens prompts cytokine release, influencing dendritic cell (DC) activity ([39]). Depending on the exposure route, APCs in the affected epithelial layer – whether in the lungs, skin, or gut – engulf the allergen. These cells then travel to nearby lymphoid tissues, where they process the allergen into peptide fragments and present them on Major Histocompatibility complex (MHC) Class II molecules. This presentation activates T helper (Th) cells, which, alongside APC-derived cytokines, release Interleukin-4 (IL-4) and Interleukin-13 (IL-13). These cytokines drive B cells to undergo class-switch recombination from IgM to IgE, a process facilitated by the interaction of CD40 and CD40L, leading to genetic restructuring and the production of allergen-specific IgE antibodies ([40]). The resulting plasma cells secrete these IgE antibodies, which then bind to high-affinity (Fc ϵ RI) and low-affinity (Fc ϵ R2/CD23) receptors on effector cells like mast cells, eosinophils and basophils. The high-affinity Fc ϵ RI receptors, predominantly found on mast cells, are primarily responsible for triggering allergic symptoms upon re-exposure, while the low-affinity CD23 receptors modulate IgE synthesis by B cells and facilitate its transport across epithelial barriers [41]). For example, milk proteins (e.g., β -lactoglobulin) are taken up by intestinal epithelial cells or specialised antigen-sampling M cells overlying Peyer's patches. These proteins

are then internalised by subepithelial DCs via endocytosis, macropinocytosis, or receptor-mediated uptake (e.g., through CD36 or mannose receptors). DCs process the allergen into peptides and present them on MHC class II molecules to naïve CD4⁺ T cells in mesenteric lymph nodes or Peyer's patches. In genetically susceptible individuals, the local cytokine milieu—rich in epithelial-derived alarmins (IL-33, TSLP, IL-25) released upon barrier disruption—skews the T-cell response toward Th2 polarisation. This results in secretion of IL-4, IL-5, IL-13, and IL-9, which drive B-cell class-switching to IgE production via CD40-CD40L interaction and STAT6 signalling ([42];[43]).

This dual-receptor system underscores the intricate regulation of allergic responses, with recent studies suggesting that environmental pollutants may enhance receptor expression, amplifying sensitization risk ([44]).

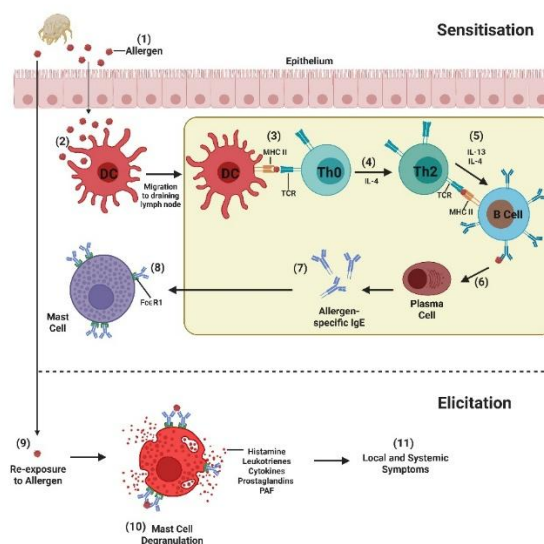


Figure 1.1 – Type I hypersensitivity mechanisms. The process of Type I hypersensitivity allergic reactions, which unfold in two key stages: sensitization and the triggering of symptoms. Taking milk as an example, the sequence begins when (1) the allergen penetrates the gut lining and is (2) engulfed by antigen-presenting cells (APCs), such as dendritic cells (DCs). These DCs break down the allergen into smaller peptide components and (3) display them on their surface using Major Histocompatibility Complex (MHC) Class II molecules. This presentation activates naïve CD4⁺ T cells, prompting (4) the secretion of IL-4, which drives their differentiation into Th2 cells. Activated Th2 cells then interact with additional allergen-MHC II complexes on B cells, releasing cytokines IL-4 and IL-13, which stimulate plasma cells to generate milk-specific IgE antibodies. These IgE molecules bind to high-affinity FcεRI receptors on effector cells, including mast cells. Upon subsequent exposure to the milk allergen, the elicitation phase is initiated. The allergen bridges the IgE antibodies attached to mast cells, inducing degranulation and the release of pro-inflammatory agents like histamine, leukotrienes, and prostaglandins. This cascade of mediators is responsible for the onset of allergic symptoms, ranging from localized irritation to systemic reactions. (Created using BioRender)

During the effector or elicitation phase, subsequent allergen exposure triggers clinical manifestations as the allergen binds to pre-existing allergen-specific IgE antibodies attached to mast cells, engaging the IgE Fragment crystallizable (Fc) regions with the receptor. This cross-linking on effector cells initiates a complex network of intracellular signalling cascades that amplify the immune reaction. Minimal receptor cross-linking on mast cells is sufficient to provoke immediate hypersensitivity, which can manifest in various tissues harbouring IgE and mast cells – such as the lungs, skin, gastrointestinal tract, eyes – or even in the bloodstream via basophils, potentially leading to a widespread anaphylactic response ([45]). The signalling process triggered by IgE cross-linking activates tyrosine kinases, which in turn stimulate a range of enzymes, including protein kinase C and mitogen-activated protein kinases (MAPKs) ([46]). These enzymes activate transcription factors like Nuclear Factor kappa B (NF- κ B), driving the production of pro-inflammatory cytokines, while also engaging lipases such as phospholipase D, which controls granule release, and phospholipase A2, which catalyses the conversion of arachidonic acid into lipid mediators like leukotrienes (LT) and prostaglandins ([47]). Mast cells and basophils release leukotrienes, including potent anaphylaxis mediators LTC₄, LTD₄ and LTE₄, which are particularly prominent in severe allergic episodes ([48]). Additionally, these cells secrete other bioactive molecules, such as platelet-activating factor (PAF) and chemokines, alongside granule contents like histamine, heparin and proteases, which orchestrate further immune responses in allergic inflammation ([49]). Histamine release promotes vascular leakage and smooth muscle tightening, leading to symptoms like swelling or respiratory distress, while prostaglandins induce vasodilation and additional muscle contraction. Cytokines such as IL-1 and TNF- α contribute to systemic anaphylaxis, whereas IL-4 and IL-13 boost further IgE production [50]). PAF triggers platelet aggregation and degranulation, exacerbating the reaction. Collectively, these mediators cause oedema, heightened vascular permeability and vasodilation, resulting in clinical signs including itching, nasal congestion, airway narrowing, nausea and skin rashes ([45]). Symptoms can emerge swiftly due to the immediate release of histamine and leukotrienes, but a delayed late-phase response may follow hours later. This secondary phase involves

cytokines like TNF- α and IL-1, which upregulate adhesion molecules on endothelial cells, recruiting eosinophils and neutrophils to sustain prolonged inflammation ([51]).

Altogether, the effector phase is primarily driven by the strong binding affinity between IgE and its high-affinity receptor Fc ϵ RI, which coordinates a swift and intense immune reaction. This process is initiated when IgE cross-linking on mast cells prompts their rapid degranulation, unleashing a surge of inflammatory mediators that underpin the clinical manifestations of an allergic response ([52]). Recent studies also highlight the role of this interaction in amplifying secondary signalling pathways, enhancing the recruitment of additional immune cells to sustain the reaction ([53]).

1.1.5 Treatment of Type I hypersensitivity

Beyond the significant health challenges posed by Type I hypersensitivity, individuals often face adverse effects on their dietary habits and social interactions. The unpredictable nature and potential severity of allergic episodes underscore the urgent need for effective management strategies. While a cure for allergies remains elusive, a growing array of approaches are available to control symptoms and improve quality of life. Traditional management includes allergen avoidance and the use of emergency treatments like epinephrine auto-injectors and antihistamines. For food allergies, this entails meticulous exclusion of culprit foods and comprehensive education on emergency medication use. However, this strategy can lead to nutritional imbalances due to limited food variety, particularly affecting the growth of young children, and may impose social limitations, such as difficulties dining in public settings or attending social gatherings ([54]).

In the early 2000s, oral immunotherapy emerged as an innovative therapeutic approach for managing food allergies, offering a disease-altering strategy that involves gradually introducing controlled doses of the offending food at scheduled intervals to foster tolerance ([55]). This method elevates the threshold at which the allergen triggers a reaction, providing a protective effect. Clinical trials have demonstrated its efficacy in pediatric patients with allergies to cow's milk, wheat and eggs, significantly improving their quality of life ([56]). Similarly, allergen-specific immunotherapy (SIT), which employs subcutaneous injections of escalating allergen doses, operates on

comparable principles to build immunological tolerance and stimulate the production of IgG4 blocking antibodies over time ([57]). This technique has proven successful in addressing allergic rhinitis, insect venom hypersensitivity, drug-related allergies and mild asthma cases. The underlying mechanism is believed to hinge on the activation of T regulatory (Treg) cells, which release suppressive cytokines such as IL-10 and TGF- β , facilitating a shift from a Th2-dominated immune response to a Treg-mediated state ([58]). This shift helps dampen allergic inflammation and promote long-term desensitization. However, a notable drawback of these immunotherapies is the potential for adverse reactions, as patients may experience allergic symptoms during each allergen dose escalation, necessitating careful monitoring and safety protocols [59].

Another promising therapeutic option is the anti-IgE monoclonal antibody Omalizumab, which has demonstrated clinical efficacy in raising the tolerance threshold for both food and airborne allergens. Omalizumab functions by attaching to the C ϵ 3 domain of IgE, effectively blocking its interaction with the Fc ϵ RI receptor on immune effector cells. This mechanism not only inhibits IgE from binding to these receptors but also displaces pre-bound IgE, preventing the cross-linking that triggers allergic responses [60]. By disrupting this cascade, Omalizumab reduces the release of inflammatory mediators, thereby alleviating associated symptoms. Also, Lebrikizumab, an innovative anti-IL-13 monoclonal antibody, has emerged as a targeted biologic therapy for managing allergic conditions, particularly severe asthma. This drug works by neutralizing IL-13, a key cytokine driving type 2 inflammation, which is central to allergic responses in the airways. Recent phase 3 clinical trials have yielded promising yet mixed results, with post-hoc analyses indicating significant benefits for patients with elevated eosinophil levels, suggesting a potential niche for its use ([61]). Additional biologic therapies include Etokimab, which targets IL-33 to suppress its pro-inflammatory effects ([62], Dupilumab, designed to block IL-4 signalling and modulate Th2-driven inflammation ([63]), and Mepolizumab, which inhibits IL-5 to reduce eosinophilic activity in allergic conditions ([64]). These monoclonal antibodies represent a growing frontier in allergy management, particularly for severe cases. However, their application, especially for food allergies, remains

relatively new, and extensive studies with larger patient groups are needed to fully validate their effectiveness and long-term safety ([65]).

For managing aeroallergies, the primary therapeutic strategies include the use of antihistamines and inhaled corticosteroids, which target distinct aspects of the allergic response. Inhaled corticosteroids, such as budesonide, work by dampening the cell-mediated inflammatory processes in the respiratory tract. They achieve this by inhibiting the production and activity of pro-inflammatory cytokines (e.g., IL-5, IL-13), chemokines that recruit immune cells, and adhesion molecules that facilitate leukocyte infiltration into airway tissues, thereby reducing swelling and mucus production ([66]). This suppression helps mitigate chronic inflammation, a key feature of conditions like allergic rhinitis and asthma. Antihistamines, including second-generation options like cetirizine and loratadine, counteract the physiological effects of histamine, a potent mediator released during allergic reactions. By competitively binding to H1 receptors on target cells, these drugs alleviate symptoms such as sneezing, itching and nasal congestion, offering rapid symptom relief without significant sedative effects ([67]). Recent advancements have also explored combination therapies, integrating antihistamines with leukotriene receptor antagonists (e.g. montelukast) to address both histamine-driven and leukotriene-mediated pathways, enhancing overall control of aeroallergic symptoms. These treatments remain foundational, though efficacy can vary, prompting ongoing research into personalized dosing and adjunctive therapies ([68]).

1.2 Immune cells in allergic sensitization

1.2.1 Epithelial cells

The epithelium, a thin membranous layer coating the inner surfaces of most organs, serves as a critical physical shield, safeguarding underlying tissues. Its selective permeability is governed by an array of receptors, transport proteins, and tight junctions, which meticulously control the passage of substances across this barrier ([69]). Epithelial cells in the respiratory tract, skin, and gastrointestinal system express PRRs, including Toll-like receptors (TLRs) and NOD-like receptors, enabling them to detect specific allergens and

initiate defensive response. Upon allergen recognition, these cells release a diverse range of signaling molecules, such as cytokines and growth factors, which modulate the activity of adjacent immune cells, including DCs ([70]). For example, food allergens may traverse the epithelial barrier through mechanisms like transcytosis, paracellular diffusion or receptor-mediated endocytosis ([71]). Certain allergens, such as Der p 1 from house dust mites, possess potent protease activity that can compromise epithelial integrity, leading to tissue damage and inflammation. In response, epithelial cells produce alarmins, including IL-25, IL-33, and thymic stromal lymphopoietin (TSLP), which play pivotal roles in shaping immune responses ([72]). These molecules influence DC behaviour, promoting allergic sensitization by recruiting and activating Th2 cells, while IL-23 further amplifies Th2 cytokines production, such as IL-4 and IL-13, intensifying the allergic cascade. The dynamic interplay between epithelial cells and DCs is central to orchestrating a Th2-biased immune reaction. Allergen detection by PRRs triggers the release of chemokines that draw DCs to the site, alongside cytokines that drive DC maturation and antigen presentation ([73]). Moreover, intraepithelial DCs can establish tight junctions with epithelial cells, enhancing their ability to capture and process allergens directly. This close collaboration not only facilitates allergen uptake but also amplifies the downstream Th2 response, contributing to the persistence of allergic inflammation. Recent studies also suggest that environmental factors, such as pollution, such as particulate matter (PM2.5 and PM10), ozone and diesel exhaust particles, may exacerbate this process by upregulating PRR expression, further complicating the epithelial-immune axis ([74]).

1.2.2 Dendritic cells

DCs play a crucial role in the progression of Type I hypersensitivity allergic responses, serving as the initial sentinels in the sensitization process that underpins adaptive immunity. The recognition and internalization of antigens by these innate immune cells mark the critical first step in triggering an allergic cascade, shaping the subsequent immune reaction ([75]). Positioned as vigilant scouts, DCs actively survey their environment to engulf antigens, playing a pivotal role in launching both cellular and humoral immunity, while

offering protection against pathogens and malignancies. As key intermediaries, DCs seamlessly connect the innate and adaptive arms of the immune system. When allergens are encountered at mucosal surfaces, immature DCs (iDCs) capture them via mechanisms such as receptor-mediated endocytosis, micropinocytosis or phagocytosis, subsequently maturing and migrating to nearby lymph nodes for further processing ([76]). Within these lymph nodes, DCs break down allergens into peptide fragments, presenting them on Major Histocompatibility Complex (MHC) Class II molecules to engage T cell receptors (TCRs), thereby igniting the adaptive immune response. In response to allergen exposure, iDCs upregulate CCR7 receptors, which bind to chemokines CCL21 and CCL19. These chemokines, respectively produced by lymphatic endothelium and T cell zones in lymph nodes, guide DCs to these critical sites for immune coordination ([77]; [78]). iDCs also express an array of C-type lectin receptors, such as the mannose receptor and dendritic cell-specific ICAM-3-grabbing non-integrin (DC-SIGN), which enhance their ability to recognize and internalize diverse allergens ([79]). As the most potent APCs, DCs are essential for activating naïve T cells, steering differentiation into Th2 cells – a hallmark of allergic sensitization. This activation involves extensive molecular interactions, including adhesion molecules like DC-SIGN with ICAM-3, and co-stimulatory pairs such as CD80/CD86 with CD28 as well CD40 with its ligand, fine-tuning the immune outcome ([80]). DCs regulate the intensity and persistence of TCR signaling, influencing cytokine profiles that dictate whether the response leans toward tolerance, a balanced Th0 state, or polarized Th1 or Th2 pathways ([81]). Beyond T-cell modulation, DCs secrete cytokines to engage other innate immune players, such as macrophages and natural killer cells, amplifying the allergic cytokine pool. Upon allergen exposure DCs upregulate surface markers like CD40, CD80, CD86 and HLA-DR while releasing cytokines including IL-10, IL-12 and IL-6 ([82]). These changes enhance DC maturation and shape T-cell responses, with IL-10 promoting regulatory T-cell activity and IL-12 potentially skewing toward Th1 responses under certain conditions.

Human DCs are categorized into three distinct subsets: classical dendritic cells (cDCs), plasmacytoid dendritic cells (pDCs) and monocyte-

derived dendritic cells (moDCs), each with specialized roles in immune regulation ([83]). cDCs exhibit a characteristic branched morphology and are distributed across peripheral tissues and lymphoid organs, where they can be subdivided into cDC1 and cDC2 subtypes. The cDC1 population excels in presenting antigens to CD8⁺ T cells and fosters Th1 cell development by secreting IL-12 and IFN- γ , bolstering defences against intracellular pathogens like viruses and certain bacteria ([84]). This subset is critical for initiating cytotoxic T-cell responses, enhancing overall immune surveillance. In contrast, cDC2 cells are recognized as the primary APC group driving Th2-mediated immune reactions *in vivo*, a process potentially linked to their robust expression of tight junction proteins, which enhance allergen uptake across epithelial barriers ([85]). These cells are vital for the initial activation of naïve CD4⁺ T cells, guiding their differentiation into Th2 and Th17 lineages, which are central to allergic sensitization and chronic inflammation ([86]). The cDC2 subset's ability to produce cytokines like IL-4 and IL-1 β further amplifies Th2 polarization, contributing to the production of IgE and eosinophil recruitment in allergic conditions such as asthma and atopic dermatitis. Recent studies also suggest that cDC2 may interact with the microbiome, modulating their allergen-processing capacity through PRR signalling, adding a layer of complexity to their role in allergy pathogenesis ([87]).

pDCs are characterized by their rounder morphology and are distributed throughout the various tissues across the body, where they serve as key players in the immune landscape ([88]). These cells are renowned for their robust production of type I interferon (IFN) cytokines, such as IFN- α and IFN- β , which position them as frontline defenders against viral infections by enhancing antiviral defences and activating other immune cells ([89]). Beyond their antiviral role, pDCs contribute to immune regulation by fostering the development of Tregs, which help maintain homeostasis and prevent excessive inflammation ([90]). Interestingly, recent studies also suggest that pDCs may modulate allergic responses, potentially by influencing the balance between pro-inflammatory and tolerogenic signals in mucosal tissues ([91]). Their ability to adapt to their cytokine profile – shifting from IFN-dominated to IL-10 production via CD4⁺ T cells under certain conditions – further underscores

their versatility, though this plasticity may also contribute to chronic inflammatory states if dysregulated ([92]).

moDCs represent a dynamic and adaptable subset of human DCs, originating from circulating monocytes that differentiate into DCs under specific inflammatory or allergic conditions ([93]). Unlike their classical or plasmacytoid counterparts, moDCs are generated in response to environmental cues, such as allergen exposure or tissue damage, making them particularly relevant in the context of acute allergic inflammation ([94]). These cells are predominantly found in inflamed tissues, including the skin, lungs and gastrointestinal tract, where they emerge as a critical bridge between innate and adaptive immunity during allergic sensitization. moDCs are characterized by their plasticity, enabling them to efficiently engulf allergens through mechanisms such as phagocytosis, macropinocytosis, and receptor-mediated uptake, facilitated by surface molecules like CD209 (DC-SIGN) and CD206 (mannose receptor) ([95]). Upon allergen internalization, moDCs process these antigens and present them on MHC class II molecules, migrating to draining lymph nodes where they activate CD4⁺ T cells. This interaction is enhanced by the upregulation of chemokine receptor CCR7, which responds to CCL19 and CCL21 gradients, guiding moDCs to lymphoid tissues for T-cell priming ([96]). Their role in driving Th2 responses is pronounced, as they secrete cytokines such as IL-6, IL-1 β and TSLP, which skew the immune environment toward a pro-allergic phenotype, promoting IgE production and eosinophil recruitment ([97]). Beyond Th2 polarization, moDCs contribute to Th17 differentiation through release of IL-23 and IL-1 β , linking them to chronic allergic inflammation and conditions like severe asthma and atopic dermatitis ([98]). Their adaptability is further highlighted by their ability to form transient tight junctions with epithelial cells, enhancing allergen capture and presentation efficiency ([99]). Recent research also indicates that moDCs can be influenced by the gut microbiome, with bacterial components modulating their cytokine profiles, potentially offering a therapeutic target for allergy management ([100]). Moreover, moDCs upregulate co-stimulatory molecules like CD80, CD86 and CD40 upon maturation, amplifying T-cell activation and

fine-tuning the immune response, which can range from tolerance induction to robust allergic sensitization depending on the cytokine context ([101]).

1.2.3 Naïve T cells

Naïve T cells, a subset of lymphocytes circulating in a quiescent state, play a pivotal role in the initial stages of allergic sensitization, serving as a cornerstone of the adaptive immune response. These uncommitted T cells, of both CD4⁺ and CD8⁺ subsets, reside in secondary lymphoid organs, such as lymph nodes and spleen, awaiting activation by APCs ([102]). Their selective activation is triggered when allergens, processed and presented on MHC class II molecules by DCs, engage the T-cell receptor (TCR) in the context of co-stimulatory signals, such as CD28-B7 interactions ([103]). This interaction marks the transition from a resting to an activated state, setting the stage for allergic immune programming. During allergic sensitization, naïve CD4⁺ T cells are particularly susceptible to differentiation into Th2 cells under the influence of cytokine pool rich in IL-4, often secreted by innate immune cells like basophils or epithelial cells in response to allergen exposure ([104]). This Th2 polarization is critical as it drives the production of signature cytokines such as IL-4, IL-5 and IL-13, which orchestrate eosinophil recruitment, IgE class-switching in B cells and airway hyperresponsiveness [105]). The process is amplified by the presence of alarmins like TSLP and IL-33, which enhance DC maturation and skew the immune response toward a pro-allergic phenotype. Furthermore, the initial encounter with allergens can induce epigenetic modifications in naïve T cells such as histone acetylation, promoting a lasting Th2 memory that sustains allergic sensitization ([106]). The crosstalk between naïve T cells and DCs is further refined by chemokine gradients, such as CCL17 and CCL22, which guide T-cell migration to allergen-rich sites, facilitating sustained interactions ([107]). In individual predisposed to allergies, this interaction may be exaggerated due to genetic factors or environmental triggers like pollution, which upregulate co-stimulatory molecule expression on DCs ([108]). This heightened responsiveness can lead to an overactive Th2 response, contributing to the chronicity of allergic conditions.

1.2.4 CD4⁺ helper T cells

Following the internalization and processing of allergens, DCs present these antigens as peptide fragments on their MHC class II molecules, facilitating interactions with CD4⁺ helper T cells within draining lymph nodes. This encounter enables T cells to recognize the presented peptides, triggering a series of molecular events that initiate allergic sensitization ([109]). Naïve CD4⁺ T cells, initially uncommitted and residing in a resting state, possess the potential to differentiate into various lineages, including Th1, Th2, Th17 and Treg cells, influenced by the surrounding immune microenvironment and cytokine signals ([110]). This differentiation process integrates multiple inputs: TCR-mediated activation, co-stimulatory signals (e.g., CD28-CD80/86 interactions), cytokine gradients, hormonal cues, and the structural properties of the extracellular matrix, which collectively shape T-cell fate ([111]). The local cytokine environment exerts a dominant influence on this differentiation trajectory. For instance, IL-4, often derived from innate immune cells, predominantly steers naïve T cells toward a Th2 phenotype, a key driver of allergic responses, while IL-12, primarily secreted by DCs and macrophages, promotes Th1 differentiation to combat intracellular pathogens ([112]). The source of IL-4 remains less precisely defined, however recent investigations highlight natural killer T (NKT) cells and basophils as significant early producers, releasing IL-4 to initiate Th2 skewing during allergen exposure ([113]). In the context of allergic sensitization, the presentation of allergenic peptides to CD4⁺ T cells by DCs favors Th2 cell development, and an elevated presence of Th2 cells is strongly associated with the onset and persistence of allergic conditions such as asthma and eczema ([114]). Th2 cells contribute to the allergic cascade by secreting a suite of cytokines – IL-4, IL-5, IL-9, IL-13, IL-25, IL-31 and IL-33 – which orchestrate an inflammatory response characterized by IgE production, eosinophil activation and tissue remodeling ([115]). Conversely, when confronting intracellular threats like viruses or bacteria, APCs release IL-12 and IFN- γ , steering T-cell differentiation toward the Th1 lineage. Th1 cells, in turn, produce pro-inflammatory cytokines such as IFN- γ , TNF- α , and IL-2, which enhance the activity of macrophages, natural killer (NK) cells and CD8⁺ cytotoxic T cells to eliminate pathogens ([116]).

1.2.5 Regulatory T cells

Tregs are essential for upholding immune tolerance to allergens, acting as guardians that prevent overactive immune responses. As previously noted, an imbalance or dysfunction in Treg activity has been strongly associated with the emergence of and progression of allergic disorders ([117]). Peripheral tolerance, a critical protective mechanism, occurs when lymphocytes encounter harmless allergens in peripheral tissues, leading to the suppression of unwarranted immune reactions. When this peripheral tolerance falters, benign allergens can be misidentified as threats, triggering allergic sensitization and pathology ([118]). Tregs exert their suppressive effects by directly inhibiting Th2 cells and IgE-producing B cells, thereby fostering a state of allergen tolerance. The most extensively researched Treg subset, known as natural Tregs, is identified by the surface markers CD4⁺, CD25⁺, and the transcription factor Foxp3⁺, and they secrete immunomodulatory cytokines such as IL-10 and transforming growth factor-beta (TGF- β) [119]. For instance, when DCs process food allergens and migrate to mesenteric lymph nodes, they release IL-10 and TGF- β , guiding the differentiation of naïve CD4⁺ T cells into Tregs. These Tregs subsequently dampen the activity of Th2 cells and type 2 innate lymphoid cells (ILC2s), reducing the secretion of pro-allergic cytokines like IL-5 and IL-13 ([120]). The mechanisms underlying Treg-mediated suppression are multifaceted. Beyond cytokine production, Tregs downregulate the expression of co-stimulatory molecules CD80 and CD86 on DCs, disrupting the activation of allergen-specific Th2 cells and curtailing the allergic cascade ([121]). Additionally, Tregs play a pivotal role in establishing oral tolerance, a process where they mitigate anaphylactic responses and modulate Th2-driven immunity by secreting IL-10 and limiting effector T-cell proliferation through IL-2 deprivation ([122]). This suppression is enhanced by Treg interactions with the gut microbiota, which can bolster IL-10 production via bacterial components, further stabilizing tolerance ([123]). Clinical evidence from oral immunotherapy trials indicates a notable increase in Treg frequency and functionality, correlating with improved tolerance to food allergens, suggesting a therapeutic avenue for harnessing Treg potential ([124]). Recent studies also explore Treg epigenetic modifications, such as

enhanced Foxp3 stability, as a target for long-term allergy management, reflecting the evolving understanding of their role in allergy ([117]).

1.2.6 B cells

B cells are central to the initiation of allergic sensitization, largely due to their specialized capacity to generate allergen-specific IgE antibodies, which are pivotal in triggering allergic responses. These cells are primarily situated within lymphoid organs, where they collaborate with T cells and DCs to establish germinal centres, dynamic hubs of immune interaction ([125]). B cells equipped with allergen-specific B-cell receptors (BCRs) can directly capture allergens or receive them via follicular dendritic cells (FDCs), which present antigens in an organized manner. Subsequently, B cells process these allergens into peptides and present them on MHC class II molecules to CD4⁺ T cells, sparking T-cell activation. This process is amplified by the engagement of CD40 on B cells with CD40L on activated CD4⁺ T cells, coupled with Th2 cytokines like IL-4 and IL-13, which drive B-cell proliferation and differentiation into plasma cells that secrete IgE ([126]). Beyond their role in sensitization, regulatory B cells (Bregs) contribute significantly to maintaining immune tolerance to allergens, complementing the actions of Tregs. Bregs secrete anti-inflammatory cytokines, including IL-10 and TGF- β , which dampen excessive immune reactions and elevate serum levels of IgG4 antibodies, a marker of desensitization often observed in tolerant individuals ([127]). This IgG4 increase is particularly evident during oral immunotherapy, a widely adopted strategy for food allergies, where patients are gradually exposed to escalating doses of the allergen to build tolerance ([128]). Bregs also foster a synergistic relationship with Tregs, enhancing IgG4 production through cytokine-mediated crosstalk. Additionally, Bregs inhibit DC maturation by downregulating co-stimulatory molecules like CD80 and CD86, while promoting peripheral Treg differentiation, further reinforcing tolerance ([129]). The dual nature of B cells extends to their interaction with the microbiome, where gut-associated B cells may modulate allergen tolerance via bacterial signals, influencing IgA production alongside IgG4 ([130]). Recent studies suggest that epigenetic modifications, such as histone deacetylation in Bregs, enhance their suppressive capacity via IL-10

upregulation, offering a potential therapeutic target ([131]). Thus, B cells serve as a linchpin in both the initiation of allergic sensitisation and the establishment of immune tolerance, with their multifaceted roles shaping the trajectory of allergic diseases.

1.2.7. Innate Lymphoid cells

Innate lymphoid cells (ILCs) are a family of tissue-resident, lineage-negative lymphocytes that mirror the functional diversity of adaptive T-helper subsets but lack antigen-specific receptors. They are now recognised as central players in the early orchestration of type 2 immune responses, including allergic diseases such as food allergy, asthma, and atopic dermatitis. ILCs are divided into three main groups based on transcription factor expression and cytokine profile: ILC1 (T-bet⁺, produce IFN- γ), ILC2 (GATA3⁺, produce IL-4, IL-5, IL-13), and ILC3 (ROR γ t⁺, produce IL-17A/F, IL-22) ([132]). Group 2 innate lymphoid cells (ILC2s) are particularly important in allergy. ILC2s reside in the gut lamina propria, mesenteric lymph nodes, and intestinal epithelium, where they respond rapidly to epithelial-derived alarmins released upon allergen exposure. Key triggers include IL-33, IL-25, and TSLP, which are secreted by damaged or stressed intestinal epithelial cells following ingestion of food allergens (e.g., peanut, egg, milk proteins) ([133]; [134]). Upon activation, ILC2s produce large amounts of IL-5 and IL-13, which drive eosinophil recruitment, mast cell activation, goblet cell hyperplasia, and mucus production, all of which contribute to the intestinal inflammation and systemic IgE responses characteristic of food allergy ([135]). ILC2s also interact closely with adaptive immunity during food allergic sensitisation. They provide an early source of IL-4 and IL-13 that can prime DCs and promote Th2 polarisation of naïve CD4⁺ T cells in mesenteric lymph nodes. Recent studies have shown that ILC2-derived IL-13 enhances epithelial permeability, facilitating allergen uptake and subsequent presentation by DCs to T cells, thereby amplifying the allergic cascade ([136]). The rapid, antigen-independent activation of ILC2s positions them as early amplifiers of type 2 immunity in the gut, bridging innate detection of food allergens with the subsequent adaptive IgE response. Their interaction with DCs, mast cells, and T cells forms a feed-forward loop that sustains allergic inflammation. Understanding

the role of ILCs in food allergy is therefore essential for identifying new therapeutic targets, such as ILC2-specific inhibitors (e.g., anti-TSLP, anti-IL-33 biologics) or strategies to restore ILC3-mediated barrier protection. As research continues, ILCs are increasingly viewed as key orchestrators of the initial sensitisation phase in food allergy and promising targets for prevention and treatment.

[1.3.](#) Extracellular Vesicles

[1.3.1.](#) EV subtypes

Extracellular vesicles (EVs) are small, membrane-bound particles released by cells into the extracellular environment, serving as critical mediators of intercellular communication. These vesicles encompass a heterogeneous group of structures, including exosomes and ectosomes, each distinguished by their size, biogenesis and cargo content ([137]) (Fig. 1.2). Exosomes, typically ranging from 30 to 150nm, originate from the endosomal pathway through the inward budding of multivesicular bodies (MVBs), which fuse with the plasma membrane to release their contents. Ectosomes, larger in size ranging from 100 to 5µm, are formed by the outward budding and shedding of the plasma membrane, reflecting the cells immediate physiological state [138]. The diversity of EV subtypes is further underscored by their distinct molecular profiles, which include proteins, lipids, DNA and various RNA species, enabling them to influence recipient cell behaviour in health and disease ([139]). Exosomes are enriched with tetraspanins (e.g., CD63, CD81) and are implicated in long-range signalling, while ectosomes carry surface markers like integrins, reflecting their origin from the plasma membrane ([140]). This heterogeneity allows EV subtypes to participate in diverse physiological processes, from immune modulation to tissue repair and their dysregulation is increasingly linked to pathological conditions, including allergic diseases ([141]; [142]).

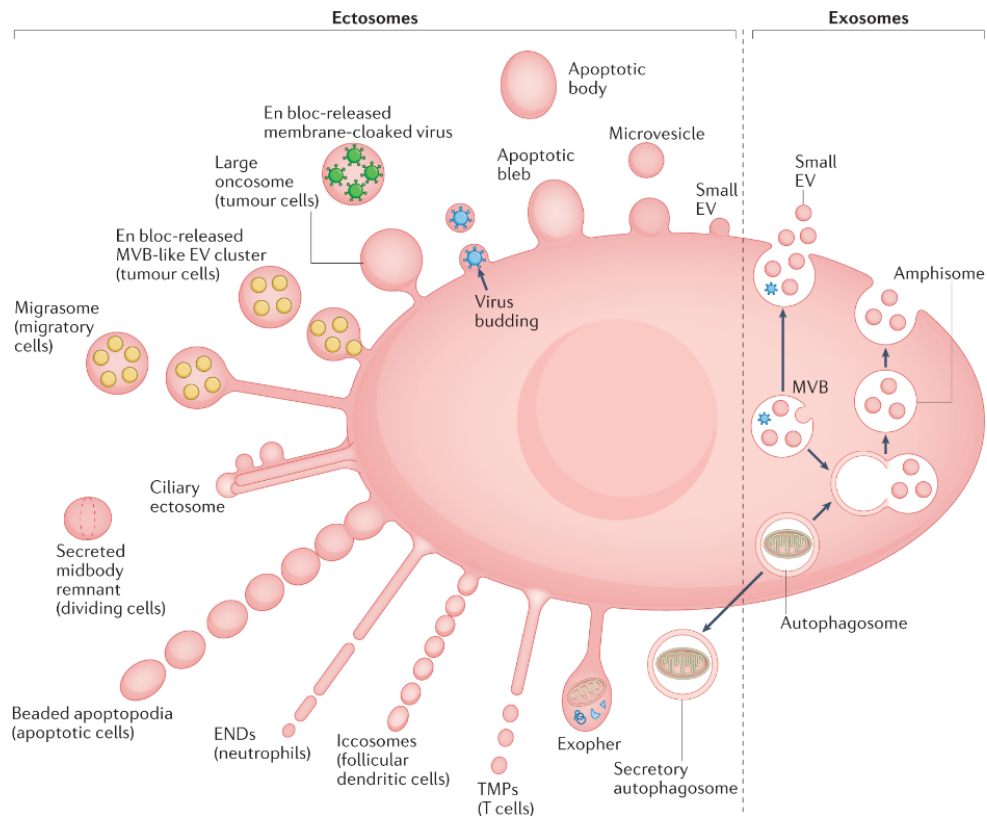


Figure 1.2 – Heterogeneity of Extracellular vesicles. Extracellular vesicles (EVs) represent a diverse group of lipid bilayer-encased particles, distinguished primarily by their distinct modes of formation into two primary categories: exosomes and ectosomes. Exosomes, originating from the endocytic pathway, are released into the extracellular space through the fusion of multivesicular bodies (MVBs) and amphisomes with the plasma membrane, where amphisomes arise from the merging of autophagosomes with MVBs. In contrast, ectosomes are produced through the outward protrusion and shedding of the plasma membrane, though certain ectosomes may also incorporate components typically associated with endosomal origins. This category encompasses a range of sizes, including smaller EVs like arrestin domain-containing protein 1-mediated microvesicles, medium-sized microvesicles, and the larger apoptotic bodies. Picture obtained from [143].

1.3.2. Biogenesis and release of exosomes

Exosomes biogenesis is a finely orchestrated process originating within the endosomal system, beginning with the inward budding of the plasma membrane to form early endosomes. These early endosomes mature into late endosomes, characterized by the development of intraluminal vesicles (ILVs) through the invagination of the endosomal limiting membrane, a process driven by the endosomal sorting complexes required for transport (ESCRT) machinery ([144]). ESCRT complex, comprising ESCRT-0, -I, -II and -III subunits, recognize ubiquitinated proteins and lipids, facilitating ILV formation, while accessory proteins like ALIX and TSG101 ensure cargo sorting and membrane deformation ([145]; [146]). As late endosomes transform into multivesicular bodies (MVBs), they accumulate ILVs, which

will eventually become exosomes upon release. The fate of MVBs – degradation via fusion with lysosomes or exocytosis – depends on regulatory factors, including Rab GTPases (e.g., Rab27a, Rab27b) and soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, which orchestrate MVB trafficking to the plasma membrane. The fusion process is calcium-dependant and involves the assembly of the SNARE complex, ensuring precise docking and membrane merger ([147]; [148]). Exosome release is tightly regulated, with the environmental cues such as allergen exposure or inflammation upregulating this process in immune cells like DCs and mast cells. The expulsion of MVBs expels ILVs into the extracellular space as exosomes, a step modulated by cytoskeletal dynamics, including actin and microtubule networks, which guide vesicle movement ([149]). Post-release, exosomes carry a diverse cargo – proteins (e.g., MHC molecules), lipids, mRNAs and microRNAs – reflecting their cellular origin and function ([150]). Recent studies highlight that exosomal biogenesis is influenced by epigenetic modifications, such as histone methylation, which alter gene expression of ESCRT components, and by the tumour microenvironment or allergic inflammation, where hypoxia and cytokine storms enhance exosome production ([151] [152] [153]). This intricate biogenesis and release mechanism underpins their potential as therapeutic targets and biomarkers in allergic disease research.

1.3.3. Biogenesis and release of ectosomes

Ectosomes are a significant subtype of EVs, ranging from 100 to 5µm in diameter, playing a crucial role in intercellular communication. Their biogenesis and release are dynamic processes originating from the plasma membrane, distinguishing them from exosomes, and involve a series of intricate molecular mechanisms that reflect the cellular state ([154]). The formation of ectosomes begins with the reorganisation of the plasma membrane, where localised changes in lipid and protein composition create sites of outward budding ([155]). This process is driven by the activation of enzymes, such as flippases and scramblases, which redistribute phospholipids like phosphatidylserine to the outer leaflet, promoting membrane curvature ([156]). The cytoskeletal machinery, including actin and myosin, is instrumental in this budding, with Rho GTPases (e.g., RhoA, Rac1) and their

downstream effectors, such as ROCK and ARF6, regulating the contractile forces and membrane remodelling necessary for ectosome generation ([157] [158]). Calcium signalling also plays a pivotal role, with intracellular calcium influx triggering the disassembly of the cortical actin cytoskeleton and facilitating vesicle shedding, a mechanism amplified in activate immune cells like mast cells during allergic responses ([159]). The cargo loading into ectosome is selective, guided by adaptor proteins like ALIX and the ESCRT-independent tetraspanin-enriched microdomains (e.g., CD9, CD81), which sort pro-inflammatory mediators, including IL-6, leukotrienes and allergen peptides into these vesicles ([160]). The release of ectosomes is a tightly controlled event, initiated by the fission of the budding membrane, a process mediated by the endocytic protein dynamin and the exocyst complex, which ensures vesicle detachment from the cell surface ([161]). Environmental stressors, such as allergen-induced oxidative stress or tissue damage, upregulate ectosome release from epithelial cells and immune cells, with hypoxia-inducible factors (HIFs) enhancing vesicle production in inflamed airways ([162]). Post-release, ectosomes interact with recipient cells via surface receptors like integrins, delivering their cargo to modulate immune responses, such as promoting Th2 polarisation in allergic sensitisation ([163]). Studies also suggest that epigenetic modifications, including DNA methylation of genes encoding cytoskeletal proteins, influence ectosome biogenesis rates, while microbial metabolites from the gut microbiota may alter their cargo composition, potentially affecting allergic inflammation ([164] [165]). The regulation of ectosome release is further refined by post-translational modifications, such as phosphorylation of key signalling proteins by kinases like PKC, which enhance vesicle shedding in response to cytokine storms ([166] [167]). In allergy this process is heightened, with ectosomes from damaged skin cells in atopic dermatitis carrying damage-associated molecular patterns (DAMPs), such as S100A8 and S100A9 that perpetuate inflammation ([168]). The exploration of mechanisms detailing ectosome biogenesis and release could lead to an understanding of allergic disease progression and their potential as therapeutic targets.

1.3.4. Tetraspanins associated with EVs

EVs are notably abundant in tetraspanins, a class of integral membrane proteins characterised by a conserved four-transmembrane topology that serves as a scaffold for organising cellular membranes ([169]). These proteins traverse the lipid bilayer four times, creating two extracellular loops of varying lengths, a short intracellular loop, and cytoplasmic N- and C-terminal tails, enabling them to anchor and regulate a diverse array of molecular interactions ([170]). As transmembrane organisers, tetraspanins possess the capacity to assemble into multimolecular complexes, engaging with an extensive range of membrane-bound and cytosolic signalling molecules, thereby establishing specialised membrane microdomains, known as tetraspanin-enriched microdomains (TEMs) ([171]). These TEMs act as platforms that enhance signal transduction and facilitate the recruitment of adaptor proteins, such as integrins and growth factor receptors, amplifying cellular responses. Tetraspanins exhibit a widespread presence across various tissues, with specific members like CD9, CD81, CD63 and CD82 being particularly concentrated within the membranes of exosomes, reflecting their role in vesicle biogenesis and cargo sorting ([172]). Beyond exosomes, tetraspanin expression extends to the plasma membrane, suggesting their presence on vesicles derived from direct membrane budding, such as ectosomes ([173]).

Tetraspanins engage with a diverse array of receptor and signalling molecules, orchestrating the formation of specialised TEMs that are instrumental in multiple facets of EV biology ([174]). These microdomains play a pivotal role in EV biogenesis, the selective packaging of cargo, the adhesion and internalisation of EVs by recipient cells, and the presentation of antigens by EVs during immune activation ([175]). Tetraspanins are especially vital in the biogenesis and release of exosomes, predominantly through ESCRT-independent mechanisms, where they influence membrane dynamics and vesicle formation ([176]). The structural properties of tetraspanins enable them to modulate membrane curvature, a key determinant in EV generation. Research has highlighted the unique cone-shaped configuration of CD9, which induces bending in lipid bilayers, suggesting that the aggregation of tetraspanin molecules can drive the curvature necessary for EV budding ([177]). This clustering not only facilitates membrane deformation but also allows the cytoplasmic tails of tetraspanins to interact with cytoskeletal components like actin and microtubules, potentially aiding in the fission process that detaches EVs from parent cell membrane ([178]). Such interactions are enhanced by calcium-dependant signalling pathways, which stabilize the cytoskeletal network during vesicle release ([179]). Beyond structural roles, tetraspanins are critical regulators of cargo sorting into EVs,

influencing the inclusion of proteins, RNAs and lipids. Metalloproteinases, a group of enzymes involved in extracellular matrix remodelling, are selectively targeted to EVs through their association with TEMs, where they bind to tetraspanins like CD9 ([180]). For instance, the metalloproteinase CD10, which supports B-cell maturation and their migration into circulation, exhibits a strong affinity for CD9, as demonstrated by studies showing a significant reduction in CD10 levels in EVs following CD9 knockout ([181]). This selective association underscores the role of tetraspanins in fine-tuning EV content. Additionally, the recruitment of MHC Class I and II molecules to EVs, essential for antigen presentation, is mediated by their integration into TEMs, with EVs derived from B cells, T cells and DCs showing high levels of tetraspanins (CD9, CD63, CD81 and CD82) alongside these MHC complexes ([182] [183]). Furthermore, in the absence of CD9 in DCs, achieved through knockout models, resulted in diminished T-cell activation compared to their wild-type counterparts ([184]). This reduction is attributed to a decrease in MHC Class II surface expression on CD9-deficient DCs, stemming from disrupted exocytosis process. Additionally, the internalisation of antigens is impaired, highlighting CD9's specific role in modulating antigen presentation by overseeing the intracellular trafficking of MHC Class II molecules. DCs are prolific producers of exosomes, which serve to enhance both adaptive immune responses involving T cells and innate responses mediated by NK cells. These exosomes are enriched with a distinctive protein profile, including CD9, all major antigen-presenting complexes (MHC-I and II, CD1a, b, c and d), and the co-stimulatory molecule CD86 ([185]). The crosslinking of CD9 on DC surfaces triggers their activation, prompting the release of cytokines that regulate inflammation, and is indispensable for effective antigen presentation, a process critical for modulating immune responses. Research has revealed that MHC Class II molecules co-localise with CD9 within luminal vesicles, which are later released as exosomes, a pathway active in iDCs and significantly enhanced during cognate interactions with antigen-specific CD4⁺ T cells ([186]). This process highlights a sophisticated mechanism by which DCs leverage exosome secretion to amplify immune responses.

The mechanisms governing the targeting and internalization of EVs by recipient cells are not fully understood, yet evidence suggests that molecules embedded in TEMs of both EVs and target cells- such as tetraspanins, integrins and additional adhesion molecules – play a central role in facilitating binding, membrane fusion, and the selective uptake of EVs ([187]). This selective uptake is finely tuned, ensuring that EVs deliver their cargo to specific cell types based on receptor compatibility.

Although tetraspanins are predominantly utilised as EV markers, as endorsed by the ISEV, emerging research increasingly highlights their multifaceted roles in EV biology, spanning biogenesis, cargo sorting and cellular uptake ([188]). For instance, DCs harvested from CD9 knockout mice exhibited decreased volume of EV release, and CD9 influences the wingless-related integration site (Wnt) signalling pathway by modulating the EV packaging of β -catenin ([189]). Additionally, tetraspanin involvement extends to regulating endocytic pathways and cytoskeletal dynamics during EV release ([190]). This underscores a significant role for tetraspanins in EV biogenesis. Despite these advances, the precise functional contribution of tetraspanins across these processes remain underexplored, necessitating further studies to unravel their full impact on EV-mediated immune modulation, particularly in allergic sensitisation.

1.4 EVs in allergies

1.4.1 Cargo of EVs in allergies

The cargo encapsulated within EVs in allergic diseases represents a diverse and dynamic collection that significantly influences immune responses, making it a focal point for understanding allergic sensitisation and inflammation. EVs carry a complex mixture of biomolecules – proteins, lipids, DNA and various RNA species – that reflect their cellular origin and orchestrate intercellular signalling in allergic conditions ([191]). Protein content within EVs is particularly notable, with DC-derived EVs transporting MHC Class II-allergen peptide complexes, which can induce Th2-like cytokine response in allergic individuals ([192]). Additionally, EVs from mast cells and epithelial cells contain pro-inflammatory proteins such as IL-6, TSLP and alarmins, like IL-33, amplifying Th2 cytokine production and contributing to airway hyperresponsiveness in asthma ([193] [194]). Lipid components of EV cargo, including sphingolipids and prostaglandins, play a regulatory role by modulating membrane fluidity and signalling pathways in recipient cells, enhancing allergic inflammation in mucosal tissues ([195]). The nucleic acid profile, particularly RNA, is equally critical, with EVs enriched in microRNAs (e.g., miR-146a, miR-155, miR-92b) that can either suppress or promote allergic responses through reduction of IL-6 expression or enhancing Th2 polarisation and DC maturation ([196]). DNA fragments within EVs, often derived from damaged epithelial cells during allergen exposure or bacterial

origin, may activate innate immune receptors like TLR9, triggering inflammatory cascades in conditions such as atopic dermatitis ([197]). The cargo composition varies by EV subtype and disease context, with ectosomes from activated mast cells carrying higher levels of histamine and leukotrienes, driving immediate hypersensitivity reactions, while exosomes from tolerogenic DCs may carry TGF- β and IL-10, supporting immune tolerance ([198] [199]). Environmental factors, such as air pollution and pollen exposure, further shape EV cargo, with pollutants inducing the packaging of oxidative stress-related proteins and miRNAs that exacerbate allergic rhinitis ([200] [201]). EVs from mast cells can stimulate IgE production in B cells through the CD40-CD40L interaction without the use of T cells, a key step in sensitisation, while interactions with the gut microbiota may enrich EVs with microbial metabolites that modulate Treg activity ([202] [203]). Emerging research underscores the therapeutic potential of EV cargo manipulation, with engineered EVs loaded with anti-inflammatory miRNAs showing promise in reducing allergic inflammation ([204]). As such, EV cargo plays an important role in immune dysregulation but also exhibits a prospective application in personalised allergy management. More information of how EVs interact with allergies is provided in Chapter 3.

1.4.2 DNA in EVs in allergies

The presence of DNA within EVs in allergic diseases represents an expanding area of research, offering insights into the mechanisms underlying immune activation and allergic sensitization ([205]). EVs encapsulate DNA fragments that originate from damaged or stressed cells, such as epithelial cells exposed to allergens, and serve as carriers that deliver these genetic materials to recipient immune cells ([206]). This DNA cargo, often consisting of double-stranded or mitochondrial DNA, is packaged during EV biogenesis, with studies suggesting that it is sorted into EVs via interactions with the ESCRT machinery or through TEMs during membrane budding ([207]; [208]). The incorporation process is enhanced under inflammatory conditions, where allergen-induced oxidative stress upregulates DNA release from apoptotic or necrotic cells, subsequently enriching EV content [209] [210]. Mechanistically, the DNA within EVs can activate innate immune receptors, notably TLR9,

which is expressed on DCs, macrophages and B cells ([211] [212]). Upon EV uptake, this DNA triggers signalling cascades, via the MyD88-dependant pathway, leading to the production of pro-inflammatory cytokines, such as IL-6 and TNF- α , which amplify Th2-biased immune responses central to allergies like asthma and atopic dermatitis ([213] [214] [215]). A study indicated that allergic rhinitis patients contained elevated levels of mitochondrial DNA in their nasal lavage fluid, potentially correlating with increased TLR9 activation and IL-33 release, hinting at a direct contribution to allergic inflammation [216]. The transfer of EV-encapsulated DNA to recipient cells is facilitated by endocytic pathways, including phagocytosis and micropinocytosis, with surface integrins and lectins on EVs enhancing targeting specificity to immune cells ([217]). Gut microbiota interactions may modulate EV DNA content, with microbial DNA fragments potentially influencing epithelial barrier integrity and modulating immune responses ([218]), which might play a role in the development of food allergies. Recent investigations have explored the therapeutic potential of manipulating EV DNA, with engineered EVs loaded with CpG DNA and OVA showing promise in enhancing Th1/Th2 balance in inducing IgG antibodies specific to OVA, in turn alleviating allergic symptoms in allergic individuals with allergic rhinitis ([219]). Furthermore, research has revealed that EVs present in bronchoalveolar lavage fluid exhibit significantly increased levels of mitochondrial DNA in asthmatic patients compared to a control group, indicating that EVs may serve as a promising biomarker for diagnosing and monitoring allergic diseases ([220]). Together, this highlights the important roles and mechanisms of DNA in EVs, which could lead to advances in the understanding of allergic disease progression and innovative therapeutic strategies.

1.4.3 RNA in EVs in allergies

The presence of RNA within EVs in allergic diseases serves as a pivotal mechanism influencing immune responses and driving allergic sensitisation, positioning it as a key area of investigation. EVs, including exosomes and ectosomes, harbor a variety of RNA types – such as messenger RNA (mRNA), microRNA (miRNA), long non-coding RNA (lncRNA) and transfer RNA

(tRNA) fragments – originating from donor cells like epithelial cells, DCs and mast cells during allergen exposure ([221]). The encapsulation of RNA into EVs occurs during biogenesis, mediated by RNA-binding proteins and the ESCRT machinery, which selectively loads RNA based on cellular stress or inflammatory cues ([222]). Allergen-induced inflammation, particularly in the respiratory tract or skin, boosts this process, with increased activity of RNA export factors facilitating miRNA enrichment in EVs ([223] [224]). Mechanistically, EV-encapsulated RNA alters gene expression in recipient cells, playing dual roles in allergies. A landmark study by Okoye et al., showed that exosomes derived from Tregs are enriched with miR-34a and let-7b, which suppress pro-inflammatory pathways and enhance Treg differentiation, thereby mitigating allergic responses in a murine asthma model ([225]). In contrast, Levänen et al., demonstrated that EVs in bronchoalveolar lavage fluid from asthmatic patients contained elevated levels of miR-21 and miR-487b, fostering Th2 cytokine production (e.g., IL-13) and intensifying airway inflammation ([226]). The transfer of this RNA occurs through endocytic uptake, with EV-associated miRNAs engaging TLR7 in recipient cells, triggering NF- κ B signalling and amplifying allergic inflammation, as supported by evidence of miRNA-mediated immune activation ([227] [228]). Furthermore, a study by Wang et al., demonstrated that exosomes derived from human nasal epithelial cells (HNECs) of AR patients carry elevated levels of lncRNA NEAT1, which contributes to the progression of AR by promoting inflammation and apoptosis via the modulation of the miR-511/NR4A2 axis ([229]). This suggests a significant role of exosomal NEAT1 in perpetuating the inflammatory responses in AR. Similarly, Zhu et al., identified that plasma exosomes from children with atopic dermatitis contain RNA, including microRNAs, which promote keratinocyte apoptosis and the secretion of inflammatory factors, contributing to the inflammatory milieu and potential barrier dysfunction in the disease ([230]). A recent study showed that engineered EVs derived from human umbilical cord mesenchymal stem cells loaded with miR-146a-5p successfully alleviated allergic airway inflammation via downregulation of IRAK1 and TRAF6 expression, leading to decreased production of IL-6, TNF- α , IL-1 β and IL-18 ([196]). Understanding the

mechanisms and roles of RNAs in EVs could lead to advancements in therapeutic and diagnostic strategies for allergic conditions.

1.4.4 Proteins of EVs in allergies

The protein content within EVs represents a dynamic component that significantly shapes the immune landscape of allergic diseases, offering a window into the molecular drivers of inflammation and potential therapeutic avenues. A key investigation by Möbs et al., underscores the critical involvement of EV-associated proteins, including tetraspanins (e.g., CD63, CD81) and cell adhesion molecules, in facilitating cross-talk between immune cells, thereby amplifying allergenic signals that underpin conditions such as asthma and atopic dermatitis, positioning these proteins as potential modulators in allergy treatment strategies ([204]). The functional roles of these proteins are deeply tied to their involvement in immune regulation and cellular interactions. Research by Morelli et al., revealed that proteins like ICAM-1 and integrins (CD51, CD61) on DC-derived exosomes enhance their internalization by other immune cells, promoting allergen presentation to T cells ([185]). Similarly, Hong et al., demonstrated that proteins such as α -hemolysin within *Staphylococcus aureus*-derived EVs trigger atopic dermatitis-like inflammation by stimulating the release of pro-inflammatory cytokines (e.g., IL-4, IL-5), highlighting the pathogenic influence of microbial protein cargo in allergic skin disorders ([231]). This finding challenges the conventional focus on allergen exposure alone, suggesting a broader microbial contribution to allergic pathology. Environmental cues further modulate the protein profiles within EVs, influencing disease progression. Lässer et al., found that EVs from lung tissues in mice with allergen-induced eosinophilic inflammation are laden with immune-related proteins, reflecting the inflammatory milieu and potentially exacerbating asthma pathology through enhanced immune activation ([232]). The uptake of these protein-rich EVs by target cells is mediated by endocytic routes, with studies by Tkach et al., indicating that the specific protein composition dictates the nature of immune cell responses, where iDCs-derived larger EVs induce Th2-like cytokines, while smaller EVs induce Th1-like cytokines, offering a nuanced understanding of protein-driven immune modulation ([233]). This adaptability suggests opportunities for therapeutic manipulation. This is evidence that points to the therapeutic promise of EV

proteins, with engineered vesicles providing a novel approach. Fischer et al., showed that proapoptotic proteins like Bim in EV cargo can regulate immune cell survival, hinting at the potential to tailor EV protein content to dampen allergic inflammation [234]. Additionally, Hong et al., illustrated that modifying *S. aureus*-derived EV proteins, such as reducing α -hemolysin levels, alleviates atopic dermatitis severity, supporting the feasibility of protein-targeted interventions ([235]). A study by Yang et al., proposed that antibodies targeting bacterial EV-specific proteins in patient serum could serve as biomarkers, enabling non-invasive tracking of atopic dermatitis condition ([236]). Together, this highlights the importance of understanding the complex functions of EV proteins and how this could lead to the unravelling of allergic disease mechanisms and pioneer innovative therapeutic and diagnostic applications.

1.4.5 EVs as biomarkers in allergy

The potential of EVs as biomarkers in allergic diseases has garnered significant attention, offering a non-invasive means to monitor disease progression, severity and therapeutic response through the analysis of their molecular cargo. EVs encapsulate a rich array of biomolecules, making them promising candidates for diagnostic and prognostic tools in conditions such as asthma, allergic rhinitis and atopic dermatitis. A seminal study by Lässer et al., demonstrated that nasal lavage fluid contains EVs that carry distinct protein and RNA signatures, suggesting their utility as biomarkers to differentiate allergic phenotypes based on inflammatory profiles ([237]). The mechanisms underlying EVs as biomarkers hinge on their ability to mirror the allergic microenvironment, with their cargo serving as a snapshot of ongoing immune responses. Research by Wagner et al., demonstrated that plasma-derived EVs (pEV) from individuals with type-1 allergies exhibit a distinct pattern of 10 immune mediators, including upregulated cytokines (TNF- α , IL-4, IL-5, IL-6, IL-17F) and chemokines (CCL2, CCL17), alongside downregulated factors (IL-11, IL-27, CCL20), enabling discrimination of allergic patients from non-allergic controls and suggesting their potential as diagnostic biomarkers for allergy assessment ([238]). Additionally, a study by Wu et al., revealed that EVs in nasal mucus from patients with allergic rhinitis display elevated levels of miR-30-5p and miR-199b-3p, which are implicated in regulating allergic

inflammation, potentially through targeting genes involved in immune activation, thus providing a foundation for using EV-derived miRNAs as biomarkers to assess disease severity ([239]). Environmental and immunological factors further enhance the biomarker potential of EVs, with their composition adapting to disease states. In human studies, the levels of miR-125b and miR-126 within EVs have been shown to be elevated in asthmatic patients compared to a control group, with emerging evidence suggesting a potential correlation between these EV-encapsulated RNAs and the severity of asthma ([240] [241]). These findings challenge the established narrative that relies heavily on traditional biomarkers like total IgE, suggesting that EV-based markers provide a more comprehensive view of allergic disease dynamics.

1.4.6 EVs as therapeutics in allergy

The advent of EVs as therapeutic agents in allergic diseases marks a transformative shift in immunological intervention, harnessing their natural capacity to modulate immune responses with precision. Unlike traditional treatments, EVs offer a biocompatible platform to deliver bioactive molecules, presenting a novel paradigm for addressing the complex interplay of inflammation and immune dysregulation in conditions such as asthma, allergic rhinitis and atopic dermatitis. A pivotal study by Fang et al., showcased the anti-inflammatory powers of mesenchymal stem cell (MSC)-derived EVs, which, when administered in a murine asthma model, curtailed lung inflammation by limiting inflammatory cell infiltration and altering alveolar macrophage polarisation, thereby mitigating airway hyperresponsiveness ([242]). This underscores the potential of EV-based therapies to target key inflammatory pathways with minimal systemic toxicity. The therapeutic mechanisms of EVs in allergy hinge on their ability to fine-tune immune cell activity through sophisticated cargo delivery. A recent study explored the innovative use of intranasally administered bone marrow-derived mast cell EVs (BMMC-EVs) loaded with *Dermatophagoides farinae* extracts (BMMC-EVs-DfE) in a murine asthma model, demonstrating their ability to reduce allergic airway inflammation by decreasing BALF cell counts (including macrophages and eosinophils), attenuating goblet cell hyperplasia and MUC5AC expression, suppressing DfE-specific IgE and IgG3 antibodies. This

also increased IgG1 and IgG2a antibodies, lowered histamine levels and shifted the Th1/Th2 balance towards Th1 by elevating IFN- γ and reducing IL-4, thus offering a promising EV-based platform for allergen delivery and tolerance induction [243]. Furthermore, a study by Zhou et al., demonstrated that EVs from commensal skin microbiota, *Staphylococcus epidermidis*, when applied topically in an atopic dermatitis mouse model, restored skin homeostasis by suppressing pro-inflammatory cytokine release (e.g., IL-6, TNF- α), highlighting the role of microbial EV proteins in modulating local immune tolerance [244]. The versatility of EVs extends to their adaptability as allergen delivery systems, enhancing immunotherapy efficacy. A notable investigation by Asadirad et al., evaluated the immunomodulatory and delivery potential of adipose tissue-isolated MSC-derived exosomes as a prophylactic regimen through a sublingual route in an OVA-induced allergic asthma murine model, exhibiting significant reduction in IgE levels and IL-4 production, alongside elevated TGF- β levels, while showing limited cellular infiltration, ultimately modulating immune responses and inhibiting allergic OVA sensitisation, offering a safer alternative to traditional allergen immunotherapy [245]. The biogenesis of these therapeutic EVs involves ESCRT machinery and lipid raft interactions, ensuring efficient cargo encapsulation and release, a process that can be optimised to enhance therapeutic outcomes. Environmental and microbial influences further enrich the therapeutic landscape of EVs. For instance, bacterial EVs derived from *Lactobacillus plantarum* could alleviate skin inflammation by reducing IL-4 cytokines in the atopic dermatitis mouse model [246]. Similarly, bacterial EVs from *Bifidobacterium longum* could decrease the incidence of diarrhoea in food allergy by causing apoptosis in mast cells [247]. These findings challenge the established narrative that prioritises synthetic drugs, advocating instead for nature-derived EV therapies that align with the body's intrinsic regulatory networks. Although EVs hold considerable promise as therapeutic agents in allergic diseases, their clinical translation faces several important disadvantages and challenges that must be carefully addressed. One of the most significant issues is reproducibility and batch-to-batch variation. EV production depends heavily on the source cells, culture conditions, passage number, and isolation method. Even minor changes in pH, oxygen levels, cell confluency, or the use of serum-free media can lead

to substantial differences in EV yield, size distribution, protein, lipid, and RNA cargo, as well as functional potency. This makes it difficult to achieve the consistency required for GMP-compliant manufacturing of clinical-grade products. EVs are also intrinsically heterogeneous, encompassing different subtypes (exosomes, microvesicles, apoptotic bodies) with varying cargo that depends on the cell state, stimulation, and harvest time. This heterogeneity can result in unpredictable therapeutic effects, as EVs may carry both beneficial anti-inflammatory components and potentially harmful molecules such as pro-inflammatory cytokines or DAMPs. Scalability and manufacturing pose further hurdles: current isolation techniques, such as ultracentrifugation, size-exclusion chromatography, and tangential flow filtration, are labour-intensive, low-throughput, and costly at clinical scale. Achieving high yield and purity while preserving bioactivity remains technically demanding, and additional requirements for sterility, endotoxin removal, and cytokine clearance add complexity and expense.

1.5. Hypothesis and Aims of the Study

The aim of my PhD was to determine the role of extracellular vesicles in allergic sensitisation. This required the development of a new assay to show the effects of dendritic cell-derived EVs on naïve T cells. The proposed hypothesis of my PhD was that allergen-matured dendritic cell EVs could influence the shift of naïve T cells into Th2 cells. The proposed mechanism of how extracellular vesicles could affect the naïve T cells is shown in figure 1.3.

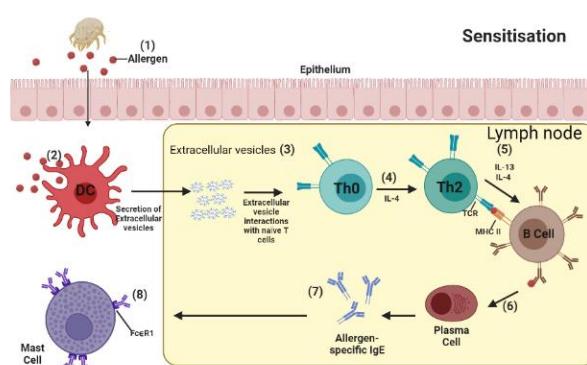


Figure 1.3 – Extracellular vesicles in allergic sensitization. Once the allergen crosses the epithelial barrier, DCs internalize the allergen and release EVs carrying the allergen. EVs interact with naïve T cells and influence them to shift towards Th2 cells and activates them. Th2 cells then present the allergen to B cells, which transform into antibody producing plasma cells. The Abs are deposited onto the surface of mast cells.

Chapter 2: Materials and methods

2.1. Materials

2.1.1. Culture media

Roswell Park Memorial Institute (RPMI)-1640 culture media containing 2mM L-glutamine (Cat no. R6504, Merck, UK), X-VIVO 15 Serum-free Hematopoietic cell media containing L-Glutamine (Cat no. 02-060Q, Lonza, UK), ImmunoCult-ACF Dendritic Cell media (Cat no. 10987, StemCell, UK) and StemXVivo serum-free dendritic cell media (Cat no. CCM003, R&D systems, UK) were used for monocyte growth and differentiation to DCs. AIM-V culture media containing L-glutamine, streptomycin sulfate at 50 µg/ml, and gentamicin sulfate (Cat no. 12055083, Thermofisher, UK) at 10 µg/ml was used for T cell assays. All medias were supplemented with Penicillin (100 IU)-Streptomycin (100µg/ml) (Cat no. P0781, Merck, UK) and 10 mM HEPES (Cat no. H0887, Merck, UK).

2.1.2. Human blood samples

Ethical approval was obtained from the Medical School Ethics committee. Healthy donors over 18 were recruited by poster advertisement and, before blood collection, informed consent was given. Ethical approval for allergic individuals was obtained from (FMHS REC ref 121-1706). The allergic donors were recruited by Cripps Health centre by contacting the allergic individuals directly either by email or text message. The eligible donors were then booked for an appointment at Cripps Health Centre for blood donation and informed consent was given by the participants by signing the consent form. It was required of the participants to come back after 1 week for a 2nd blood donation. Blood was collected in heparin-coated vacutainers (Cat no. 455051, Greiner Bio-One, UK) at The Queen's Medical Centre (QMC) or Cripps Health Centre. Blood samples were then transported to the tissue culture lab for use in cell assays. The PBMCs were isolated in two separate ways, one using SepMate 50 (Stemcell Technologies, UK) and the other method by using Histopaque (Cat no. 10771, SLS, UK) and layering the blood directly on the surface. Before isolation, the blood was diluted with Sterile Dulbecco's phosphate buffered saline (PBS) (Cat no. D8537, Merck, UK).

2.1.3. Immunofluorescent staining for flow cytometry

The University of Nottingham's Flow Facility provided access to flow cytometers. Initially, as the scale of the flow panels were smaller, the BD CANTO 2 (Beckman Coulter, UK) and Cytoflex (Beckman Coulter, UK) were utilized. In the 3rd year the flow panel was expanded, resulting in using the ID7000 (Sony, UK) spectral cytometer for DC (table 2.1) and T cell (table 2.2) marker expression. The ImageStreamX Mk2 (Luminex Corporation) was used to image DC and T cell interactions, in addition measuring the EVs stained with calcein-AM and tetraspanins (table 2.3).

DC assay optimization required the use of Annexin V and PI staining kit for cell viability (Cat no. 130-092-052, Miltenyi, UK).

Extracellular and intracellular staining required washing of cells to remove excess antibodies with phosphate buffer albumin (PBA). PBA was made in-house by adding bovine serum albumin (BSA) (Cat no. A7284, Merck, UK) and Sodium Azide (Cat no. S2002, Merck, UK) to PBS. The PBS was made by dissolving PBS tablets (Cat no. BR0014G, Thermofisher, UK) in deionized water. The solution was sterile-filtered and stored at 4C.

Fixing of cells was done by utilizing fixation buffer purchased from Biolegend (Cat no. 420801, UK). In order to permeabilize the cells, a permeabilization buffer was purchased from Thermofisher (Cat no. 00-8333-56, UK).

Cytokine staining requires the use of protein transport inhibitor cocktail 10x (Thermofisher, UK).

Table 2.1 – Dendritic cell flow panel. Antibodies used for DC marker expression with their fluorophore, brightness, company bought from.

Antibody	Fluorophore	Brightness	Company	Cat No.
CD14	PerCP	1	Biolegend	325632
CD80	FITC	3	Biolegend	305206
CD83	BV605	4	Biolegend	305336

CD86	PE	5	Biolegend	374206
CD40	BV421	5	Biolegend	334332
HLA-DR	PE/Cy5	5	Biolegend	307608
CD209	PE/Cy7	4	Biolegend	330114
Zombie	NIR	4	Biolegend	423106

Table 2.2 - T cell marker expression. Antibodies used for T cell marker expression with their fluorophore, brightness, company bought from.

Antibody	Fluorophore	Brightness	Company	Cat No.
CD3	PerCP-CY5.5	3	Biolegend	344808
CD4	APC/Fire 750	2	Biolegend	344638
CD8	AF700	2	Biolegend	301028
CD56	BV785	4	Biolegend	362550
CD69	PE/Fire640	4	Biolegend	310960
CD27	PE-Cy7	4	Biolegend	302838
CD45RA	AF488	3	Biolegend	304114
CCR7	BV421	5	Biolegend	353208
CD28	PE/Dazzle 594	5	Biolegend	302942
CD294	BV711	5	Biolegend	350124
Zombie	Aqua	2	Biolegend	423102
TNF-a	PE-eFluor610	4	Thermofisher	61-7349-42
IL-2	PE	5	Biolegend	500307
IFN-g	APC	4	BD Bioscience	554702

IL-4	BV605	4	Biolegend	500828
------	-------	---	-----------	--------

Table 2.3 - EV imagestreamX flow panel. Antibodies used for EV identification with their fluorophore, brightness, company bought from.

Antibody	Fluorophore	Brightness	Company	Cat No.
CD81	APC	5	Miltenyi Biotec	130-119- 787
CD63	PE	5	Miltenyi Biotec	130-118- 077
CD9	Vioblue	4	Miltenyi Biotec	130-118- 808
Calcein-AM	FITC	4	Biolegend	425201

2.1.4. Immunomagnetic Cell Separation

Performing immunomagnetic separation of cells required a MACS Multistand (Cat no. 130-042-303, Miltenyi, UK) with a QuadroMACS separator (Cat no. 130-090-976). MACS buffer was necessary for cell isolation, which was made by mixing 30% BSA, 2mM Ethylenediaminetetraacetic acid (EDTA) (Merck, UK) and sterile PBS.

Positive isolation of CD14⁺ monocytes was done by utilizing the CD14 UltraPure Microbeads (Cat no. 130-118-906, Miltenyi, UK). Negative isolation of naïve T cells was done by utilizing Naive CD4⁺ T Cell Isolation Kit II (Cat no. 130-094-131, Miltenyi, UK), which required a two-step process to isolate naïve T cells. The PBMCs were stained with biotinylated CD45RO, CD8,

CD14, CD15, CD16, CD19, CD25, CD34, CD36, CD56, CD123, anti-TCR γ/δ , anti-HLA-DR, and CD235a, subsequently Anti-Biotin Microbeads were then added for depletion of these cells.

2.1.5. Allergen

For stimulation of dendritic cells with an allergen, 50mg of OVA (Cat no. A7641) was bought from Sigma-Aldrich, UK.

2.1.6. T cell stimulation

Anti-CD3 2 μ g/mL (Cat no. SAB4700040-10, Sigma-Aldrich, UK) and anti-CD28 2 μ g/mL (Cat no. 40-0289-U500, Tonbo Bioscience, UK) were used to stimulate T cells.

2.1.7. Extracellular vesicles isolation and concentration

In order to perform size-exclusion chromatography (SEC) the Automatic Fraction Collector (AFC) V2 was bought from Izon and qEV columns. After SEC it was necessary to concentrate the EV solution, where Amicon Ultra-15 10 kDA concentration columns were utilized (Cat no. UFC801096D, Merck, UK).

2.1.8. Nano-tracking analysis

Nano-tracking analysis (NTA) was performed on solutions containing EVs in order to confirm their sizes and presence. NTA was considered as the gold standard for sizing EVs. The ZetaView (Particle Matrix) was used for NTA.

2.1.9. Super-resolution microscopy

Super-resolution microscopy was done utilizing ONI Nanoimager S mark III (ONI, Oxford, UK). ONI EV Profiler 2 kit and ONI assay-chip (ONI, Oxford, UK) was necessary to stain EVs. CODI software was used to analyse the data.

2.1.10. Enzyme-Linked Immunosorbent Assay

It was necessary to perform Enzyme-Linked Immunosorbent Assay (ELISA) to evaluate the presence of cytokines after SEC. IL-6 and IL-8 ELISA kits were bought from R&D systems.

2.2. Methods

2.2.1. PBMC isolation

50ml blood samples were mixed with Phosphate Buffered Saline (PBS) + 2% Fetal Bovine Serum (FBS) as a 1:1 ratio. 17 ml of diluted sample was slowly pipetted down the side of each SepMate tube containing 15 ml of Histopaque. SepMate tubes were centrifuged at 1200g (Eppendorf centrifuge 5920 R) for 10 minutes with 3 acceleration (3 up) and 3 decelerations (3 down). The PBMCs 'Buffy' layer was pipetted off in a separate tube. Tubes were then topped up with PBS + 2% FBS and gently mixed, then washed at 300g 3 up and 3 down for 8 minutes (Hettich zentrifugen Rotina 46). Supernatant was then poured off, cells were gently tapped to resuspend, and 15 ml of cell culture medium was added. A cell count was performed, and then cells were washed again at 300 g for 5 min, 3 up and 3 down, and resuspended at a concentration of 2 million cells per ml in cell culture media.

The other method of separating PBMCs was as follows; the blood sample were diluted 2:1 in cell culture medium. In a different tube, histopaque was added according to the blood mixture volume, 5 ml of Histopaque per 15 ml of the mix. Diluted blood was gently layered onto the histopaque, followed by centrifugation at 800 g for 22 min at 1 up and 0 down. The PBMCs were collected from the 'buffy' layer into a new tube, topped up with culture medium, and then washed at 300 g for 10 min 3 up and 3 down. Supernatant was poured off, and the cell pellet gently resuspended. Culture medium was added based on 1:3 ratio with original blood volume. Cell count was performed, and cells were washed in a centrifuge for 5 min at 300g, 3 up and 3 down. The centrifuged tube was emptied of supernatant and resuspended at 2 million cells per ml in cell culture media.

2.2.2. Monocyte isolation

After PBMC isolation had been performed, cells were re-suspended in MACS buffer, and anti-CD14 microbeads were added. The cells were incubated in the fridge at 4°C for 15 min, followed by a wash with MACS buffer at 300g for 10 min. Cells were re-suspended in 500 µl of MACS buffer, and run through LS column by adding 3ml of MACS buffer 3 times to collect CD14⁻ cells. After the last wash 5ml of MACS buffer was added and plunged through the LS column to collect CD14⁺ cells. CD14⁺ cells were centrifuged at 300g for 5 min, followed by re-suspension in cell culture media at 0.5 million/cells per ml. To test the optimal culture medium for dendritic cell maturation the following cell culture mediums were tested: RPMI-1640 (supplemented with 10 mM HEPES, 100 IU/ml penicillin, 100 mg/ml streptomycin and 10% FBS or 10% of exosome-depleted FBS), X-VIVO™ 15 Serum-free Hematopoietic Cell Medium (Lonza), ImmunoCult™-XF T Cell Expansion Medium (Stemcell), AIM V™ serum-free medium (ThermoFisher). Prior to isolation, all MACS columns, MACS separators and MACS buffers were kept in the fridge to keep cool.

2.2.3. Dendritic cell culture

After monocyte isolation had been performed the cells were plated in 24-well tissue culture plates at 1×10^6 /ml concentration. The monocytes were then stimulated with 50 ng/ml of GM-CSF and 20 ng of IL-4 and incubated for 3 days at 37 °C. After 3 days 500 µl of media was replaced with fresh media and re-adding the cytokines at 25 ng/ml of GM-CSF and 10 ng of IL-4 and incubated for 3 more days. To ensure that the monocytes had become immature DCs, cells were removed from the well by pipetting 200 and staining was performed. Fluorescently conjugated antibodies against CD80, CD83, CD86, CD40, HLA-DR, CD209 and CD14 (table 2.1) were added following the method described below (2.2.5) antibody staining procedure and ran on a spectral flow cytometer. The remaining cells were placed in a new 24 well tissue culture plate with fresh media and matured by either adding LPS at 100 ng/ml or OVA at 10 µg/ml and

incubated for either 24h or 48h at 37°C (Figure 2.1). Method was adapted from previously published work in the lab ([248];[249])

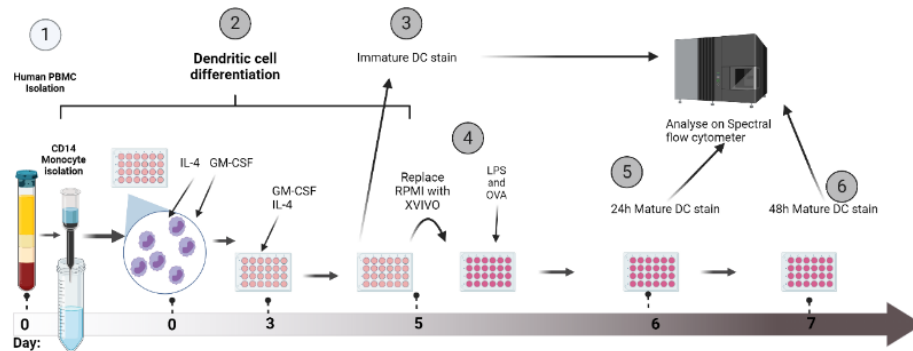


Figure 2.1 - Dendritic cell expansion. Human PBMC isolation and CD14⁺ monocyte isolation was performed, and cells were plated and stimulated with 20ng/ml of IL-4 and 50ng/ml of GM-CSF (1). Monocytes differentiated to immature dendritic cells over a 5-day period, refreshing the media and cytokines on day 3 (2). Immature dendritic cells were then stained to confirm the differentiation, and the remaining cells were plated with fresh X-VIVO media and stimulated with LPS and/or OVA (4). After maturing DCs, staining was performed to evaluate the maturation after 24h (5) and 48h (6).

2.2.4. Cell count

It was necessary to perform cell counts after isolation to ensure that the correct cell concentration was achieved. In the beginning of my PhD this was done by manually counting the cells under the microscope. This required 20µl of cell suspension mixing with 20µl of trypan Blue making it 1:1 ratio. 10-20µl of the mix was then dispensed onto a haemocytometer covered with a coverslip. Using a microscope the 3 out of 4 grids were counted and the cell concentration was calculated using the following equations:

$$\text{Cells/mL} = \text{Average No. of cells counted} \times \text{dilution factor} \times 1 \times 10^4$$

$$\text{Total cells} = \text{Cells/mL} \times \text{volume of cell suspension}$$

Once an automated cell counting machine was purchased, the Countess 3 Automated Cell Counter (Thermofisher, UK) was used to perform all cell counts. This was done by mixing 10µl of cell suspension and 10µl of trypan Blue, followed by pipetting 10µl of the mix into a countess disposable slide. The slide was inserted into the Countess 3, then the appropriate brightness and focus of the cells was selected, following the manufacturer's instructions.

2.2.5. Immunofluorescent staining for flow cytometry

2.2.5.1 Extracellular staining

To perform extracellular cell staining, the cells were pipetted into FACS tubes and 1 ml of PBA was added to each tube and then washed at 300g for 5 min, 3 up and 3 down. The supernatant was discarded, and the cell pellet was resuspended by tapping the tube. Antibodies were then added to the FACS tubes and incubated at 4°C for 30 min. After the incubation, 2 ml of PBA was added to FACS tubes and then washed at 300g for 5 min, 3 up and 3 down. Supernatants were discarded, and then 300 µl of fixation buffer was added to all the FACS tubes. The samples were then analysed by flow cytometry or stained for intracellular markers (see below). Flow results were analysed in Kaluza software.

2.2.5.2 Intracellular staining

To perform intracellular cytokine staining, 1x protein transport inhibitor cocktail was added to the cells for 14h at 37°C before cell harvesting. After extracellular staining was completed (as above), the cells were fixed for 30 min at 4°C with 500µl of fixation buffer. In order to wash off the fixation buffer, the cells were centrifuged for 5 min at 300g 9up and 9down. The supernatant was discarded, and the cells were resuspended by gently tapping the bottom of the FACS tubes. To permeabilize the cells it was necessary to add 2ml of 1x permeabilization buffer and incubate for 30 min at 4°C. The permeabilization buffer was washed off by centrifugation for 5 min at 300g 9up and 9down, followed by aspirating the supernatant and resuspending the cells by tapping. Antibodies were then added to the samples and incubated for 30 min at room temperature, in the dark. Cells were then washed twice by adding 2ml of PBA and centrifuged at 500g for 5 min, discarding the supernatant each time and tapping to resuspend. Finally, 300µl of fixation was added and the sample was analysed by spectral flow cytometry.

2.2.6. Annexin and Propidium Iodide (PI) staining

Annexin and PI kit was purchased from Miltenyi Biotec. The manufacturer's protocol was followed. Briefly, to make up 1x Binding Buffer, 1 ml of 10x Binding Buffer was added in 9 ml of deionized water. The cells

were first washed in 1 ml of 1x Binding Buffer per 10^6 cells in a centrifuge at 300g for 10 min. Supernatant was aspirated completely. Cells were then resuspended in 100 μ l of 1x Binding Buffer per 10^6 cells. 10 μ l of Annexin V-FITC was added per 10^6 cells, mixed, and then incubated for 15 min in the dark at room temperature. Cells were then washed by adding 1 ml of 1x Binding Buffer per 10^6 cells and centrifuged for 10 min at 300g, and supernatant aspirated completely. The cell pellet was then resuspended in 300 μ l of 1x Binding Buffer per 10^6 cells and 0.33 μ l of PI solution was added immediately prior to analysis on the flow cytometer.

2.2.7. T cell activation

96 well cell culture plates were coated with anti-CD3 by adding 100 μ l of 2 μ g/ml of anti-CD3 diluted in PBS in each well and incubated for 90 min at 37°C. After the incubation, the 96 well plates were washed 2 times using cold PBS, 1st time 100 μ l per well, 2nd time 200 μ l per well. Once the plates were washed, cell suspension was added to each well. Soluble anti-CD28 was added directly to all the wells at 2 μ g/ml final concentration. The plate then was incubated at 37°C.

2.2.8. Extracellular vesicle isolation

EVs were isolated by size-exclusion chromatography (SEC) utilizing automatic fraction collector (AFC). Prior to SEC, it was necessary to discard the cells, and their debris. This was achieved by centrifuging the cell suspension at 300g for 8 min, and the supernatant collected without disturbing the cell pellet. SEC was performed following the manufactures' instructions. Briefly, the qEVoriginal IZON column was inserted into the AFC machine and washed with sterile PBS. The excess PBS on the top of the column was discarded with a Pasteur's pipette. The supernatant containing EVs was then pipetted into the column, after the solution had passed through the 1st layer, PBS was added to ensure that the column did not dry. AFC then automatically collects the desired fractions of EVs after they have passed through the entirety of the qEVoriginal column. Fractions from 1 to 7 were pooled and added to Amicon Ultra-15 concentrations tubes and centrifuged at 4000g for 10 min in

order to concentrate the EVs. The solution containing EVs were then transferred to an Eppendorf for freezing, staining or added to naïve T cells.

2.2.9. Enzyme-Linked Immunosorbent Assay

After SEC had been performed, each of the fractions (1 to 7) were collected and used in ELISA to evaluate the effectiveness of SEC clearing capabilities of cytokines from EV solutions.

96-well plates were coated with IL-6 and IL-8 capture antibodies by adding 100µl of coating solution in each well, ensuring that the solution covers the bottom of each well evenly. The plate was then incubated overnight at 4 °C to allow the capture antibodies to adsorb on the bottom of the wells. After incubation the coating solution was discarded, and the wells were washed with PBS+0.05%Tween20. In order to prevent non-specific binding of proteins to the plate, the plates were blocked with blocking buffer for 1h. After the blocking of the plate, the blocking buffer was removed, and the wells were washed with PBS+0.05%Tween20. The sample was then added to the wells and incubated for 2h at room temperature, to allow the cytokines to bind to the capture antibodies. The wells were then washed with PBS+0.05%Tween20 to remove unbound substances and minimize the background noise. Horseradish peroxidase (HRP) Streptavidin was added to the wells and incubated for 1h at room temperature. The wells were washed again. 3,3',5,5' tetramethylbenzidine (TMB) was added to the wells to initiate a reaction with HRP and allowed to incubate for 20 min in the dark at room temperature. The stop solution (Sulfuric acid) was added to cease the reactions between HRP and TMB. The plate was then inserted into a microplate reader to measure the absorbance at 450nm and 570nm. The results from the standards were then plotted in a standard curve. Utilizing the standard curve, it was possible to calculate the concentrations of IL-6 and IL-8 in the solution.

2.2.10. TEM and confocal microscope preparation

To confirm presence of DC-generated EVs, samples were stained with either calcein-AM or the lipid dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt (DID). Calcein-

AM was prepared according to the manufacturer's instructions. Briefly, 50 μ l of DMSO was added to 50 μ g of calcein-AM resulting in 500 μ g/ml, and vortexed until calcein-AM was fully dissolved. Cells were removed from the samples by centrifugation, and EVs isolated utilizing size exclusion columns collecting particles from 35nm to 350nm size. EVs were then fixed in 3% glutaraldehyde solution in cacodylate buffer for 30 minutes. 10ul of sample was added to poly-L-Lysine treated carbon film slot grids (EM resolutions) and left to settle for 15 minutes. Samples were then washed twice with ddH₂O and stained with 1% uranyl acetate for 5 minutes, excess was removed using blotting paper. TEM was carried out using a Tecnai Biotwin-12 with an accelerating voltage of 100 kV. In TEM EVs appeared to be cup-shaped, collapsed due to being dried during the preparation of the sample for TEM. Differentiating EVs from other particles was done by looking for curved, darker inner circle suggesting that there was a membrane present before it was dried.

2.2.11. Nano-tracking analysis

Confirming the presence of DC-generated EVs, nano-tracking analysis was performed by utilizing ZetaView. NTA was done on samples undergone SEC or after the cells were span out. The ZetaView requires calibration before samples can be run on the machine. Once the machine was turned on and allowed to heat up to its operational temperature, the flow cell was flushed through with particle-free water thoroughly to remove any debris or leftover particles from previous runs. The machine then requires calibration, which was done by utilizing 100nm polystyrene beads. The EV samples were then diluted in PBS and utilizing a syringe loaded into the ZetaView. The machine then auto-aligns and auto-focuses the EVs in the sample. ZetaView then determines the optimal sensitivity before running the samples. The data was then collected and analysed by ZetaView software. After analysis, the flow cell was flushed thoroughly with particle-free water in order to clean it.

2.2.12. Extracellular vesicles staining

To confirm presence of EVs using flow cytometry, the calcein-AM in DMSO solution in varying concentrations (0.1, 0.2, 1, 2, 10, 20, 100, and

200 μ M) was added to the samples containing EVs and incubated at 37C for 1h. Antibodies against three tetraspanins, namely CD9, CD63 and CD81, were added after 30 min of incubation with calcein-AM, for the final 30 min incubation at 37C. In order to reduce the high concentrations of EVs in the solution, the samples were diluted in PBS to guarantee smooth running onto the imaging flow cytometer. The EVs were then analysed using ImageStreamX and IDEAS application.

2.2.13. Super-resolution microscopy

DCs were cultured as above, and stimulated with 100ng/ml LPS to produce EVs. The EVs were isolated using SEC and concentrated utilizing 10kDa Amicon concentration columns. To stain EVs for ONI nanoimager, the ONI EV Profiler 2 kit was used with anti-CD9 AF488, anti-CD63 AF568 and anti-CD81 AF647, these antibodies were diluted in 1:100 in staining buffer. 10 μ l of EV solution was mixed with the antibody mixture for 50 min at room temperature with gentle rocking. The excess antibodies were washed by centrifugation. 20 μ l of fixation buffer was used to stabilize the labeled EVs for 5 min and washed again. It was necessary to prepare an ONI assay chip by applying 10 μ l of surface reagent to each lane, incubating for 15 min with rocking, then washing with wash buffer. Then 10 μ l of capture reagent was applied for 15 min. 10 μ l of stained EVs was added to the chip and incubated for 75 min, followed by washing unbound EVs with wash buffer, and applying 20 μ l of fixation buffer for 10 min. The chip was then ran on ONI nanoimager S for analysis. The data was analyzed using company's software CODI.

2.2.14. Statistical analysis

All statistical analyses were performed using GraphPad Prism p-values <0.05 were considered significant for experiments. The majority of the tests used were Two-way ANOVA's and mixed effects analysis, involving Tukey's multiple comparison tests. The tests used for specific data are detailed throughout the results sections.

Chapter 3: Systematic review ‘The role of extracellular vesicles in allergic sensitization’

The systematic review presented in Chapter 3 was undertaken to situate the current PhD research within the broader scientific landscape of allergic diseases and the emerging role of EVs in immune regulation, specifically allergy. By systematically evaluating the existing literature, the review aimed to establish a solid conceptual foundation, identify established methods for EV isolation, characterisation, and functional analysis, and highlight key gaps that the subsequent experimental work could address. This step was essential for several reasons. Allergic diseases, including food allergies, asthma, and atopic dermatitis, represent a growing global health burden, with complex and incompletely understood mechanisms of sensitisation and chronicity. EVs have only recently been recognised as potent mediators of intercellular communication in immunity, capable of transferring allergens, cytokines, miRNAs, and other bioactive molecules that can either promote or suppress type 2 inflammatory responses. A comprehensive review was therefore necessary to clarify the current state of knowledge, map the dual (pro- and anti-allergic) roles attributed to EVs, and delineate the predominant routes (airway, skin, gastrointestinal) and cellular sources (epithelial cells, dendritic cells, mast cells) studied to date. Furthermore, the review allowed critical appraisal of methodological heterogeneity in the field. Many earlier studies employed non-standardised isolation protocols, inconsistent characterisation (often lacking MISEV compliance), and predominantly murine models, limiting generalisability to human allergy. By summarising these limitations alongside the most robust findings, the review provided a clear rationale for the methodological choices made in Chapters 4–6, particularly the emphasis on human primary cells, MISEV-compliant isolation (size-exclusion chromatography), and multi-modal characterisation (NTA, super-resolution microscopy, flow cytometry).

3.1. Introduction

Atopic allergies, also known as immediate or type I hypersensitivities, affect between 10% and 30% of the population [[250]]. Allergies affect people's quality of life, impacting their work and personal lives; in addition, the treatments for allergies bear a heavy burden on healthcare systems across the globe. It has been reported that it costs the NHS over GBP one billion per annum [251]. However, the underlying mechanism of how an allergy develops is still poorly understood. The most frequent clinical manifestations of allergies are asthma and allergic rhinitis, but other atopic diseases include urticaria and anaphylactic shock.

Several factors can impact whether someone develops atopic allergy, such as environmental exposure, genetic predispositions or lifestyle factors, such as a Westernized diet, low vitamin D intake, antibiotic use, and the route of birth; all these form an intricate balance that determines the development of type 1 hypersensitivity [[252]].

Allergic sensitization, the first phase of atopic allergy development, is central to the initiation of atopic diseases. Typically, proteins within allergen sources initiate allergic sensitization through recognition by dendritic cells (DCs) and presentation to naïve T cells, which results in a cascade of reactions shifting the response in a Th2-dominant manner. Dendritic cells can be classified into four subsets—human plasmacytoid DC (pDC), conventional DC1 (cDC1), conventional DC2 (cDC2) and monocyte-derived DC (mo-DC) [[253]].

Subsequently, allergen-specific immunoglobulin E (IgE) is bound on mast cells through the FcεRI receptor. Conversely, naïve T cells can differentiate into Th1 or Treg cells, resulting in immune tolerance, which normally happens in healthy individuals. Recent research has suggested other components can drive allergic sensitization, such as lipids [[254] [255]], carbohydrates [256], and most recently, EVs, which can, in fact, also carry proteins, lipids and carbohydrates [[257] [258]]. Further information of allergies have been provided in Chapter 1 'Introduction'.

EVs are small lipid-bilayer particles that are released naturally from cells within the body and by other sources, such as bacteria. They are key inter-cellular communicators, and their size can range from 20 nm to 10 µm [[259]]. EVs can transport proteins, nucleic acids (deoxyribonucleic acid (DNA),

messenger ribonucleic acid (mRNA), and non-coding RNA), lipids, metabolites and specific cell surface markers depending on the cells from which they originate [[260]]. They influence recipient cells due to their capacity to transfer this ‘cargo’ [[261]]. When discriminating between EVs, their origin is considered; if they develop internally and are then released through the plasma membrane, they are called exosomes, where their size is usually smaller than 200 nm. If they bud off from the plasma membrane, they are called ectosomes, where their size is 100 nm to 10 µm. The recommendations for carrying out research into EVs are formulated in Minimal Information For Studies of Extracellular Vesicles (MISEV) 2023 [[262]]. Importantly, EVs and their contents represent the cell they come from, as well as the current condition of the cell [[263]]. Due to their ability to reflect the condition of their cellular origin, they are often used as biomarkers for diseases; however, studies have also examined their involvement in the pathogenesis of diseases [[263]]. Indeed, recent studies have been trying to understand the role of EVs in allergy. For example, in a study done by Fang et al. in 2020, it was found that by utilizing human mesenchymal stem cell (MSC) EVs, it was possible to alleviate inflammation in the lungs by restricting the infiltration of inflammatory cells and epithelial goblet cells by changing recruitment and polarization of the alveolar macrophages [[242]]. In asthma, EVs can be used as a diagnostic tool between healthy and asthmatic patients, and EVs can promote inflammation and airway remodeling, suggesting EVs also play a role in the pathogenesis of asthma, which has been systematically reviewed in a study done by Sangaphunchai et al., 2020 [[264]]. In this systematic review, the focus is on the role of EVs in allergic sensitization. The role of EVs generated from the host (termed Host-derived EVs) and from exogenous sources, such as bacteria (termed exogenous EVs), is discussed, and whether these EVs induce sensitization or tolerance.

3.2 Materials and methods

3.2.1 Search strategy

This review was conducted with the implementation of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. The search strategy was constructed around two key terms:

‘extracellular vesicles’ and ‘allergy’. In this review, two databases were searched to find relevant papers: PubMed and Embase, from their inception to 31 December 2023, using the following search terms:

PubMed—(exosomes or exosome* or extracellular vesicle* or ectosome or microparticle or microvesicle or shedding vesicle*) AND (allergic sens* or allergic sensitization or type 1 hypersensitivity or allergy)

EMBASE—(exosomes or exosome* or extracellular vesicle* or ectosome or microparticle or microvesicle or shedding vesicle*) AND (allergic sens* or allergic sensitization or type 1 hypersensitivity or allergy).mp. [mp = title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word].

3.2.2 Selection of studies

This systematic review aimed to examine the evidence on the role and function of EVs in allergic sensitization, with the hypothesis that EVs from various origins contribute to the development of allergy. For this review, other reviews, books, editorials, and conference proceedings were excluded, language was restricted to English, and there was no restriction on the date of publication. Publications were screened for their relevance against pre-defined inclusion and exclusion criteria (Table 3.1), and a random subset (10%) of these studies was reviewed independently by a second reviewer.

Table 3.1 Selection criteria for papers included in the systematic review.

Inclusion	Exclusion
IgE-mediated food allergy	Asthma
IgE-mediate inhalant allergy	Non-IgE-mediated allergies
Allergic sensitisation	Non-English language publications
English language	Elicitation phase
Full-text available	Reviews
Experimental data	Conference abstracts
Healthy subjects	Contact hypersensitivity
Allergic subjects	Delayed hypersensitivity
Human models	Artificially created EVs
Animal models	EVs used as biomarkers

3.2.3. Study Quality Assessment

To assess the robustness of the studies included in this systematic review and to highlight the current research methodology in this field, a scoring system was developed, with specific criteria set in Table 3.2. Each study's score was assessed by the criteria: model, robustness of model, sample size, EV isolation, EV characterization, and sensitization method. Scores from the studies were summed and divided by the highest possible total score to calculate the study bias score. Studies that received lower scores are not less reliable but are missing key methods in researching EVs, such as ensuring pure and intact EV populations through isolation.

Table 3.2 Scoring system to assess the included studies in this systematic review.

Category	Reasoning for scores
Model	If Multiple models are used, then a combined score is given - Human (Ex vivo) (4) - Cell Culture (In vitro - human) (3) - Murine Model (In vivo) (2) -Cell Culture (In vitro - murine) (1)

Robustness of model	If Multiple models are used, then a combined score is given Human model (Ex vivo): Allergic Status not specified or not clearly defined (0) Allergic Patients were sought from a Clinical Setting (1) Allergic patients were sought from Clinical Setting AND had a positive testing to allergen (Specific IgE or Positive allergen Challenge) (2) Allergic patients were sought from a Clinical Setting with a healthy control group (3) Allergic patients were sought from Clinical Setting AND had a positive testing to allergen (Specific IgE or Positive allergen Challenge) with a healthy control group (4) Murine Model (In vivo): Sensitization of mice not specified or defined (0) Sensitization of mice partially defined (Discloses some but NOT all the following: exposure method, dosing, and duration) (1) Sensitization of mice fully defined outlining (exposure method, dosing, and duration) (2) Sensitization of mice partially defined (Discloses some but NOT all the following: exposure method, dosing, and duration) with a negative control group (3) Sensitization of mice fully defined outlining (exposure method, dosing, and duration) with a negative control group (4) Cell Culture (In vitro): If both used the greater Model score will be used. Immortalized Cell Line using partially defined exposures (Discloses some but NOT all the following: exposure method, dosing, and duration) (1) Immortalized Cell Line using fully defined exposures (exposure method, dosing, and duration) (2) Primary Cell Line using partially defined exposures (Discloses some but NOT all the following: exposure method, dosing, and duration) (3) Primary Cell Line using fully defined exposures (exposure method, dosing, and duration) (4)
---------------------	---

Sample size	If multiple models are used, then a combined score is given. Human model (Ex vivo): Number of participants not defined (0) 5 or less participants per group (1) 6 to 10 participants per group (2) 11 or more participants per group (3) Murine Model (In vivo): Number of mice per group not defined (0) 5 or less mice per group (1) 6 to 10 mice per group (2) 11 or more mice per group (3) Cell Culture (In vitro): n not specified (0) n = 1 (1) n = 3 (2) n > 3 (3)
EV isolation	No Isolation - precipitation only techniques Studies looking direct in a liquid with no isolation applied (usually done on very low volume samples). (0) Poor – ultra centrifugation at one speed or serial UC without sucrose cushion (1) Fair - size exclusion chromatography (without a precipitation or concentration step), Immuno-capture beads for investigation of non-specific populations (2) Good - Size exclusion chromatography with a precipitation or concentration step or the use of a specific isolation method such as exosome EV isolation, purification kits or Immuno-capture beads utilizing a unique marker. (3)
EV characterisation	No Characterization – No attempt made to profile or characterise EVs or Exosomes. (0) Poor – Use of Just one characterization technique (Quantification, sizing, biomarkers, cargo analysis) (1) Fair – The use of multiple complimentary characterization techniques and at least 1 biomarker. (2) Good – Everything mention prior as well as appropriate controls (E.g. robust profiling of culture conditions such as media, inclusion of positive and negative controls such as those recommended by MISEV2018) (3) Very Good – Everything mentioned in prior as well the addition of extra biomarkers (3+) or the utilization of any other novel techniques. (4)
Overall quality score	The combined score of the categories above divided by the highest possible score of depending on model used (24/31/37)

3.3 Results

3.3.1 PRISMA Search and Selection

A PRISMA diagram was used to track the search details for this systematic review (fig.3.1). The search resulted in 1356 papers: 1044 from PubMed and 312 from EMBASE. After the removal of duplicated publications, 1271 titles and abstracts of papers were examined for relevance. After the title and abstract review, 336 papers were kept as potential interest for the review. After selecting papers using the inclusion and exclusion criteria, 22 studies were included in the systematic review, which were relevant to understanding the role of EVs in allergic sensitization.

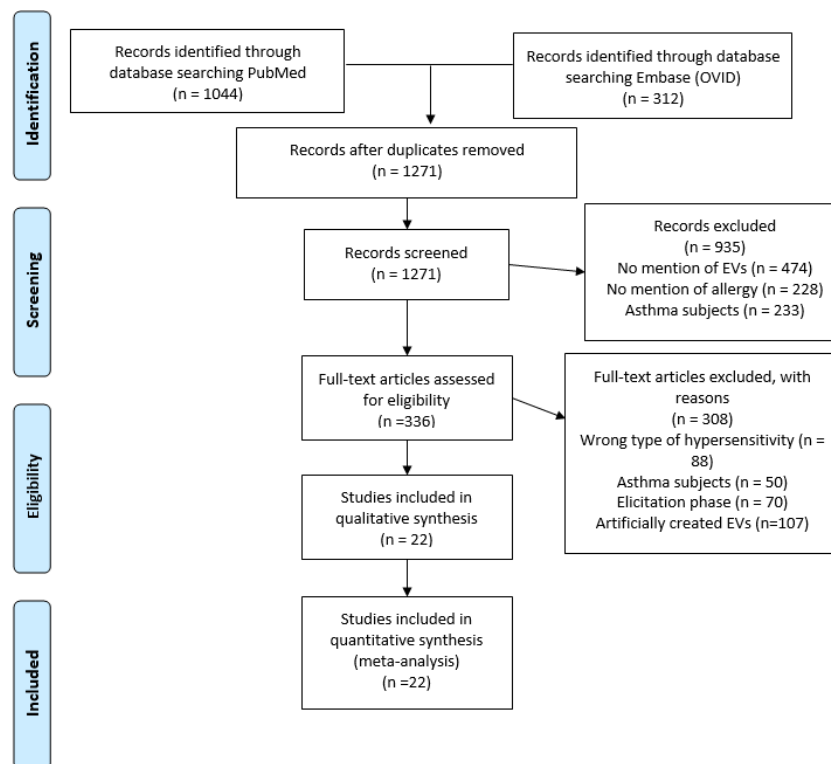


Figure 3.1 **PRISMA diagram of article search and selection.** A PRISMA diagram detailing the process of searching and selecting EVs studies in allergic sensitization, using PubMed and **Embase** (OVID) from 1974–2023.

The first publication relevant to EVs in allergic sensitization was published in 2007; however, 60% of the papers were published in the last 8 years (fig.3.2A).

The results highlight two main types of EVs based on their source of origin: EVs that were produced from the host (host-derived) or from external sources (exogenous), such as bacteria (fig.3.2B). Thus, the results will be discussed in

terms of host-derived or exogenous EVs and their potential to either promote allergic sensitization or the tolerance of allergens.

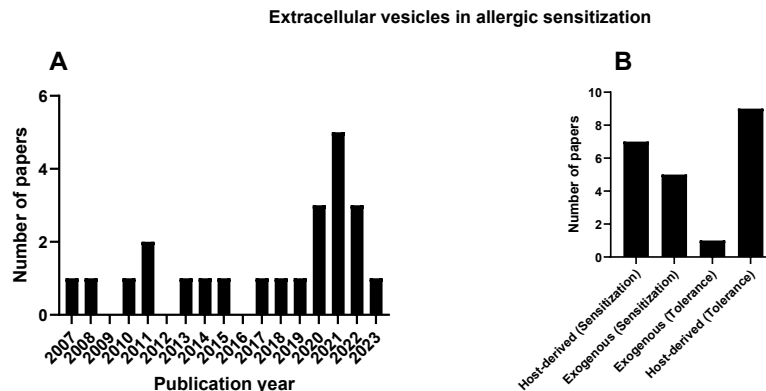


Figure 3.2 Publication on EVs in allergic sensitization through the years (A). Publications concerning the Host-derived EVs – sensitization and tolerance, and exogenous source EVs (B).

3.3.2 Host-derived Extracellular vesicles

3.3.2.1 Promotion of allergic sensitization

In the allergic sensitization process, naïve T cells differentiate into Th2 cells, resulting in interleukin 4 (IL-4) production and IgE deposition on mast cells, but what drives this shift to Th2 is poorly understood. Publications suggesting that host-derived EVs can promote Th2 differentiation and, thus, the sensitizations were identified and summarized in **Table 3**.

Seven studies examined the role of EVs in promoting Th2-like cytokine production and inducing the differentiation to Th2 cells [[265] [266] [267] [268] [192] [229] [269]]. B-cell-derived EVs had allergen peptide-loaded major histocompatibility complex (MHC) molecules on their surface and were capable of stimulating T cells to proliferate and produce IL-5 and IL-13 [[265]]. A different study observed that plasma EVs have antigen-presenting capabilities and induced Th2 cell differentiation and increased levels of IL-13 [[266]]. One in vitro study found that thymic stromal lymphopoietin (TLSP)-activated DC EVs induced a higher number of Th2 cells by elevating IL-4 concentration and decreasing interferon-gamma (IFN- γ) concentration, thus reducing Th1 cells [[267]]. Investigating the effect of T follicular helper cell (Tfh) EVs found that these EVs could regulate DC maturation through miR-142-5p/CDK5/STAT3 pathway; however, most importantly, Tfh EVs promoted Th2 responses in naïve mice through elevated levels of IL-13 and

IgE, while simultaneously decreasing IFN- γ and IL-17 levels [[268]]. Another study showed that DC-derived EVs could carry Fel d 1 and in co-culture with peripheral blood mononuclear cells (PBMCs) induced higher levels of IL-4 production [[192]]. By researching lncRNA Nuclear Paraspeckle Assembly Transcript 1 (NEAT1) it was found that it was responsible for the promotion of allergic rhinitis (AR) pathogenesis via EVs, which interacted with human nasal epithelial cells (HNECs) and induced IL-13 production and apoptosis, which can lead to damage to the epithelium and increase the likelihood of allergen exposure and development of allergic sensitization [[229]]. Epithelium-derived EVs carried long non-coding RNA growth arrest-specific 5 (GAS5), which can suppress Th1 differentiation by downregulating the enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2) and T-bet, where IFN- γ concentration and Th1 responses were decreased, while also stimulating IL-4 production and Th2 differentiation [[269]].

Table 3.3 Studies involving allergic sensitization and host-derived EVs in this systematic review.

Author (Ref)	Title	Year	Objective	Isolation technique	Identification of EVs	Findings
Admyre et al., 2007 [265]	B cell-derived exosomes can present allergen peptides and activate allergen-specific T cells to proliferate and produce TH2-like cytokines	2007	To investigate the role of antigen-presenting cell derived exosomes in allergen presentation and T-cell stimulation.	ultracentrifugation - 300g x, 3000g 20 min, 10k g 30 min at 4C, 110k g 1h at 4C	adsorbed onto 4,5 μ m Dynabeads precoated with anti-MHC class 2. ELISA	These results demonstrate for the first time that exosomes isolated from B cells can present allergen-derived peptides and thereby induce T-cell proliferation and TH2-like cytokine production.
Fang et al., 2021 [242]	Plasma EVs Display Antigen-Presenting Characteristics in Patients With Allergic Rhinitis and Promote Differentiation of Th2 Cells	2021	Role of plasma EVs in allergic rhinitis	differential ultracentrifugation - 600g 10 min, 2000g 20 min, 12000g 30 min, 110k g 2h and 70 min	ELISA, NTA, TEM, Western blot for EV markers. CFSE stained EVs in flow cytometry	Plasma EVs derived from patients with AR exhibited antigen-presenting characteristics and promoted differentiation of Th2 cells, thus providing novel understanding of the pathogenesis of AR.

Huang et al., 2018 [267]	Exosomes from Thymic Stromal Lymphopoietin-Activated Dendritic Cells Promote Th2 Differentiation through the OX40 Ligand	2019	This study aimed to investigate whether exosomes from DCs activated by thymic stromal lymphopoietin (TSLP) play a role in T-helper cell differentiation through the OX40 ligand	ultracentrifugation of DC S/Ns, re-suspended in PBS and stored at -80 °C.	transmission electron microscope to observe, Bradford dye assay for protein concentration, Western blot for CD63	TSLP activated DCs produce OX40L and sOX40L and EVs were capable of transporting it. TSLP-DEXs were promoting Th2 response in CD4+ T cells. Blockade of OX40L in DC-derived exosomes could inhibit exosome-mediated CD4+ T proliferation and Th2 differentiation.
Teng et al., 2022 [268]	Tfh Exosomes Derived from Allergic Rhinitis Promote DC Maturation Through miR-142-5p/CDK5/STAT3 Pathway	2022	To explore the mechanism of Tfh on DC maturation in AR. In this study, a murine AR model was induced by challenging with ovalbumin (OVA).	Ultracentrifugation 100k g 90 min.	TEM	Tfh-derived exosomes induce DC maturation by regulating miR-142-5p/CDK5/STAT3 signaling pathway, thereby promoting the occurrence of AR. AR-Tfh-exos promoted TH2 responses in naïve mice, promoting both nasal inflammation and affecting naïve T cells
Vallhoy et al., 2015 [192]	Dendritic cell-derived exosomes carry the major cat allergen Fel d 1 and induce an allergic immune response	2015	The study investigated whether DC-derived exosomes can present the major cat allergen Fel d 1 and whether they thereby contribute to the pathogenesis of allergic disease.	serial ultracentrifugation 90k 90 min.	iEM with gold particles. NTA. Sulfate-aldehyde latex microspheres labeled with CD63 and CD81 run on FACSCalibur. ELISA. TEM	The results demonstrate that exosomes are able to present aeroallergens and thereby induce T-cell T(H)2-like cytokine (IL-4) production in allergic donors. Thus, these exosomes may be important immune-stimulatory factors in allergic immune responses and important targets or engineered tools in immunotherapy.
Wang et al., 2021 [229]	Exosomal lncRNA Nuclear Paraspeckle Assembly Transcript 1 (NEAT1) contributes to the progression of allergic rhinitis via modulating microRNA-	2021	In this study the function and molecular mechanisms of NEAT1 in AR by Evs was explored	ExoQuick precipitation kit, centrifuged 3k g 15 min, 30 min at 4C, then 1.5k 30 min at 4C	Western blot for CD9 and CD63, TEM	In conclusion, this data revealed that exosomal NEAT1 contributed to the pathogenesis of AR through the miR-511/NR4A2 axis. These findings might offer novel strategies for the prevention and treatment of AR. Exposing HNECs with AR induced HNECs-derived exosomes resulted in inflammatory responses

511/Nuclear Receptor Subfamily 4 Group A Member 2 (NR4A2) axis								
Zhu et al., 2020 [269]	Exosomal long non-coding RNA GAS5 suppresses Th1 differentiation and promotes Th2 differentiation via downregulating EZH2 and T-bet in allergic rhinitis.	2020	The paper hypothesizes that under AR conditions, nasal epithelium-derived exosomes encapsulate LncGAS5, and transport them into CD4+T cells, promoting allergic reactions via suppressing Th1 differentiation and increasing Th2 differentiation.		ultracentrifugation - 12k g 45 min at 4C, 110k g 2h at 4C, filtered 0.22µm then 110k g 70 min at 4C	TEM, western lot for CD63 and CD81	In summary, it is demonstrated that LncGAS5 in AR epithelium-derived exosomes is the key mediator in Th1/Th2 differentiation, providing a possible therapeutic target of AR. OVA-EXO suppresses Th1 differentiation promotes Th2 differentiation.	

Of the seven papers describing host-derived EVs driving allergic sensitization, all studies used human samples for the isolation of EVs and applied them in an in vitro model [[265] [266] [267] [268] [192] [229] [269]], whereas one study also used mice and an in vivo model [[268]]. Regarding the EVs isolation methodology, six studies used serial ultracentrifugation [[265] [266] [267] [268] [192] [269]], while one study used the ExoQuick precipitation kit, which utilized size exclusion chromatography (SEC) [[229]]. The identification process was different across the studies; however, six studies used TEM to detect the presence of EVs [[266] [267] [268] [192] [229] [269]], and four studies used Western blotting to confirm the presence of tetraspanins [266] [267] [229] [269]. Three papers used flow cytometry to detect tetraspanins on the EVs [265] [266] [192]. Two studies used NTA to measure the size of EVs [266] [192]. The papers were scored based on their quality using specific criteria created for this systematic review (Table 3.2). Overall, the identification of EVs was sufficient in three papers [266] [267] [192] as two or more methods were used, while in the other papers [265] [268] [229] [269]

where only one identification method was used (TEM). Two papers received high scores of 63% [266] [192] due to their thorough characterization of EVs, while three studies had the lowest scores of 52% [265] [267] [269] because of the limited work done in EV isolation and characterization. A full assessment of the papers is presented in Table 3.4.

Table 3.4 – Assessment of papers of allergic sensitization from host-derived EVs.

Reference	Model (n/6)	Robustness of Model		Sample Size		Sensitization (n/3)	EV Isolation (n/3)	EV Characterization (n/4)	Total Score (n/27) or (n/34)	Bias score
		Murine Model (n/4)	Cell Culture (n/4)	Murine Model (n/3)	Cell Culture (n/3)					
Admyre et al., 2007 [265]	Human (<i>in vitro</i>) (3)		Fully defined exposures (4)		More than 3 repeats (3)	Allergen defined, its conc. Measured (2)	Ultracentrifugation (1)	One method used (1)	14/27	52%
Fang et al., 2021 [266, 270]	Human (<i>in vitro</i>) (3)		Fully defined exposures (4)		More than 3 repeats (3)	Allergen defined, its conc. Measured (2)	Ultracentrifugation (1)	Multiple complimentary techniques, plus a novel technique utilized (4)	17/27	63%
Huang et al., 2018 [267]	Human (<i>in vitro</i>) (3)		Fully defined exposures (4)		3 repeats (2)	Allergen defined, its conc. Measured (2)	Ultracentrifugation (1)	Multiple complimentary techniques (2)	14/27	52%
Teng et al., 2022 [268]	Human (<i>in vitro</i>) and murine (<i>in vivo</i>) (5)	Fully defined mice sensitization (4)	Fully defined exposures (4)	5 or less mice (1)	3 repeats (2)	Allergen defined, its conc. Measured (2)	Ultracentrifugation (1)	One method used (1)	21/34	62%
Vallho et al., 2015 [192]	Human (<i>in vitro</i>) (3)		Fully defined exposures (4)		More than 3 repeats (3)	Allergen defined, its conc. Measured (2)	Ultracentrifugation (1)	Multiple complimentary techniques, plus a novel technique utilized (4)	17/27	63%
Wang et al., 2021[29]	Human (<i>in vitro</i>) (3)		Fully defined exposures (4)		3 repeats (2)	Allergen defined, its conc. Measured (2)	Exoquick isolation kit (size chromatography) (3)	One method used (1)	15/27	56%
Zhu et al., 2020 [269]	Human (<i>in vitro</i>) (3)		Fully defined exposures (4)		More than 3 repeats (3)	Allergen defined, its conc.	Ultracentrifugation (1)	One method used (1)	14/27	52%

Overall, all seven studies show the potential of EVs influencing the immune system to shift towards a Th2 response by increasing the differentiation towards Th2 cells and Th2-like cytokine production, which could potentially reveal how EVs influence allergic sensitization. By understanding allergy development, it may be possible to prevent its occurrence.

3.3.2.2 Promotion of immune tolerance

Naïve CD4 T-cells can differentiate into Th1, Th2 or Treg cells; if it is a Th1- or Treg-dominant response, immune tolerance is promoted. Papers suggesting that EVs can drive immune tolerance are compiled in Table 3.5.

Table 3.5 - Studies in host-derived EVs and allergic tolerance in this systematic review.

Author (Ref)	Year	Objective	Isolation Technique	Identification of EVs	Findings
Intestini et al., 2011 [271]	2011	Can IEC-derived exosomes carry $\alpha\beta6$ and antigens and whether such exosomes are able to facilitate the generation of tolerogenic DCs and antigen-specific Tregs; and what the role is of $\alpha\beta6$ and antigen-carrying exosomes in the activation of LTGF β	serial centrifugation - 300g 10 min, 1200g 20 min, 10k g 30 min, 100k g 1h	Bradford assay, TEM	<i>In vivo</i> administration of $\alpha\beta6$ /OVA-laden exosomes induced the generation of Tregs and suppressed skewed Th2 responses toward food antigen in the intestine.

Pen g et al., 2022 [272]	Mesenc hymal stromal cells- derived small extracell ular vesicles modulat e DC function to suppress Th2 respons es via IL- 10 in patients with allergic rhinitis	20 22	MSC-derived small extracellular vesicles (MSC-sEV) are increasingly considered as one of the main factors for the effects of MSCs on immune responses. However, the effects of MSC- sEV on DCs in allergic diseases remain unclear.	2000g 20 min at 4C, anion exchange chromatog raphy using Econo-Pac column, then concentrat ed using Pierce Protein Concentrat or	Brad ford assa y, NTA, TEM, West ern blott ing	The paper identified that sEV-mDCs suppressed the Th2 immune response by reducing the production of IL-4, IL- 9, and IL-13 via IL-10. Furthermore, sEV-mDCs increased the level of Treg cells.
Prad o et al., 2010 [273]	Bystand er suppress ion to unrelate d allergen sensitiza tion thro ugh intranasa l administ ration of toleroge nic exos omes in mouse.	20 10	The aim of the study was to investigate whether such nanovesicles specific to Ole e 1 can also prevent the sensitization to other unrelated allergen, as Bet v 1 from birch pollen	filtration on 0.22qm pores and ultracentrif ugation at 100k 1h	Prot ein conc mea sure d by micr o- bicin chon inic acid assa y	ExoTol specific to Ole e 1, in addition to inhibit specific immune response to this allergen, blocked the allergic response to a second unrelated allergen such as Bet v 1. The <i>in vivo</i> "bystander suppression
Prad o et al., 2008 [274]	Exosome s from broncho alveolar fluid of tolerized mice prevent allergic r eaction.	20 08	In the present study, the effect of allergen-specific exosomes from tolerized mice on the development of allergen-induced allergic response was determined using a mouse model.	ultracentrif ugation	TEM, micr obici ncho nic acid assa y, SDS- PAG E and	BALF-derived Exotol inhibit IgE and induce IgG2a cytokine production. These observations demonstrate that exosomes can induce tolerance and protection against allergic sensitization in mice. Thus, exosome-based vaccines could represent an alternative to conventional therapy for allergic diseases in humans.

					Western blotting, coated beads on FACS	
Wahlund et al., 2017 [275]	Exosomes from antigen-pulsed dendritic cells induce stronger antigen-specific immune responses than microvesicles <i>in vivo</i>	20 17	To investigate both MVs and exosomes from Ovalbumin (OVA)-pulsed dendritic cells for their immunostimulatory potential side by-side <i>in vivo</i>	differential centrifugations - 300g 10 min, 3000g 30 min, 10k g for 40 min to get MVs, S/N 100k for 90 min,	Transmission Electron Microscopy	DC-derived MVs and exosomes differ in their capacity to incorporate antigen and induce immune responses. Exosomes were more efficient in inducing CD8 T cells and IgG production than microvesicles.
Yu et al., 2020 [276]	Specific antigen-guiding exosomes inhibit food allergies by inducing regulatory T cells	20 20	To suppress experimental FA by inducing Tregs through the employment of modified exosomes (mExosomes). In this study, mExosomes were prepared by incubating dendritic cells with interleukin (IL)-2 and ovalbumin (OVA, used as a specific antigen) in the culture	serial ultracentrifugation - 300g 10 min, 1200g 20 min, 10k 30 min, 100k for 1h	Bradford assay	Administration of mExosomes induced Tregs in the intestinal tissues and efficiently suppressed FA in mice. We conclude that the mExosomes can suppress FA in mice through inducing Tregs. The data suggest that the mExosomes have translational potential in the treatment of FA and other allergic disorders.
Zeng et al., 2020 [277]	Exosomes carry IL-10 and antigen/MHC II complex to induce antigen-specific	20 20	To investigate the role of VIP in the immune tolerance development in the intestine.	ultracentrifugation - 300g 10 min, 1200g 20 min, 10k g 30 min, 100kg 1h, CD9 coated magnetic beads	Bradford assay	IL10CARs (produced from IEC exposed to VIP and OVA) induced Tr1 in healthy CD4 T cells the data show that IL10CARs are capable of suppressing experimental FA by inducing antigen-specific Tr1 cells, which has the translation potential for FA treatment.

	oral tolerance					
Zhou et al., 2021 [278]	HMSC-Derived Exosome Inhibited Th2 Cell Differentiation via Regulating miR-146a-5p/SERP	20 21	The present study aimed to investigate the role of HMSC-exos in the pathogenesis of AR.	Exoquick, serial centrifugation - 500g 10 min, twice 2000g 15 min twice 10k g 30 min, 70k g 1h at 4C	Western blotting for CD6 3, CD8 1, TEM	HMSC-exos could inhibit the differentiation of Th2 cells via the regulation of the miR-146a-5p/SERPINB2 pathway. miR-146a-5p and SERPINB2 could be applied as potential targets for AR treatment.
Asadirad, et al. 2023 [279]	Sublingual prophylactic administration of OVA-loaded MSC-derived exosomes to prevent allergic sensitization	2 0 2 3	This study evaluated the immunomodulatory and delivery potential of adipose tissue-isolated MSC-derived exosomes as a prophylactic regimen through a sublingual route in the ovalbumin (OVA)-induced allergic murine model.	Exospin isolation kit (SEC)	NTA, SEM, immunophenotyping of CD9 and C63	Significant reduction in the IgE levels and IL-4 production, along with elevated TGF- β levels, were observed. Also, limited cellular infiltrations and perivascular and peribronchiolar inflammation in the lung tissues and normal total numbers of cells and eosinophils in the NALF were reported.

Nine studies suggested that host-derived EVs were capable of suppressing Th2 responses, resulting in immune tolerance [[271-279]]. Four of these studies researched the influence of EVs on food allergy [[271, 276, 277, 279]], one of which found intestinal epithelial-cell-derived integrin $\alpha\beta 6$ and OVA-carrying EVs interacting with T cells could induce T cell differentiation to Treg cells, through the increased production of TGF- β [[271]]. Furthermore, EVs from IL-2 and OVA-stimulated DCs, with MHC class II molecules, interacting with OVA-specific CD4⁺ T cells induced differentiation into Tregs [[276]]. Another study relating to food allergy showed that EVs carry the allergen-MHC class 2 complexes and IL-10 and could induce Tr1 differentiation in healthy CD4⁺ T cells [[277]]. Another study found that MSC-derived EVs containing OVA pre-

exposure in mice resulted in tolerance to OVA, with decreased levels of IgE and IL-4, along with elevated TGF- β levels [[279]].

Three studies examined the role of EVs in allergic rhinitis [[272, 274, 278]]. In these studies, it was found that when using EVs, it was possible to inhibit the Th2 cell responses and induce Treg differentiation by limiting the production of IgE and increased expression of TGF- β and IgG2a [[274]]. Another study showed that MSC-derived EVs also suppressed the Th2 response by reducing the production of IL-4, IL-9, and IL-13 via IL-10 production, thus increasing the levels of Tregs in the system [[272]]. The last allergic rhinitis study showed that human mesenchymal stem cells (HMSC)-EVs co-cultured with CD4⁺ T cells inhibited the Th2 differentiation by regulating the miR-146a-5p/SERPINB2 pathway [[278]].

Two studies looked at the role of allergens and EVs in immune tolerance; one of the studies looked at the differences between microvesicles and exosomes and their capacity to influence the immune responses, where it was found that exosomes induced a stronger response, which generated a Th1-like response by inducing the production of allergen-specific IgG abs and expansion of OVA-specific CD8⁺ T cell population [[275]]. The other study observed the potential of ‘bystander’ tolerance of allergens by EVs, where it was found that EVs inhibited the immune responses to the specific allergen in addition to another unrelated allergen by limiting the production of IL-5 and IL-13 and reducing the inflammatory responses in the lung [[273]].

Of the nine papers describing host-derived EVs in immune tolerance, seven studies used mice as their source of EVs [[271, 273-277]], and two studies used human samples and human cell lines as their EV source [[272, 278]]. EV isolation protocols were similar across the papers, where seven of the studies used some form of serial ultracentrifugation [[271, 273-278]], one study used anion exchange chromatography [[272]], and one study utilized an Exospin isolation kit, which uses SEC [[279]]. Various methods were used to confirm the presence of isolated EVs; four studies used the Bradford assay [[271, 272, 276, 277]], five studies used TEM [[271, 272, 274, 275, 278]], and one used SEM [[279]]. Two studies used Western blotting to detect the presence of tetraspanins [[272, 278]], and one study used immunophenotyping [[279]]. Overall, the identification and characterization of EVs in these studies were lacking. The

highest bias score was 63%, but this study lacked the use of multiple models [[272]], whilst the lowest score achieved was 44%, resulting from the limited use of models and inferior isolation technique [[274]]. Six out of nine studies scored over 50% [[272, 273, 275-278]]. The overall scores were compiled into a table below (Table 3.6).

Table 3.6 - Scoring assessment of papers in host-derived EVs and allergic tolerance in this systematic review.

Author (Ref)	Model (n/10)	Robustness of Model		Sample Size		Sensitization (n/3)	EV Isolation (n/3)	EV Characterization (n/4)	Total Score (n/27)	Bias score
		Murine Model (n/4)	Cell Culture (n/4)	Murine Model (n/3)	Cell Culture (n/3)					
Chen et al., 2011 [271]	Murine (in vitro and vivo) (3)	Mice defined, no negative control (2)	Fully defined exposures (4)	5 or less mice (1)	3 repeats (2)	Allergen defined, its conc. Measured (2)	Ultracentrifugation performed (1)	One method used (1)	16/34	47%
Peng et al., 2012 [272]	Human (in vitro) (3)		Fully defined exposures (4)		More than 3 repeats (3)	Allergen defined, its conc. Measured (2)	Anion exchange chromatography (3)	Multiple complementary techniques (2)	17/27	63%
Prado et al., 2010 [273]	Murine (in vitro and vivo) (3)	Fully defined mice sensitization (4)	Fully defined exposures (4)	5 or less mice (1)	Less than 3 repeats (1)	Allergen defined, its conc. Measured (2)	Ultracentrifugation performed (1)	One method used (1)	17/34	50%
Prado et al.,	Murine (in	Fully defined mice		5 or less		Allergen defined, its conc.	Ultracentrifugation	Multiple complementary	12/27	44%

2008	vivo) (2)	sensitization (4)		mice (1)		Measured (2)	performed (1)	y techniques (2)		
[274]										
Wahlund et al., 2017	Murine (in vitro and vivo) (3)	Fully defined mice sensitization (4)	Fully defined exposures (4)	5 or less mice (1)	3 repeats (2)	Allergen defined, its conc. Measured (2)	Ultracentrifugation performed (1)	One method used (1)	18/34	53%
[275]										
Yu et al., 2020	Murine (in vitro and vivo) (3)	Fully defined mice sensitization (4)	Fully defined exposures (4)	6 to 10 mice per group (2)	3 repeats (2)	Allergen defined, its conc. Measured (2)	Ultracentrifugation performed (1)	One method used (1)	19/34	56%
[276]										
Zeng et al., 2020	Murine (in vitro and vivo) (3)	Fully defined mice sensitization (4)	Fully defined exposures (4)	6 to 10 mice per group (2)	More than 3 repeats (3)	Allergen defined, its conc. Measured (2)	Ultracentrifugation performed (1)	One method used (1)	20/34	59%
[277]										
Zhou et al., 2021	Human (in vitro) (3)		Fully defined exposures (4)		3 repeats (2)	Allergen defined, its conc. Measured (2)	Exoquick isolation kit (size chromatography) (3)	One method used (1)	15/27	56%
[278]										

Overall, these nine studies showed that EVs were capable of suppressing Th2 immune response and skewing towards Th1 responses, as well as the induction of Treg cells, which in turn reduce the inflammatory cytokine and IgE production in food allergy and allergic rhinitis. Thus, these results show the potential of using EVs as a vehicle for alleviating and/or preventing allergies.

3.3.3 Exogenous extracellular vesicles

3.3.3.1 Promotion of allergic sensitization via the skin epithelium

In allergic sensitization, the first ‘defense’ against allergens is epithelial cells, either as skin cells, lung cells or intestinal cells. EVs generated from exogenous sources, such as bacteria, must thus cross this first barrier in order to interact with the immune system and influence the allergic sensitization process. Publications suggesting that exogenous EVs can also promote sensitization resulting in allergies, were identified (Table 3.7).

Table 3.7 - Studies involving external EVs and allergic sensitization in this systematic review.

Author (ref)	TITLE	YEA R	OBJECTIVE	ISOLATION TECHNIQUE	Identificati on of EVs	FINDINGS
Asano et al., 2021 [280]	Extracellular vesicles from methicillin resistant <i>Staphylococcus aureus</i> stimulate proinflammatory cytokine production and trigger IgE-mediated hypersensitivity	2021	The study demonstrated that purified EVs from a clinical isolated methicillin-resistant <i>S. aureus</i> (SaEVs) significantly stimulated proinflammatory cytokine production in mouse immune cells and induced host cell death	ultracentrifuge - 5k g 20 in, 100k g 90 min at 4C, purified using OptiPrep density gradient, 100k g 16h at 4C	TEM, qNano, Measurment net Electrolytes used in NP150 nanopore membrane s. SD-Page, Bradford protein assays, Western blot	These results suggested that MRSA-derived EVs act as an immunostimulant that induces inflammatory response and IgE-mediated hypersensitivity after MRSA infection.
Hong et al., 2014 [281]	An important role of α -hemolysin in extracellular vesicles on the development of atopic dermatitis induced by <i>Staphylococcus aureus</i> .	2014	An important role of α -hemolysin in EVs on the development of atopic dermatitis induced by <i>Staphylococcus aureus</i> .	Filtered, ultracentrifugation 150k g 3h	BCA assays, Confocal microscopy, western blot	Taken together, α -hemolysin secreted from <i>S. aureus</i> , particularly the EV-associated form, induces both skin barrier disruption and AD-like skin inflammation, suggesting that EV-associated α -hemolysin is a novel diagnostic and therapeutic target for the control of AD.
Hong et al., 2011 [231]	Extracellular vesicles derived from <i>Staphylococcus aureus</i> induce atopic dermatitis-like	2011	Although <i>S. aureus</i> infection has been linked with atopic dermatitis (AD), the identities of the causative agents	Ultracentrifugation 150k g. Specifically, 0.45 μ m vacuum filtration,	Bradford assays, ELISA	These results indicate that <i>S. aureus</i> EV induce AD-like inflammation in the skin and that <i>S. aureus</i> -derived EV are a novel diagnostic and

	skin inflammation		from <i>S. aureus</i> are controversial. Therefore, the study evaluates whether <i>S. aureus</i> -derived EV are causally related to the pathogenesis of AD.	concentrated by QuixStand Benchtop System, then 0.22 µm vacuum filter before ultracentrifugation.		therapeutic target for the control of AD.
Kim et al., 2018 [246]	Lactobacillus plantarum-derived Extracellular Vesicles Protect Atopic Dermatitis Induced by Staphylococcus aureus-derived Extracellular Vesicles	2018	The study aims to compare the bacterial EV composition between AD patients and healthy subjects and to experimentally find out the beneficial effect of some bacterial EV composition.	Multiple ultracentrifugation and purification processes	TEM, SDS-Page, Zetasizer Nano ZS	The study suggests the protective role of lactic acid bacteria in AD based on metagenomic analysis. Experimental findings further suggest that <i>L. plantarum</i> -derived EV could help prevent skin inflammation
Staudenmaier et al., 2022 [282]	Bacterial membrane vesicles shape Staphylococcus aureus skin colonization and induction of innate immune responses	2022	Membrane vesicles (MVs) are released by pathogenic bacteria and might play an essential role in the long-distance delivery of bacterial effectors such as virulence factors.	Size exclusion chromatography. Specifically, filtered, 3000g for 30 min, ExoQuickTC, 1500g 30 min	Western blotting	The data underline the complex interplay in host- and bacterial derived factors in <i>S. aureus</i> skin colonization and the important role of bacterial derived MVs and their membrane lipid and protein A content in skin inflammatory disorders.

Five studies showed that EVs generated by bacteria could damage the skin barrier, leading to easier colonization of the skin, which in turn may influence allergic sensitization and AD [[231, 246, 280-282]]. Five studies were experimenting with *S. aureus* EVs and their influence on the induction of atopic dermatitis-like skin inflammation [[231, 246, 280-282]]; however, one paper showed that *Lactobacillus plantarum*-derived EVs could prevent damage to the skin from *S. aureus* EVs [][246]]. In one of these studies, the toxin α -Hemolysin produced by *S. aureus* was more potent in EV form and could induce IL-6 production associated with Th17 response, which caused skin barrier disruption and AD-like skin inflammation [][281]]. After investigating the potential effects of methicillin-resistant *Staphylococcus aureus* (MRSA)

EVs in mice, it was found that the EVs act as immunostimulant that induces inflammatory responses, producing cytokines such as IL-4, IL-5 and IL-6 and triggering IgE-mediated hypersensitivity after MRSA infection [280].

The isolation protocols for the five studies were similar, where filtration and serial ultracentrifugation were used [231, 246, 280-282], which is not efficient enough to provide pure and intact EVs [283]. One study used filtration and an ExoQuickTC kit that utilized SEC [282]. The identification methods used in most studies were also not comprehensive, where 3 studies only used one method of identification for characterizing their EVs [231, 246, 282], while only one study utilized novel techniques on top of the standard methods [280]. Papers in external sources scored higher than the other sections in this systematic review. The highest scoring paper was 79% [280], due to the use of a novel identification method utilizing electrolytes in NP150 nanopore membranes on top of other conventional techniques such as Western blots, SDS-page gels and Bradford protein assays. The lowest scoring paper was also found in this section, with 38% [231], because of the poor isolation and characterization of their EVs, plus the use of only a murine in vivo model. The full assessment of papers can be found in Table 3.8.

Table 3.8 -Scoring assessment of studies in exogenous EVs and allergic sensitization in this systematic review.

Reference	Model (n/10)	Robustness of Model			Sample Size		EV Isolation (n/3)	EV Characterization (n/4)	Total Score (n/24) (n/31) (n/38)	Bias score
		Murine Model (n/4)	Cell Culture (n/4)	Human Model (n/4)	Murine Model (n/3)	Cell Culture (n/3)				
Asano et al., 2021 [280]	Murine (in vitro and vivo) (3)	Fully defined mice sensitization (4)	Fully defined exposure (4)		5 or less mice (1)	3 replicates (2)	Ultracentrifugation (1)	Multiple complementary techniques, plus a novel technique utilized (4)	19/24 (4)	79%

Hong et al., 2014 [281]	Human (<i>in vitro</i>) and Murine (<i>in vivo</i>) (5)	Fully defined mice sensitization (4)	Fully define d exposu res (4)	5 or less mic e (1)	3 rep eats (2)	Ultracentrif u gation (1)	Multiple compliment ary techniques (2)	19/31	61%	
Hong et al., 2011 [231]	Murine (<i>in vivo</i>) (2)	Fully defined mice sensitization (4)		5 or less mic e (1)		Ultracentrif u gation (1)	One method used (1)	9/24	38%	
Kim et al., 2018 [246]	Human (<i>in vitro</i>) and Murine (<i>in vivo</i>) (5)	Fully defined mice sensitization (4)	Fully define d exposu res (4)	5 or less mic e (1)	3 rep eats (2)	Ultracentrif u gation (1)	Multiple compliment ary techniques (2)	19/31	61%	
Staudenmaier et al., 2022 [282]	Human (<i>Ex vivo</i> and <i>in vitro</i>) (7)		Fully define d exposu res (4)	Fully define d exposu res (4)	3 rep eats (2)	5 or less partici pants (1)	Exoquick isolation kit (size chromatogr aphy) (3)	One method used (1)	22/31	71%

Overall, in these five studies, bacterial EVs were shown to damage the epithelial cell barrier, which can lead to increased susceptibility to developing an allergic reaction.

3.3.3.2 Promotion of immune tolerance

It has been shown that immune cells can recognize and interact with these exogenous EVs to alter their responses to stimuli [[284]]. Only one study showed that exogenous EVs promote immune tolerance (Table 3.9). Observing that EVs produced by Gram-negative bacteria in indoor dust can induce neutrophilic pulmonary inflammation and increased production of IFN- γ and IL-17 associated with both Th1 and Th17 cell response. Furthermore, dust EVs from Gram-negative bacteria induced tumor necrosis factor alpha (TNF- α) and IL-6 production in macrophages [[285]].

Table 3.9 - Studies involving external EVs and allergic tolerance in this systematic review.

Author (ref)	Title	Year	Objective	Isolation technique	Identification	Findings
--------------	-------	------	-----------	---------------------	----------------	----------

						met hod
	Extracellular vesicles, especially derived from Gram-negative bacteria, in indoor dust induce neutrophilic pulmonary inflammation associated with both Th1 and Th17 cell responses	To evaluate whether EVs in indoor air are related to the pathogenesis of pulmonary inflammation and/or asthma.	10k g 15 min, 0.45µm vacuum filter and concentrated using ultrafiltration QuixStand Benchtop System, filtered 0.22µm vacuum filter, 150k g 3h at 4C	TEM , NTA	Indoor dust EV, especially derived from Gram-negative bacteria, is a possible causative agent of neutrophilic airway diseases. In summary, the present data indicate that inhalation of indoor dust EV induces both Th1 and Th17 cell responses and neutrophilic inflammation in the lung.	

The one study showing that an exogenous source of EVs can tolerize the immune system used a mice model and a human in vitro model [[285]]. The isolation method utilized in this paper was ultracentrifugation [[285]]. The paper used NTA to size the EVs and used TEM [[285]] to confirm the morphology of the EVs. This paper scored well at a 58% bias score, as it utilized multiple model systems and fully described them [[285]]. A full breakdown of the scoring can be found in Table 3.10.

Table 3.10 - Assessment of studies involving exogenous EVs and allergic tolerance.

Reference	Model (n/10)	Robustness of Model		Sample Size		Sensitization (n/3)	EV Isolation (n/3)	EV Characterization (n/4)	Total Score (n/34)	Bias score
		Murine Model (n/4)	Cell Culture (n/4)	Murine Model (n/3)	Human (n/3)					
Kim et al., 2013 [285]	Human (in vitro) and Murine (in vitro and vivo) (6)	Fully defined mice sensitization (4)	Fully defined exposure (4)	5 or less mice (1)	3 people (2)	N/A	Ultra-centrifugation (1)	On the method used (1)	18/31	58%

In summary, this paper shows that exogenous-sourced EVs were capable of shifting the immune response towards a Th1-like response.

3.4 Discussion

Allergies are a worldwide issue with an increasing number of cases in the population [[286]], yet the mechanisms behind the development of an allergy are still poorly understood. However, recently, there has been a focus on research on EVs and their role in allergies and their development. In this systematic review, we highlight the increasing evidence that EVs can promote allergic sensitization; conversely, there is also much evidence for their role in promoting tolerance to allergens.

Of the 22 studies relevant to EVs in allergic sensitization, 64% of studies [[192, 229, 231, 246, 265-269, 280-282, 285]] showed evidence of EVs enhancing the allergic sensitization process and shifting towards Th2-type responses, compared to 36%, which reported EVs could prevent allergic sensitization and shift the immune response towards a Th1 and Treg-like immune tolerance [[271-278]]. In terms of sensitization, this systematic review highlights that EVs promote the differentiation of naïve T cells into Th2 cells by interacting with immune cells to influence cytokine production, such as increasing IL-4, IL-13 and IL-5 concentration and decreasing IFN- γ levels [[192, 229, 265-269]]. In terms of tolerance, we highlight that EVs can induce immune tolerance by stimulating Th1 and Treg differentiation by increasing concentrations of TGF- β , IL-2 and IL-10 cytokines while suppressing the production of IL-4, IL-9, and IL-13 cytokines, leading to lower levels of Th2 differentiation [[271-278]]. In addition, this review highlights the origin of EV impacts, whether the EV promotes tolerance or sensitization, where EVs generated internally by the body influence both Th1 and Th2 responses, but exogenous EVs generated by bacteria mainly promote allergen exposure by increasing the permeability of the skin, thus resulting in increased chance of allergens interacting with immune cells [[231, 246, 280-282, 285]].

Furthermore, bacteria-derived EVs can act as the ‘allergen’ and trigger allergic

sensitization itself by stimulating IL-4, IL-5, and IL-6 production, and then, on re-exposure, trigger an IgE-mediated response [[280]].

One of the key mechanisms driving allergic sensitization suggested is EVs produced by DCs, B cells, Tfh, and epithelial cells that can affect the cytokine production in immune cells by promoting IL-4, IL-5, and IL-13 production while simultaneously decreasing the levels of IFN- γ and IL-17. For example, DC-derived EVs induced Th2 differentiation and suppressed Th1 responses, which can lead to allergic sensitization [[192, 265-269]]. Currently, what induces naïve T cells to differentiate into Th2 cells during allergic sensitization is poorly understood [[109]]; however, the evidence presented here shows that EVs generated by immune cells can act as the signal to trigger the Th2 differentiation. Further research in this area could lead to developing new treatments to stop naïve—cell differentiation into Th2 cells, thus stopping allergic sensitization. Conversely, it is possible to reduce Th2 responses by inducing ‘desensitization’ by immunotherapy, where allergen-specific IgG, more specifically IgG4, antibodies are produced and compete with IgE and show inhibitory capacity for IgE-dependent events [[287]]. IgG4 can bind to the allergen specifically, thus blocking it from interacting with IgE, meaning decreased levels of IgE-allergen deposits on mast cells and, in turn, decreased histamine levels.

Furthermore, it has been shown that IgG4 competition can block IgE-allergen complexes binding to B cells, thereby inhibiting IgE-facilitated antigen presentation to T cells, which is a major driving force for generating allergen-specific Th2 responses [[288]]. Recent research has been exploring the potential of EVs used in therapies utilizing the ‘desensitization’ process [[289]]. Another mechanism by which EVs can promote allergic sensitization is by damaging the epithelial barrier by carrying lncRNA NEAT1, which interacts with HNECs to promote IL-13 and apoptosis, thus enhancing the likelihood of allergen exposure to immune cells [[229]].

In addition, a study by Gon et al. found that EVs released in the bronchoalveolar lavage fluid (BALF) were enriched with miRNAs once exposed to house-dust mites, and these EVs could upregulate Th2 cytokines

[[290]], highlighting the importance of EVs as carriers of effector molecules. Furthermore, a study found key evidence where bronchial epithelial cells secreted by EVs carry a pivotal protein tissue factor (TF) in asthma and are involved in its pathogenesis [[291]]. This highlights the importance of studying the contents of EVs, as their cargo can be essential in their function.

As aforementioned, it is also possible for EVs to induce Th1 and Treg responses, thus inducing immune tolerance towards the allergen. One of the key mechanisms suggested is inducing Tregs in the immune system by increasing the production of TGF- β [[271-277]]. Furthermore, it is suggested that EVs carrying integrin $\alpha\beta 6$ and OVA could interact with T cells and induce Treg differentiation, thus suppressing allergic sensitization [[271]].

Another mechanism of suppression of Th2 differentiation was by HMSC-derived EVs influencing the miR-146a-5p/SERPINE2 pathway in healthy CD4⁺ T cells [[278]], where it can be speculated that EVs can affect intrinsic pathways in the cells that they interact with either by activating the recipient cells receptors, thus initiating intracellular signaling pathways or cargo by fusion with the plasma membrane of target cells [[292]]. In addition, previously published literature has found how EVs can affect specific cells through intrinsic signaling pathways; for example, a study done by Salem et al. showed that EVs generated from bronchial fibroblasts could promote epithelial cell line remodeling through the TGF- $\beta 2$ signaling pathway in severe asthma [[293]], suggesting that EVs are affecting the target cells' intrinsic pathways and thus altering their function.

Another study found that EVs can also induce immune tolerance towards unrelated allergens, as well as specific allergens, by limiting the production of IL-5 and IL-13, thus inducing 'bystander tolerance' [[273]].

Recently, there has been more focus on the microbiome of humans and how it can affect the immune system [[294]]. It was found that EVs produced by bacteria, specifically *S. aureus*, could be the leading cause of damage to the skin and induction of AD-like symptoms, which are most likely caused by the EV cargo. For instance, the toxin α -Hemolysin was found to be more potent in EVs than in soluble form, causing further skin damage [[231, 246, 281, 282,

285]]. Damaged skin barrier can lead to increased susceptibility to allergic sensitization of allergens. This is due to the compromised barrier releasing defensins, which can predispose to allergic sensitization by subsequent interactions with T cells and can thus lead to atopic dermatitis [[295]]. In this systematic review, a major function of EVs was suggested by a study regarding MRSA-derived EVs, where EVs acted as immunostimulants, inducing IL-4, IL-5, and IL-6 cytokines in mice. Furthermore, following subsequent exposure to MRSA, an IgE-mediated hypersensitivity developed [[280]]. This suggests that EVs were able to sensitize the mice towards MRSA, directly suggesting that EVs can trigger allergic sensitization without additional signals from the mice.

It is worth noting that many of the studies examined had low-quality assessment scores, as many of the included studies had poor methods of EV isolation and characterization. The papers providing evidence for host-derived EVs and their influence on allergic sensitization scored a mean value of 57%, which was minimally higher by 5% than host-derived EVs inducing tolerance, showing the reliability of the key mechanisms was similar in these sections. However, the highest scoring section was exogenous EVs in promoting allergic sensitization with a 62% mean score, while exogenous EVs and tolerance had 58% scoring quality, indicating the robustness of these studies was of similar quality. As the sections do not have high variance between their scoring, there is no indication of higher reliability in the findings between the sections. The quality scoring highlights the need for further understanding of EVs and how to isolate them more efficiently to increase purity and maintain their original form without rupture. Only two studies [[192, 267]] received the highest points for EV characterization. In addition, five different studies [[268, 272, 278, 281, 282]] had the highest score of EV isolation. Thus, future research must utilize new isolation techniques to ensure the highest purity of EVs and the exclusion of cytokines, as it has been shown that cytokine presence can affect the function of the EVs.

Furthermore, fully characterizing EVs would lead to a better understanding of the differences between the sub-populations of EVs and which receptors, and what cargo, drives their role in allergy. In addition, almost all studies lacked

human ex vivo experiments, except one study [[282]], which used a foreskin puncture to show a more accurate representation of the immune and epithelial cell interactions. Interestingly, almost all studies had high scores regarding the robustness of the model, as they clearly described what cell type was used, and the duration, exposure method, and dosing of the allergen were fully explained. All the studies received the same score for material used for sensitization as the allergen was clearly described and measured; however, none of the studies performed endotoxin measurement to ensure the allergen was endotoxin-free.

This systematic review contained studies both using human and murine models, with 57% of studies utilizing murine models for the study of allergic sensitization. However, the use of mice in allergy studies is not preferred, as an allergy in mice is induced artificially and does not fully represent allergic sensitization in humans. In addition, mice have been shown to produce cytokines differently than humans; for example, IL-10 in mice is only produced by Th2 response, while in humans, it can be both Th1 and Th2 responses [[296]].

Future research in the field of EVs could open avenues for understanding the development of allergic sensitization, possibly leading to the development of new treatments and/or prevention of allergic sensitization. It is clear EVs play a role in allergies, either in their development or by induction of tolerance, and this is dependent on the cell of origin, health status, and environmental conditions in which the cells grow. In turn, this can influence the outcome of the EVs generated, resulting in different cargo encapsulations and differing cellular outcomes [[297]]. In the future, more research is required on EVs released by cells, investigating their cargo and proteins on the surface, which interact with immune cells to shift the response away from allergic sensitization and towards tolerance.

In summary, this systematic review provides evidence for the role of extracellular vesicles in allergies, where they have been shown to influence Th1, Th2 or Treg responses to allergens. This evidence suggests that EVs could be the driving force that decides the immune response to an allergen. However,

with the limited number of papers that have been published, further research is crucial in fully understanding the role of EVs in allergies.

Since the original systematic review was conducted in 2022, several noteworthy primary research studies published between 2023 and 2025 have further elucidated the role of EVs in allergic sensitization. These new contributions largely reinforce the review's main findings on EVs' dual pro- and anti-allergic potential, while introducing novel mechanisms related to microbial and environmental influences, without fundamentally altering the core conclusions. For instance, a 2024 experimental study by Long et al. explored EVs as carriers of environmental allergens in indoor dust, revealing that dust mite-derived EVs enhance allergenic activity by co-delivering proteins and lipids that boost Th2 sensitization in airway epithelial cells, reinforcing the review's section on exogenous EVs' pro-sensitizing effects and emphasizing environmental exposures as a complicating factor ([298]). Another 2023 study by Asadirad et al. evaluated sublingual prophylaxis with OVA-loaded MSC-derived exosomes in an allergic asthma murine model, showing that these EVs prevented sensitization by modulating immune responses and inhibiting Th2 cytokine production, introducing a prophylactic application that complements the review's discussion on exogenous EVs' anti-sensitizing roles ([279]). Finally, a 2023 study by Zhao et al. examined exosomes from adipose stem cells in an atopic dermatitis mouse model, finding that they ameliorated inflammation by reducing Th2 cytokines and restoring barrier function, thus supporting the review's emphasis on therapeutic potential without contradicting the pro-allergic dominance in host-derived EVs ([299]). Overall, these post-2022 primary studies strengthen the systematic review's emphasis on context-dependent EV functions, with a growing focus on microbiome and environmental interactions, but they do not substantially change the main points; instead, they underscore the need for updated analyses incorporating human data and therapeutic implications, as the field continues to evolve rapidly.

The systematic review presented in this chapter synthesises the current evidence on the role of EVs in allergic sensitisation, revealing a dual and context-dependent function. Host-derived EVs, particularly those from

epithelial cells and antigen-presenting cells, frequently promote sensitisation by transporting intact allergens, alarmins (e.g., IL-33, TSLP), and Th2-polarising signals (e.g., MHC-II–allergen complexes, miRNAs such as miR-146a), thereby facilitating DC maturation, naïve T-cell priming, and IgE production (Chapter 3.3.2). In contrast, certain exogenous EVs (e.g., from commensal bacteria or tolerogenic DCs) and some host EVs under specific conditions can induce immune tolerance through regulatory cytokine delivery (IL-10, TGF- β), Treg expansion, or suppression of effector responses (Chapter 3.3.3). Despite this emerging duality, the literature remains limited by heterogeneity in EV isolation methods, lack of standardised characterisation (often not fully MISEV-compliant), and a predominance of murine models with few human studies. Most studies focus on airway or skin routes, with considerably less attention given to gastrointestinal sensitisation—particularly relevant to food allergy. These gaps highlight the need for more human-relevant, well-controlled investigations that dissect the specific molecular cargo and cellular context determining whether EVs drive pro-allergic or tolerogenic outcomes. The findings from this review directly inform the experimental work in the subsequent chapters, where a human in vitro model was developed to test the hypothesis that DC-derived EVs can influence naïve T-cell polarisation during allergic sensitisation, providing a mechanistic bridge between the observed literature patterns and direct functional evidence.

Chapter 4: Dendritic cell culture conditions

4.1 Introduction

4.1.1 DCs as immune response modulators

Dendritic cells (DCs), a heterogeneous population of antigen-presenting cells, play a pivotal role in orchestrating IgE-mediated allergic responses. Among these, monocyte-derived DCs (moDCs) become prevalent during inflammatory states, enabling the presentation of allergens to CD4⁺ ([300]). During allergic sensitisation, moDCs encounter allergens at mucosal surfaces, internalising them through mechanisms such as endocytosis, pinocytosis, or phagocytosis. Subsequently, these cells migrate to draining lymph nodes, where allergens are processed and presented as peptide-MHC class II complexes to T cell receptors. This interaction, often termed the immunological synapse, drives the activation and differentiation of naïve T cells, thereby initiating the adaptive immune response ([288]). Crucially, effective T-cell activation requires three coordinated signals: signal 1 (TCR recognition of peptide-MHC II), signal 2 (co-stimulation via CD80/CD86 on DCs binding CD28 on T cells), and signal 3 (cytokine milieu). In the allergic context, the co-stimulatory molecules CD80 and CD86, upregulated on DCs upon allergen encounter, provide the essential second signal that prevents T-cell anergy and promotes full activation. Furthermore, moDCs secrete cytokines, including IL-1 β and IL-12, which can influence the differentiation process of naïve T cells into Th2 cells ([302]). Exposure to allergens also induces upregulation of key cell-surface molecules, such as CD40, CD80, CD86 and HLA-DR, which, alongside cytokine release, critically shape T cell responses ([303]). Collectively, these processes determine whether an individual develops sensitisation to specific allergens by generating an IgE response, underscoring the sophisticated immunoregulatory role of DCs in allergic pathology.

DCs dynamically modulate the production and composition of EVs based on their cell state and external stimuli [304]. The maturation state of DCs, whether immature or mature, along with stimuli like PAMPS, cytokines and allergens, governs EV biogenesis, release, and molecular content, thereby

shaping their immunoregulatory functions. In immature DCs, EV production is relatively low, and their cargo primarily includes MHC class II molecules and low levels of costimulatory molecules like CD86, where these EVs often promote immune tolerance, as they lack strong stimulatory signals ([305]). DCs, upon maturation, undergo significant phenotypic and functional changes that alter EV production. Mature DCs increase EV release, with exosomes enriched in MHC- II, CD86, and immunostimulatory molecules like CD40, enhancing T-cell priming and activation ([306]). This highlights the interplay between DC cell state and external stimuli and their influences on EV production, cargo composition, and immunological function. These dynamic changes position DC-derived EVs as potential critical modulators of immune responses, such as allergy development.

4.1.2 Chapter objectives

As DC cell state affects EV production, cargo composition and immunological functions, it was important to investigate the optimal culturing conditions for differentiation of monocytes into immature DCs and their subsequent maturation. To achieve this aim, several culture media were tested and cell viability examined after generation of DCs. Furthermore, monocyte seeding density was explored to ensure the highest viability and the lowest amount of contamination with platelets was present in the culture. Finally, to confirm the maturation process of DCs following stimulation, several maturation markers were measured.

4.2 Methods

4.2.1 PBMC isolation

The method was followed according to the previously described method in methods section 2.1.11.

4.2.2 CD14⁺ monocyte isolation

Following PBMC isolation, PBMCs were mixed with 80µl of MACS buffer and magnetically labelled with 20µl of CD14 microbeads per 10⁷ cells and incubated at 4°C for 15 minutes. The cells were then washed with MACS buffer by centrifugation at 300g for 10 minutes. The cell pellet was then re-

suspended in 500µl of MACS buffer and added to the LS column. The column was washed 3 times with 3mL of MACS buffer to wash away the CD14⁻ cells, after which to obtain the CD14⁺ fraction 5mL of MACS buffer was forced into a new tube.

4.2.3 DC generation

Following monocyte isolation, the monocytes were stimulated to differentiate into immature DCs. The monocytes were resuspended in different culture media (table 4.1) and cultured in a 48-well flat-bottomed tissue culture-treated plate at different seeding densities (25×10^3 , 50×10^3 and $100 \times 10^3/\text{cm}^2$).

Table 4.1 - Culture media used. Containing the name of the culture media used and from where they were acquired.

Culture Media	Company	Cat no.	In order to
RPMI-1640	Merck, UK	R6504	
X-VIVO 15	Lonza, UK	02-060Q	
StemXvivo	R&D systems, UK	CCM003	
ImmonuCult-ACF	StemCell, UK	10987	

differentiate monocytes into DCs 50 ng/mL of GM-CSF and 20 ng/mL of human IL-4 was added. The plate was incubated at 37°C for 5 days, replenishing media and cytokines on Day 3.

To ensure immature DCs were generated, some cells were removed from the wells and extracellularly stained for: CD14, CD40, CD83, CD80, CD86, HLA-DR and CD209. To test the maturation process of immature DCs following stimulation, some immature DCs were stimulated with 100ng/mL of LPS and different OVA concentrations for 24h at 37°C. These cells were then harvested and stained with the same antibodies as immature DCs to measure the changes in maturation markers.

4.2.4 Flow Cytometry

The antibodies used for assessing monocyte purity, DC maturation and cell viability are presented in Table 4.2.

Table 4.2 - Flow Cytometry Antibodies for DC Maturation Markers. The marker, fluorophore, brightness, company purchased from, and catalogue number for the antibodies utilised in the flow cytometry staining of DCs.

Antibody	Fluorophore	Brightness	Company	Cat No.
CD14	PerCP	1	Biolegend	325632
CD80	FITC	3	Biolegend	305206
CD83	BV605	4	Biolegend	305336
CD86	PE	5	Biolegend	374206
CD40	BV421	5	Biolegend	334332
HLA-DR	PE/Cy5	5	Biolegend	307608
CD209	PE/Cy7	4	Biolegend	330114
Zombie	NIR	4	Biolegend	423106

For flow cytometry staining of extracellular markers, the cells were harvested into FACS tubes with 1 mL of PBA and centrifuged at 300g for 5 minutes. The supernatant was removed, and the pellet was re-suspended by tapping, which at this point the antibodies were added to the tube and incubated at 4°C for 30 minutes. After incubation 2mL of PBA was added and washed by centrifugation at 300g for 5 minutes, to ensure any unbound antibodies were removed. Then, the supernatant was removed, and the pellet was re-suspended in fixation buffer before analysis by spectral flow cytometer SONY ID7000. To interpret the results Kaluza software was used.

4.2.5 Extracellular vesicle staining

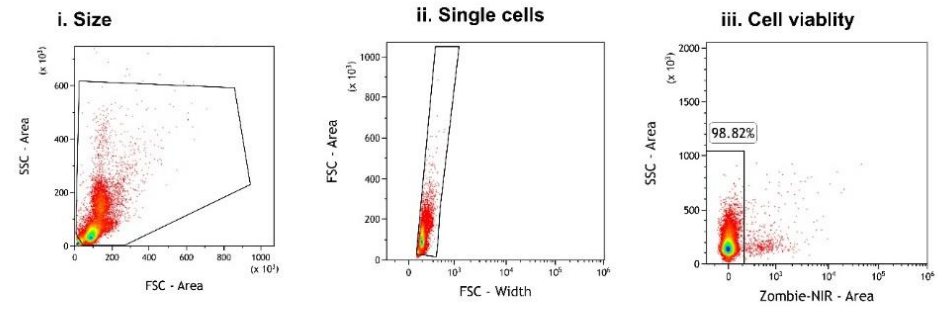
To assess the presence of EVs in media, 100µl of media was stained with 1µl of Calcein-AM and analysed on imaging flow cytometer ImageStreamX, and the results were interpreted in IDEAS software.

4.3 Results

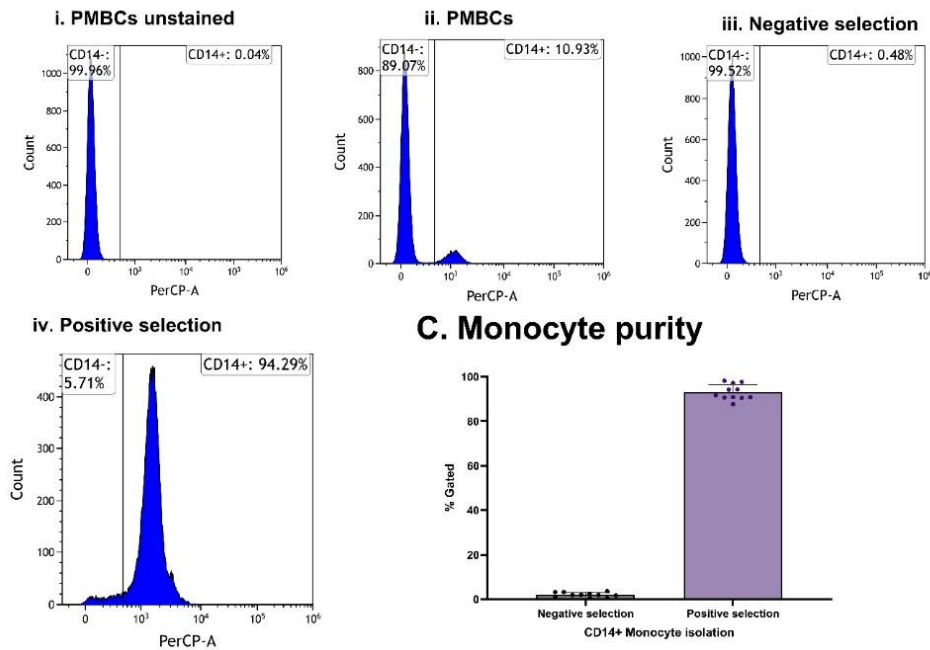
4.3.1 Gating strategies

To analyse monocyte purity, a gating strategy was developed in Kaluza. Firstly, all cells were selected according to their size and granularity (fig.4.1Ai), after which only single cells (fig.4.1Aii), and viable cells (fig.4.1Aiii) were included. Following the cell selection, cells were examined for CD14 expression, firstly by setting the cutoff of CD14⁺ cells, utilising unstained PBMCs (fig.4.1Bi). As CD14⁺ cells are known to be present in the PBMCs, they were stained for CD14, showing ~10% of PBMCs were CD14⁺ (fig.4.1Bii). After immunomagnetic isolation was performed on PBMCs, two fractions were generated; the CD14 negative fraction, which, as expected, had no CD14⁺ cells (fig.4.1Biii) and the CD14 positive fraction, that was ~95% CD14⁺ cells (fig.4.1Biv). Monocyte purity was tested multiple times after each CD14 magnetic isolation, where it was observed that monocyte purity was very high (fig.4.1C).

A. Cell selection



B. CD14⁺ Monocyte isolation purity



C. Monocyte purity

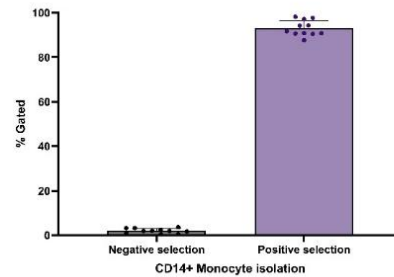


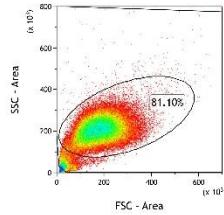
Figure 4.1 - Monocyte purity gating strategy. After PBMCs have been isolated from whole blood, CD14 positive selection of monocytes was performed. Cells of interest first were selected based on SSC and FSC values (Ai), then single cells (Aii), finally selecting only viable cells (Aiii). To select CD14⁺ monocytes, unstained PBMCs control was used (Bi), to ensure the starting presence of monocytes in PBMC prep CD14 staining was done (Bii). After CD14⁺ isolation was performed, both the negative (Biii) and positive (Biv) selections were stained. Monocyte purity was recorded for each experiment (C). n=15

To analyse DC maturation, a gating strategy was also developed in Kaluza. Firstly, cells were selected based on their size and granularity (fig.4.2Ai), afterwards only single and viable cells were selected (fig.4.2Aii, iii). After the DCs were selected, to observe their maturation, the relative mean fluorescence intensity (rMFI) of each maturation marker was calculated. To determine rMFI, the MFI of the unstained sample and the MFI of the stained sample were determined, then the sample MFI value was divided by the unstained MFI value, to give the rMFI of each maturation marker. Representative histograms of unstained, immature and matured dendritic cells were used to monitor the

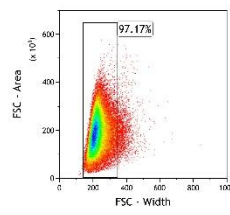
positive shift of each marker (fig. 4.2B).

A. Dendritic cell gating

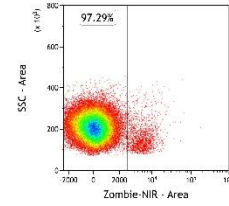
i. Size selection



ii. Singles

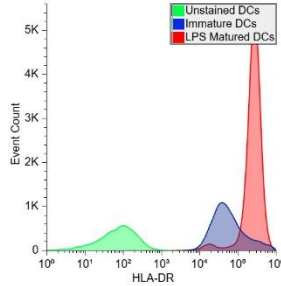


iii. Viability

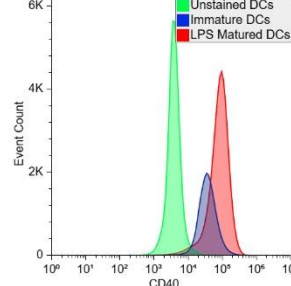


B. Dendritic cell maturation markers

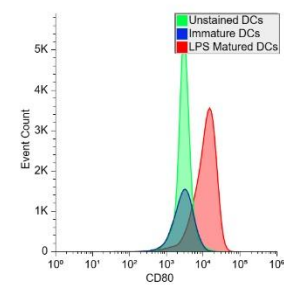
i. HLA-DR



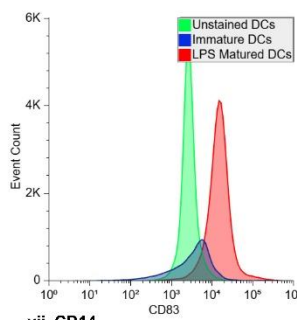
ii. CD40



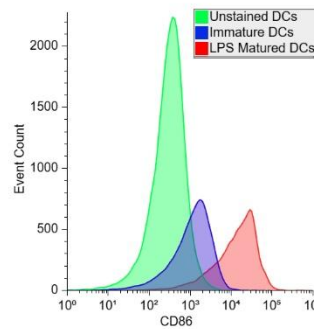
iii. CD80



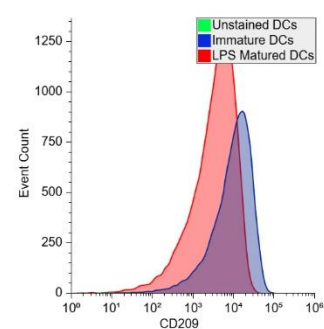
iv. CD83



v. CD86



vi. CD209



vii. CD14

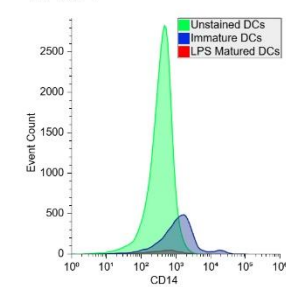


Figure 4.2 - Dendritic cell selection. Dendritic cells were selected based on their SSC and FSC values (Ai), then only singles were selected (Aii), and then only viable cells (Aiii). Maturation was measured by rMFI, which was calculated by dividing median fluorescence of sample with unstained median fluorescence. Representative histograms were included for each marker, comparing unstained, immature and matured DCs (B). HLA-DR (Bi), CD40 (Bii), CD80 (Bii), CD83 (Biv), CD86 (Bv), CD209 (Bvi), CD14 (Bvii).

4.3.2 Optimal culture media for DCs

In order to follow MISEV guidelines, it was necessary to find a culture media that did not have contamination with EVs, but still maintained high cell viability, as well as enabling DC maturation. Monocytes were plated in 4

culture media at $25 \times 10^3/\text{cm}^2$ seeding density – RPMI+10% FBS, X-VIVO, IMMUNOCULT and STEMXVIVO (fig.4.3). After 5 days of differentiation to immature DCs, cell viability was assessed by using zombie dye NIR, and again after 24h and 48h of stimulation with LPS and OVA to examine mature DC viability. Following the MISEV guidelines, it was necessary to have at least 60% viable cells. Both RPMI and X-Vivo media achieved the desired viability during all the stages – immature, after 24h and 48h stimulation with both LPS and OVA. Immunocult media had less than 60% viability at the 48h stage, and StemXVIVO media did not meet the 60% requirement at any of times tested. Due to low viability, StemXVIVO was dropped from future experiments, while the other media was further assessed for the most optimal culture media to use for culturing DCs.

A. Dendritic cell viability across media

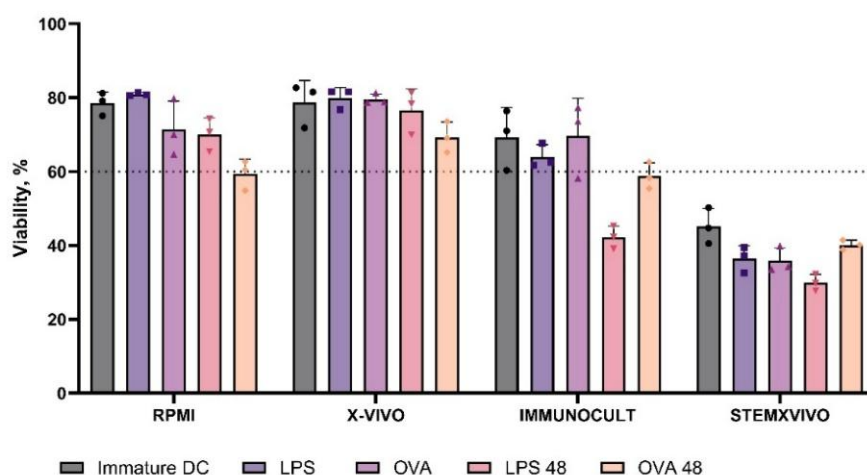
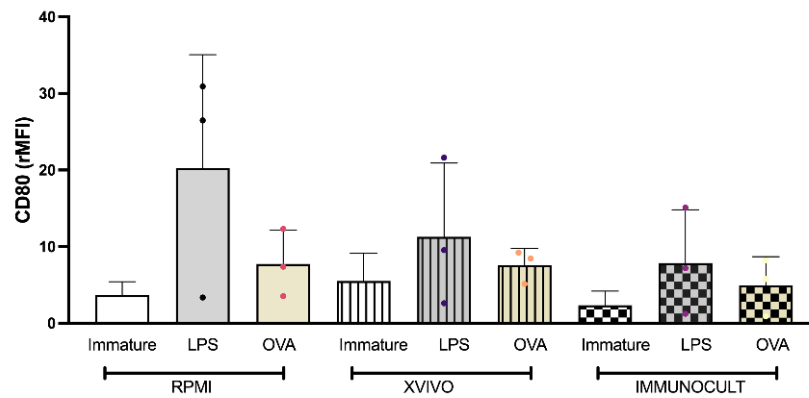


Figure 4.3 - Dendritic cell viability after stimulation in different media. DCs were cultured in 4 medias - RPMI+FBS, and 3 serum-free medias - X-VIVO, Immunocult, StemXVIVO. Viability was measured at immature stage and after 24h and 48h stimulation with 100ng/ml LPS and 200ng/ml OVA by zombie dye NIR. For EV work, MISEV guidelines recommend cell viability above 60%. n=3.

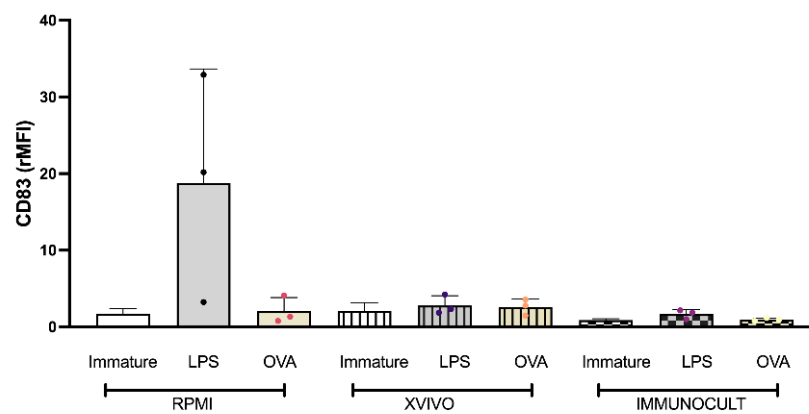
After examining cell viability, it was necessary to assess the maturation of DCs following stimulation with LPS and OVA using each media. Comparing CD80, CD83 and CD40 expression on immature DCs and after stimulation with LPS and OVA would allow examination of the maturation of DCs following culture in each media, as all of these markers are known to be upregulated on mature DCs (fig.4.4). All culture media achieved higher CD80 expression after stimulation with LPS and OVA compared to immature DCs

(fig.4.4A). Only RPMI media had higher CD83 expression following LPS stimulation, while X-VIVO and Immunocult media DCs did not express more CD83 following stimulation (fig.4.4B). CD40 expression was higher in the stimulated DCs in all media (fig.4.4C). Together, this shows DC maturation after stimulation with LPS and OVA, where the maturation markers, as expected, were more highly expressed in LPS-stimulated DCs.

A. DC CD80 expression



B. DC CD83 expression



C. DC CD40 expression

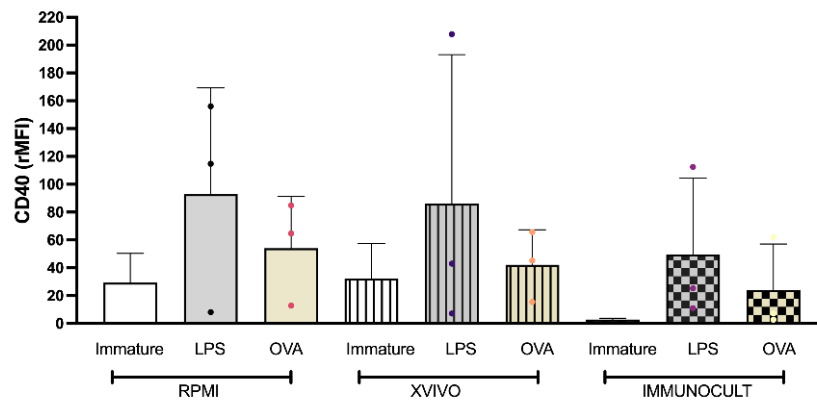


Figure 4.4 - Maturation markers on DCs across media. DCs were cultured in 3 media - RPMI+FBS, and serum-free XVIVO and Immunocult. Monocytes were seeded at $25 \times 10^3/\text{cm}^2$ concentration. Immature DCs were stimulated with 100ng/ml LPS and 200ng/ml OVA. DC maturation markers CD80 (A), CD83 (B) and CD40 (C) were measured by calculating rMFI for each marker. $n=3$.

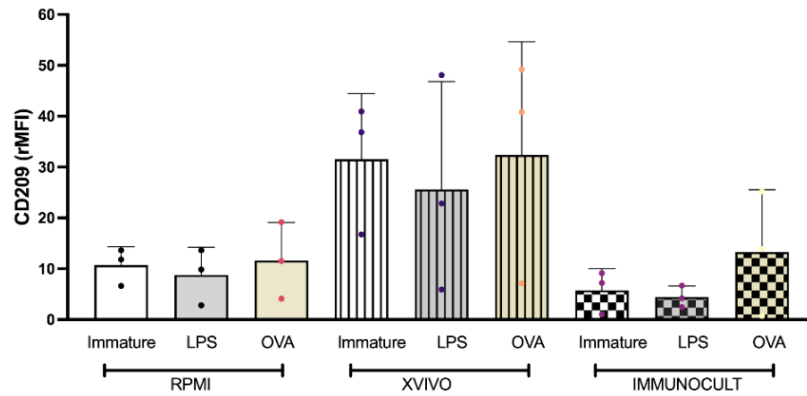
To further assess the maturation of DCs, CD209, HLA-DR and CD14 markers were measured following culture in each media (fig.4.5). CD209, also known as DC-SIGN, is expressed on both immature and mature DCs, but the expression is reduced on mature DCs. Interestingly, DCs cultured in RPMI

and Immunocult media had low expression of CD209 following stimulation, showing maturation, while DCs cultured in X-VIVO media still highly expressed CD209 during all stages (fig.4.5A). HLA-DR is a type of MHC class II molecule, and its expression is a key indicator of DC maturation. Importantly, HLA-DR was highly expressed in LPS-stimulated DCs within RPMI media, but in X-VIVO media it was expressed during all stages, while Immunocult DCs had lower expression of HLA-DR, except following culture with OVA (fig.4.5B). CD14 is expressed by monocytes and some immature DCs but is typically not found on mature DCs. CD14 expression was low across all media and all stages (fig.4.5C). Measuring these maturation markers raised questions on achieving maturation with both X-VIVO and Immunocult media.

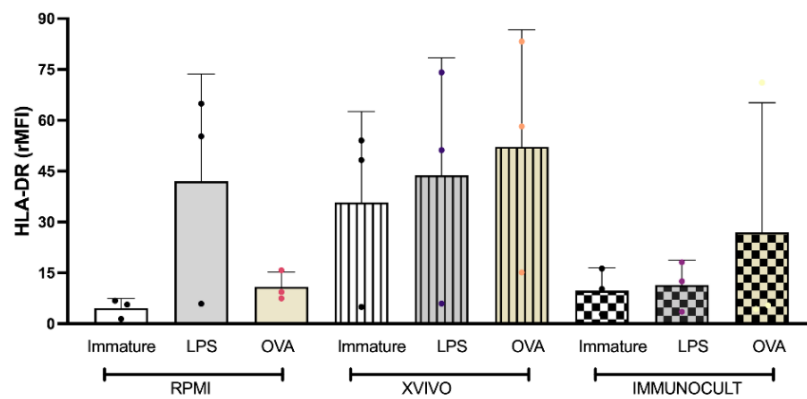
Taken together, assessing all the maturation markers of DCs cultured in the 3 media showed high maturation rates of DCs stimulated with LPS with RPMI media, while OVA-stimulated DCs showed low maturation rates. Furthermore, the decreased cell viability with Immunocult, and not observing upregulation of maturation markers, the media was excluded from further experiments. By not achieving the desired results of using either the serum-free media X-VIVO or the RPMI media supplemented with 10% FBS, it was

necessary to further examine cell viability and maturation by modifying the seeding density.

A. DC CD209 expression



B. DC HLA-DR expression



C. DC CD14 expression

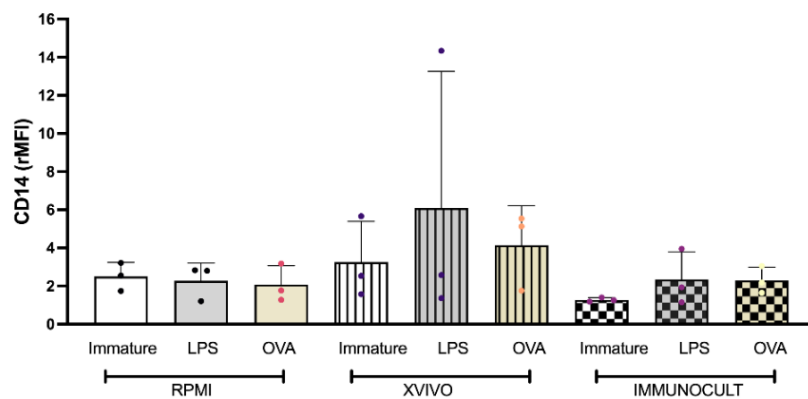
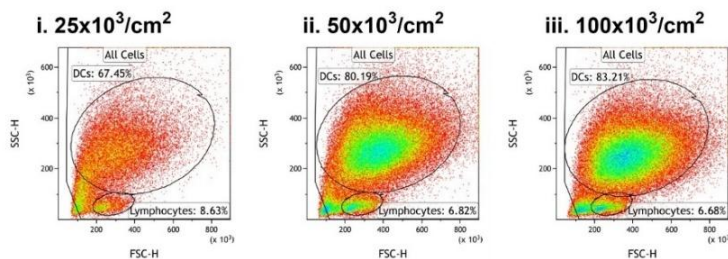


Figure 4.5 - Maturation markers on DCs across media. DCs were cultured in 3 media - RPMI+FBS, and serum-free XVIVO and Immunocult. Monocytes were seeded at $25 \times 10^3/\text{cm}^2$ concentration. Immature DCs were stimulated with 100ng/ml LPS and 200ng/ml OVA. DC maturation markers CD209 (A), HLA-DR (B) and CD14 (C) were measured by calculating rMFI for each marker. $n=3$.

4.3.3 Cell seeding density and platelet counts

Due to the DC maturation and viability results of the previous experiments with culture media, it was decided to assess the seeding density of monocytes in an attempt to improve cell viability and maturation. Furthermore, at this stage, it was identified that platelet contamination may be an issue due to EVs released from platelets. As such, monocytes were seeded in 3 concentrations – 25×10^3 , 50×10^3 and $100 \times 10^3/\text{cm}^2$ – and differentiated into immature DCs and subsequently stimulated with 100 ng/mL of LPS to achieve maturation, to assess both the DCs generated (fig.4.6A) and percentage of platelets in the culture (fig.4.6B). It was observed that the previous seeding density $25 \times 10^3/\text{cm}^2$ had a lower percentage of DCs generated (fig.4.6Ai) and a higher percentage of platelets present in the culture (fig.4.6Bi). While the highest seeding density of $100 \times 10^3/\text{cm}^2$ achieved both higher percentage of DCs generated after stimulation with LPS (fig.4.6Aiii) and lower percentage of platelets present in culture (fig.4.6Biii). Considering these results, it was decided that using higher seeding densities would achieve higher yield of DCs generated.

A. Monocyte seeding density



B. Platelet presence

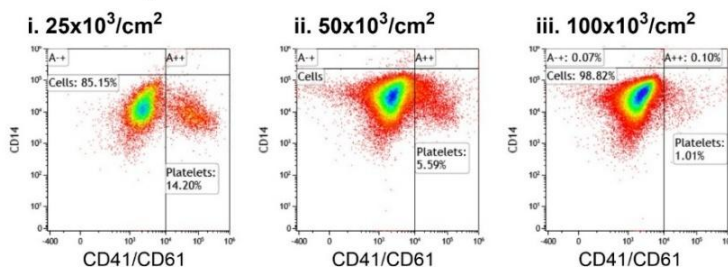
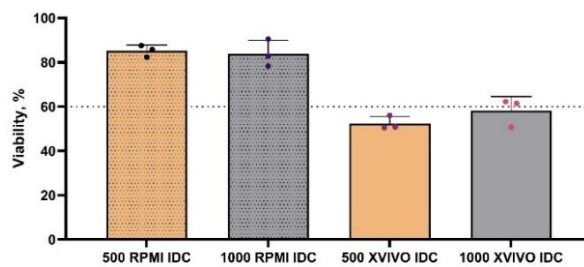


Figure 4.6 - Seeding density of monocytes and platelet contamination. Monocytes were seeded in 3 concentrations - $25 \times 10^3/\text{cm}^2$, $50 \times 10^3/\text{cm}^2$ and $100 \times 10^3/\text{cm}^2$ in RPMI+FBS. Monocytes were differentiated into immature dendritic cells. DCs were matured following 100 ng/mL LPS stimulation. Dendritic cell presence was monitored by SSC and FSC values across seeding densities (Ai, Aii, Aiii). Platelet contamination was tested at the same time across seeding densities (Bi, Bii, Biii).

4.3.4 DC seeding density and optimal culture media

Following the seeding density experiments with the higher yield of DCs with higher seeding densities, the two most optimal culture media were re-assessed, using RPMI + 10% FBS and serum-free X-VIVO media, with 50×10^3 and $100 \times 10^3/\text{cm}^2$ seeding densities. DCs were cultured for 5 days, and cell viability was measured using zombie dye AQUA during the immature stage and after stimulation with 100ng/mL of LPS for 24h (fig.4.7). RPMI media achieved higher cell viability during the immature stage than the X-VIVO media in both seeding densities (fig.4.7A), with DCs cultured in X-VIVO media not meeting the MISEV guideline of more than 60% viability. After stimulation with LPS, RPMI media still had higher cell viability than X-VIVO media in both seeding densities (fig.4.7B). From this, it was evident that DCs had higher survival rates in RPMI media compared to X-VIVO media.

A. Immature dendritic cell viability



B. Matured dendritic cell viability

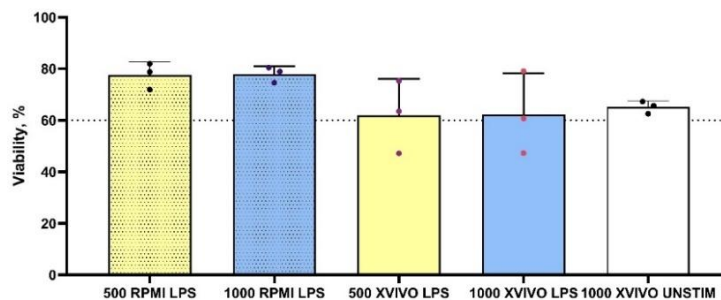
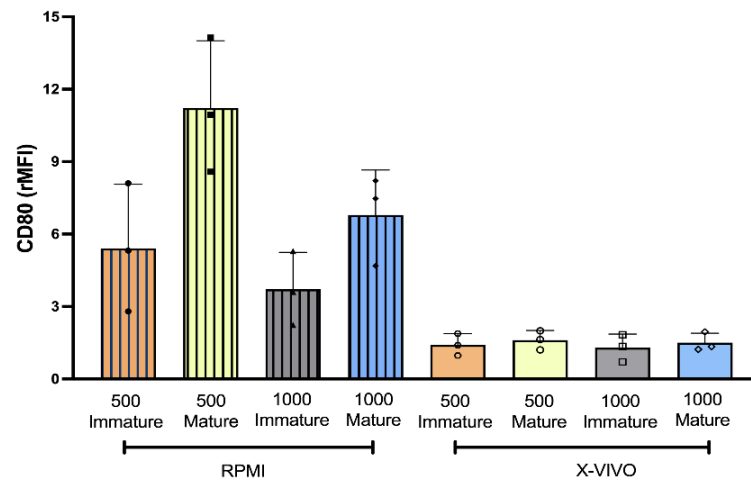


Figure 4.7 - Comparison of DC viability across media. DCs were cultured in RPMI+FBS or X-VIVO media at $50 \times 10^3/\text{cm}^2$ and $100 \times 10^3/\text{cm}^2$ seeding density. Viability was measured by zombie dye AQUA. DCs were stimulated with 100 ng/ml of LPS for 24h. DCs viability was measured at immature stage (A) and after 24h stimulation (B). $n=3$.

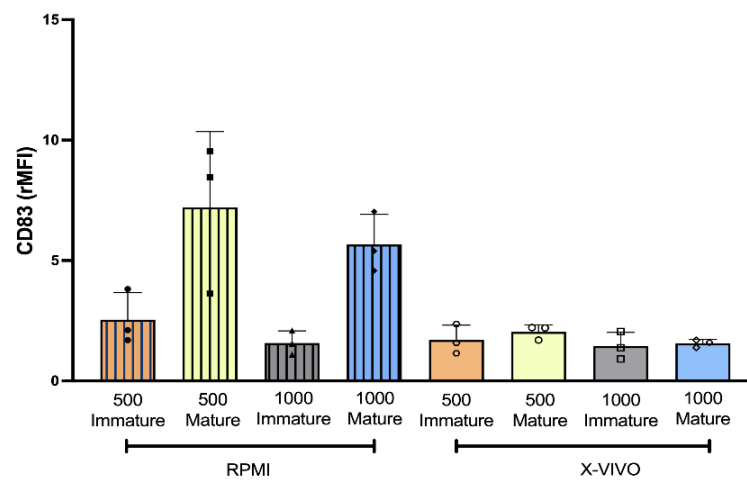
As before CD80, CD83 and CD40 markers were used to also measure the maturation rate of DCs in RPMI and X-VIVO media (fig.4.8). DCs in RPMI

media highly expressed CD80 after stimulation with LPS in both seeding densities, while X-VIVO media had low expression of CD80 (fig.4.8A). The same trend was observed for both CD83 and CD40 markers, where following LPS stimulation DCs cultured in RPMI media, both markers were highly expressed in both seeding densities, while X-VIVO DCs had low expression of both markers (fig.4.8BC). From these 3 maturation markers, it suggests DCs were maturing in RPMI media after LPS stimulation, while in X-VIVO media, they did not achieve maturation.

A. DC CD80 expression



B. DC CD83 expression



C. DC CD40 expression

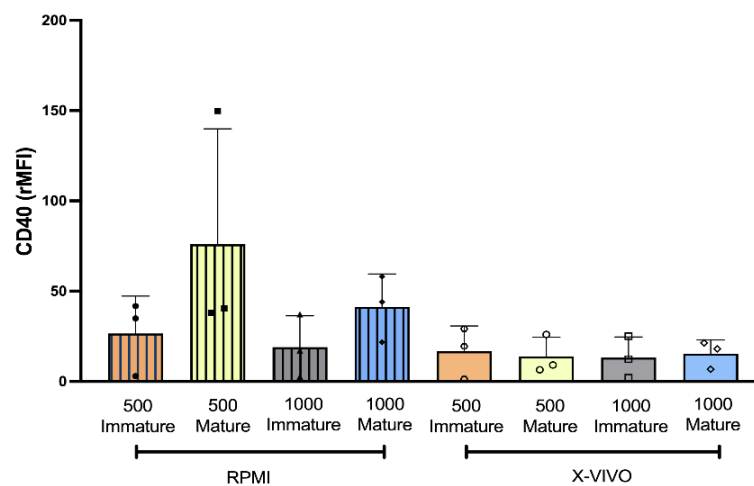
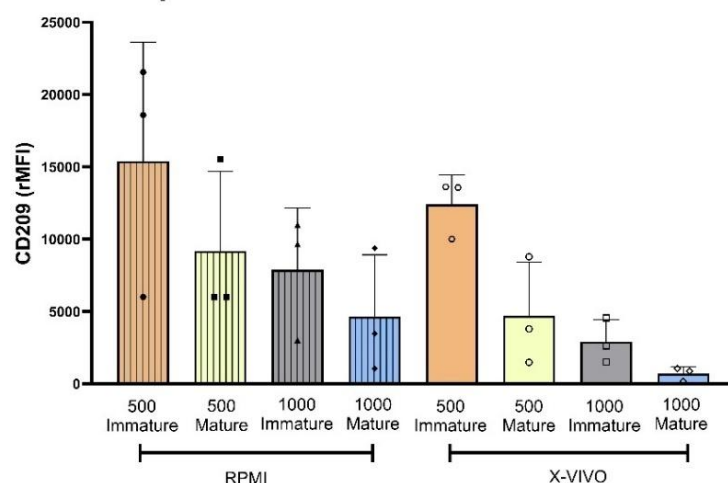


Figure 4.8 - Maturation markers on DCs in RPMI and XVIVO. DCs were cultured in 2 media - RPMI+FBS, and serum-free XVIVO. Monocytes were seeded at $50 \times 10^3/\text{cm}^2$ and $100 \times 10^3/\text{cm}^2$ concentration. Immature DCs were stimulated with 100ng/ml LPS. DC maturation markers CD80 (A), CD83 (B) and CD40 (C) were measured by calculating rMFI for each marker. $n=3$.

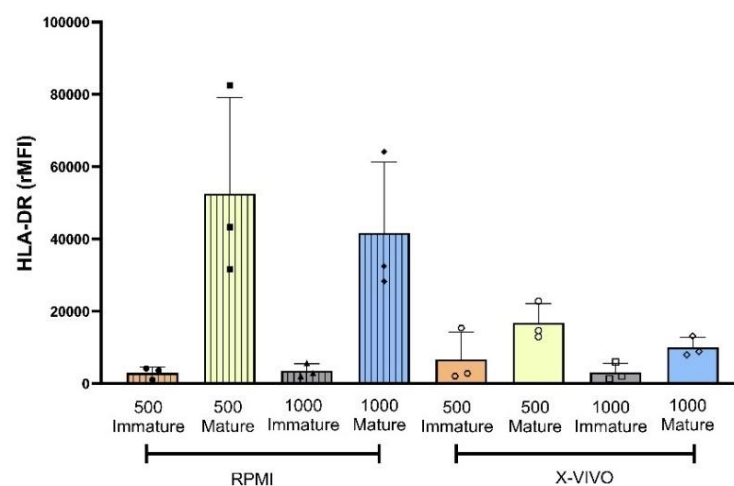
To further assess the maturation of DCs, CD209, HLA-DR and CD14 expression were measured on cells cultured in RPMI and X-VIVO media (fig.4.9). CD209 expression was decreased in both densities in RPMI and X-VIVO media following stimulation with LPS (fig.4.9A). LPS-stimulated DCs highly expressed HLA-DR in both seeding densities in RPMI media, while DCs in X-VIVO media had slightly increased expression of HLA-DR after stimulation (fig.4.9B). CD14 expression was almost undetected across both seeding densities and both media following stimulation (fig.4.9C). These maturation markers suggested that maturation rates were higher in RPMI media than X-VIVO media.

In summary, after assessing all 6 maturation markers, DCs achieved a higher rate of maturation in RPMI media with the highest seeding density of $100 \times 10^3/\text{cm}^2$ compared to X-VIVO media. However, as FBS contains EVs and MISEV guidelines suggest the use of serum-free media for work related to EVs, a culture media swap was then tested.

A. DC CD209 expression



B. DC HLA-DR expression



C. DC CD14 expression

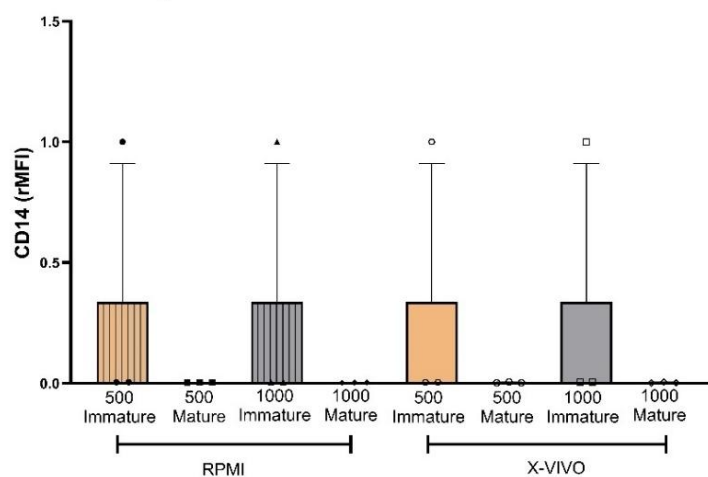


Figure 4.9 - Maturation markers on DCs in RPMI and X-VIVO. DCs were cultured in 2 media - RPMI+FBS, and serum-free X-VIVO. Monocytes were seeded at $50 \times 10^3/\text{cm}^2$ and $100 \times 10^3/\text{cm}^2$ concentration. Immature DCs were stimulated with 100ng/ml LPS. DC maturation markers CD209 (A), HLA-DR (B) and CD14 (C) were measured by calculating rMFI for each marker. $n=3$.

Following achieving poor cell viability and maturation of DCs using a serum-free X-VIVO media, monocytes were cultured within RPMI +10% FBS media to achieve a high yield of DCs, but the media was swapped to serum-free X-VIVO media before maturation to minimise the EV contamination. To ensure EVs were removed following a media swap, EV numbers were tested before and after swapping the media (fig.4.10). From the results it was observed that swapping the media to a serum-free media lowered the contamination of EVs before stimulation, but that DCs were still capable of secreting EVs following stimulation.

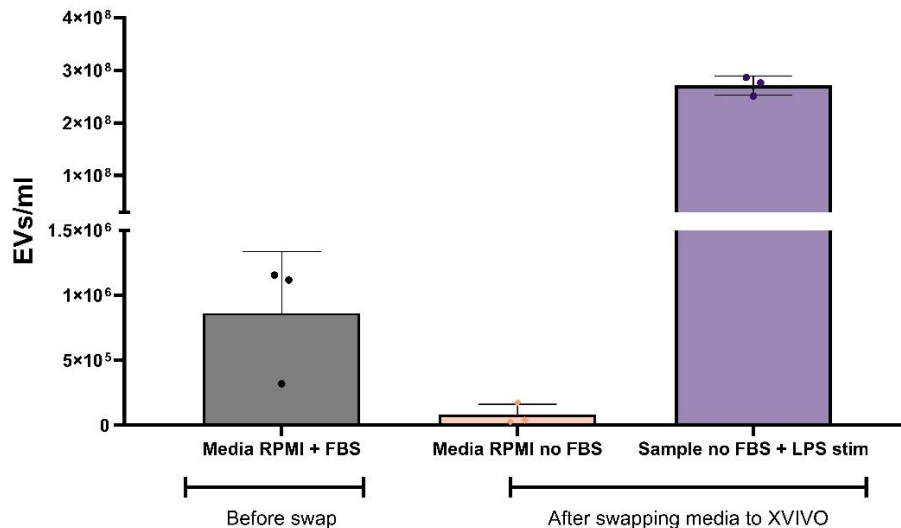


Figure 4.10 - Assay preparation. In order to ensure minimal contamination of FBS EVs, the media was swapped to serum-free media at 5th day before stimulation of DCs. Samples containing only media showed decreased number of EVs after the swap when compared to a sample containing DC produced EVs, the media contamination was minimal (A). $n=3$.

4.3.5 DC maturation rate using different OVA concentrations

After establishing that culturing DCs in RPMI + 10% FBS generated a high yield, viability and maturation following stimulation with 100ng/mL LPS, but that 200ng/mL OVA stimulation did not achieve the same high rate of maturation, the OVA concentration of 0.2 μ g/mL was increased to 10, 20 and 40 μ g/mL, and maturation was measured by using CD80, CD83 and CD40 maturation markers (fig.4.11). As before LPS-stimulated DCs achieved high expression of all 3 maturation markers of CD80, CD83 and CD40, while all the OVA concentrations did not achieve higher expression of these markers than the unstimulated DCs, except for a slight increase in CD83 expression (fig.4.11ABC). From these results, it was clear that LPS-stimulated DCs were matured, while OVA-stimulated DCs did not achieve the same maturation rate.

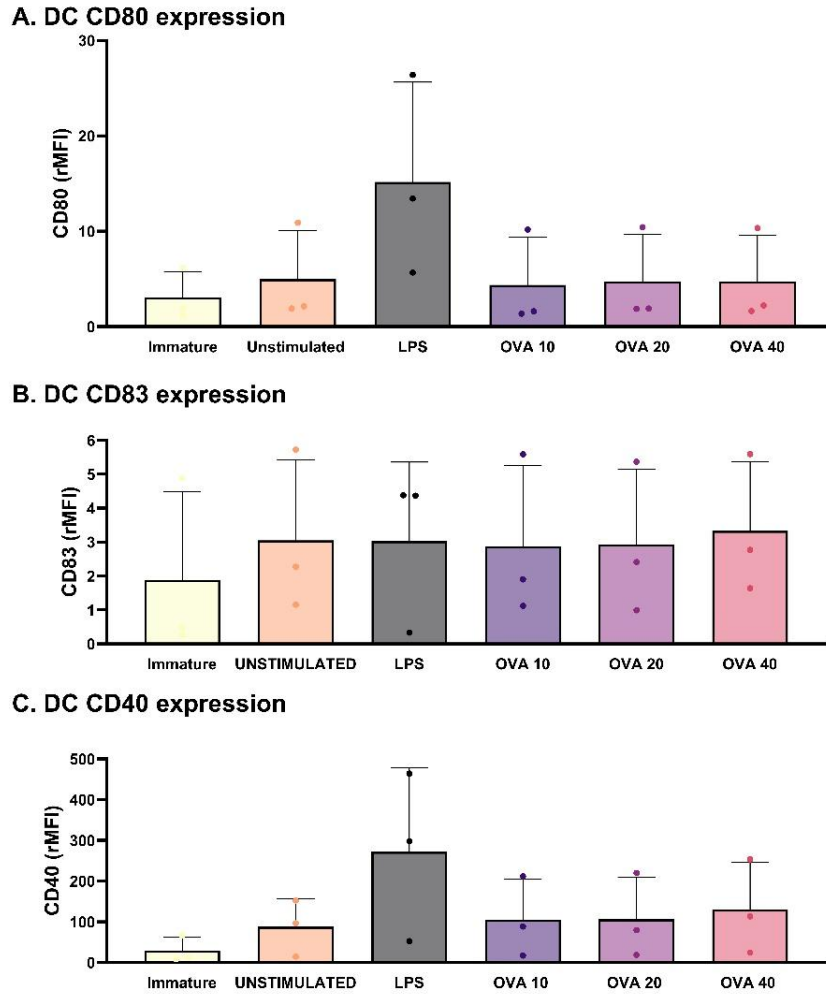


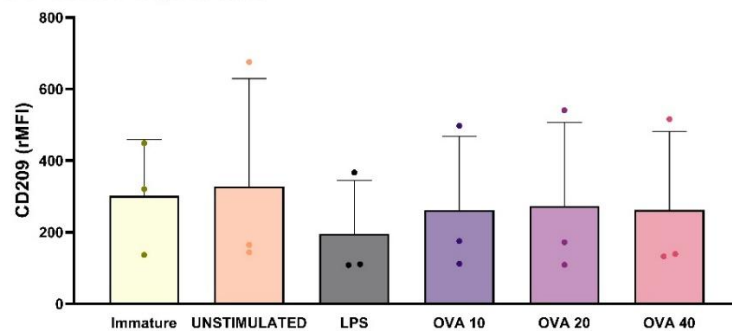
Figure 4.11 - Maturation markers on DCs with increasing OVA conc. DCs were cultured in RPMI+FBS and then swapped on the 5th day to serum-free XIVO media. Monocytes were seeded at $100 \times 10^3/\text{cm}^2$ concentration. Immature DCs were stimulated with 100ng/ml LPS, OVA (10 $\mu\text{g}/\text{ml}$), OVA 200 (20 $\mu\text{g}/\text{ml}$) and OVA 400 (40 $\mu\text{g}/\text{ml}$). DC maturation markers CD80 (A), CD83 (B) and CD40 (C) were measured by calculating rMFI for each marker. $n=3$.

To further assess the maturation of DCs, CD209, HLA-DR and CD14 expression was also measured following increasing concentrations of OVA (fig.4.12). Only LPS-stimulated DCs had lower expression of CD209 when compared to the immature DCs (fig.4.12A). HLA-DR was highly expressed in LPS-stimulated DCs, with also increased expression observed in OVA-stimulated DCs, however only the 40 $\mu\text{g}/\text{mL}$ concentration had a slightly increased value when compared to unstimulated DCs (fig.4.12B). CD14 expression was low across all stimulations (fig.4.12C). The 3 maturation

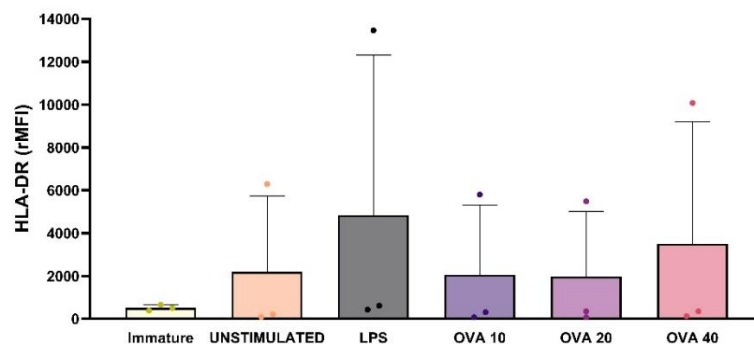
markers show again that LPS-stimulated DCs achieve a high rate of maturation, however OVA-stimulated DCs, regardless of concentration, do not.

Altogether, examining all 6 maturation markers, LPS-stimulated DCs showed a high level of maturation while OVA-stimulated DCs demonstrated a low level of maturation even with increased concentrations. From these findings, it was apparent that OVA alone was not enough to stimulate DCs to fully mature.

A. DC CD209 expression



B. DC HLA-DR expression



C. DC CD14 expression

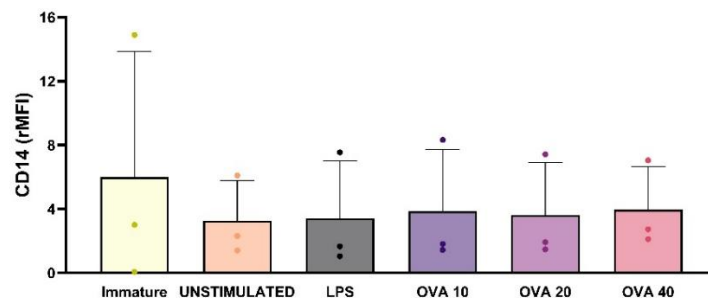
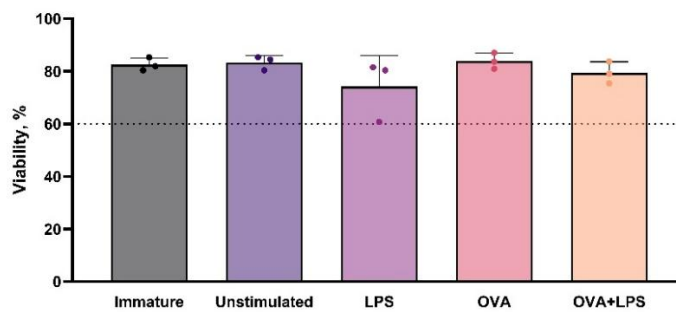


Figure 4.12 - Maturation markers on DCs with increasing OVA conc. DCs were cultured in RPMI+FBS and then swapped on the 5th day to serum-free XVIVO media. Monocytes were seeded at $100 \times 10^3/\text{cm}^2$ concentration. Immature DCs were stimulated with 100ng/ml LPS, OVA (10 $\mu\text{g}/\text{ml}$), OVA 200 (20 $\mu\text{g}/\text{ml}$) and OVA 400 (40 $\mu\text{g}/\text{ml}$). DC maturation markers CD209 (A), HLA-DR (B) and CD14 (C) were measured by calculating rMFI for each marker. $n=3$.

4.3.6 Final DC assay

Following the previous experiments, it was determined that OVA alone could not achieve full maturation, and increasing concentration did not influence maturation. Next, we tested co-stimulation of DCs with a decreased concentration of LPS at 50ng/mL, and 10 μ g/mL OVA, to test if this achieved higher cell viability and maturation (fig.4.13). Following DC culture and 24h stimulation with LPS, OVA and OVA + LPS, the cell viability remained high and above the 60% requirement of MISEV guidelines (fig.4.13A). After 48h of stimulation only LPS-stimulated DCs consistently remained above 60%, while unstimulated and OVA were on the cutoff, and OVA + LPS-stimulated DCs fell below 60% viability (fig.4.13B). These results suggest that DCs remain highly viable after 24h stimulation, while after 48h the viability starts to drop.

A. DC viability after 24h stimulation



B. DC viability after 48h stimulation

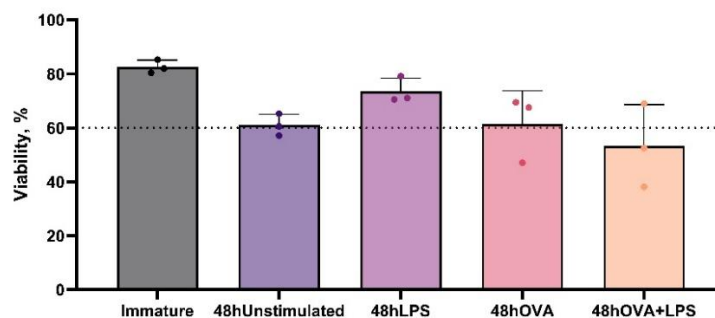
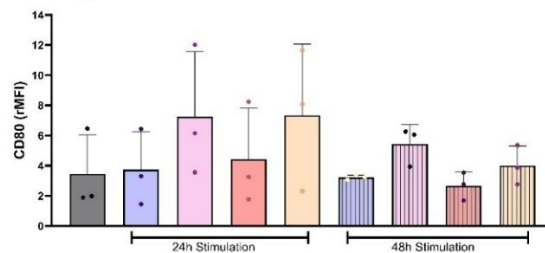


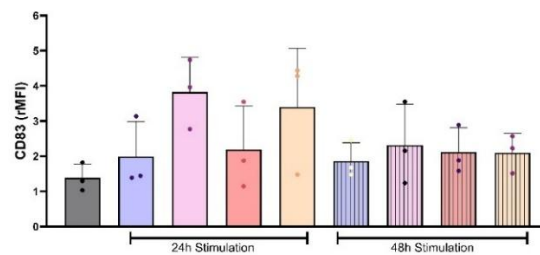
Figure 4.13 - Comparison of DC viability with different stimulations. DCs were cultured in RPMI+FBS at 100x10³/cm² seeding density and then swapped at 5th day for XVIVO media. Viability was measured by zombie dye AQUA. DCs were stimulated with 100 ng/ml of LPS, 10 μ g/ml of OVA and 50ng/ml LPS + 10 μ g/ml OVA for 24h and 48h. DCs viability was measured at immature stage, 24h stimulation (A) and 48h stimulation (B). n=3.

As before CD80, CD83 and CD40 markers were used to assess the maturation of DCs following stimulation with LPS, OVA and OVA + LPS after 24h and 48h (fig.4.14). CD80 was highly expressed in both LPS and OVA + LPS-stimulated DCs after 24h, while OVA-stimulated DCs had the same expression as the immature and unstimulated DCs. 48h stimulation followed the same results as their 24h counterparts (fig.4.14A). LPS and OVA+LPS stimulated DCs highly expressed both CD83 and CD40 markers after 24h, while after 48h stimulation, only LPS-stimulated DCs had increased CD40 expression (fig.4.14BC). These findings suggest that both LPS and OVA + LPS stimulated DCs achieved maturation after 24h, while after 48h stimulation the maturation rate decreased, and OVA alone showed low level of maturation regardless of the duration of the stimulation.

A. DC CD80 expression



B. DC CD83 expression



C. DC CD40 expression

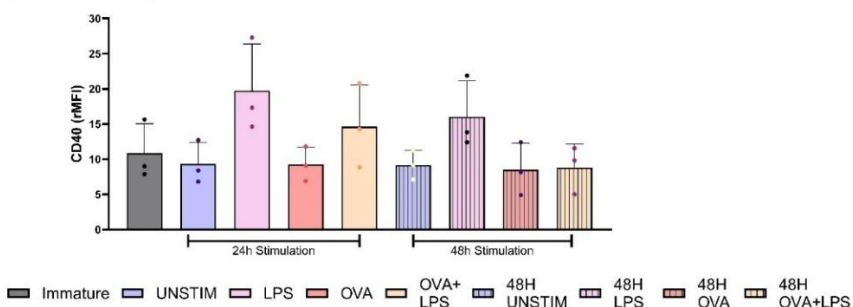


Figure 4.14 - 24h and 48h Maturation markers on DCs with different stimulations. DCs were cultured in RPMI+FBS and then swapped on the 5th day to serum-free XIVO media. Monocytes were seeded at $100 \times 10^3/\text{cm}^2$ concentration. Immature DCs were stimulated with 100ng/ml LPS, OVA ($10 \mu\text{g}/\text{ml}$), and 50ng/ml LPS + $10 \mu\text{g}/\text{ml}$ OVA. DC maturation markers CD80 (A), CD83 (B) and CD40 (C) were measured by calculating rMFI for each marker. $n=3$.

To further assess the maturation of DCs, CD209, HLA-DR and CD14 expression were also investigated following stimulation with LPS, OVA and OVA + LPS for 24h and 48h (fig.4.15). Interestingly, CD209 expression was quite varied between the repeats, with only LPS-stimulated DCs after 24h and 48h stimulation showing decreased expression (fig.4.15A). In both LPS and OVA + LPS-stimulated DCs, HLA-DR expression was increased following 24h, while after 48h the expression was increased further (fig.4.15). CD14 expression was low in all conditions following stimulation (fig.4.15C). These results further suggest that DCs stimulated with LPS and OVA + LPS achieved maturation after 24h and 48h, while OVA alone had low levels of maturation regardless of the duration of the stimulation.

In summary, the 6 maturation markers showed that both LPS and OVA + LPS stimulation successfully induced maturation in DCs after 24h, while 48h stimulation showed mixed results of maturation, and lower levels of viability.

It was thus determined that for future work, 24h stimulation would be used to achieve a high yield of DCs and induce their maturation.

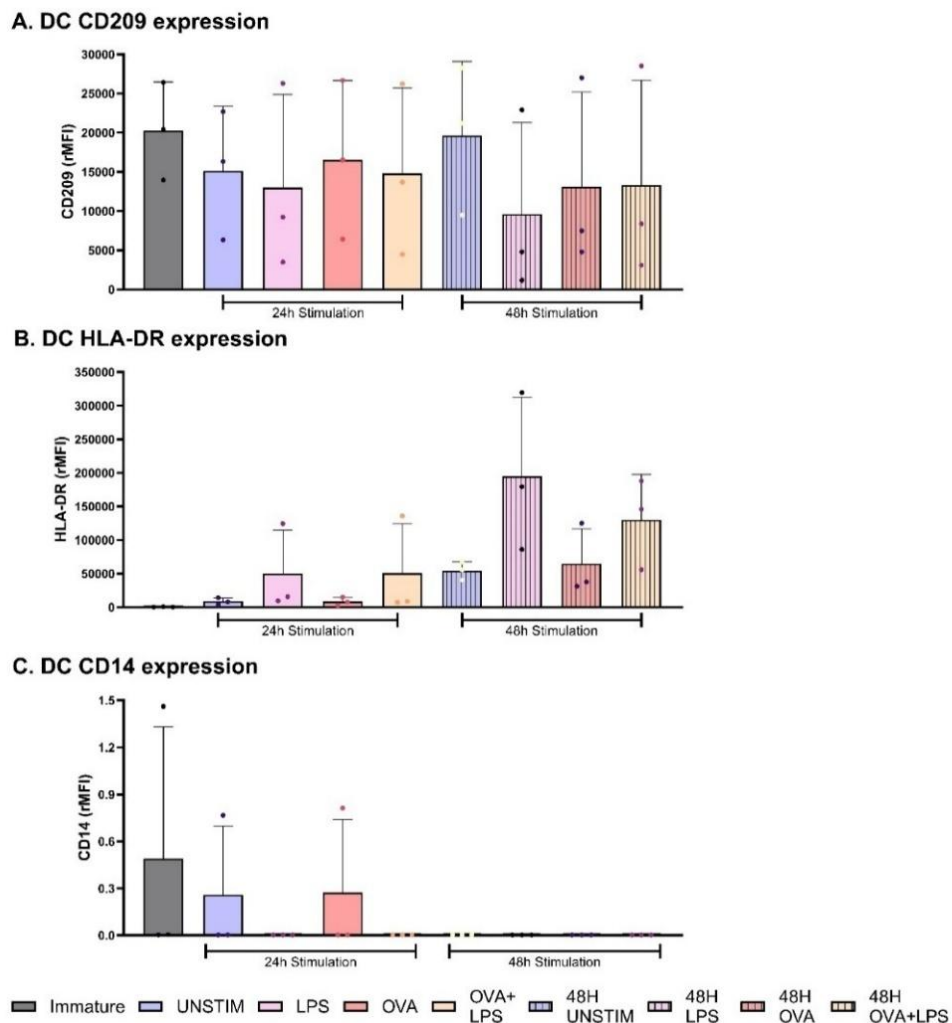


Figure 4.15 - 24h and 48h Maturation markers on DCs with different stimulations. DCs were cultured in RPMI+FBS and then swapped on the 5th day to serum-free XVIVO media. Monocytes were seeded at $100 \times 10^3/\text{cm}^2$ concentration. Immature DCs were stimulated with 100ng/ml LPS, OVA (10 $\mu\text{g}/\text{ml}$), and 50ng/ml LPS + 10 $\mu\text{g}/\text{ml}$ OVA. DC maturation markers CD209 (A), HLA-DR (B) and CD14 (C) were measured by calculating rMFI for each marker. $n=3$.

By combining all the previous findings, culturing DCs in RPMI + 10% FBS at $100 \times 10^3/\text{cm}^2$ and then swapping to serum-free X-VIVO media before stimulation achieved the highest yield of DCs, and stimulation of LPS and OVA + LPS for 24h had the highest maturation rate. These culture conditions were used for any future experiments.

4.4 Discussion

To ensure the successful growth and differentiation of human monocytes into DCs, it was necessary to optimize the culture conditions. Several culture media were tested – RPMI + 10% FBS, X-VIVO, StemX-VIVO and Immunocult. It was found that DC viability was the highest in RPMI + 10% FBS, however, to follow MISEV guidelines, it is necessary to use serum-free media to minimise the contamination of EVs present in the culture media. As such, other methods were explored to improve the viability of DCs, such as seeding densities of monocytes, where it was found that $100 \times 10^3/\text{cm}^2$ increased the yield of DCs generated in both RPMI + 10%FBS and X-VIVO serum-free media. These observations align with earlier reports that higher seeding densities enhance monocyte survival and DC generation without causing excessive aggregation or differentiation defects ([307]. Furthermore, by increasing the seeding density, the percentage of platelets present in the culture decreased relative to the percentage of DCs present. However, once attempting to mature the immature DCs by stimulating them with LPS or OVA in both RPMI and X-VIVO media, it was observed that the DCs in the X-VIVO media did not show signs of maturation by not expressing the maturation markers tested, which coincided with previous work where serum-free cultured DCs expressed lower levels of maturation markers ([308] [309]). To minimise the contamination of FBS-derived EVs in the culture, a new method was tested, where human monocytes were differentiated in RPMI + 10%FBS media for 5 days, and before maturation, the media was swapped to X-VIVO serum-free media. By testing the EVs present in the media before and after swapping, it was observed that the EV numbers were lower once swapped into the X-VIVO serum-free media. Furthermore, the EVs produced by DCs following LPS stimulation were in much higher quantities than the EVs present in the media, accounting for less than 1%, which is considered acceptable by MISEV guidelines.

Once the most optimal culturing conditions were found for culturing DCs from monocytes, it was necessary to optimise the concentration of OVA used for maturation, as during the initial experiments, it was observed that 200 ng/ml was not sufficient to mature DCs. However, increasing the OVA concentrations to 10, 20, and 40 µg/ml also was not successful in maturing the

DCs. Once consulting previously published literature it was found that increasing OVA past 10µg/ml could lead to adverse genetical changes in DCs ([310]), which was not desired as it could lead to changes in EV production and affect their composition. It was thus decided to use 10µg/ml to minimise these effects and add LPS co-stimulation to try to induce maturation in DCs with OVA present. Here, we showed that exposing DCs to both LPS alone and LPS plus OVA for 24 hours resulted in fully matured DCs with high viability.

In summary, the most optimal way of culturing human DCs was by differentiating human monocytes in RPMI + 10%FBS media for 5 days at $100 \times 10^3/\text{cm}^2$ density, and then before maturation, fully swapping the media with serum-free media X-VIVO. Maturation was achieved by stimulation of LPS and/or OVA following 24h exposure.

Chapter 5: Optimization of EV techniques

5.1 Introduction

5.1.1 Current techniques for EV research

EVs are present in various bodily fluids, such as blood, urine, breast milk, and saliva. These nanoscopic particles are diverse in their biogenesis, size, origin, surface markers, and cargo [311]. EVs play a role in numerous physiological and pathological processes, making them promising non-invasive biomarker sources. However, their small size and heterogeneity pose challenges for EV research. As a result, ongoing efforts focus on improving methods for isolating and characterizing EV populations. The International Society for Extracellular Vesicles (ISEV) has introduced the Minimal Information for Studies of Extracellular Vesicles (MISEV) guidelines to try to address these challenges ([312]). These guidelines outline ISEV's recommendations for the “minimum experimental standards for defining extracellular vesicles and their functions” ([312]).

A significant hurdle in EV research is achieving complete purification or isolation of EVs from other entities [312]. Highly purified EVs are crucial for accurately linking specific functions or biomarkers to vesicles rather than other particles. Various methods have been employed to isolate and enrich EVs from biological samples. Differential ultracentrifugation remains a widely used technique for EV isolation and concentration, but it has drawbacks, such as low yield, potential vesicle damage, and contamination with non-EV particles or proteins ([313-315]). Alternative isolation methods include density gradients, precipitation, filtration, size exclusion chromatography, and immunoisolation ([312] [316]). The MISEV 2024 guidelines advise selecting a separation method based on the intended downstream applications and research questions, as no single method is optimal, and common EV isolation protocols cannot fully separate non-vesicular entities from EVs [312]. A more comprehensive overview of the major EV isolation techniques and their specific limitations is warranted to contextualise the optimisation work described later in this chapter. Differential ultracentrifugation (DUC) is historically the most common

method, involving sequential centrifugation steps at increasing speeds to pellet EVs based on size and density. While effective for concentrating EVs from large volumes, DUC often results in low recovery rates (typically 10–30%), significant vesicle damage or aggregation due to high g-forces, and co-isolation of non-EV contaminants such as protein aggregates and lipoproteins. Density gradient ultracentrifugation (e.g., iodixanol or sucrose gradients) offers improved purity by separating EVs according to buoyant density, but it is time-consuming (often >18 hours), requires expensive equipment, and still suffers from variable recovery and potential loss of fragile subpopulations. Precipitation methods (e.g., polyethylene glycol or commercial kits like ExoQuick) are simple, high-throughput, and do not require ultracentrifugation, making them attractive for small-volume samples; however, they frequently co-precipitate non-EV material (e.g., proteins, lipoproteins), resulting in lower purity and potential functional artefacts. Size-exclusion chromatography (SEC) separates EVs from soluble proteins and other particles based on size using porous columns, providing high purity and preserving vesicle integrity, but it is limited by low throughput, sample dilution, and the risk of column clogging with viscous biofluids. Immunoaffinity capture uses antibodies against EV-specific surface markers (e.g., CD63, CD81) to selectively pull down subpopulations; while highly specific, it is biased toward marker-positive EVs, misses marker-negative subsets, and can be expensive and low-yield. Filtration-based methods (e.g., ultrafiltration) are rapid and scalable but often co-isolate non-EV contaminants and can cause vesicle damage due to shear stress. Overall, no single method achieves complete separation of EVs from non-vesicular entities, and the choice of isolation technique must be guided by the downstream application, as emphasised by MISEV 2024 [295]. The limitations of these methods—particularly co-isolation of contaminants, low yield, and potential vesicle damage—directly informed the optimisation experiments described in subsequent sections of this chapter, where SEC was ultimately selected for its superior balance of purity and EV retention.

A variety of methods have been employed to characterize the size, count, and molecular profile of EVs. These include TEM ([317], cryo-TEM ([318], confocal microscopy ([319], dynamic light scattering (DLS) ([320],

direct stochastic optical reconstruction microscopy (d-STORM) ([321]), nanoparticle tracking analysis (NTA) ([322]), resistive pulse sensing ([321]), and flow cytometry ([320]), among others. Electron microscopy (EM), particularly TEM, is a standard approach for EV characterization, providing high-resolution images to assess size and morphology ([323]). A widely used alternative, Nano Tracking Analysis (NTA), relies on light scattering from particles undergoing Brownian motion to accurately determine size distribution for both monodisperse and polydisperse nanoparticle populations ([324] [325]). NTA also enables assessment of EV concentration and phenotype. Other light-scattering techniques, such as flow cytometry, can measure particle counts. There are different types of high-throughput flow cytometers, including traditional flow cytometry (FCM), imaging flow cytometry (IFC), and nanoflow cytometry (nFCM). FCM offers high throughput but lacks the resolution to precisely characterize individual EVs, especially smaller ones ([326]). IFC, with lower throughput than FCM but higher than nFCM, capture images of individual cells and particles, allowing detection of submicron EVs, as shown in previous studies ([327, 328] [329] [330]). However, IFC struggles to detect low-abundance EV populations. nFCM, designed for small particle analysis, operates at slower flow rates to maintain sensitivity for nanoparticle detection, but is limited by lower sample throughput, reduced multiplexing capabilities, and complex, time-consuming sample preparation.

Additionally, EVs encompass various subtypes that differ in size and cellular origin. Currently, no specific or universal molecular markers exist to distinctly characterize each EV subtype. The MISEV guidelines currently advise that, for protein-based characterization of EVs, at least one protein from multiple categories should be analysed to confirm the EV nature and assess the purity of an EV preparation ([312]). Category 1 proteins include transmembrane or GPI-anchored proteins linked to the plasma membrane and/or endosomes, such as non-tissue-specific proteins like tetraspanins (CD63, CD81, CD82) and MHC class I, as well as tissue-specific proteins including CD9, CD90, CD45 and MHC class II. Category 5 proteins are soluble extracellular proteins that can bind to receptors on the surface of EVs, including cytokines, growth factors, and extracellular matrix components. Non-

protein markers are also frequently used to identify EVs. These include dyes activated by intracellular EV components. For instance, Calcein-AM, a cell-permeant non-fluorescent pro-dye, is cleaved by intravesicular esterase into fluorescent Calcein, EV-impermeant form, enabling differentiation of intact EVs from membrane fragments ([331]).

5.1.2 Chapter objectives

This chapter aims to provide the optimise techniques for size and characterisation of EVs, as well as establishing working protocols for cell culture, EV staining and isolation techniques.

5.2 Methods

5.2.1 PBMC isolation

The same method was followed as previously described in Chapter 2 methods sections 2.2.11.

5.2.2 T cell activation

96-well u bottom plate was coated with 2µg/ml of anti-CD3 for 90 min at 37°C and washed twice with cold PBS. PBMCs were diluted to 2×10^6 cells/ml, and then 200µl were plated in 96-well u bottom wells. 2µg/ml of anti-CD28 was added directly to the wells and the cells were incubated at 37°C for 24h or 48h.

5.2.3 CD14⁺ Monocyte isolation

PBMCs were mixed with 80µl of MACS buffer and with 20µl of CD14⁺ microbeads per 10^7 cells and incubated at 4 C for 15 min. Cells were washed with MACS buffer at 300g for 10 min. The pellet was re-suspended in 500µl of MACS buffer and ran through LS column. The column was washed 3 times with 3ml of MACS buffer and eluted into a sterile tube with 5ml of buffer, to recover the CD14⁺ monocytes.

5.2.4 Dendritic cell culture

CD14⁺ monocytes were re-suspended in RPMI + 10% FBS and cultured in 48 well flat-bottomed tissue culture-treated plate at 1×10^6 cells/ml. To differentiate monocytes into iDCs, 50 ng/ml of GM-CSF and 20 ng/ml of IL-4 were added. The cells were incubated at 37 °C for 5 days, replenishing 50% of media and cytokines on day 3. On day 5 the media was changed to serum-free media X-VIVO, before stimulating with 100 ng/ml of LPS and/or 10 µg of OVA, in order to mature the iDCs.

5.2.5 Primary human bronchial epithelial cell culture

MucilAirTM, a fully differentiated bronchial epithelial model derived from primary human cells of healthy donors, was obtained from Epithelix Sàrl. The samples were provided as 24-well transwell inserts, which were placed into a sterile 24-well plate containing phenol red-free and serum-free MucilAirTM culture medium (Epithelix Sàrl). These were maintained in a humidified incubator at 37°C with 5% CO₂, with the medium refreshed every 2–3 days. The aspirated medium, containing extracellular vesicles (EVs), was collected for subsequent analysis. This work was carried out by Dr Georgina Hopkins.

5.2.6 Size exclusion chromatography

Cell supernatants were removed from wells and centrifuged at 300g for 8 minutes to remove any cell debris. EVs were then isolated by SEC using qEV original 35 columns with an automated fraction collector (Izon Science). Fractions 1-7 of 500 µL were collected, pooled, and concentrated by centrifuging at 4000g for 10 minutes using 10 kDa amicon columns (Merck). The result is ~250 µL of EVs in solution, ranging from 35 nm to 350 nm in size.

5.2.7 Transmission electron microscopy

EVs isolated via SEC were applied to carbon-coated 300 Cu mesh TEM grids for 15 minutes, followed by fixation with 2% paraformaldehyde for 10

minutes. The grids were rinsed five times with deionized water and then stained with 4% uranyl acetate for 10 minutes. Excess stain was removed, and the grids were allowed to dry completely before imaging with a Tecnai BioTwin transmission electron microscope (TEM) operating at 80 kV. Images were captured using a Gatan Orius camera.

5.2.8 Super-resolution microscopy

SEC isolated EVs were stained with tetraspanin antibodies tagged with blinking fluorophores (EV profiler Kit, EV-MAN-1.0, Oxford Nanoimaging) (CD81-647, CD63-561 and CD9-488) for 60 min at 4°C. Pre-coated slide with capture antibodies was provided by the company, which was then blocked with 5% BSA. The labelled EVs were then placed on the slide and incubated for 60 min at room temperature. To ensure higher clarity of data acquired, the slides were fixed with 4% formaldehyde for 10 min. Setting up the ONI supermicroscope was done according to the manufacturer's instructions. Briefly, focus, laser power, exposure and frame rate were adjusted based on the established protocols set by the manufacturer to utilise the single particle tracking (SPT). The data acquired was analysed using the manufacturer's software CODI.

5.2.9 Nanoparticle tracking analysis

EVs isolated via SEC were diluted 1:1000 in 0.22 μ M filtered PBS and loaded into an LM10/14 Nanosight instrument (Nanosight, Malvern Panalytical, UK) for particle size distribution and quantification. Before analysis, the instrument's sensitivity was calibrated using a 1:10 dilution of 100 nm carboxylated polystyrene nanoparticles (IZON) and a 1:1000 dilution of 200 nm polystyrene nanoparticles (Malvern Panalytical). Automatic settings were used for minimum expected particle size, minimum track length, and blur. The instrument was configured with a sensitivity of 78, a gain of 26.88, and a temperature of 21 °C. National Institute of Standards and Technology (NIST) beads were analyzed at a sensitivity of 65. Data processing and particle size distribution analysis were conducted using NTA Software 3.3 Dev build 3.3.301 (Malvern Panalytical).

5.2.10 Sizing of EVs

Polystyrene size standards certified by the National Institute of Standards and Technology (NIST) (60 nm, 80 nm, 101 nm, 125 nm, and 151 nm; ThermoFisher 3060A, 3080A, 3125A, 3100A, and 3150A) were employed on the ImageStream X Mk II, CytoFLEX S, and nanoparticle tracking analysis (NTA) systems. The beads were diluted in 0.22 μm sterile-filtered PBS, with dilutions adjusted for each instrument: 1×10^{-4} to 1×10^{-5} for ImageStream X MkII, 1×10^{-5} for CytoFLEX S, and 1×10^{-6} for NTA. For the CytoFLEX Nano, NIST-certified polystyrene size standards (80 nm, 101 nm, 200 nm, 300 nm, and 450 nm; ThermoFisher 3080A, 30100A, 3200A, 3300A, and 3450A) were used at a dilution of $2.5 \times 10^{-7}/\text{mL}$. Light scattering signals from NIST bead populations analyzed on the CytoFLEX S and CytoFLEX Nano were processed with Mie theory using FCM PASS software. The manufacturer-provided refractive index for each bead was entered into the software. Effective scattering cross sections of the calibration beads were determined by integrating amplitude scattering matrix elements across 576 collection angles. Both data and theoretical values were log10-transformed to align the data with the theory using a least-square-fit approach.

For the ImageStream X MkII, the vFC™ extracellular vesicle (EV) analysis assay kit (Cellarcus Biosciences) was utilized to assess EV size, following the manufacturer's protocol. Initially, the instrument was calibrated with vCal™ nanoRainbow beads using the provided IDEAS layout from the manufacturer. Then, 5 μL of either the Lipo100™ synthetic vesicle size standard or primary epithelial cell supernatant was stained with vFRed™ membrane dye at a 1X concentration in vFC™ staining buffer, in a total volume of 50 μL , and incubated for 1 hour at room temperature in the dark. Samples were subsequently diluted 1:30 in staining buffer prior to analysis on the ImageStream X MkII. Data files were imported into IDEAS software, exported as FCS files, and analyzed using FCS Express (DeNovo Software) with the manufacturer's provided layouts. The Lipo100™ size standard, with a size range of 80–140 nm as established by nanoparticle tracking analysis (NTA), was used to select vFRed-positive events. These were calibrated in

FCS Express using the transformation tool and a surface area equation provided by the manufacturer's layout. This calibration was then applied to primary epithelial cell samples to determine EV sizes.

5.2.11 Tetraspanin Staining of EVs

Cell culture supernatants were collected from wells and centrifuged at 300g for 8 minutes to eliminate cellular debris. Either EV-containing supernatants or isolated EVs, in a 50 μ L volume, were stained with calcein-AM (Biolegend) at a final concentration of 10 μ M, vortexed for 1 minute, and incubated for 1 hour at 37°C in the dark. For tetraspanin expression analysis, anti-tetraspanin monoclonal antibodies (CD9 APC, CD63 PE, and CD81 PE-Vio615) were added at a final concentration of 2.5 μ g/mL simultaneously with calcein-AM. Samples were then diluted in 0.22 μ M filtered PBS and analyzed using the CytoFLEX S, ImageStream X MkII, or CytoFLEX Nano.

5.3 Results

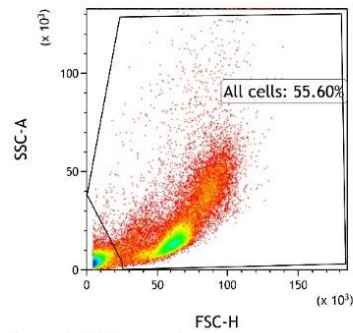
5.3.1 T cell optimizations

T cells were obtained by PBMC isolation from human whole blood and cultured in 96-well plates in the presence of anti-CD3 and anti-CD28 to induce T cell activation and produce EVs. It was necessary to find the optimal culture media to achieve the highest T cell viability and to activate T cells to maximize EV production.

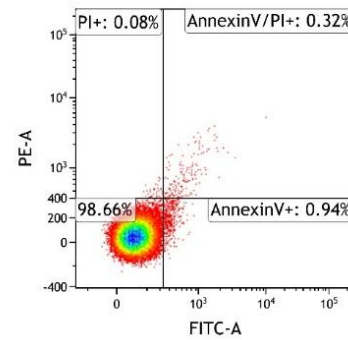
Firstly, the cell preparation was stained with Annexin and PI to determine the viability of the cells by analyzing the cells on a flow cytometer. Gating was selected on unstained samples to determine the gate locations for cells (fig.5.1Ai) and Annexin/PI (fig.5.1Aii). The gate locations were used on stained samples (fig.5.1Bi, ii) to determine viability.

A. Unstained AnnexinV/PI control

i. Selection of cells

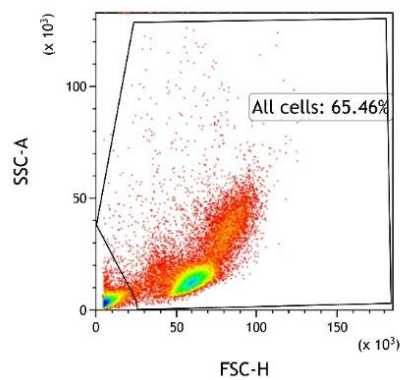


ii. Annexin/PI



B. AnnexinV/PI sample

i. Selection of cells



ii. Annexin/PI

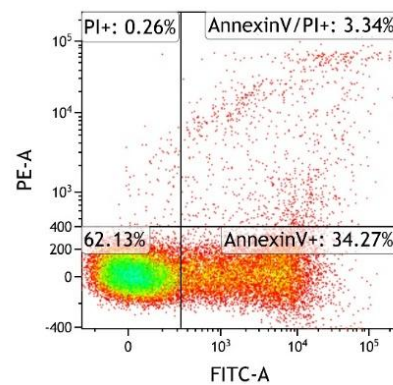


Figure 5.1 - T cell annexin/pi gating strategy. All cells in the sample were selected, excluding the debris (A.i.). Quadrants for annexin and PI were selected based on unstained sample (A.ii.). Stained sample utilized the gates from unstained sample (B).

Next, it was necessary to evaluate the activation levels of T cells, which was achieved by staining for early and late activation markers CD69 and CD25, respectively. T cells were run through a flow cytometer for analysis, where it was required to build a gating strategy to analyse the cells of interest. First, the cell population of interest was selected – lymphocytes (fig.5.2A), and to increase accuracy of the results only single cells were gated (fig.5.2B). Following this only CD3⁺ T cells were selected to evaluate the activation levels, which were gated according to unstained controls for CD3 (fig.5.2Ci,

Di) and CD25/CD69 (fig.5.2Cii, Dii).

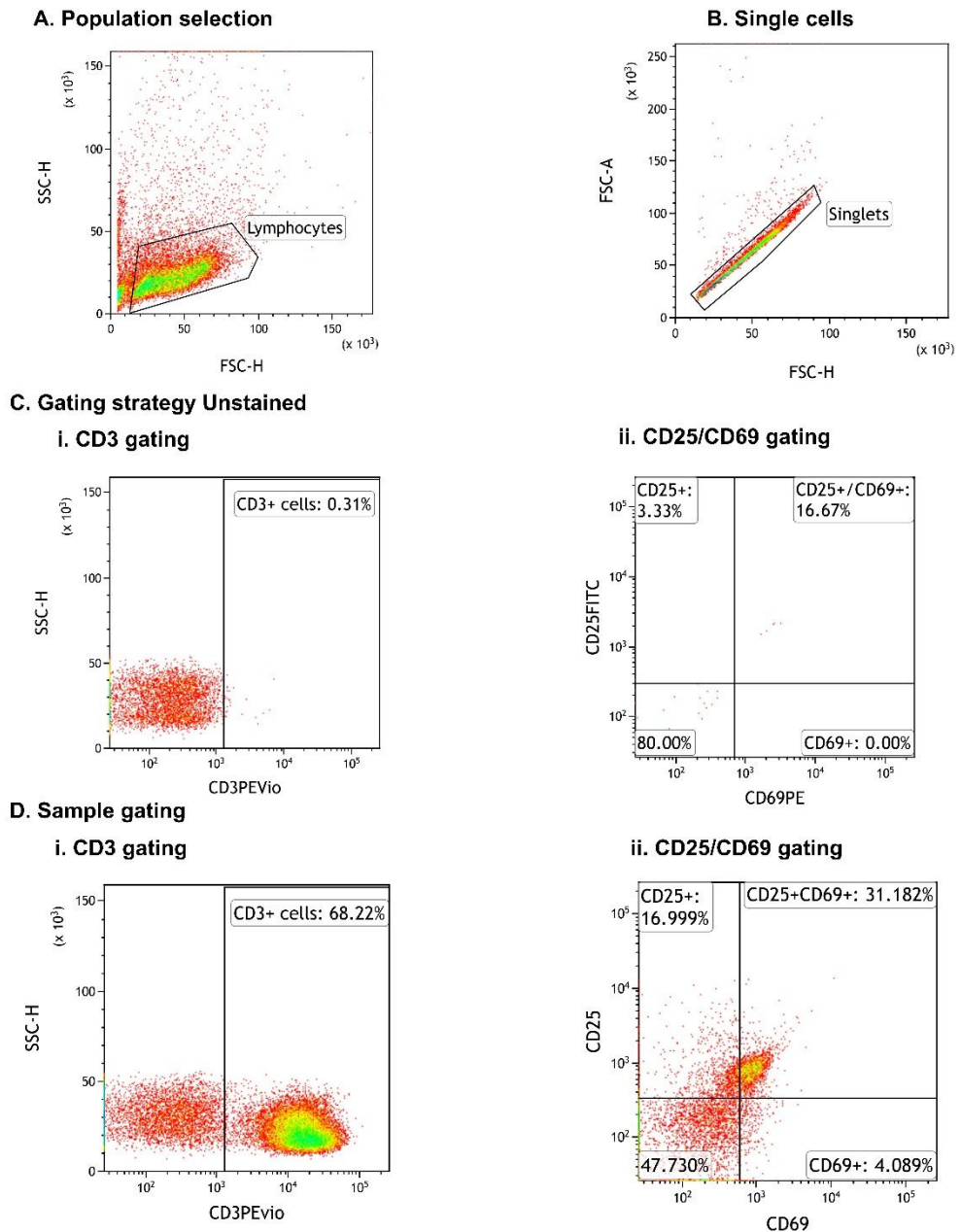
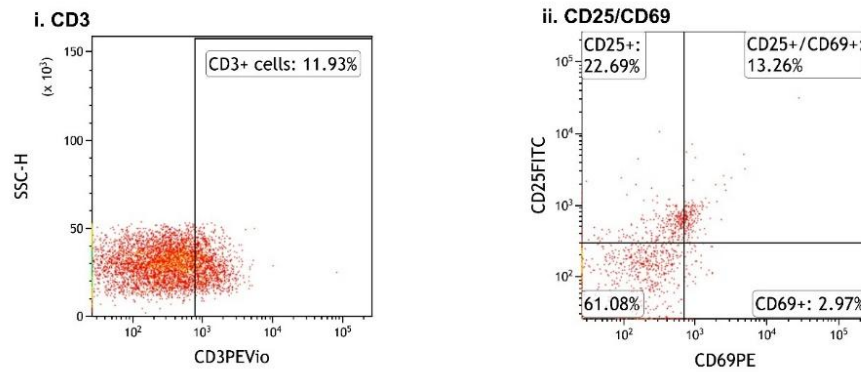


Figure 5.2 - Measuring activation levels on T cells. The cells of interest - Lymphocytes were selected based on SSC and FSC (A). To ensure more accurate results, only single cells were selected (B). To further select the cells of interest - T cells were gated by CD3+ expression (Ci)). To measure the activation level of T cells, early activation CD25 and late activation CD69 were used (Cii) Gates set by unstained and FMOs controls were applied onto fully stained samples (D).

Optimising the T cell activation via CD3/CD28 stimulation, 2 ways of using anti-CD3 were tested, 1st by adding anti-CD3 directly to the cells – soluble, and 2nd by coating the plate with anti-CD3 before adding cells – immobilised. However, once the cells were analysed it became apparent that using soluble

anti-CD3 impaired the selection of CD3⁺ T cells in the analysis (fig.5.3Ai), while immobilised anti-CD3 had no such issues (fig.5.3Bi), which made evaluating T cell activation via CD25/CD69 expression challenging when using soluble anti-CD3 (fig.5.3Aii).

A. Stimulation with Soluble anti-CD3



B. Stimulation with Immobilised anti-CD3

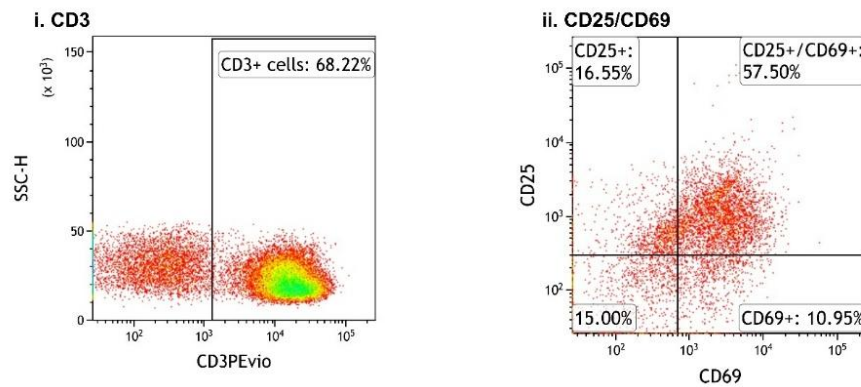
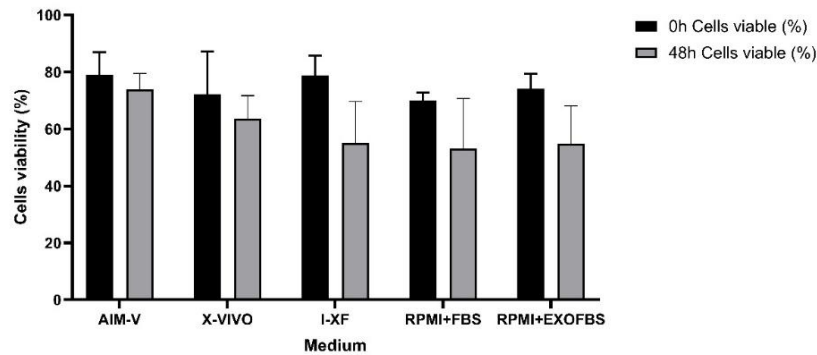


Figure 5.3 - Soluble and immobilised anti-CD3 activation of T cells. As before the lymphocytes were gated based on their SSC and FSC values and then only single cells selected. T cells were then selected on their CD3 expression (Ai., Bi.). To measure the activation levels CD25 and CD69 were used (Aii, Bii.).

Once gating strategies were developed, several T cell culture media were used to culture T cells – AIM-V, X-VIVO, I-XF, RPMI+FBS and RPMI + exosome-depleted FBS. By analysing cell viability of each culture media after 48h stimulation, it was found that AIM-V serum-free culture media had the highest cell viability (fig.5.4A). Furthermore, it was observed that soluble anti-CD3 resulted in decreased activation levels of T cells after 24h and 48h (fig.5.4B). Taken together, future experiments examining T cell-derived EVs used AIM-V media and immobilised anti-CD3.

A. T cell viability in different media (AnnexinV/PI)



B. T cell activation

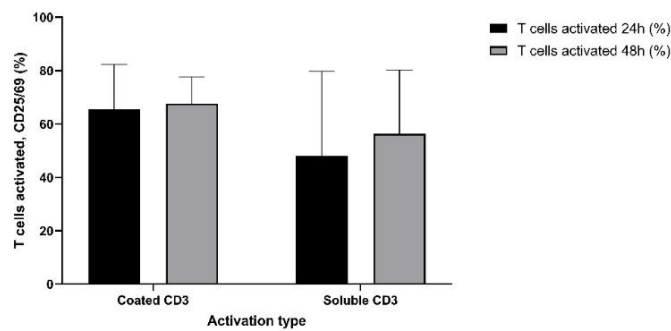


Figure 5.4 - T cell viability and activation levels. PBMCs were plated in 96 well plates coated with anti-CD3 across different media and supplemented with anti-CD28. Cells were stained with annexin/PI at 0h and 48h (A). To find the optimal activation method of T cells, anti-CD3 was either coated on plates or added directly to the well (Soluble). T cell activation levels were measured with CD25 and CD69 after 24h and 48h stimulation with anti-CD3 and anti-CD28 (B). $n = 3$.

5.3.2 Imaging flow cytometry optimization

Once the optimal T cell culture conditions were found, it was necessary to analyse the EVs they produce. As such a gating strategy was developed on ImageStreamX software IDEAS. It was designed using nanobeads as a point of reference for the selection of EVs. Several nanobeads were used – 220nm, 450nm, 880nm, 1340nm and 3 μ m and analysed on ImagestreamX (fig.5.5i). Following this, anything below the 220nm nanobeads was selected as EVs (Nb. NIST beads do not directly relate to the size of an EV but at this stage of the study FCMPass Software was not available) (fig.5.5ii). Utilising the built-in restrictions of ImageStreamX, where its brightfield channel cannot capture particles below 1 μ m in size, a Spot Count of 0 was used as a gating strategy to ensure EVs only smaller than 1 μ m in size would be included in further analysis

(fig.5.5iii). Finally, to ensure only single calcein+ EVs were selected IDEAS software feature a single Calcein spot count was utilized (fig.5.5iv).

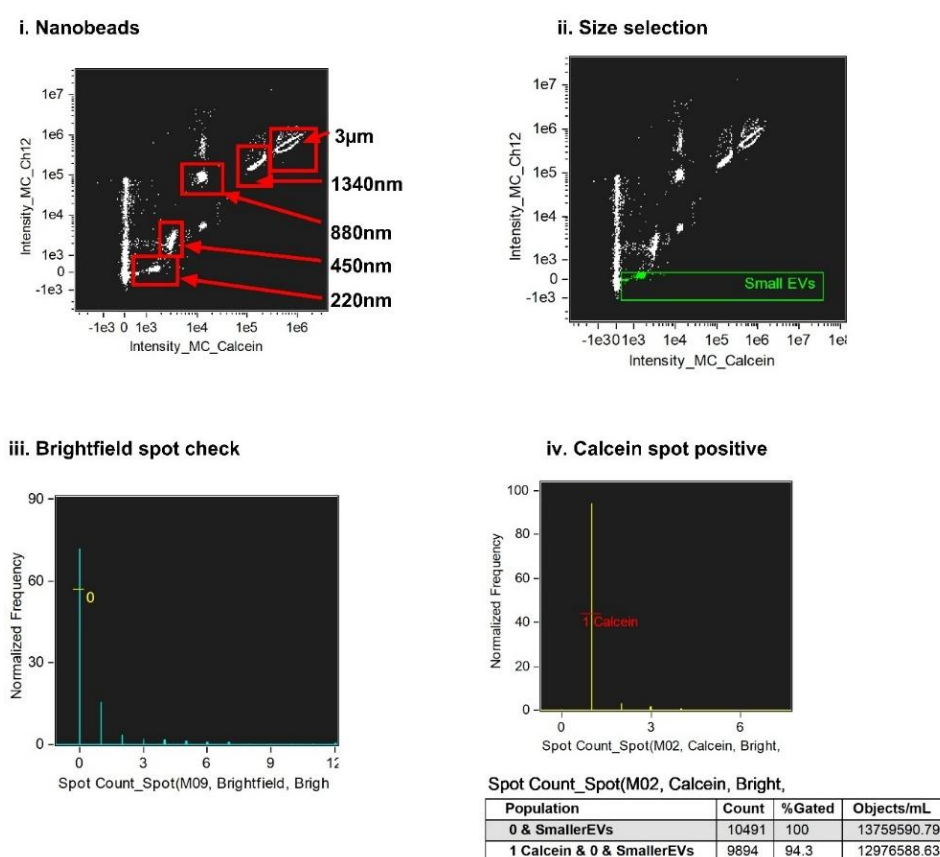
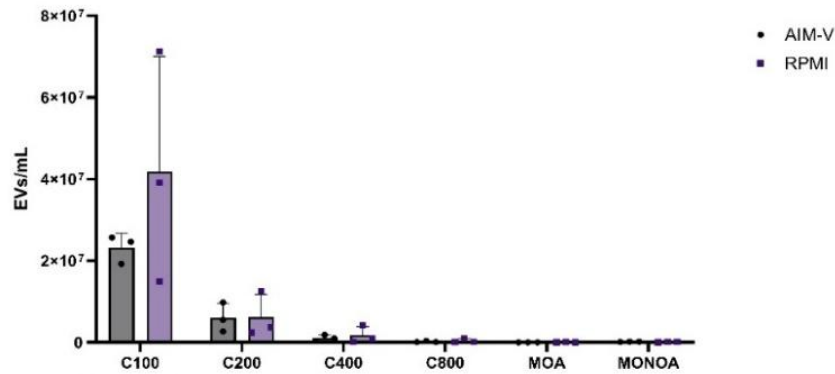


Figure 5.5 - Early ImageStream gating strategy. A set of nanobeads of known values were run on ISX to determine their positioning on the machine (i.). Based on the interest of only in small EVs, everything below 220nm beads was selected (ii.). Knowing that ISX brightfields minimum pixel range is $0.9\ \mu\text{m}$, it was possible to create a gate ensuring objects larger than $0.9\ \mu\text{m}$ would be excluded (iii.). To ensure objects selected were intact EVs, a calcein+ gate was introduced (iv.).

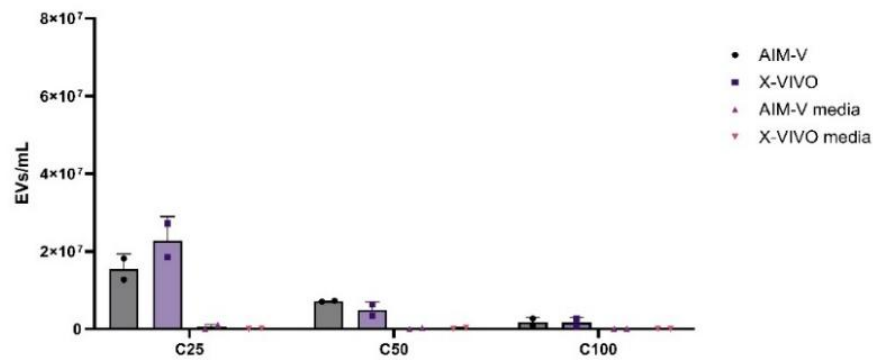
It was then required to find the most optimal calcein-AM staining concentrations to ensure the highest % of staining of EVs. T cells were cultured in several culture media – AIM-V, X-VIVO, I-XF, RPMI + FBS and RPMI + exosome depleted FBS, and activated with anti-CD3 and anti-CD28 for 24h. Following this, several calcein-AM concentrations were added directly to the wells - C25= $0.2\ \mu\text{M}$, C50 = $0.1\ \mu\text{M}$ C100 = $0.05\ \mu\text{M}$, C200 = $0.025\ \mu\text{M}$, C400 = $0.0125\ \mu\text{M}$, C800 = $0.00625\ \mu\text{M}$. The wells were then incubated for 1h in 37°C in the dark. The cells and EVs were then harvested and analysed on ImageStreamX. It was observed that the highest number of EVs recorded was achieved using $0.05\ \mu\text{M}$ calcein-AM in both AIM-V and RPMI media

(fig.5.6A, B). However, once analysed by using different culture media in a following experiment, it was observed that 0.1 μ M calcein-AM had a higher yield of EVs across all the media used (Fig.5.6C).

A. Lower calcein concentrations



B. Higher calcein concentrations



C. Calcein conc across media

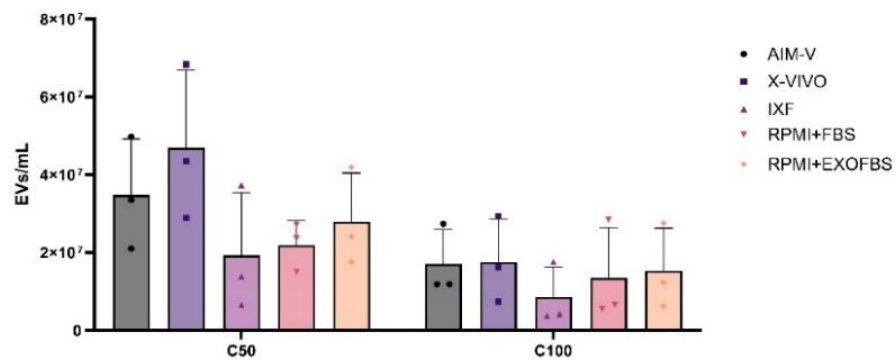
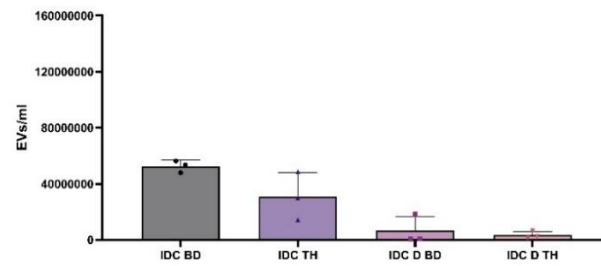


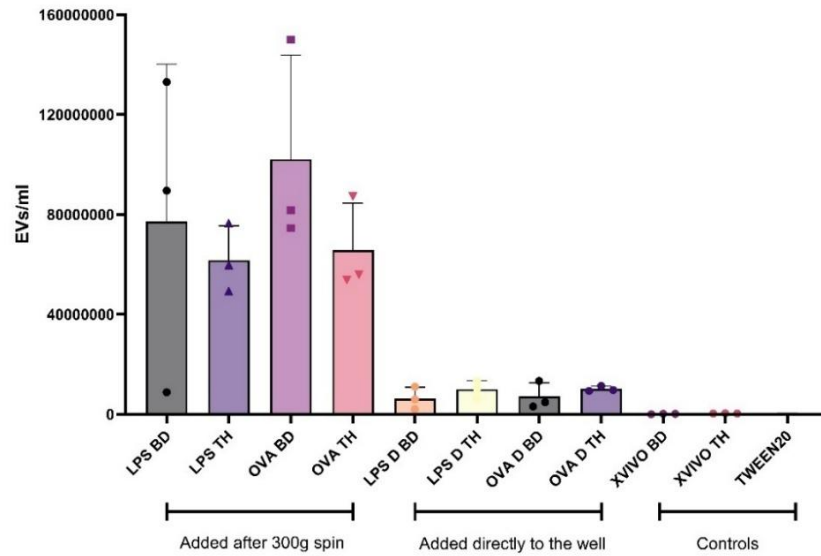
Figure 5.6 - Calcein concentrations across different media. T cells were cultured in 96-well plates coated with 2 μ g/ml of anti-CD3 and added 2 μ g/ml of anti-CD28. T cells were left for 24h and EVs harvested. To ensure the optimal concentration of Calcein-AM to stain extracellular vesicles, several concentrations were used. Lower and higher concentrations of Calcein-AM was used to determine the optimal concentration of Calcein-AM (A, B). C25= 0.2 μ M, C50 = 0.1 μ M C100 = 0.05 μ M, C200 = 0.025 μ M, C400 = 0.0125 μ M, C800 = 0.00625 μ M. Two concentrations of C50 and C100 were used across all potential media to grow T cells (C). n = 3.

Once a baseline protocol was established for staining EVs from T cells, it was necessary to develop a similar protocol for DC-derived EVs. DCs were cultured in RPMI + FBS media and activated with either 100ng/ml of LPS or 200ng/ml of OVA. Calcein-AM was acquired from both Thermofisher and Biolegend, and each calcein-AM was added either directly to the wells or added to S/N after a 300g spin for 10 min at 0.05 μ M and incubated in 37°C in the dark for 1h. It was observed that in all instances it was more efficient to add calcein-AM after centrifugation, regardless of the DC maturity state (fig.5.7A, B, C). Furthermore, calcein-AM procured from Biolegend achieved higher staining numbers of EVs than calcein-AM from Thermofisher (fig.5.7A, C).

A. IDC staining



B. 24h DC staining



C. 48h DC staining

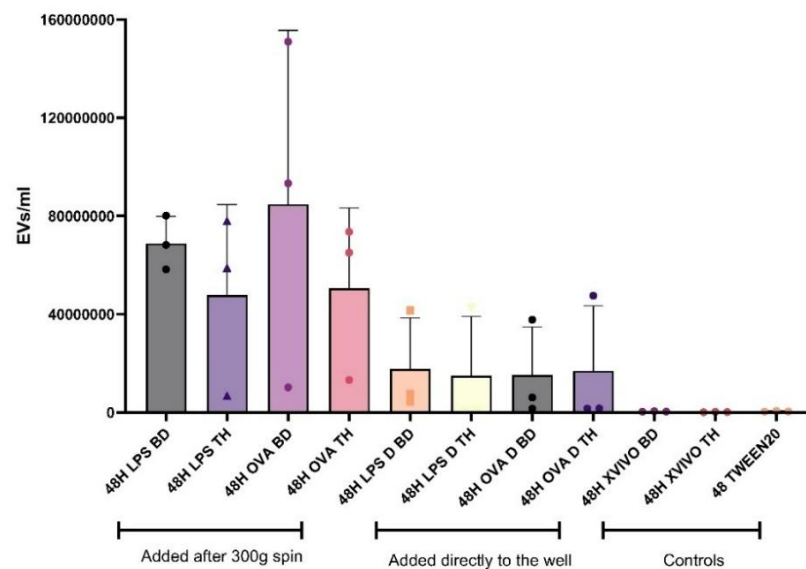


Figure 5.7 - DC-derived EV staining of calcein-AM. Dendritic cells were cultured in 24-well plates with RPMI media containing 50 ng/ml GM-CSF and 20ng/ml IL-4. Immature dendritic cells were stimulated with 100 ng/ml LPS or 200 ng/ml OVA. Calcein-AM was added at 0.05 μ M either directly to the well or after spinning out the cells at 300g for 10 min. Calcein-AM used was sourced from Thermofisher or Biolegend. n=3.

Once the DC EV protocol was established, it was decided to use smaller nanobeads, so 130nm nanobeads were run on the ImageStreamX (fig.5.8i). EVs were then selected as everything below the 130nm nanobeads (fig.5.8ii, iii). As before, to ensure that only EVs smaller than 1µm were selected, the brightfield was utilized (fig.5.8iv). To ensure only single calcein+ EVs were selected, spot count feature was used (fig.5.8v). Furthermore, to ensure higher accuracy in the EVs analysed, a new gate was introduced to only select EVs which were in focus (fig.5.8vi).

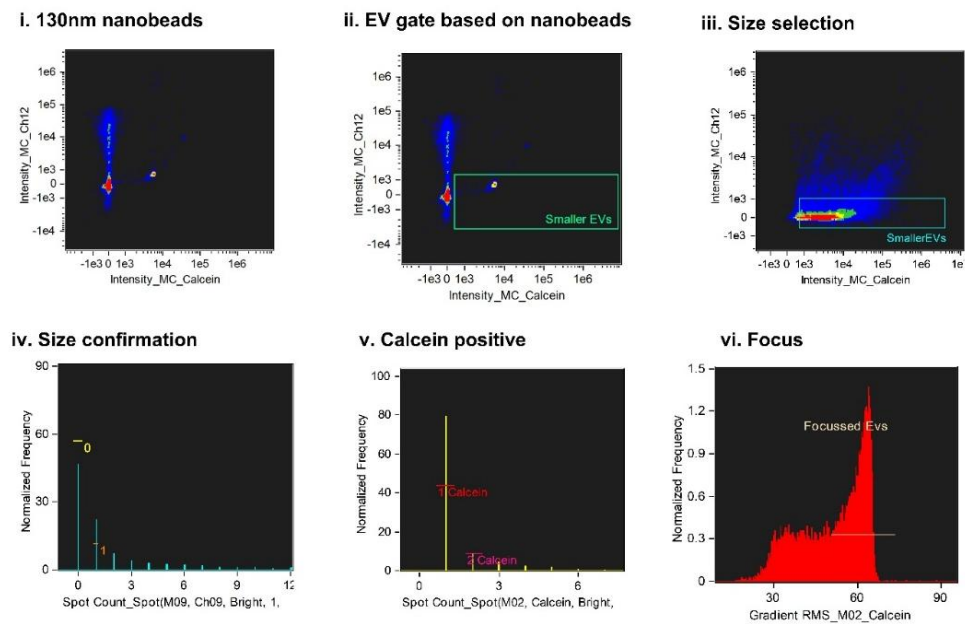
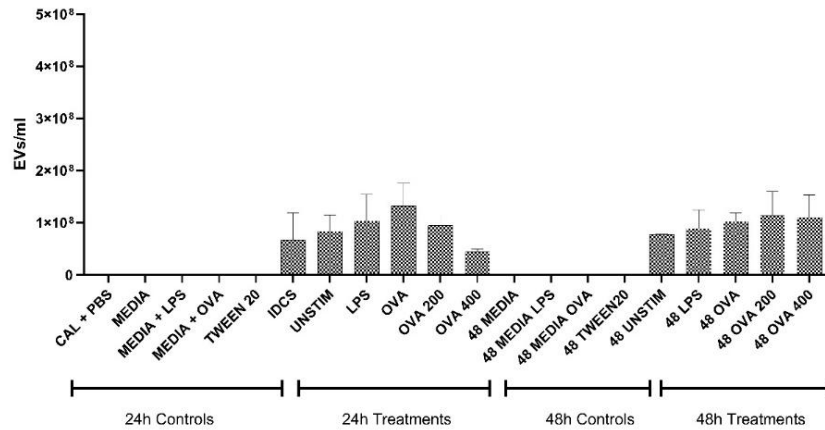


Figure 5.8 - Optimal imagestream gating strategy. Nanobeads of known refractive index and sized at 130nm were run on ISX to determine their positioning on the machine (i.). Based on the interest of only in small EVs, everything below 130nm beads was selected (ii.). The sizing gate was then applied to EV samples (iii.). Knowing that ISX brightfields minimum pixel range is 0.9 µm, it was possible to create a gate ensuring objects larger than 0.9 µm would be excluded (iv.). To ensure objects selected were intact EVs, a calcein+ gate was introduced (v.). In order to reduce artifacts and increase accuracy of tetraspanin expression on EVs, only in-focus EVs were selected (vi.).

Following the new gating strategy on ImageStreamX it was compared to the old gating strategy on the yield of EVs collected (fig.5.9). DCs were cultured with LPS or OVA for 24h and 48h to produce EVs. The EVs were harvested by spinning out the cells at 300g for 10 min. The S/N were collected and stained with 1 µM of calcein-AM and incubated at 37°C for 1h in the dark, the calcein-AM concentration was increased due to poor staining of EVs in previous

experiments. Following the analysis of the EVs collected on ImageStreamX it was apparent that the old gating strategy was excluding a lot of potential EVs in the analysis (fig.5.9A) when compared to the new gating strategy utilized (fig.5.9B).

A. Old gating strategy on ISX



B. New gating strategy on ISX

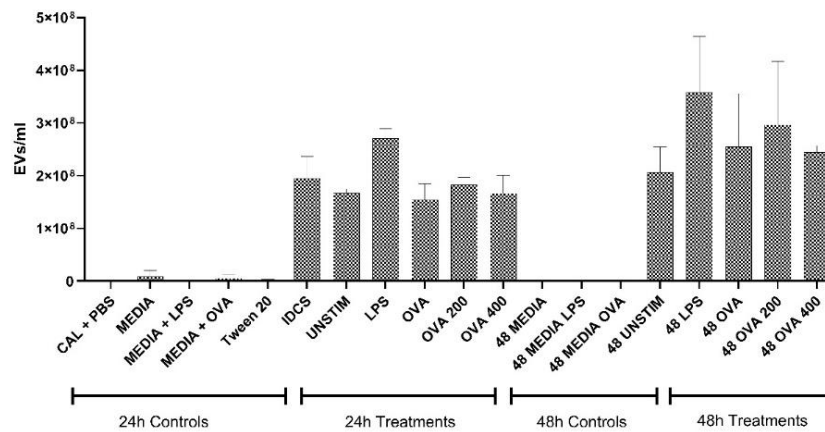


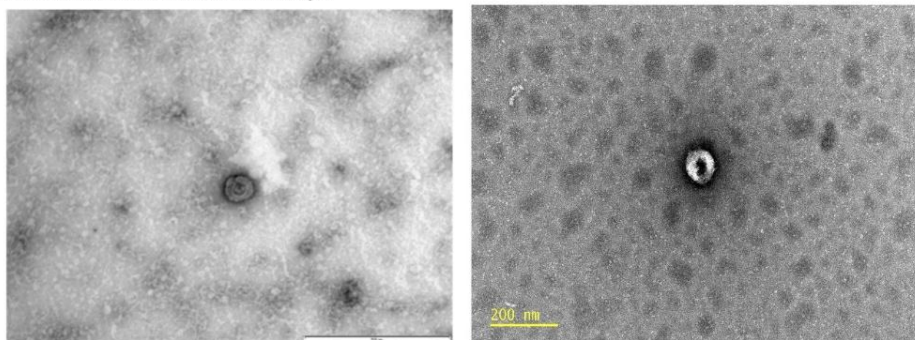
Figure 5.9 - Comparison of ISX gating strategies. Dendritic cells were cultured with different stimulations to ensure varied EV production. EVs were harvested from DCs and stained with 1 μ M of calcein-AM measured with old gating strategy (A) and with new gating strategy (B). n=3.

5.3.3 Confirmation of EV presence

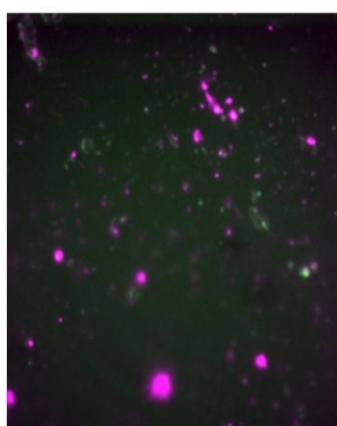
Following the successful development of DC-derived EV culturing protocol, it was necessary to confirm the presence of EVs in the S/N with other technologies other than calcein-AM-stained EVs on the ImageStreamX. The presence of EVs was confirmed in 3 way – TEM, where the expected cup shaped, collapsed structure was seen (fig.5.10A), confocal microscope, where

the EVs stained with lipid dye DID (purple) and calcein-AM stained (green) was present (fig.5.10B) and super-resolution microscope, where the tetraspanin – CD81, CD63, CD9 – was detected by the super-resolution microscope ONI (fig.5.10C).

A. Transmission Electron Microscope



B. Confocal microscope



C. Super-resolution microscope (ONI)

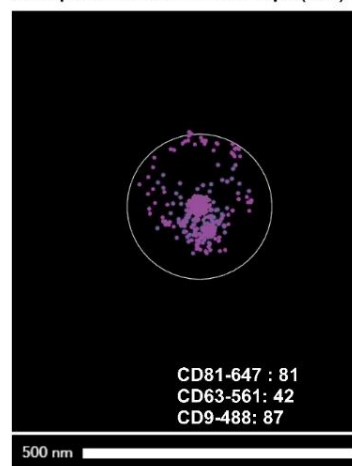


Figure 5.10 - Extracellular vesicles under different microscopes. EVs were observed under TEM (A). Utilizing confocal microscopes features, calcein-AM stained and lipid dye DID stained EVs were overlaid together (B). Super-resolution microscope (ONI) allowed the staining of tetraspanins CD81, CD63 and CD9, to observe the number of copies on each EV in the sample (C).

5.3.4 EV sizing

After verifying the presence of EVs, the focus shifted to determining their sizes using multiple approaches, with nanoparticle tracking analysis (NTA) recognized as a highly regarded method for size measurement of EVs. NTA employs Brownian motion to assess particle dimensions, allowing size sensitivity adjustments through the ‘detect particle sensitivity’ function, initially validated by measuring NIST beads, which NTA accurately detected

with peak sizes aligning closely with their true dimensions (Fig. 5.11i). By optimizing the ‘detect particle sensitivity’ function, a setting of 78 was established for epithelial EVs (Fig. 5.11ii), enabling NTA to successfully identify particles as small as 20 nm, with peak measurements at 100 nm, consistent with the anticipated EV size range (Fig. 5.11iii).

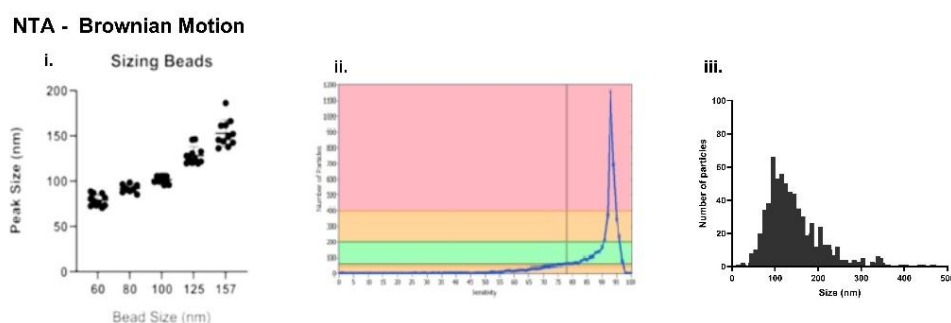


Figure 5.11 NTA EV sizing. Several NIST beads were run on NTA to monitor the accuracy of sizing measurements (i.), and the sensitivity of the machine was set to 78 due to the automatic sensitivity feature (ii.). Using Brownian motion, NTA sized EVs derived from primary human epithelial cells (iii.).

Subsequently, the decision was made to assess EV sizes using both conventional and imaging flow cytometry techniques (Fig. 5.12), where the Cytoflex S successfully detected NIST beads ranging from 80 nm to 151 nm, though the 60 nm bead was obscured by the machine’s background noise (Fig. 5.12Ai). By processing data from the four measurable NIST beads with FCMPASS software and applying Mie theory, the refractive index and light scattering properties were calculated and translated into EV size estimates (Fig. 5.12Aii); however, due to the inability to detect the 60 nm bead and the 80 nm bead corresponding to an EV size of 126 nm, smaller EVs remained undetected, limiting Cytoflex S sizing to a range of 126 nm to 500 nm (Fig. 5.12Aiii). To address this limitation, EV sizing was also attempted with the imaging flow cytometry platform, ImageStreamX Mk II (ISX), necessitating the acquisition of a commercial kit that estimates sizes by quantifying lipid dye incorporation against a well-characterized synthetic lipid size standard, validated by NTA to span 80-140 nm (Fig. 5.12Bi). This standard was stained with a membrane dye and calibrated using the FCS Express transformation tool with a surface area equation from the manufacturer’s protocol (Fig. 5.12Bii), enabling the interpolation of EV sizes based on vFRed™ intensity, revealing the detection of EVs as small as 55 nm in the sample. This demonstrated that

imaging flow cytometry offers greater sensitivity for EV size detection compared to conventional flow cytometry, leading to the preference for ISX in future experiments.

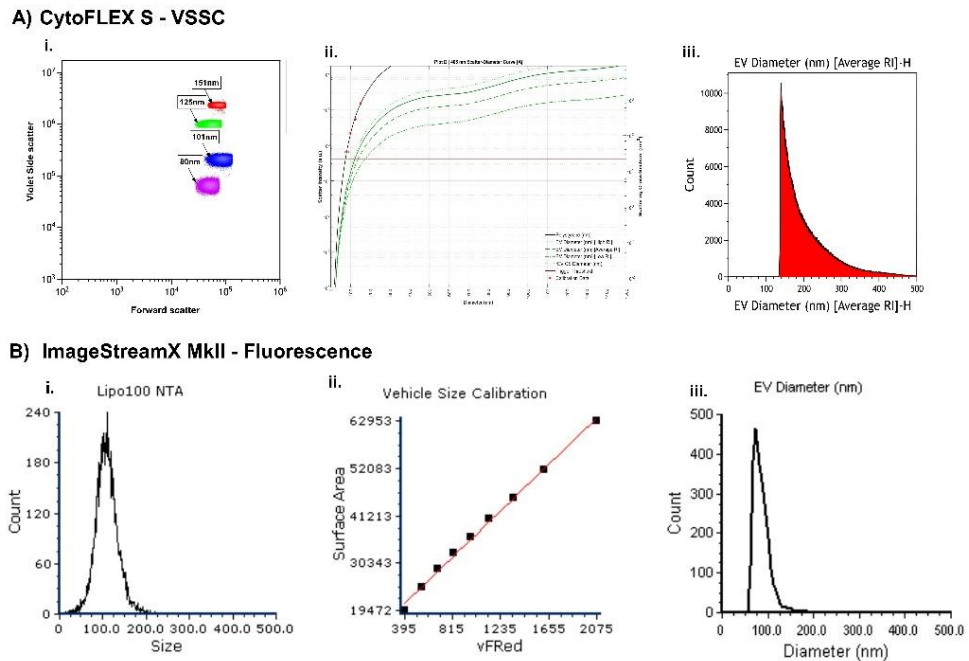
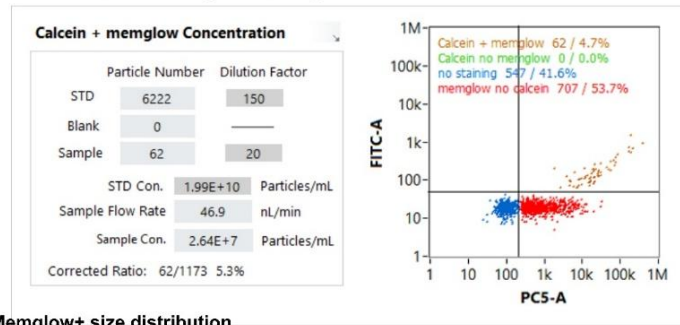


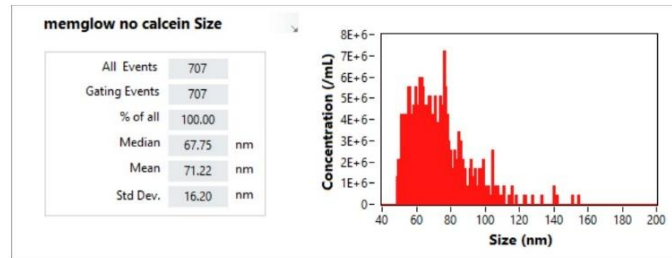
Figure 5.12 Cytoflex S and ImagestreamX EV sizing. NIST beads were run on Cytoflex S FC using violet side scatter (Ai.), to then be used for Mie theory conversion (Aii.), which allowed the sizing of EVs from epithelial cells (Aiii.). ImagestreamX employed a vesicle size standard confirmed by NTA (Bi.) to then be used for calibration with a fluorescent membrane stain (Bii.). The calibration file could then be applied to primary epithelial EV samples stained with the VFRed dye for sizing (Biii.).

Another EV sizing technique was tested – NanoFCM (fig.5.13). The EVs were harvested from DC culture after 24h stimulation with 100ng/ml of LPS, and the cells were spun out after a 300g for 10 min, furthermore, the EVs had to be isolated, which was achieved by SEC. Following SEC isolation, the EVs were stained with 1 μ M calcein-AM and 100 nM lipid dye Memglow and incubated at 37°C for 1h in the dark. The EVs were then analysed on NanoFCM. As expected, there were no calcein⁺ only EVs, as the lipid dye Memglow should stain anything with a lipid bilayer, so any intact EV would be positive for both calcein and Memglow (fig.5.13A). Furthermore, Memglow stained much smaller particles (fig.5.13B) than calcein-AM (fig.5.13C). Indicating that calcein-AM works as intended and selectively stains only intact EVs.

A. NanoFCM calcein/memglow staining



B. Memglow+ size distribution



C. Calcein+ size distribution

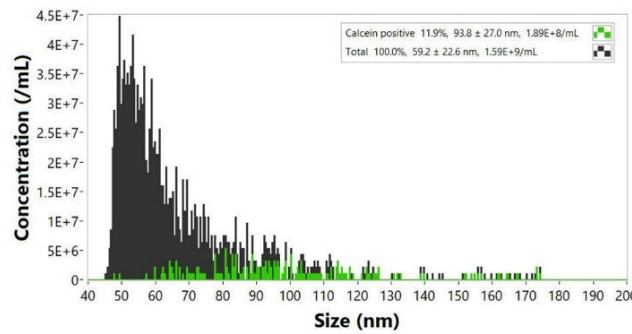


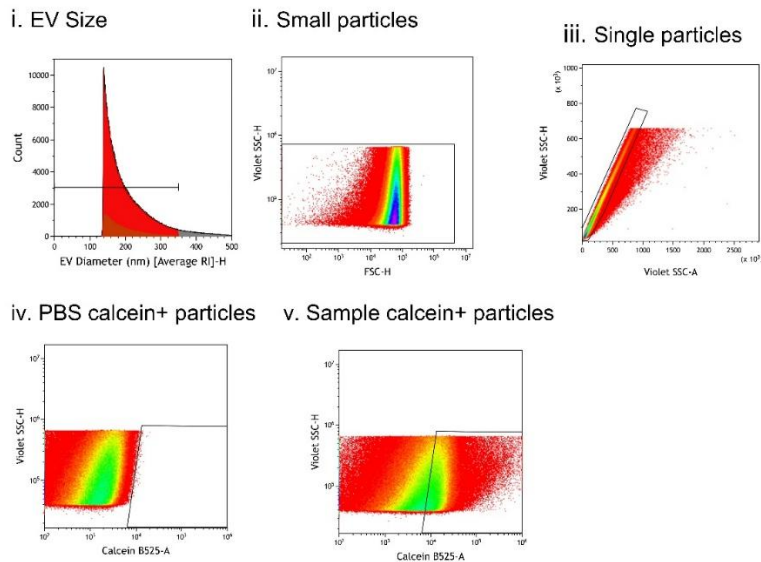
Figure 5.13 - NanoFCM calcein and memglow staining. EVs were stained with calcein-AM and memglow (A). Calcein-AM and memglow stained EV size distribution was observed (B, C).

5.3.5 Optimal calcein-AM concentrations

After further experimentation, it was determined that testing a range of calcein-AM concentrations was necessary to identify the most effective staining condition, with the Cytoflex S chosen for its user-friendly sample processing compared to the ImageStreamX. A gating strategy was established using Kaluza software to analyse EVs (Fig. 5.14A), leveraging FCMPASS software data to define a selection gate for small particles within the sample (Fig. 5.14Ai, Aii), ensuring accuracy by isolating single particles (Fig. 5.14Aiii), and setting the gate for calcein+ particles based on a PBS + calcein-AM control run on the Cytoflex S (Fig. 5.14Aiv), which was then applied to EV samples to capture calcein+ events (Fig. 5.14Av). EVs were stained with

eight calcein-AM concentrations, spanning 0.1 μM to 200 μM , and analysed on the Cytoflex S, with all titration values corrected for background autofluorescence by subtracting PBS + calcein sample readings. Results indicated that calcein+ EVs reached a plateau at 1 μM calcein-AM (Fig. 5.14Bi), yet the optimal staining index was attained at 10 μM (Fig. 5.14Bii), leading to the adoption of 10 μM calcein-AM for subsequent experiments. Serial dilutions of the sample, stained with 10 μM calcein-AM, demonstrated a linear reduction in EV counts with increasing dilution, validating the dye's effectiveness across varied concentrations (Fig. 5.14Biii), thus confirming 10 μM as the preferred concentration for optimal EV staining in future analyses.

A. Cytoflex gating strategy



B. Calcein titrations

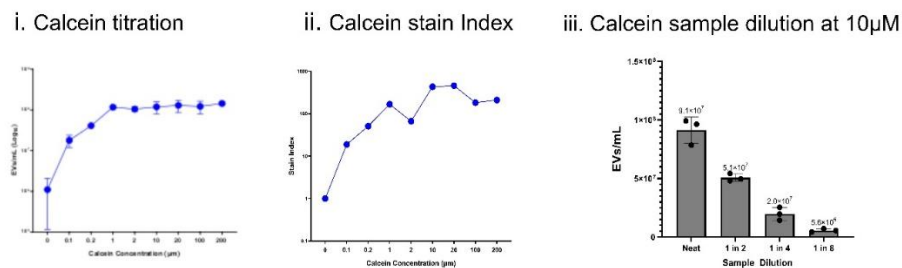


Figure 5.14 - Calcein Titrations. (A) EV gating strategy on CytoFLEX S. (i) Data is first run through FCM PASS to obtain the size distribution of EVs. EVs less than 300nm were then gated. (B) (ii) Sterile-filtered PBS and MucilAir supernatant were stained with increasing concentrations of calcein-AM and run on CytoFLEX S. (iii) Serial dilutions of primary epithelial cell supernatant were performed and stained with 10 μM of calcein to determine any swarming at high concentrations of EVs, before analysing. ($n=3$).

5.3.6 Detergent optimization

Adhering to the MISEV2024 guidelines, it was imperative to incorporate appropriate controls, notably ensuring that EVs disintegrate and vanish from analysis upon detergent application, confirming the observed particles are indeed EVs. To this end, various detergents were evaluated (Fig. 5.15), with EVs harvested and stained with Calcein-AM as previously outlined, followed by the addition of Tween20 at concentrations of 10%, 20%, 30%, 40%, and 50% directly to the EV preparation; however, analysis revealed that EVs persisted in the samples (Fig. 5.15A). Subsequently, a range of detergents—SDS-PAGE, TritonX, and Tween20—were tested, including combinations to enhance their efficacy, and it was determined that preparing the optimal detergent concentration in PBS prior to adding it to the EV preparation in a larger volume produced more consistent outcomes, a finding validated in an experiment with LPS-stimulated DC-derived EVs (Fig. 5.15B). The refined methodology was further assessed across different stimulation conditions—OVA and unstimulated—using DC-derived EVs after 24 and 48 hours, noting that longer stimulation increased EV yield, yet the new approach with detergents SDS-PAGE and Tween20 effectively disrupted EVs even in higher volumes, as demonstrated across the tested conditions (Fig. 5.15C, D), with notable success in removing most EVs even after 48h stimulation (Fig. 5.15Di, ii), confirming that pre-mixing detergents with PBS enhances reproducibility and efficacy.

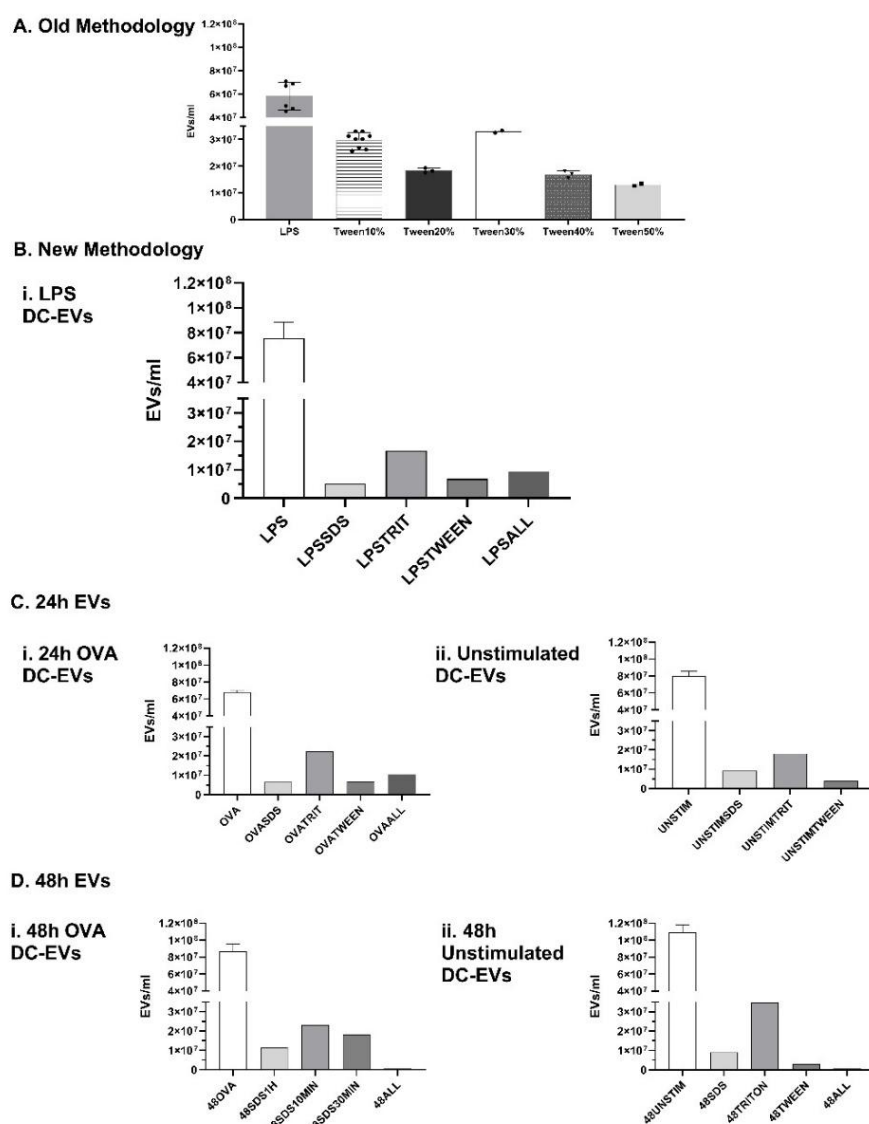


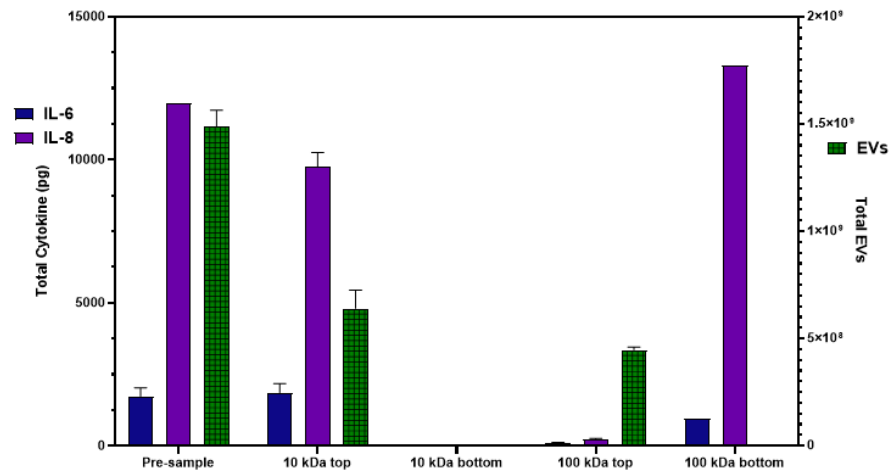
Figure 5.15 Detergent use on EVs. Tween20 was used on EVs by directly adding the desired concentration of Tween20 to the sample (A). Exploring different detergents was considered and the methodology was adjusted to mix the desired concentration of detergent of interest and then adding it to the sample (B). 24h stimulation and 48h stimulation of DC-derived EV detergent use effectivity across 2 conditions OVA and unstimulated (Ci,ii and Di,ii). Detergents used SDS-Page, TritonX, Tween20, and combinations.

5.3.7 Cytokine removal

The central aim of this PhD is to elucidate the role of EVs in allergic sensitization, where the presence of cytokines produced by DCs could significantly skew the outcomes of downstream experiments. Thus, obtaining a pure EV population devoid of contaminating cytokines is essential to accurately assess their effects on naïve T cells. Two potential methods for cytokine removal - Amicon concentration columns and SEC - were evaluated, with the added requirement that the chosen method should maximize EV yield

(Fig. 5.16). To facilitate cytokine experiments, EVs were derived from primary human epithelial cells, and standard solutions of IL-6 and IL-8 were introduced to spike the EV samples prior to processing with Amicon concentration and SEC. Cytokine levels were quantified using ELISAs, calibrated against standard curves, while EVs were labeled with 10 μ M Calcein-AM and analyzed using the ISX platform with IDEAS software. The results revealed that both IL-6 and IL-8 persisted in the top chamber of Amicon filters alongside EVs, irrespective of whether 10 kDa or 100 kDa filters were used (Fig. 5.16A), indicating that these columns were inadequate for cytokine removal. Moreover, centrifugation of both filter types resulted in a noticeable loss of EVs. In contrast, SEC proved highly effective, with no detectable cytokines in fractions 1 through 9, while retaining the majority of EVs, predominantly concentrated in fractions 1 to 5 (Fig. 5.16B). Consequently, SEC was deemed the preferred method for isolating EVs from cytokines, ensuring an uncontaminated sample to accurately investigate naïve T-cell responses following EV exposure without cytokine interference.

A. Cytokine removal using Amicon columns



B. Cytokine removal using SEC

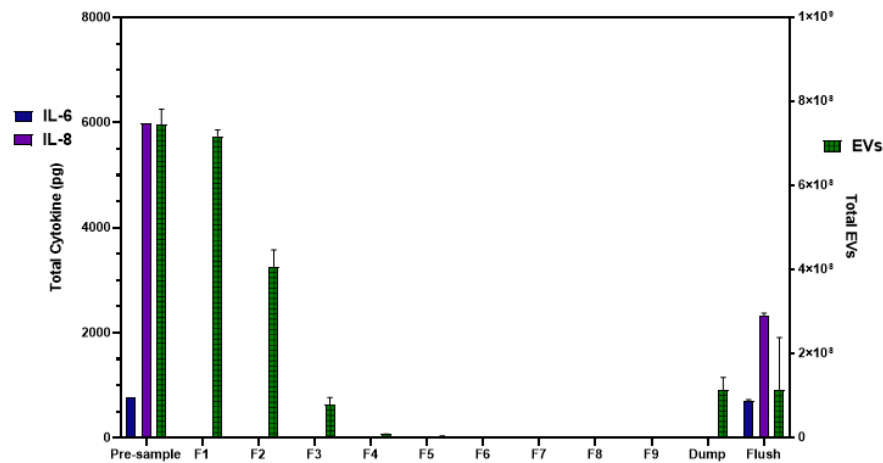


Figure 5.16 - Methods to separate EVs from cytokines. The ability to separate EVs and cytokines within epithelial cell supernatants, using Amicon filter columns and automated SEC. EVs were detected by calcein staining and analysing using the ISX. ELISAs were performed to analyse the presence of IL-6 and IL-8 before and after isolation methods. (A) Total cytokine and EV numbers before and after centrifugation using 10kDa and 100 kDa Amicon columns, in both the top and bottom fractions of the column. (B) Total cytokine and EV numbers before automated SEC and in each of the 9 collected fractions, as well as the 'dump' and 'flush' reservoirs. N=3

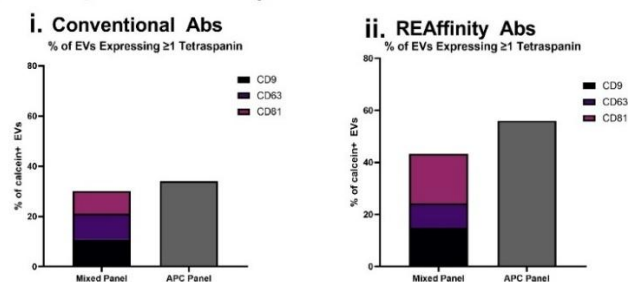
5.3.8 EV Tetraspanin profile

Prior to tetraspanin characterisation, antibodies from two manufacturers were tested to determine their effectiveness for EV staining. Traditional mouse monoclonal antibodies were compared with REAfinity antibodies (recombinant human antibodies engineered for high purity and featuring a modified Fc region to reduce background) in their ability to detect tetraspanins (fig.5.17A).

EVs were labeled with calcein-AM and stained using either a mixed fluorophore panel (APC, PE, and PE-vio615) or a uniform pan-APC panel. Despite maintaining a consistent antibody concentration of 2.5 $\mu\text{g/mL}$, REAfinity antibodies demonstrated significantly higher tetraspanin detection (mixed = 43.24% gated of EVs, pan = 56.05% gated of EVs) compared to conventional antibodies (mixed = 30.05% gated of EVs, pan = 34.11% gated of EVs). Additionally, the mixed fluorophore panel exhibited lower sensitivity for tetraspanin detection than the pan-APC panel for both antibody types, indicating that a pan-APC antibody panel enhances the sensitivity of tetraspanin detection on EVs.

The mixed fluorophore panel with REAfinity tetraspanin antibodies was selected for future experiments, as although it offers lower sensitivity than the pan-APC panel, it enables further differentiation of EVs based on the expression of three distinct tetraspanins. Without requiring prior EV isolation, supernatants from healthy and allergic individuals' DCs were collected, stained with calcein-AM and the mixed REAfinity tetraspanin panel, and then analyzed using the ImageStream X MkII (fig.5.17Bi). Thanks to the optimized tetraspanin panel, it was possible to observe single, double and triple tetraspanin expression on DC-derived EVs from healthy and allergic individuals (fig.5.17Bii). Interestingly, CD9 and CD81 expression was higher in both healthy and allergic individuals DC-derived EVs, which was consistent with the super-resolution microscopy results.

A) Tetraspanin Antibody Panels



B) Tetraspanin Detection

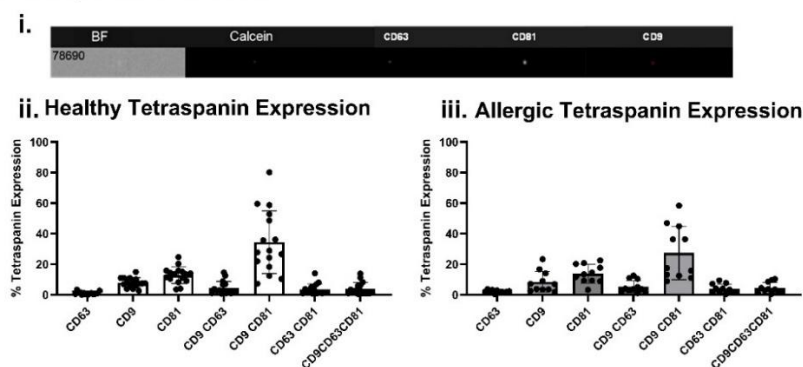


Figure 5.17 - Tetraspanin Profiling. (A) A comparison between (i) conventional antibodies and (ii) REAfinity antibodies, as both a mixed fluorophore panel (CD9 APC, CD63 PE, and CD81 PE-Vio615) and a pan-APC panel ($N=1$). (B) (i) ImageStream X MkII imaging of a single EV showing the EV brightfield image (BF), and calcein, CD63 PE, CD81 PE-Vio 615, and CD9 APC fluorescence. (B) (ii) % of tetraspanin expression on DC-derived EVs from healthy and allergic individuals (iii).

5.4 Discussion

In this chapter, it was necessary to optimise techniques for cell culture and EV analysis. As such, techniques were developed for T cell culture and activation prior to EV analysis to ensure the generation of EVs in serum-free conditions. Staining procedures for EV enumeration utilising calcein-AM were tested using conventional and imaging flow cytometry. Using microscopy, it was possible to confirm the presence of EVs in the sample preparations ensuring that samples analysed contained EVs. Thanks to the techniques developed it was possible to size EVs on several machines without prior isolation. It was also confirmed that SEC isolation completely removes cytokines from the EV preparation ensuring that only downstream effects of EVs will be observed.

The most optimal T cell activation was achieved by using immobilised anti-CD3 instead of soluble anti-CD3, as the soluble form interfered with

CD3⁺ T cell selection during analysis, most likely due to the epitope competition between anti-CD3 and fluorophore-tagged CD3 Antibodies. Hence, immobilised anti-CD3 was chosen for future experiments. AIM-V serum-free media had the highest T cell viability after 24h and 48h, which was essential as MISEV2024 guidelines require the use of serum-free media, as FBS contains residual EVs ([312]).

Developing a calcein-AM protocol for DC-derived EVs proved challenging, as the same protocol as for the T cell-derived EVs did not stain all DC-derived EVs. As such, the first step was to determine the optimal staining procedure, which was by spinning the cells out from the preparation at 300g for 10 min, and then adding calcein-AM. Furthermore, by further fine-tuning the gating strategy in IDEAS software by the addition of new nanobeads, a greater yield of EVs was detected.

For the detection and quantification of EVs by flow cytometry, calcein-AM dye was employed in this study because it distinguishes biologically active, intact EVs from debris by being hydrolysed into a fluorescent form by intravesicular esterases. Calcein-AM has been effectively used for EV staining in prior studies ([332]). Optimised protocols for identifying calcein-positive EVs using conventional and imaging flow cytometers were also developed. The calcein-AM dye was evaluated at eight different concentrations, with 10 μ M identified as the optimal concentration based on the highest stain index. This dye can be combined with fluorescent tetraspanin antibodies to both quantify EVs and characterize their marker expression.

Once the concentration of calcein-AM to stain all EVs was determined, it was required by MISEV2024 guidelines to prove the presence of EVs in the sample preparation ([312]). By utilising TEM, confocal microscope and super-resolution microscope ONI, it was confirmed that sample preparations contained EVs. Furthermore, by optimising the detergent use, by mixing the detergent with PBS before addition to the EV sample, this successfully reaffirmed the findings of microscopy, as all the EVs present in the sample were ‘popped’ once the detergent was added. The most effective detergents were shown to be Tween20 and SDS-page.

Multiple analytical techniques were compared for EV sizing: NTA (Zetaview), conventional FC (CytoFLEX S), and imaging FC (ImageStream X MkII) and NanoFCM. NTA has a particle concentration-dependent sensitivity profile across 20-500 nm detection range, which is considered the most commonly used and the gold standard in sizing EVs (S [333]). NTA utilises light scattering and Brownian motion to measure particle sizes, where the measured EVs were ranged from 100-200nm which falls in the typical size ranges for exosomes (30-150nm) and smaller microvesicles. However, when using the conventional flow cytometer Cytoflex S it was observed that due to the machine's limitations, it could only measure EVs starting from 126nm, losing all the smaller EVs, even when utilising violet laser for side scatter measurements for increased sensitivity. The findings collaborate with previously published literature where conventional flow cytometers are not sensitive enough to measure smaller EVs ([334]). ImageStreamX was also tested, but to size EVs, it required the use of a fluorescent lipid dye, as the data files were not compatible with FCMPass. Using a liposome size standard correlated with surface area-to-fluorescence of a membrane dye, vFRed, the ImageStream X MkII estimated sizing of EVs to be as small as 55 nm. Overall, the sizing results suggest imaging flow cytometry was more appropriate for small EV analysis compared to current conventional FCs, and this will be used for the EV analysis in this thesis.

The removal of cytokines from EV samples is a vital step to ensure that the specific cellular responses are generated from EVs themselves, as opposed to other constituents in the cell supernatants. Cytokines, which are often produced by cells in culture, can independently activate signalling pathways in cells of interest, which could potentially mask or amplify the effects of EVs ([312]). To ensure that cytokines are not present in the EV preparation when the final assays were conducted, three methods were tested to monitor their effectiveness in removing cytokines from the samples, namely 10 kDa, 100 kDa Amicron concentration columns and qEV SEC columns. This showed that 10 kDa Amicron concentration columns were ineffective in the removal of cytokines IL-6 and IL-8 from the EV sample, while the 100 kDa was more efficient, it did not remove all the cytokines. Furthermore, the 10 kDa and 100

kDa Amicon columns eliminated a large proportion of the EVs in the process, which was highly undesirable as the goal was to ensure complete removal of cytokines while keeping the EVs in the sample preparation. EV samples that had undergone qEV SEC showed that the collected fractions 1 through 9 contained no IL-6 or IL-8 cytokines and had kept the EVs through the isolation process. This would ensure there would be no contamination of cytokines present and maximise the number of EVs in the sample. SEC purification of sample thus enhances the downstream analyses, ensuring that the observed cellular responses are due to EVs influence, either through their cargo or marker expression, and not cytokines present in the sample. For example, studies have shown that residual cytokines like IL-6 or TNF- α in EV samples can affect inflammatory responses in target cells, complicating the mechanisms of how EVs influence the recipient cells ([335]). Thus, the complete removal of cytokines by qEV SEC ensures that the changes in recipient cells are generated from EVs, which would enable higher accuracy and reproducibility in functional studies of EVs.

Overall, it was possible to develop several techniques for characterising and sizing EVs, utilising an optimised calcein-AM dye concentration to differentiate between cell debris and intact EVs by analysing EVs on an imaging flow cytometer with optimised gating. Confirmation of the presence of EVs was achieved by TEM, confocal and super-resolution microscopy, and the successful use of detergents. Sizing EVs could be achieved by both conventional and imaging cytometers, which each have their own advantages and disadvantages. Most importantly, through SEC it was possible to completely remove the presence of cytokines, which is essential in downstream analysis of EVs.

Chapter 6: DC-derived EVs influence on naïve T cells

6.1 Introduction

6.1.1 Dendritic cell communication as APCs for naïve T cells

Dendritic cells (DCs) are uniquely qualified for the role as ‘professional’ APCs due to their positioning in mucosal and epithelial areas, such as the airways and skin, where they can interact with allergens, like pollen, dust mites, or pet dander, capturing, processing and presenting these allergens to naïve T cells ([336]). DCs are activated by allergen-associated signals, like thymic stromal lymphopoietin (TSLP) or danger molecules, to migrate to lymphoid tissues, where they present the peptide-MHC class 2 complexes with co-stimulatory molecules to naïve T cells. In the case of allergy, skewing them towards a Th2 phenotype, leading to allergic inflammation ([337]). The interaction is often described as an immunological synapse, which results in a cascade of Th2-driven cytokine production, such as IL-4 and IL-13, which drives inflammation in allergic diseases ([338]). Naïve T cells play a critical role in the development of allergic sensitization, which leads to allergen-specific immune responses ([339].) Naïve T cells are primarily in secondary lymphoid organs, remaining dormant until activated by APCs, such as DCs. Recently, studies have shown that distinct EV subtypes may modulate the balance between Th2 and regulatory T-cell responses depending on the cargo and co-stimulators of EVs ([233]). The crosstalk between DC-naïve T cells, either through cell-to-cell contact or EV-mediated signaling, could define the allergic immune responses in driving allergic sensitization and chronic allergic diseases. In food allergy, the role of DCs in priming naïve T cells is particularly critical due to the gastrointestinal route of allergen exposure. Upon ingestion of food allergens (e.g., egg, peanut, milk proteins), intestinal epithelial cells release alarmins such as IL-33 and TSLP in response to barrier disruption or protease activity of allergens. These alarmins activate subepithelial DCs, which then migrate to mesenteric lymph nodes or Peyer’s patches to present allergen peptides on MHC class II molecules to naïve CD4⁺ T cells. The co-stimulatory molecules CD80 and CD86, upregulated on DCs following allergen and alarmin exposure, provide signal 2

to prevent anergy and promote full activation, while the local cytokine environment (high IL-4, IL-25, TSLP) drives Th2 polarisation. This DC-mediated priming is essential for initiating allergen-specific Th2 responses and IgE production by B cells in the gut-associated lymphoid tissue, a process that is amplified in genetically susceptible individuals and underlies the development of food allergic sensitisation ([42]; [340]). Several studies have also reported alterations in circulating naïve T cell numbers or phenotypes in allergic patients, including those with food allergies. For example, children with persistent food allergy (including egg and peanut) show reduced frequencies of circulating naïve CD4⁺ T cells and increased memory/effector T cells compared to healthy controls or those who outgrow their allergy, suggesting accelerated differentiation and loss of the naïve pool due to chronic allergen exposure ([341]; [342]). These changes in naïve T cell homeostasis may reflect the heightened priming activity of DCs in allergic individuals, further supporting the importance of understanding DC–naïve T cell interactions in the context of food allergy.

6.1.2 EVs as intercellular communicators

The emerging evidence of EVs acting as potent intercellular communicators has changed the understanding of allergic sensitization, by showing a potential new mechanism of DC interaction with naïve T cells beyond cell-to-cell direct contact to influence the allergen-driven immune responses [312]). EVs are nanoscale vesicles released by nearly all cells, including DCs, and can carry a wide variety of cargo, such as proteins, lipids, and nucleic acids, plus allergen-loaded MHC molecules and co-stimulatory signals, for example, CD86 and ICAM-1. The role of EVs is not fully understood due to their heterogeneity, as different subtypes, differentiated by size or biogenesis, could lead to promotion of Th2 polarization or regulatory T-cell induction, depending on their molecular composition ([233]). The nature of EV-mediated communication also offers a spatially and temporally flexible pathway for allergen dissemination within lymphoid tissues ([343]).

6.1.3 Chapter objectives

To further understand the role of DC-derived EVs in driving Th2 responses, the optimized experimental assay was utilized. DCs from allergic and healthy individuals were cultured with LPS, Ovalbumin or a combination, and their EVs harvested after 24h. Subsequently, matched naïve T cells were exposed to these isolated EVs from both healthy and allergic patients. Naïve T cell activation and cytokine levels were measured to understand the effect of DC-derived EVs on naïve T cells in allergic and healthy patients. Furthermore, the effect of EVs was examined by comparing the naïve T cell populations when EVs were added, to naïve T cells with no exposure. This would lead to an understanding of the key differences generated between healthy and allergic naïve T cell populations and the direct effect of EVs in these two populations.

6.2 Methods

6.2.1 PBMC isolation

The same method was followed as previously described in Chapter 2 methods sections 2.2.11..

6.2.2 CD14⁺ Monocyte isolation

The method was followed as previously described in Chapter 2 methods sections 2.2.12.

6.2.3 Dendritic cell culture

The method was followed as previously described in Chapter 2 methods sections 2.2.13.

6.2.4 Naïve T cell isolation and culture

Naïve T cells were isolated through negative selection from matched whole blood. PBMCs were isolated as previously described, and then 10 μ L of naïve CD4⁺ biotin-Abs cocktail II per 10^7 cells was added and incubated at 4°C for 5 min. Following the incubation, 30 μ L of MACS buffer and 20 μ L of naïve CD4⁺ microbeads cocktail II per 10^7 cells was added and incubated in the fridge for 10 min. The solution was then run through an LS column and washed with 3 mL of MACS buffer, the solution containing the naïve T cells was pelleted by centrifugation for 5 min at 300g. The cells were then re-suspended in serum-free AIM-V media at 1×10^6 cells/mL, and 200 μ L of cells were placed in 96 well u-bottomed tissue culture plates.

6.2.5 EV isolation

After 24h stimulation of DCs, the supernatant was collected from wells and centrifuged for 8 min at 300g to pellet the cells. The supernatants were then used in size-exclusion chromatography, utilising IZON AFC and IZON 350nm qEV columns. Prior to SEC, the qEV columns were washed with filtered PBS, surplus PBS was then pipetted off, and the supernatant was added on top of the column. As soon as the S/N was absorbed into the column, extra PBS was added. Following SEC, 9 fractions containing the EVs were concentrated by using 10 kDa Amicon concentration columns by

centrifugation at 4000g for 10 min. The top solution containing concentrated EVs was collected.

6.2.6 DC-derived EVs and T cell assay

The isolated DC-derived EVs were isolated as described above and then added to the naïve T cell culture every 24h for up to 3 days. Naïve T cells with EVs were then incubated for a further 30h at 37 C, at which point a protein inhibitor cocktail was added, and left for 12h. In addition, 96-well plates were pre-coated with 2µg/ml of anti-CD3 and 2µg/ml of anti-CD28 was added directly to the wells, along with the isolated EVs. The naïve T cells were then harvested and stained for extracellular and intracellular markers.

6.2.7 T cell staining

Antibodies against extracellular markers were added after washing the cells with PBA for 10 min at 300g. Naïve T cells were then incubated in the fridge for 30 min, followed by a wash with PBA at 300g and then fixed with 500µl of fixation buffer for 30 min. Once the cells were fixed, the fixation buffer was removed by centrifugation at 500g for 5 min. The cells were then washed with 2ml of 1x permeabilization buffer at 500g for 5 min. Antibodies against the intracellular markers were then added at for 30 min at room temperature. This was followed by 2 washes with PBA at 500g for 5 min, and then the cells were fixed with 300µl of fixation buffer and stored in the fridge. Once all the naïve T cells were stained and fixed, the naïve T cells were run on a Sony ID7000 spectral flow cytometer for 2 min, collecting all the data. The overall assay process is visualised in Figure 6.1.

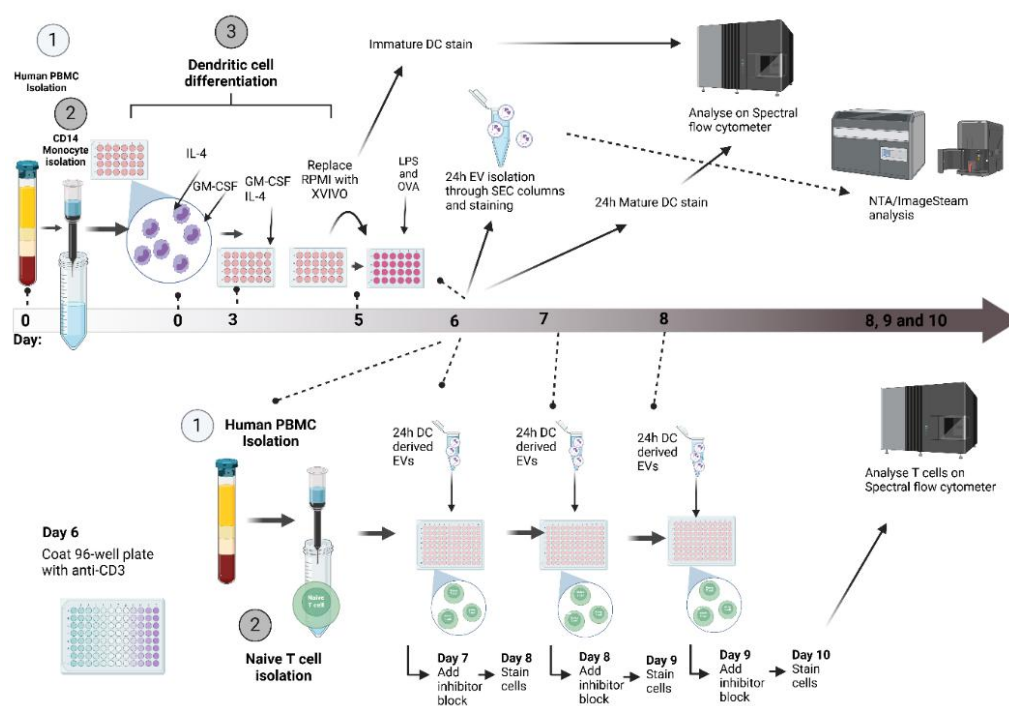


Figure 6.1 – DC-EV and T cell assay setup. On day 0 human whole blood was collected, and PBMCs were isolated by density gradient (1), followed by CD14⁺ monocyte isolation using magnetic separation (2). Monocytes were cultured in 48-well tissue culture plates with GM-CSF and IL-4 stimulation in RPMI + 10% FBS to initiate differentiation in dendritic cells, on day 3 the media and cytokines were replenished. On day 5 the media was swapped to serum-free media X-VIVO and stimulated with LPS, OVA and OVA+LPS (3). On day 6 EVs from DCs were collected and isolate through SEC, alongside PBMC 2nd isolation and negative selection of naïve T cells using magnetic separation (1,2). Naïve T cells were exposed to DC EVs for 3 separate doses, and the cells were collected and stained for extracellular and intracellular markers.

6.2.8 Supervised analysis

After the data was collected on the spectral flow cytometer, it was analysed by utilising Kaluza software by manually creating a gating strategy to include viable CD3⁺CD4⁺ naïve T cells and observe all the markers included in the experimental setup. All relevant marker percentages were inputted in GraphPad Prism and statistical differences were calculated by using two-way ANOVAs with Sidak multiple comparison correction, $p < 0.05$ for comparison of healthy and allergic donors, and one-way ANOVAs with Brown-Forsythe multiple comparison correction, $p < 0.05$ for comparison of conditions in both groups.

6.2.9 Unsupervised analysis

The data was also analyzed by using FlowJo v10 software where the samples were input into the software and manually gated to include viable CD3+CD4+ naïve T cells, from which a plugin ‘Downsample’ was reduced to only include 10,000 cells for analysis. Sample naïve T cells were then concatenated based on the analysis question, from which the plugin ‘Phenograph’ was used to find out the number of populations in the analysis. Once the population number was known, it was inputted into a plugin ‘Flowsom’ to generate the populations. Then, using the populations, a tSNE plot was built, and by using the ‘Cluster explorer’ plugin, a colored tSNE plot and population sizes were generated.

In order to analyze statistical differences, each healthy and allergic subjects’ percentage of naïve T cells were measured in each cluster. The data was inputted in GraphPad Prism and statistical differences were calculated using Multiple Mann-Whitney tests, and false discovery rate set at 5% using Two-stage step-up (Benjamini, Krieger, and Yekutieli) method for comparison between healthy and allergic patients, while multiple Wilcoxon tests with false discovery rate set at 5% using Two-stage step-up (Benjamini, Krieger, and Yekutieli) method for comparison of conditions in allergic patients.

6.3 Results

DC-derived EVs from different stimulations were added to matched naïve T cells from healthy and allergic patients in a dose-dependent manner. They were incubated for 30h after the last EV dose, before being harvested and stained for extracellular and intracellular markers and analyzed using Sony ID7000 spectral flow cytometer. The data generated was analyzed in 2 ways – supervised and unsupervised – utilizing Kaluza and FlowJo software, respectively. Naïve T cells were analysed to answer two key questions; firstly, is there a difference in T cell phenotype between healthy and allergic patients following exposure to DC-derived EVs, and secondly, is there a difference between different stimulations to generate DC-derived EVs in naïve T cell phenotype in either the healthy or allergic group.

6.3.1 Naïve T cell gating strategy

For analysis of the naïve T cell responses, a gating strategy was devised in Kaluza software. Firstly, cells were gated based on their forward scatter (FSC) and side scatter (SSC) characteristics (fig.6.2A). To ensure there were no doublets in the analysis, only singlets were selected according to FCS-Height vs FSC-Area (fig.6.2B). To ensure there were no dead cells in the analysis, only live cells were selected (fig.6.2C). Cells were then gated on CD3 (fig.6.2D) and CD4 (fig.6.2E) to only include CD3+CD4+ T cells in further analysis. Viable CD3+CD4+ T cells were then gated to examine their expression of cytokines, namely TNF- α , IL-4, IL-2 and IFN- γ (fig.6.2F, G, H and I), the cutoff for positivity was set according to the unstimulated T cells. The gating strategy was then applied to all samples.

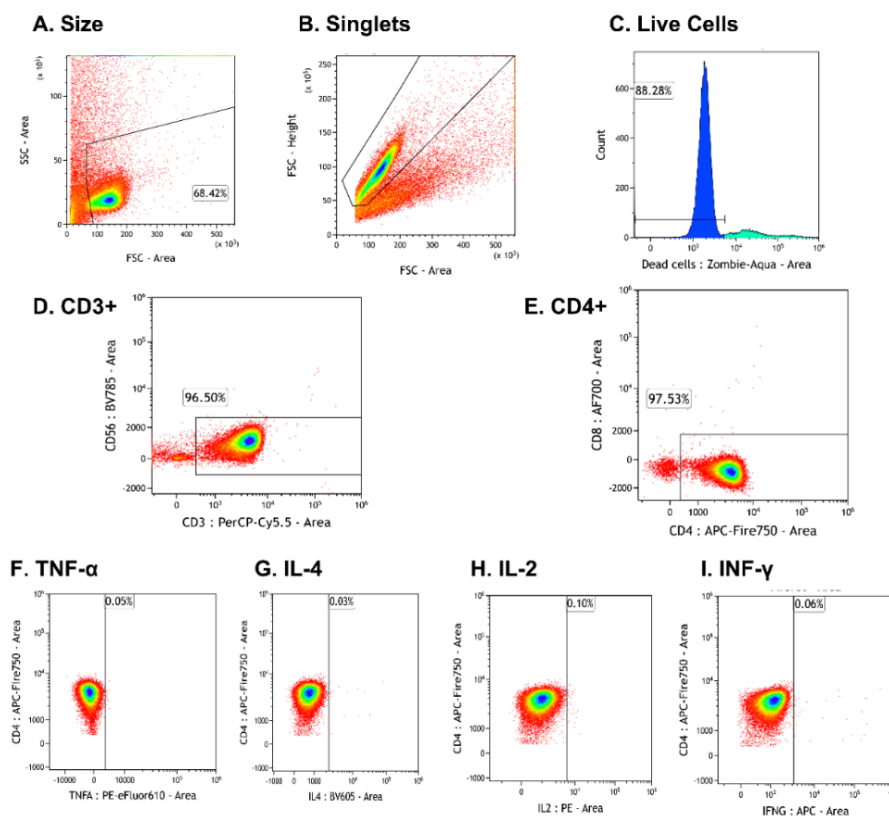


Figure 6.2 – Gating strategy for naïve T cell analysis. Naïve T cells were isolated from PBMCs through negative selection. T cells were selected according to their SSC and FSC (A). Only single T cells were selected (B). Only viable T cells were included in the analysis (C). Only CD3+ and CD4+ T cells were selected for further analysis (D, E). Cytokine production was gated on unstimulated T cells for TNF- α , IL-4, IL-2 and IFN- γ (F, G, H and I).

6.3.2. The effect of DC-derived EVs alone on naïve T cell responses

To understand the role of just DC-derived EVs on naïve T cells, the EVs were isolated and added to freshly isolated naïve CD4⁺ T cells, up to three times, and the impact of DC-EVs on viability, activation and cytokine production levels in naïve T cells was measured.

6.3.2.1. Effect of DC-derived EVs on naïve T cell viability between healthy and allergic individuals

To investigate the impact of DC-derived EVs on naïve CD4⁺ T cell viability, they were exposed to EVs derived from DCs under various stimulatory conditions – unstimulated (UNSTIM EVs), LPS-stimulated (LPS EVs), ovalbumin-stimulated (OVA-EVs), and ovalbumin/LPS co-stimulated (OVALPS EVs) – alongside a control group with no EV exposure (NO EVs), across one, two and three doses (fig.6.3). By looking at the naïve T cell viability it would be possible to monitor the effects of DC-EVs on naïve T cell survival. After one dose, naïve T cells from both healthy and allergic donors had high viability, approximately 80-90%, across all conditions, with no significant differences between groups (fig.6.3i). However, with two doses, viability slightly decreased in allergic naïve T cells, especially in OVA EV exposure and OVA+LPS EV exposure, where they were 75% and 70% respectively, compared to healthy naïve T cells, which were around 85% viable (fig.6.3ii). The trend continued in dose three, where allergic naïve T cells exposed to OVA and LPS EVs exhibited the lowest viability at 60%, whereas naïve T cells in the healthy group were at 80% viability (fig.6.3iii). Overall, the naïve T cells in the allergic group showed lower levels of viability across all the doses and conditions when compared to naïve T cells in the healthy group, but no statistically significant differences were identified, showing that DC-EVs had little or no effect on naïve T cell viability.

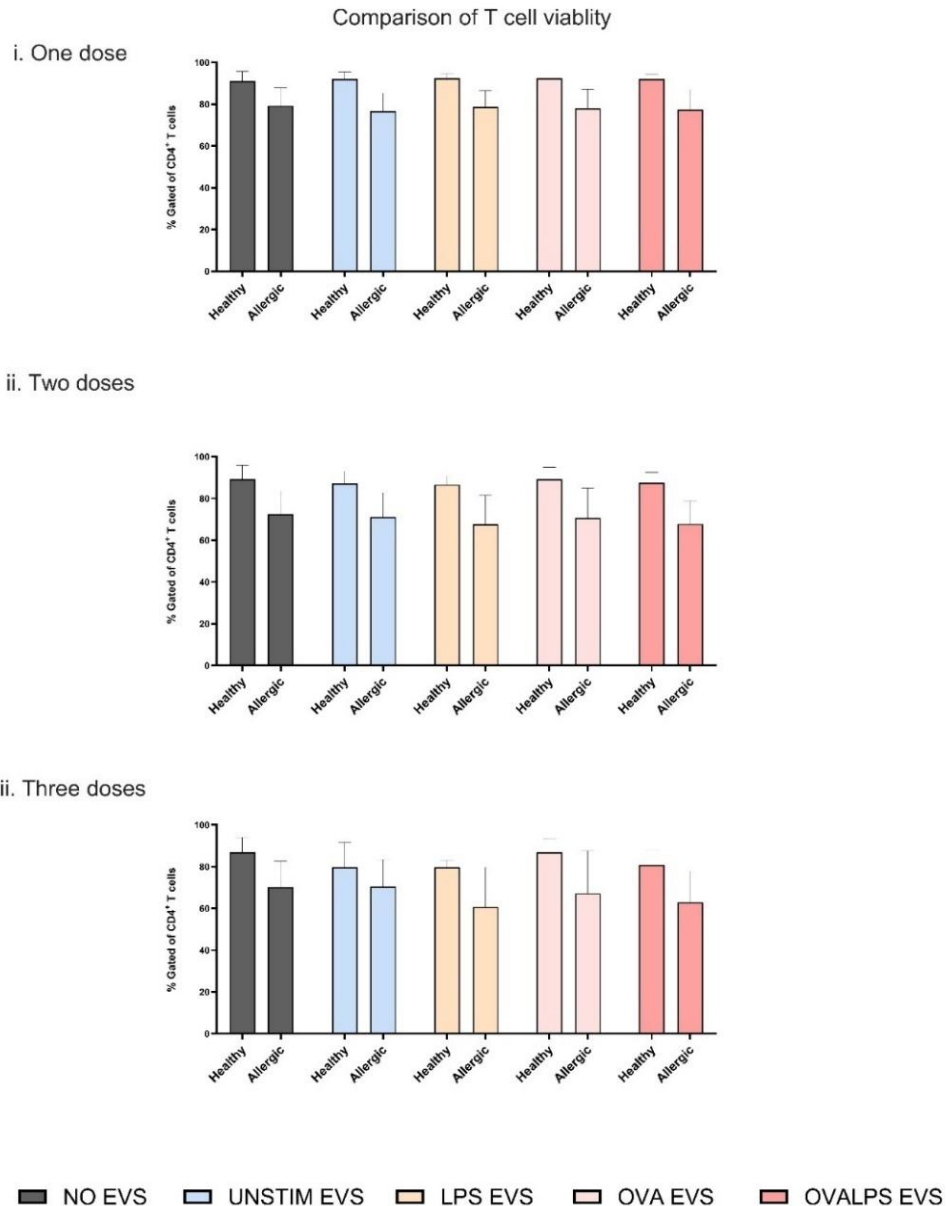


Figure 6.3 - Comparison of T cell viability in healthy and allergic participants. Statistical analysis was performed in GraphPad Prism utilising two-way ANOVA, $p < 0.05$. $n = \text{healthy} = 7$, $\text{allergic} = 5$.

6.3.2.2. Effect of DC-derived EVs on naïve T cell activation levels between healthy and allergic individuals

To assess the influence of DC-derived EVs on naïve CD4⁺ T cell activation, CD69 expression was measured on naïve T cells from healthy and allergic patients following exposure to EVs under different stimulatory conditions of dendritic cells, as stated previously (fig.6.4). CD69 is an early marker of lymphocyte activation due to its rapid appearance on the surface of the plasma membrane of naïve T cells after stimulation. After a single dose

exposure, minimal CD69⁺ T cells were observed across all conditions, with levels ranging from 2-7% in both healthy and allergic naïve T cells (fig.6.4i). With the second DC-EV exposure the percentage of CD69⁺ T cells slightly decreased across all the conditions ranging at 1-5% (fig.6.4ii). On dose three the decrease in the percentage of CD69⁺ T cells was more noticeable in all conditions in naïve T cells from both healthy and allergic individuals, ranging from 0.5-3%(fig.6.4iii). Overall, there was a low level of activation once exposed to DC-derived EVs in naïve T cells from both healthy and allergic individuals across all conditions and doses, and no significant differences between the two groups were observed. This suggested that EVs alone did not activate the naïve CD4⁺ T cells.

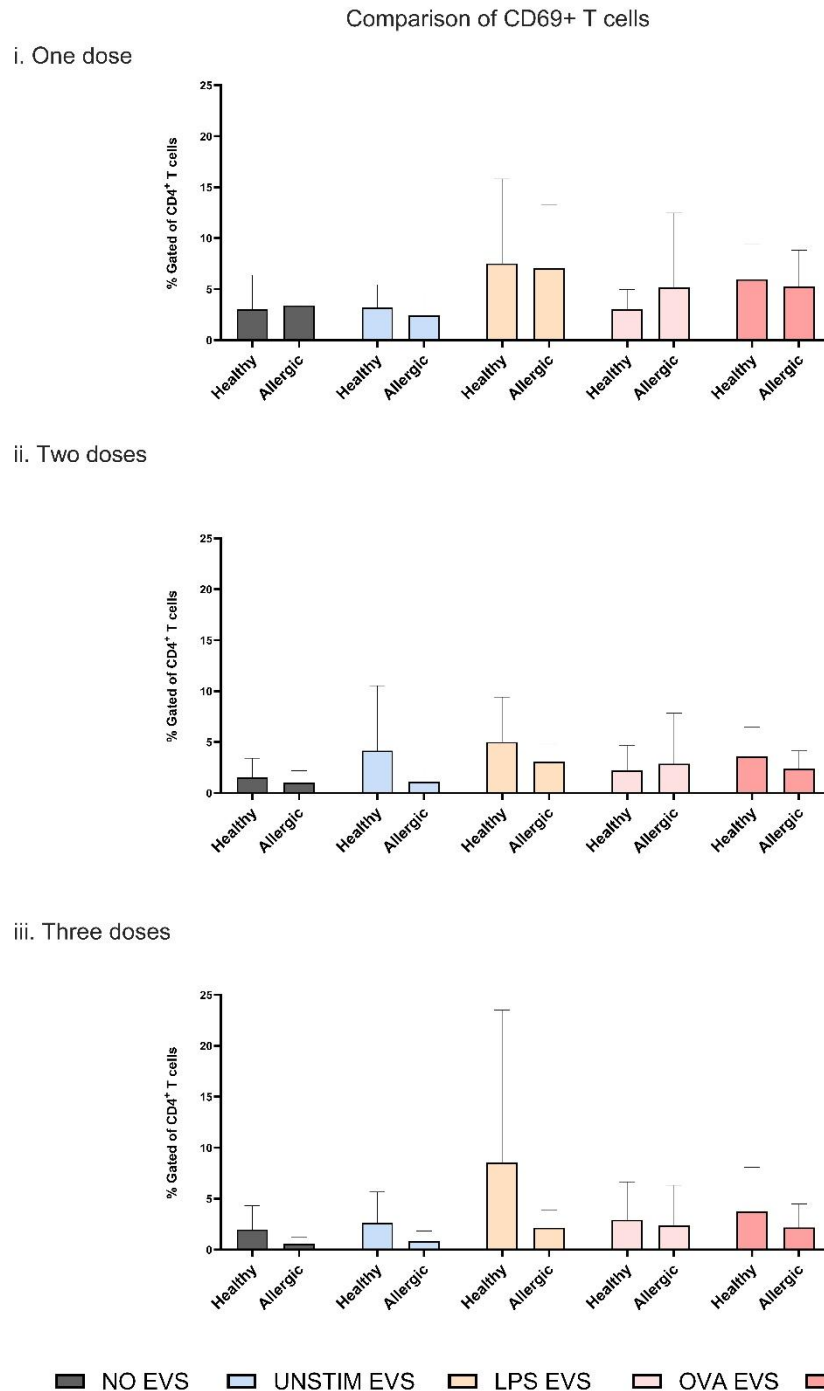


Figure 6.4 - Comparison of IFN- γ expressing T cells in healthy and allergic participants. Statistical analysis was performed in GraphPad Prism utilising two-way ANOVA, $p < 0.05$. $n = \text{healthy} = 7$, $\text{allergic} = 5$.

6.3.2.3. Effect of DC-derived EVs on naïve T cell IFN- γ production between healthy and allergic individuals

Next, IFN- γ production in naïve T cells was investigated (fig.6.5). It was important to measure IFN- γ production as it could show early commitment to Th1 cell development due to EV exposure. After one dose, the percentage

of IFN- γ ⁺ T cells was low across all conditions, with levels of 0.1-0.3% in naïve T cells from both healthy and allergic individuals (fig.5i). With two doses, the percentage of IFN- γ T cells only increased in naïve T cells from healthy individuals exposed to EVs derived from unstimulated DCs (0.75%), while other conditions showed a lower percentage of IFN- γ ⁺ T cells, ranging from 0.05-0.28% (fig.6.5ii). After three doses of EV exposure the percentage of IFN- γ ⁺ T cells remained low, with an increase in naïve T cells from healthy individuals exposed to EVs derived from LPS-stimulated DCs at 0.65% (fig.6.5iii). Altogether, the percentage of IFN- γ ⁺ T cells was low in naïve T cells in both healthy and allergic individuals across all conditions and all doses, with no significant differences between the groups identified, again suggesting EVs alone did not drive T cell activation.

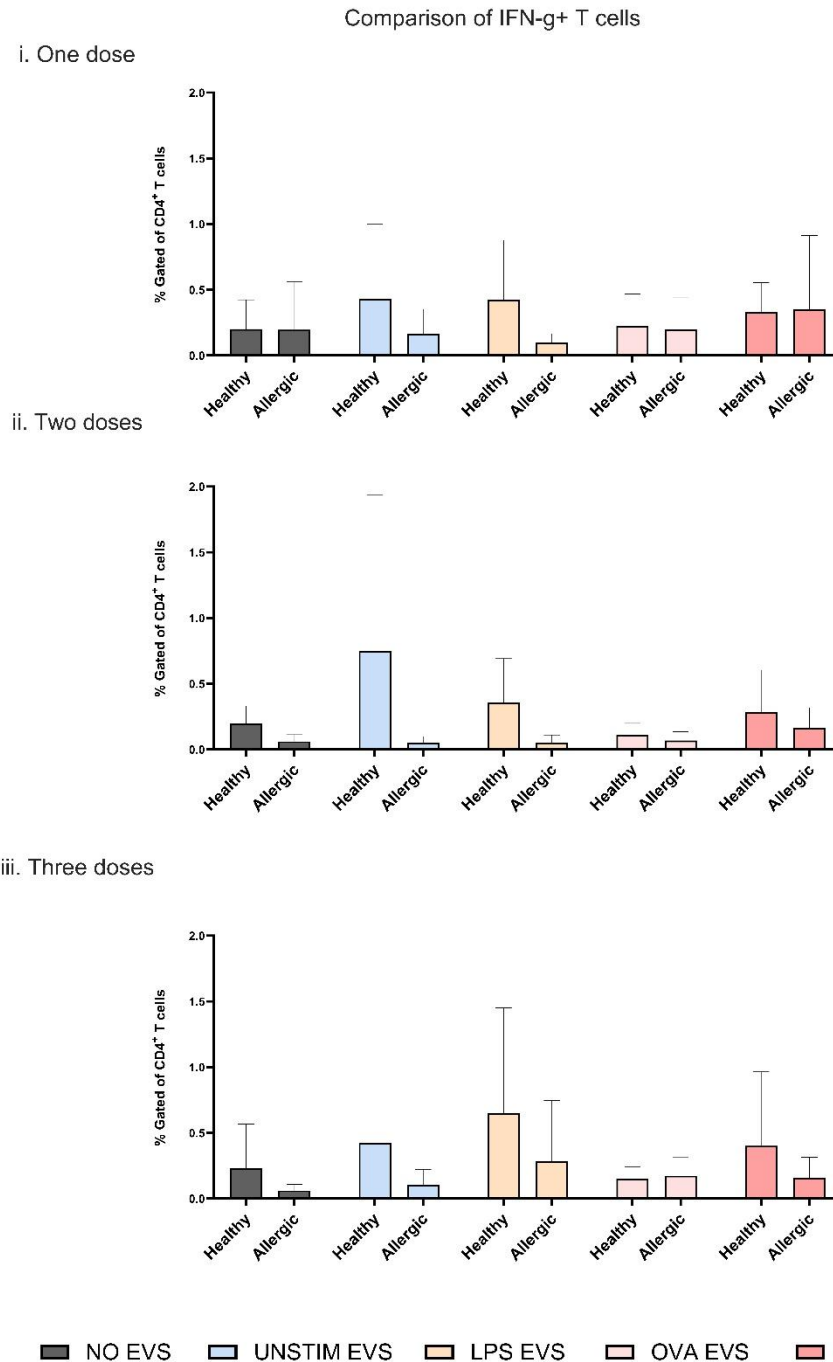


Figure 6.5 - Comparison of IFN-g expressing T cells in healthy and allergic participants. Statistical analysis was performed in GraphPad Prism utilising two-way ANOVA, $p < 0.05$. $n = \text{healthy} = 7$, $\text{allergic} = 5$.

6.3.2.4. Effect of DC-derived EVs on naïve T cell TNF- α production between healthy and allergic individuals

Subsequently, TNF- α production was observed in naïve T cells (fig.6.6). TNF- α is a powerful pro-inflammatory cytokine that plays a crucial role in the body's inflammatory response and immune system regulation. After

one dose, the percentage of TNF- α + T cells was higher in healthy individuals, ranging from 0.5-1.1%, with highest percentage observed in naïve T cells exposed to EVs derived from LPS-stimulated DCs. Interestingly, naïve T cells from allergic individuals showed little production of TNF- α , ranging from 0.01-0.03% (fig.6.6i). Following the 2nd exposure, the trend remained the same, where naïve T cells from healthy individuals produced more TNF- α + T cells than from allergic individuals (fig.6.6ii). After three doses of DC-EV exposure, the trend remained the same again, as naïve T cells from healthy individuals had a higher percentage of TNF- α + T cells, with the highest in EVs derived from LPS-stimulated DCs (fig.6.6iii). Overall, the percentage of TNF- α + cells was very low in all treatment conditions, further suggesting EVs alone did not simulate naïve CD4+ T cells.

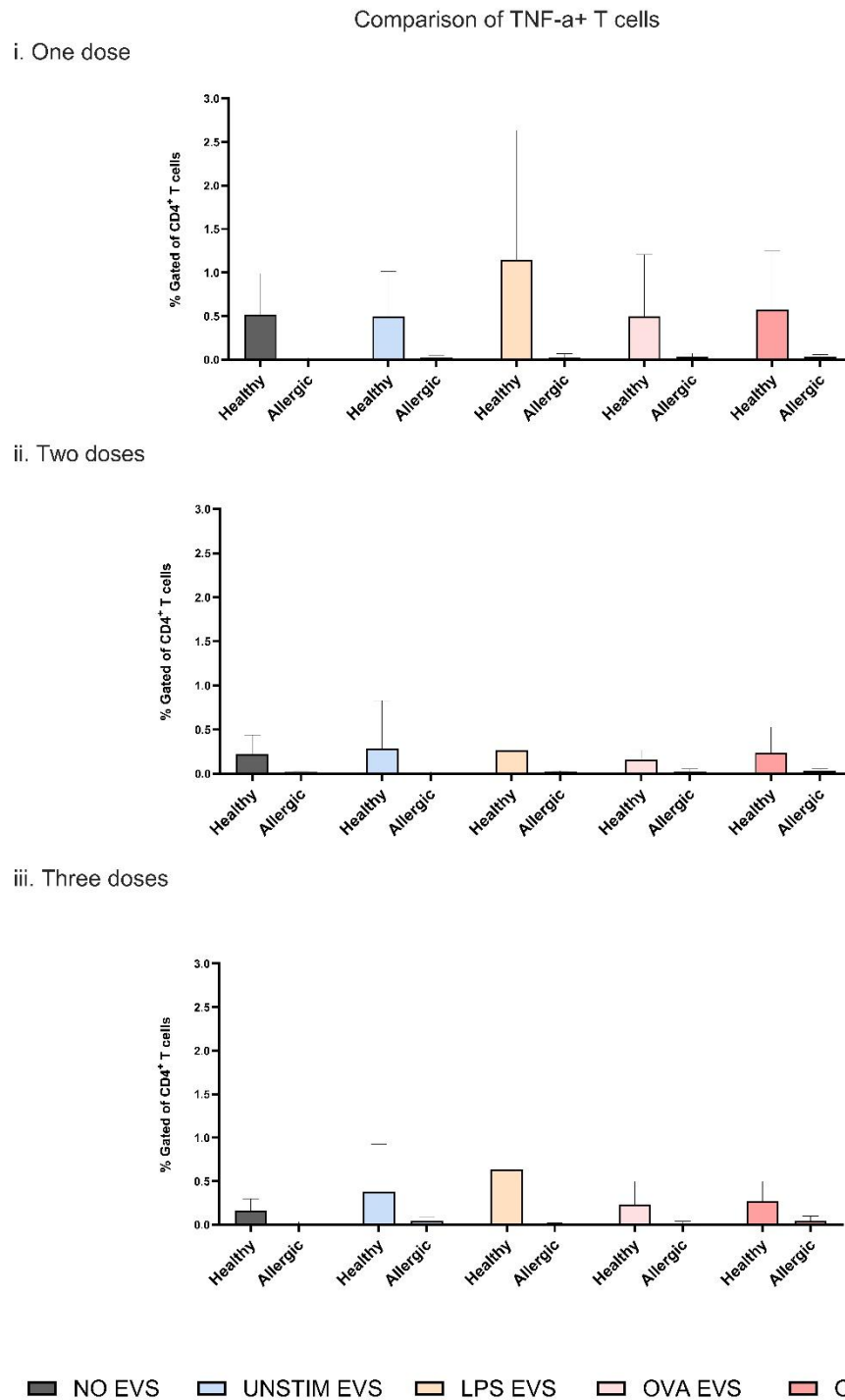


Figure 6.6 - Comparison of TNF- α expressing T cells in healthy and allergic participants. Statistical analysis was performed in GraphPad Prism utilising two-way ANOVA, $p < 0.05$. $n = \text{healthy} = 7$, $\text{allergic} = 5$.

6.3.2.5 Effect of DC-derived EVs on naïve T cell IL-4 production between healthy and allergic individuals

Next, IL-4 production was investigated in naïve T cells (fig.6.7). IL-4 plays a crucial role in allergic immune responses and drives the polarization of naïve T

cells towards Th2 phenotype, thus, by observing the effect of DC-EVs on IL-4 could potentially reveal how EVs could drive Th2 differentiation during allergen exposure. After one dose of DC-EVs, the percentage of IL-4⁺ T cells was minimal, with levels below 0.2% in naïve T cells in both groups (fig.6.7i). With two doses of DC-EVs, the percentage of IL-4⁺ T cells remained low in both groups (fig.6.7ii). After three doses of DC-EVs, the percentage of IL-4⁺ T cells was minimal in both groups across all conditions, ranging from 0.06-0.2% (fig.6.7iii). In summary, the percentage of IL-4⁺ T cells remained low in both healthy and allergic groups across all the conditions and all doses, with a slight increase in IL-4⁺ T cells from healthy individuals exposed to EVs derived from LPS-stimulated DCs, however no significant differences were observed. Taken together, this further suggests EVs alone have little effect on naïve

CD4+ T cells.

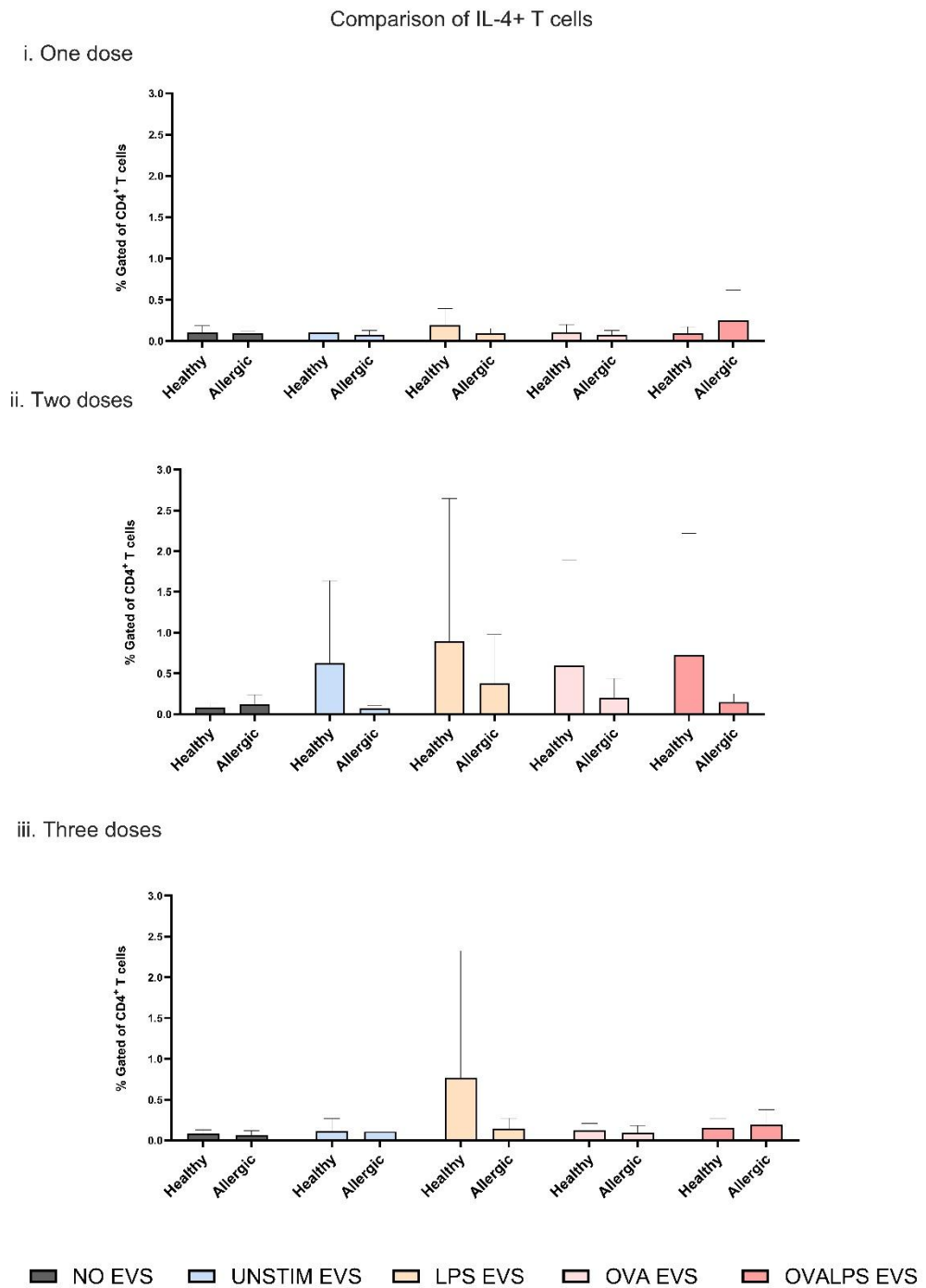


Figure 6.7 - Comparison of IL-4 expressing T cells in healthy and allergic participants. Statistical analysis was performed in GraphPad Prism utilising two-way ANOVA, $p < 0.05$. $n = \text{healthy} = 7$, $\text{allergic} = 5$.

6.3.2.6 Effect of DC-derived EVs on naïve T cell IL-2 production between healthy and allergic individuals

IL-2 production was also examined in naïve T cells (fig.6.8). IL-2 is a critical cytokine for naïve T cell early activation and clonal expansion upon

antigen encounter, and by observing the production levels it is possible to monitor DC-EVs ability to prime naïve T cells and support the survival and expansion of Th2 cells. Following the 1st dose of DC-EVs the percentage of IL-2+ producing naïve T cells was low across all conditions, ranging from 0.17-0.73% in both groups. However, most notably, a significant difference was observed between healthy and allergic naïve T cells exposed to EVs derived from OVA/LPS stimulated DCs (fig.6.8i). After two doses of DC-EVs the trend followed dose one, where the overall percentage of IL-2+ producing naïve T cells remained low, however, slightly higher percentage of IL-2+ producing naïve T cells in healthy individuals was observed, with the loss of significant difference in naïve T cells exposed to EVs derived from OVA/LPS stimulated DCs (fig.6.8ii). After three exposures to DC-EVs the trend remained the same, where the percentage of IL-2 producing naïve T cells remained higher in the healthy group than in naïve T cells in the allergic group (fig.6.8iii). Overall, the percentage of IL-2+ naïve T cells was low in both groups across all conditions and doses, with a statistically significant difference of IL-2+ naïve T cells following an exposure to EVs derived from OVA/LPS stimulated DCs after one dose, however it was lost after subsequent doses, with no other significant difference observed. This lack of IL-2 producing cells further supports the notion that EVs alone do not activate naïve CD4+ T cells.

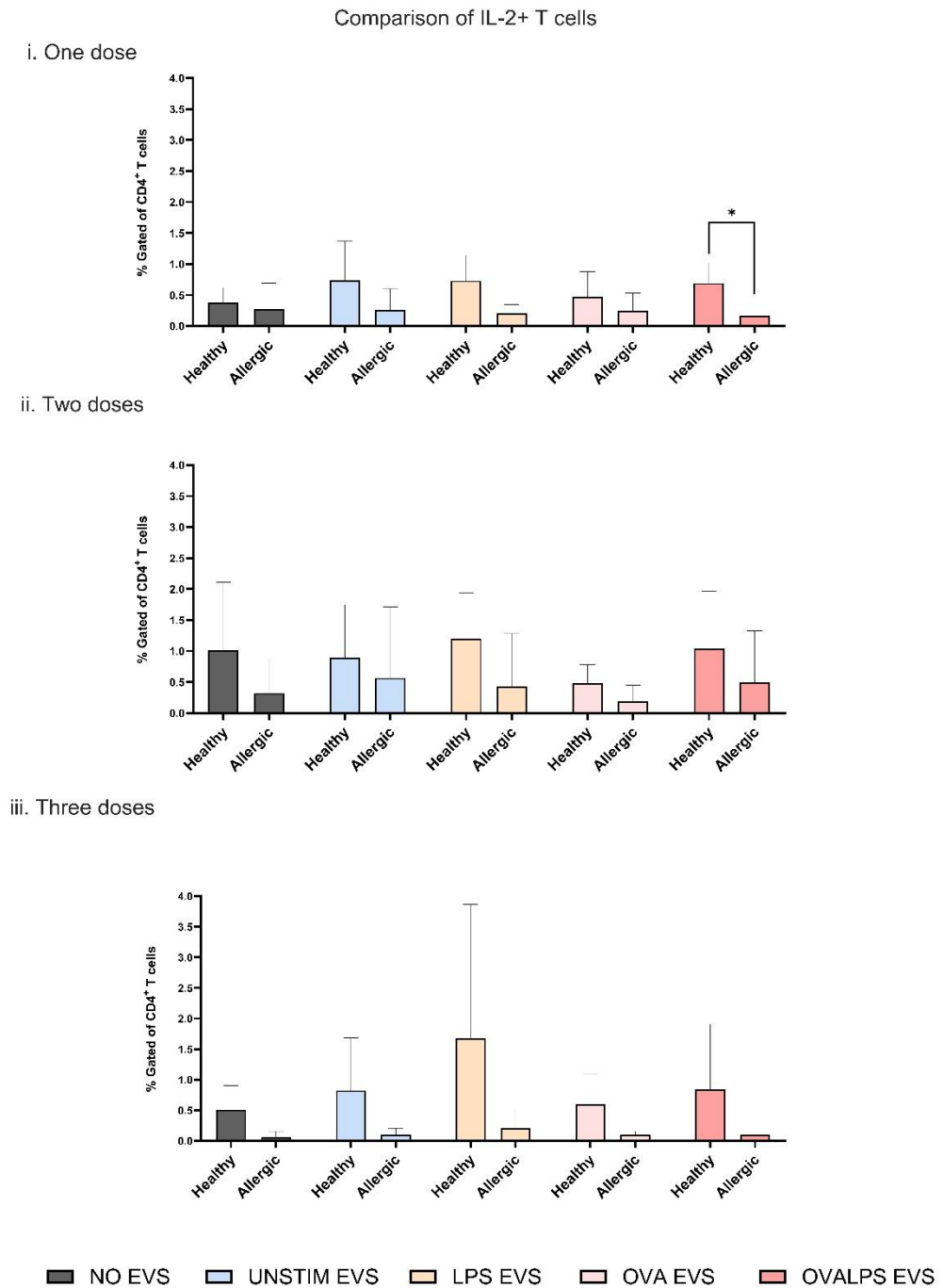


Figure 6.8 - Comparison of IL-2 expressing T cells in healthy and allergic participants. Statistical analysis was performed in Graphpad Prism utilising two-way ANOVA, $p < 0.05$. $n = \text{healthy} = 7$, $\text{allergic} = 5$.

6.3.3. The effect of DC-derived EVs, along with anti-CD3 and anti-CD28, on naïve T cell responses

As there were minimal effects of DC-EVs on naïve T cells by themselves, it was hypothesised that instead of acting as the sole signal on naïve T cell activation, DC-EVs could act as signal 3, following anti-CD3 (signal 1) and anti-CD28 (signal 2) co-stimulation. Monitoring the viability,

activation and cytokine production levels of naïve T cells co-stimulated with anti-CD3, anti-CD28 and DC-EVs would thus enable the study of the potential role of EVs as the polarising signal 3 in allergic sensitization.

6.3.3.1 Effect of co-stimulation with DC-EVs and CD3/CD28 on naïve T cell viability between healthy and allergic individuals

To investigate the effects of co-stimulation of DC-EVs and CD3/CD28 on naïve T cell viability, naïve T cells were exposed to DC-derived EVs generated following various stimulatory conditions, as previously stated, and co-stimulated with CD3 and CD28 (fig.6.9). After the 1st dose of DC-EVs co-stimulated with CD3/CD28, naïve T cells in the healthy group had higher viability than the allergic group in all conditions, with statistically significant differences in naïve T cells exposed to EVs derived from LPS and OVA/LPS stimulated DCs at 91% to 77% and 92% to 79%, respectively (fig.6.9i). Following the 2nd dose of DC-EVs to co-stimulated naïve T cells, the trend was similar to the 1st dose, where the naïve T cells in the healthy group had higher cell viability than the allergic group across all conditions, however only naïve T cells exposed to EVs derived from unstimulated DCs showed a statistically significant difference between them at 88% to 76%, respectively (fig.6.9ii). The trend continued into the 3rd dose of DC-EVs, where the healthy group naïve T cells had higher viability across the conditions, however no statistically significant differences were observed (fig.6.9iii). Altogether, the naïve T cells in the healthy group had higher cell viability than the allergic group across all conditions and doses, with statistically significant differences in 1st dose and 2nd dose once exposed to EVs derived from LPS, OVA/LPS stimulated and unstimulated DCs, potentially showing an effect on naïve T cell viability by DC-EVs.

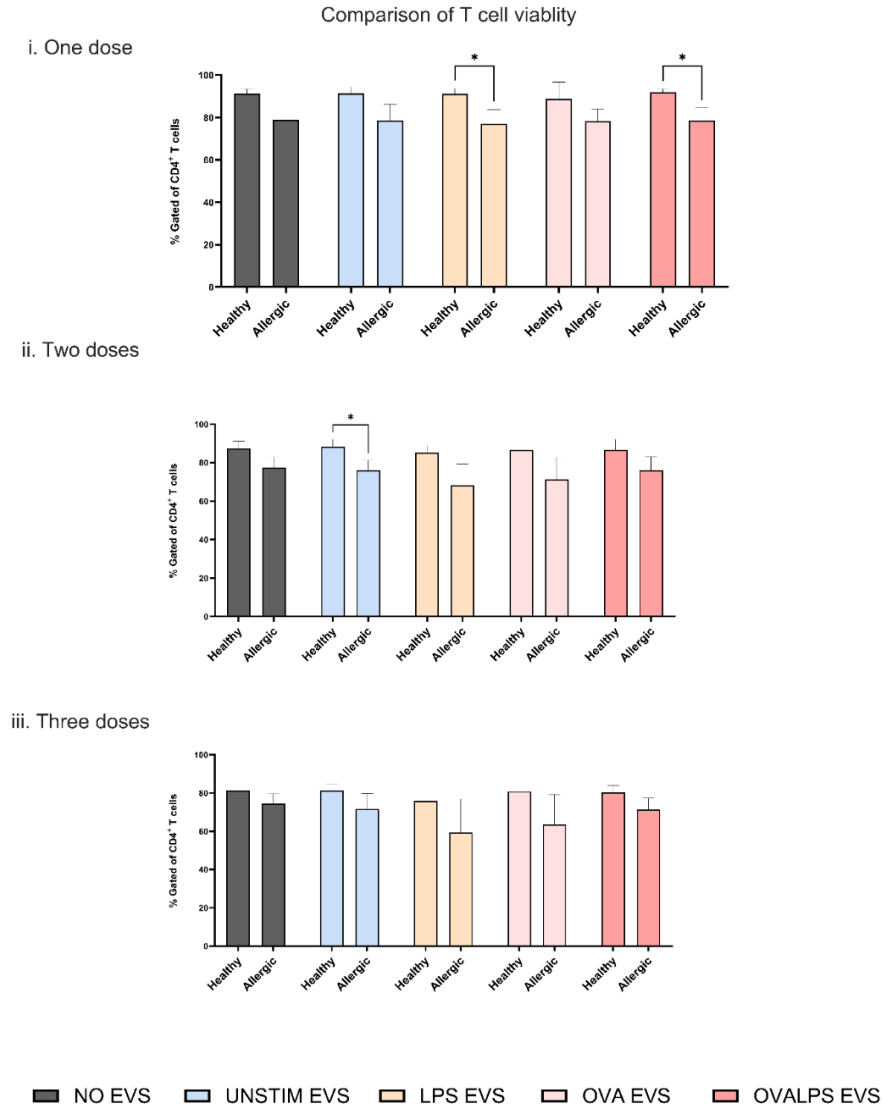


Figure 6.9 - Comparison of T cell viability in healthy and allergic participants. Statistical analysis was performed in GraphPad Prism utilising two-way ANOVA, $p < 0.05$. $n = \text{healthy} = 7$, $\text{allergic} = 5$.

6.3.3.2 Effect of co-stimulation with DC-EVs and CD3/CD28 on naïve T cell activation levels between healthy and allergic individuals

Next, naïve T cell activation was observed after co-stimulation with anti-CD3/anti-CD28 and DC-EVs by measuring the percentage of CD69+ T cells (fig.6.10). After the 1st dose of DC-EVs to co-stimulated naïve T cells, the percentage of CD69+ naïve T cells was very high in both healthy and allergic groups, ranging from 92-95% (fig.6.10i). Following the 2nd dose of DC-EVs, the CD69 expression had decreased in both groups, ranging from 72-85% (fig.6.10ii). After the 3rd dose of DC-EVs there was a statistically significant decrease in the percentage of CD69+ T cells in the allergic groups

compared to the healthy group when no EVs were added or following addition of EVs from unstimulated DCs (fig.6.10iii). This may suggest that DC-EVs from the three stimulated conditions enable naïve T cells in the allergic group to become activated, but when there are no EVs or the EVs are generated from unstimulated DCs, the T cells do not become activated.

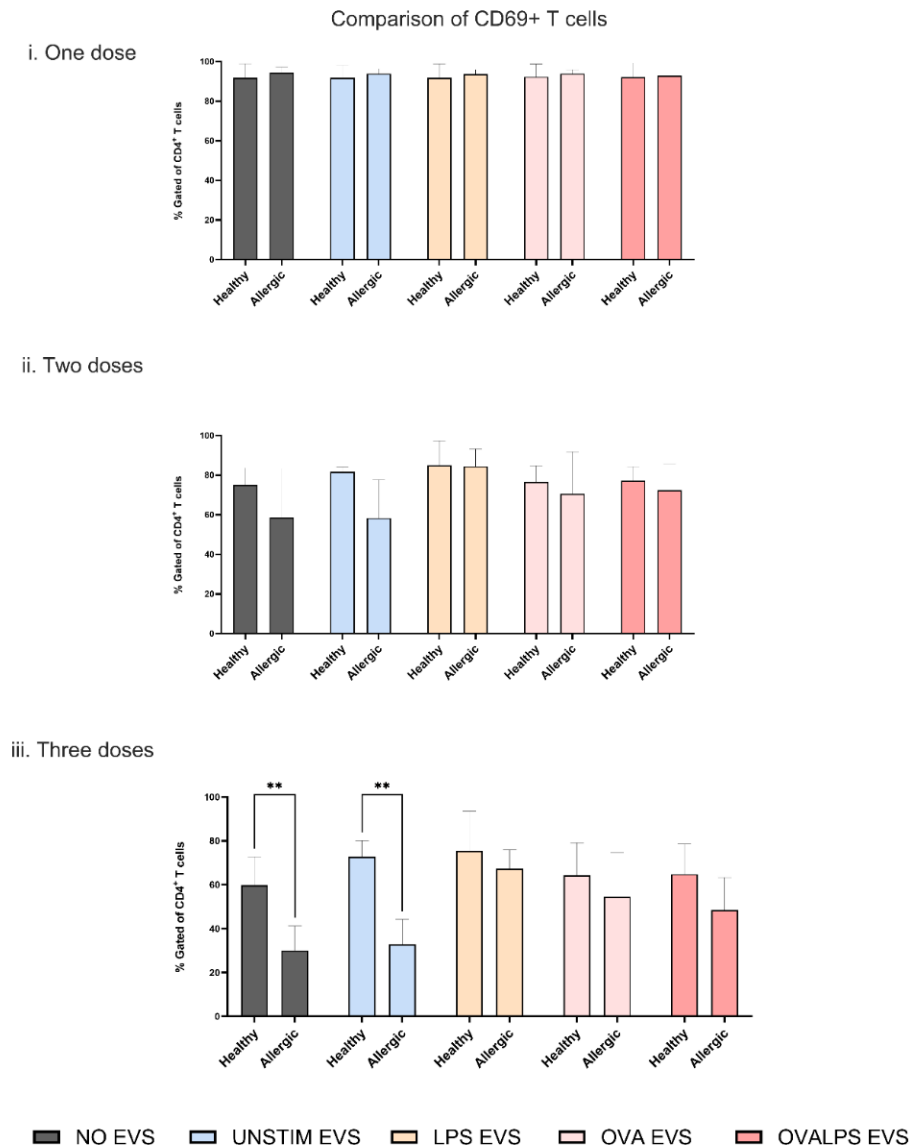


Figure 6.10 - Comparison of CD69 expressing T cells in healthy and allergic participants. Statistical analysis was performed in GraphPad Prism utilising two-way ANOVA, $p < 0.05$. $n = \text{healthy} = 7$, $\text{allergic} = 5$.

6.3.3.3 Effect of co-stimulation with DC-EVs and CD3/CD28 on naïve T cell IFN- γ production between healthy and allergic individuals

IFN- γ production was then observed in naïve T cells following addition of EVs and co-stimulation, as previously stated (fig.6.11). After the 1st dose of

DC-EVs to co-stimulated naïve T cells, the percentage of IFN- γ ⁺ naïve T cells in the healthy group was much higher than the allergic group in all conditions, ranging from 23-27% to 5-8%, respectively (fig.6.11 i). Following the 2nd dose of DC-EVs the trend was the same between the groups, however, with statistically significant differences being observed in naïve T cells exposed to EVs derived from unstimulated, OVA and OVA/LPS stimulated DCs and with no EVs (fig.6.11 ii). After the 3rd dose of DC-EVs, cells in the healthy group continued to display a higher percentage of IFN- γ ⁺ T cells than the allergic group, with statistically significant differences in naïve T cells exposed to EVs derived from OVA and OVA/LPS stimulated DCs and with no EVs (fig.6.11 iii). Overall, naïve T cells from the healthy group had higher percentage of IFN- γ ⁺ naïve T cells than the allergic group in all the conditions and doses, with statistically significant differences after the 2nd and 3rd dose of DC-EVs, indicating that naïve T cells from healthy individuals are more likely to polarize towards a Th1 phenotype, regardless of EVs.

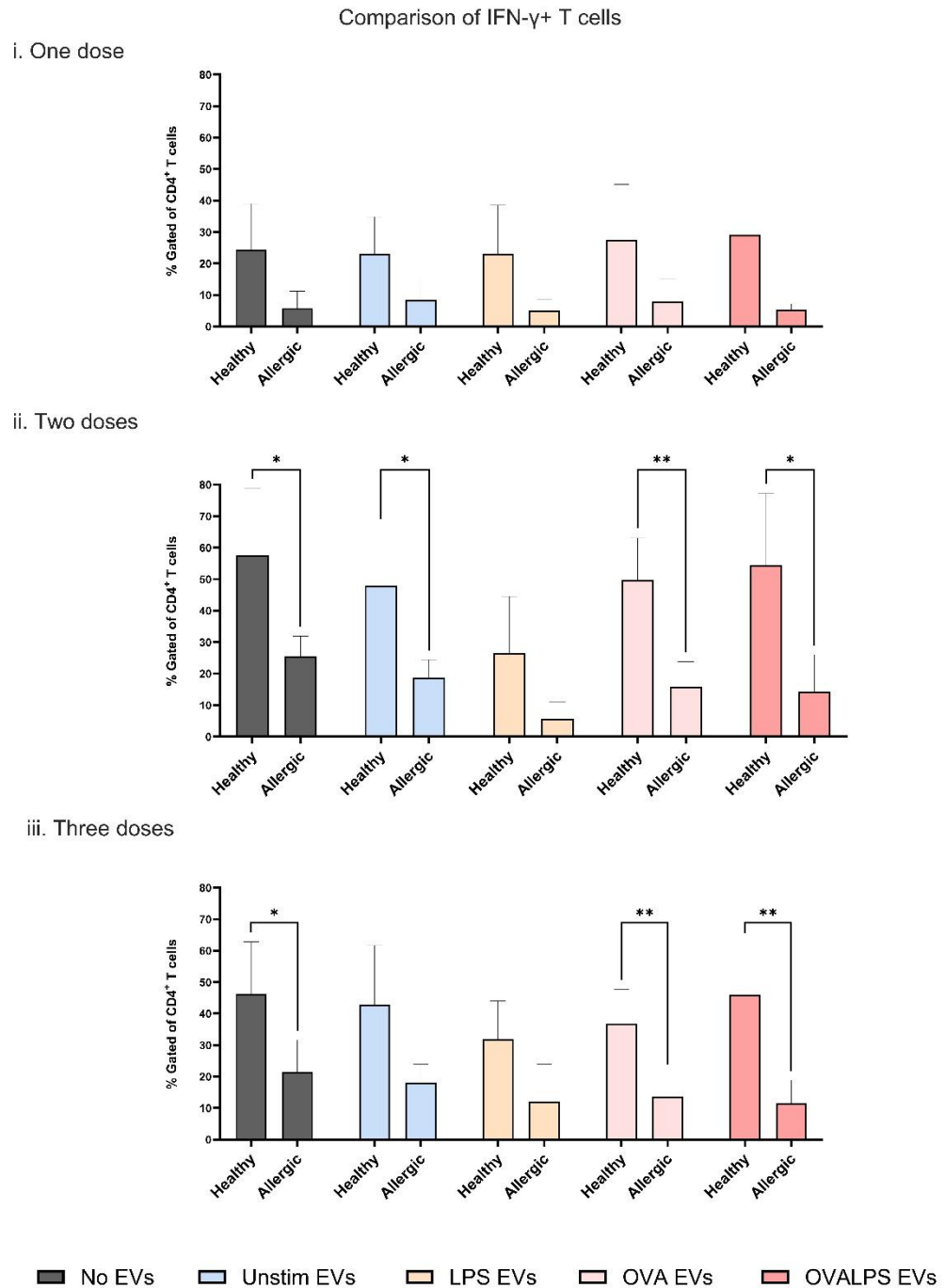


Figure 6.11 - Comparison of IFN- γ expressing T cells in healthy and allergic participants. Statistical analysis was performed in GraphPad Prism utilising two-way ANOVA, $p < 0.05$. $n = \text{healthy} = 7$, $\text{allergic} = 5$.

6.3.3.4 Effect of co-stimulation with DC-EVs and CD3/CD28 on naïve T cell TNF- α production between healthy and allergic individuals

TNF- α production was then measured in naïve T cells after co-stimulation as previously stated (fig.6.12). After the 1st dose of DC-EVs to co-stimulated naïve T cells, the healthy group had a higher percentage of TNF- α + T cells than the allergic group (fig.6.12i). Following the 2nd dose of DC-EVs

the trend was the same, as the percentage of TNF- α ⁺ T cells were higher in the healthy group than in the allergic group (fig.6.12ii). After the 3rd dose of DC-EVs, the healthy group naïve T cells continued to have a higher percentage of TNF- α ⁺ T cells than the allergic group (fig.6.12iii). Overall, the healthy group had higher percentage of TNF- α ⁺ T cells across all the conditions and doses, however, no statistically significant difference was observed, which could indicate that DC-EVs had minimal impact on TNF- α production.

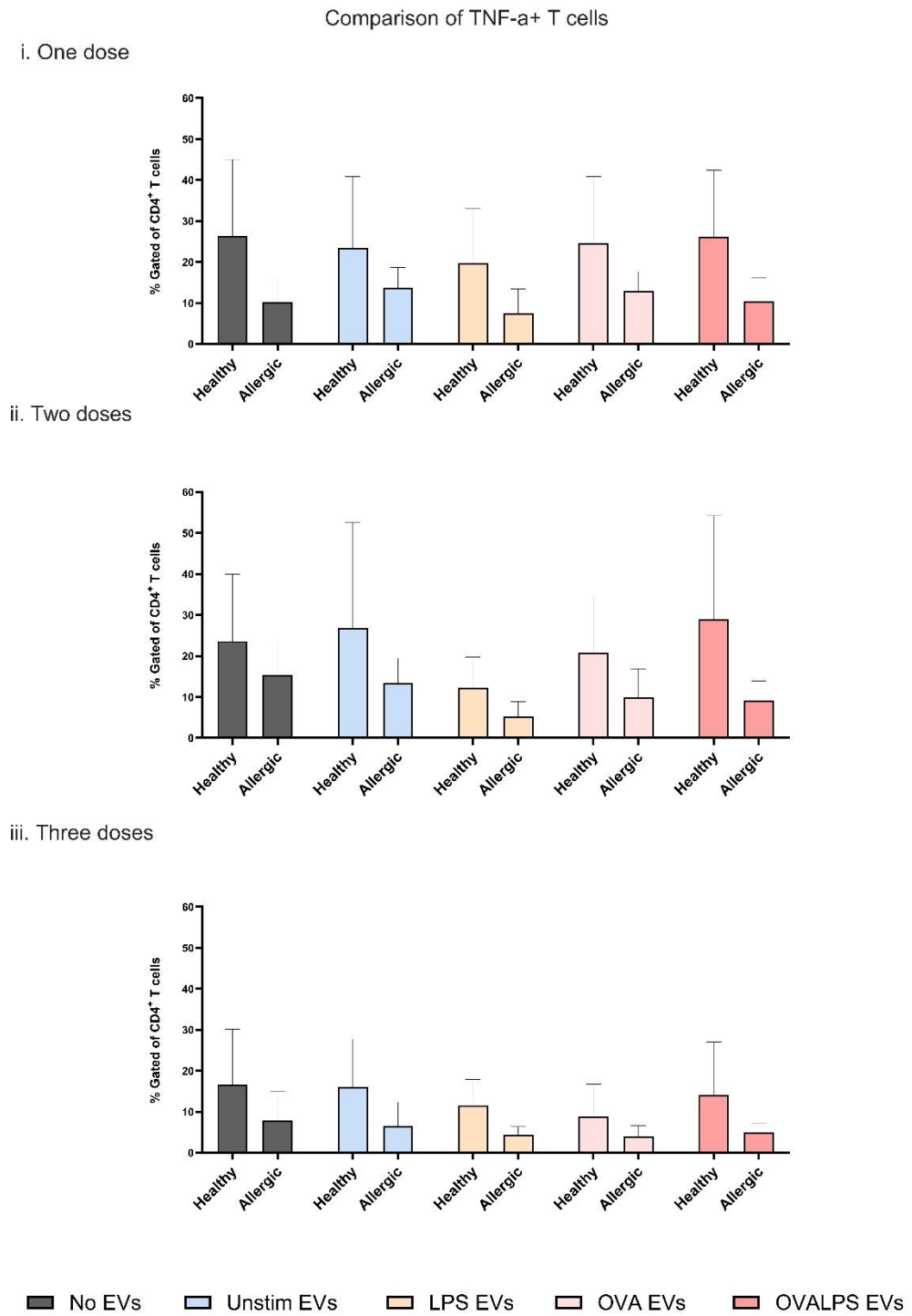


Figure 6.12 - Comparison of TNF- α expressing T cells in healthy and allergic participants. Statistical analysis was performed in GraphPad Prism utilising two-way ANOVA, $p < 0.05$. $n = \text{healthy} = 7$, $\text{allergic} = 5$.

6.3.3.5 Effect of co-stimulation with DC-EVs and CD3/CD28 on naïve T cell IL-4 production between healthy and allergic individuals

Next, IL-4 production was investigated following co-stimulation as previously stated (fig.6.13). After the 1st dose of DC-EVs with anti-CD3/anti-

CD28 co-stimulated naïve T cells, the percentage of IL-4⁺ T cells was higher in the healthy group than in the allergic group across all conditions (fig.6.13i). Following the 2nd dose of DC-EVs, the trend remained the same, with naïve T cells in the healthy group having a higher percentage of IL-4⁺ T cells than the allergic group across all the conditions (fig.6.13ii). After the 3rd dose of DC-EVs the healthy group continued to display a higher percentage of IL-4⁺ T cells than the allergic group across all conditions (fig.6.13iii). Overall, naïve T cells in the healthy group showed a higher percentage of IL-4⁺ T cells than the allergic group across all conditions and all doses, however, no statistically significant differences were observed, showing little impact on IL-4 production by DC-EVs between healthy and allergic groups.

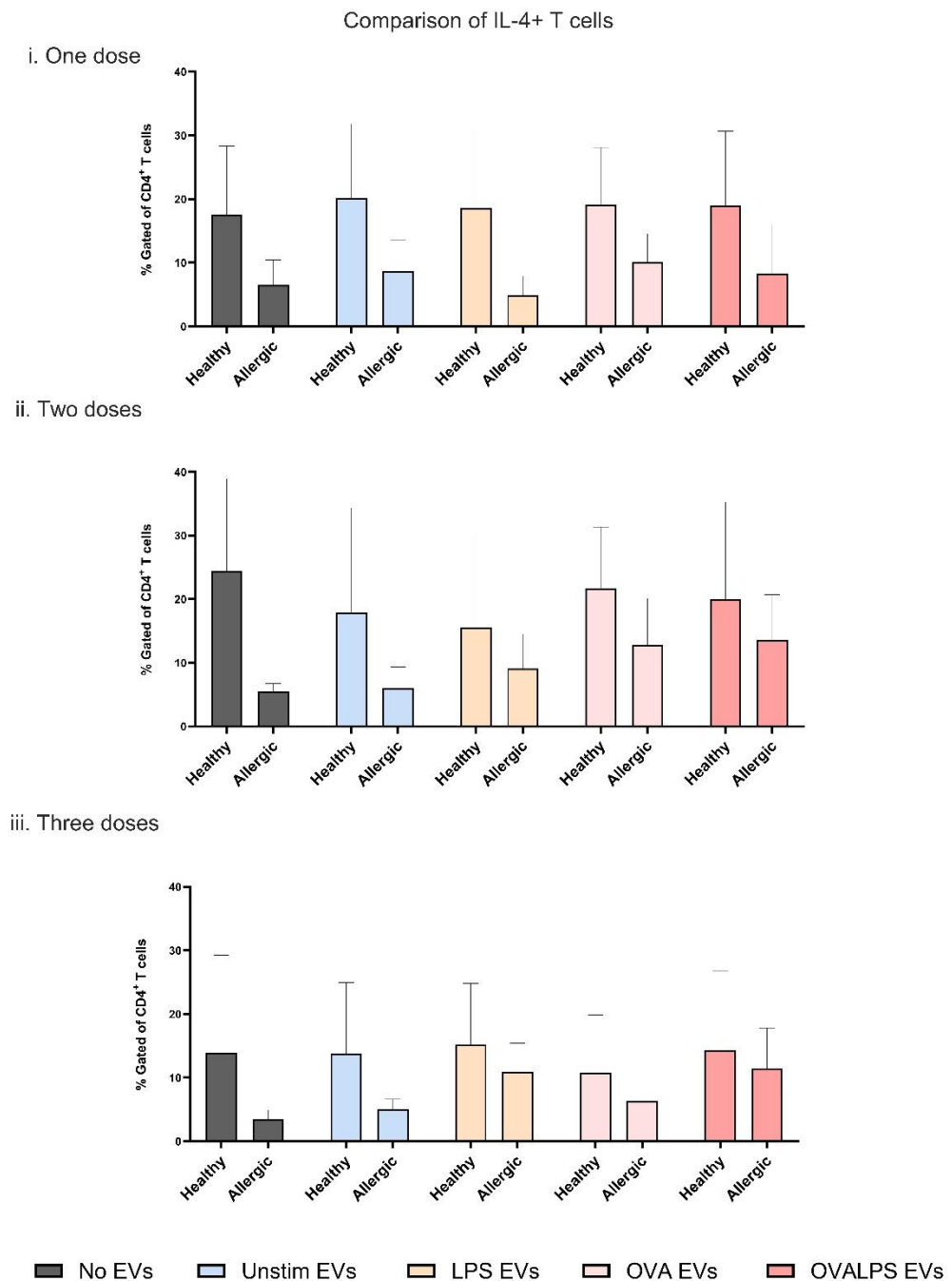


Figure 6.13- Comparison of IL-4 expressing T cells in healthy and allergic participants. Statistical analysis was performed in GraphPad Prism utilising two-way ANOVA, $p < 0.05$. $n = \text{healthy} = 7$, $\text{allergic} = 5$.

6.3.3.6 Effect of co-stimulation with DC-EVs and CD3/CD28 on naïve T cell IL-2 production between healthy and allergic individuals

IL-2 production was explored in naïve T cells after co-stimulation, as previously stated (fig.6.14). After the 1st dose of DC-EVs to naïve T cells co-stimulated with CD3/CD28, the healthy group had a higher percentage of IL-2+ T cells than the allergic group across all the conditions (fig.6.14i). Following

the 2nd dose of DC-EVs the percentage of IL-2+ T cells had similar levels between healthy and allergic groups (fig.6.14ii). After the 3rd dose of DC-EVs, naïve T cells from the healthy group had a higher percentage of IL-2+ T cells than the allergic group across all conditions, with statistically significant differences in naïve T cells with no EVs (28% compared to 8%) and EVs derived from unstimulated DCs (30% compared to 8%) (fig.6.14iii). Overall, there were more IL-2+ T cells in the healthy group than the allergic group after the 1st and 3rd dose, with statistically significant differences in the 3rd dose, but not when EVs derived from stimulated DCs are added to the culture, suggesting that EVs derived from stimulated DCs could be activating naïve T cells from allergic individuals to produce higher amounts of IL-2.

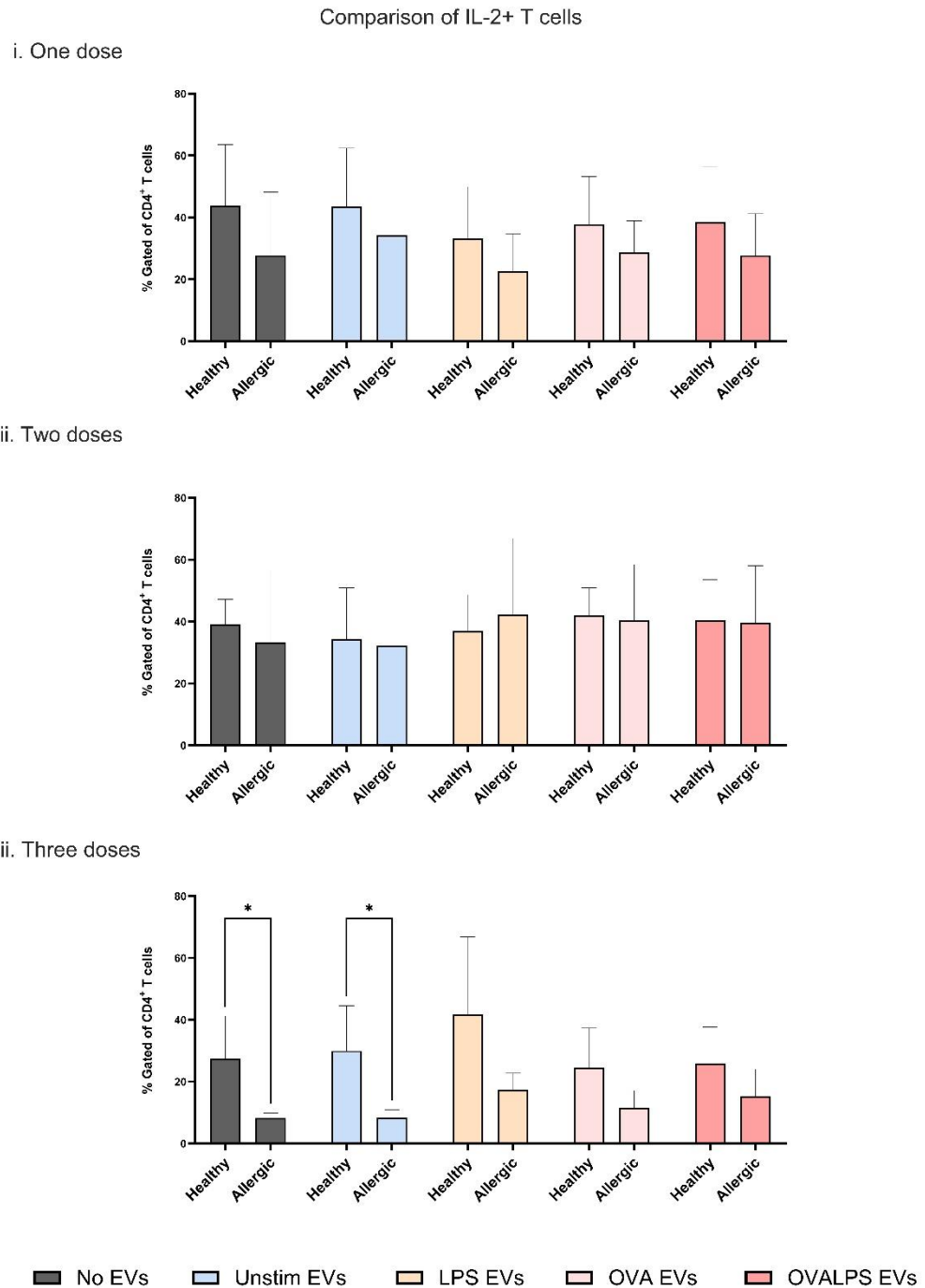


Figure 6.14 - Comparison of IL-2 expressing T cells in healthy and allergic participants. Statistical analysis was performed in GraphPad Prism utilising two-way ANOVA, $p < 0.05$. $n = \text{healthy} = 7$, $\text{allergic} = 5$.

6.3.4 Unsupervised clustering analysis of DC-EVs following co-stimulation with anti-CD3/anti-CD28 between healthy and allergic individuals

To look at all possible effects DC-EVs could have on naïve T cells when co-stimulated with anti-CD3/anti-CD28, unsupervised clustering analysis, using FlowJo, was performed to analyse naïve T cell phenotypes between subjects in an unbiased manner. Due to the number of cells collected,

this analysis could only be undertaken using samples following the 2nd dose of EVs, as there were insufficient cell numbers in the 1st and 3rd dose datasets.

6.3.4.1 Unsupervised clustering analysis of 2nd dose of LPS-stimulated DC-EVs co-stimulated with anti-CD3/anti-CD28 between healthy and allergic individuals

To examine any differences between groups when EVs from LPS-stimulated DCs were added in conjunction with anti-CD3/anti-CD28 to naïve T cells, single viable CD3/CD4⁺ naïve T cells were gated in FlowJo before equal sampling of 5000 events from each 4 healthy and 4 allergic subjects. By performing FlowSOM clustering analysis, 20 meta-clusters were identified after analysing naïve T cells with parameters CD3, CD4, CD45-RA, CD8, CD56, CD27, CD28, CCR7, CD69, IL-2, IL-4, IFN- γ and TNF- α , which were then presented on a tSNE plot (fig.6.15AB). Each marker expression across the clusters was analysed (fig.6.15C). To find out the differences between healthy and allergic groups naïve T cells phenotypes, the percentage of naïve T cells present in each cluster was measured and statistical analysis was performed on each cluster, resulting in 13 statistically significant clusters (fig.6.15D). In order to identify the marker expression in each cluster, Cluster Explorer analysis was performed and populations that were significantly higher in healthy subjects (fig.6.15Ei), and allergic subjects (fig.6.15Eii) were summarised. This shows there were many statistically different populations of naïve T cells between healthy and allergic subjects following exposure to EVs derived from LPS stimulated DCs, most notably, the largest cluster 1 (CD45-RA⁺, CD3⁺, CD4⁺, CD27⁺, CCR7⁺, CD69⁺+) was mostly present in the allergic group naïve T cells, suggesting that a large portion of naïve T cells were minimally activated and had low cytokine production.

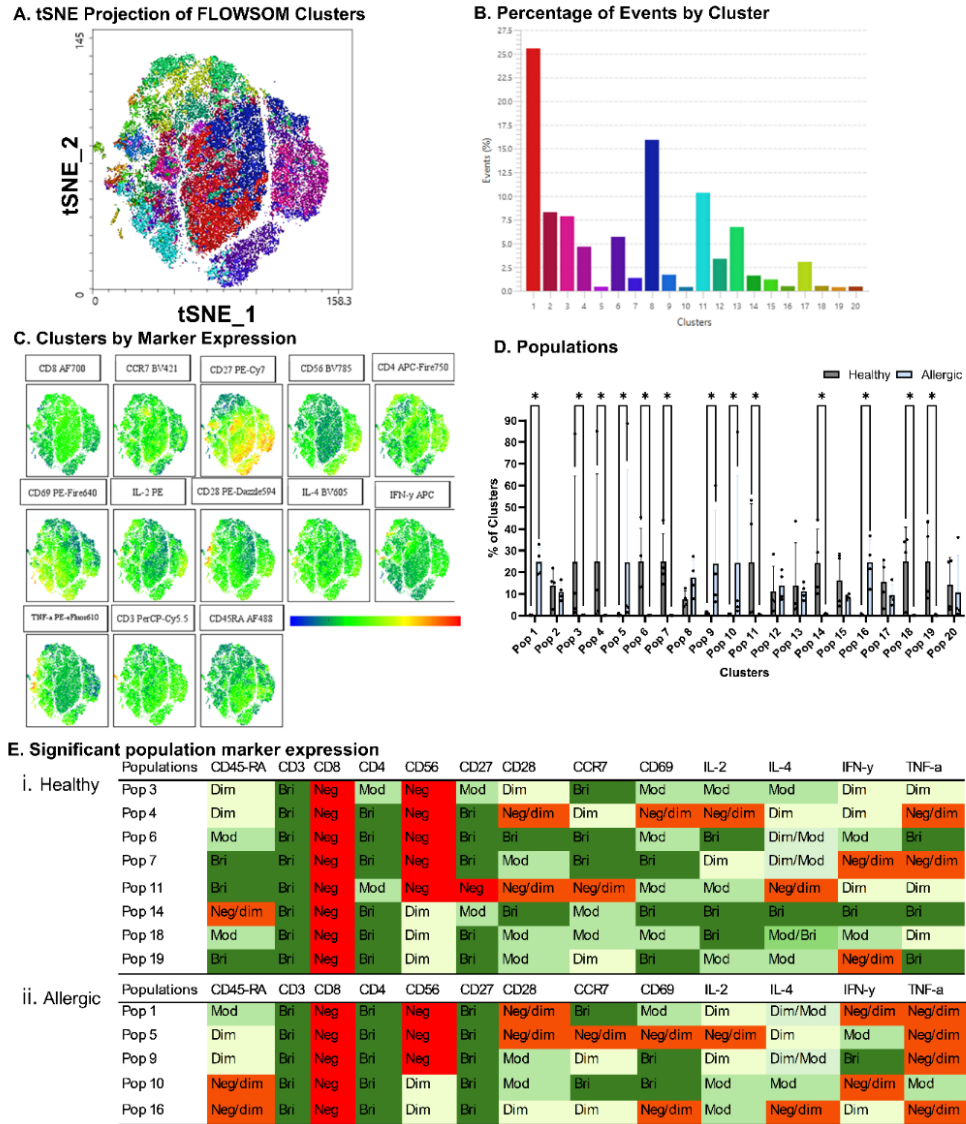


Figure 6.15 - Unsupervised cluster analysis of healthy and allergic naive T cells exposed to EVs derived from LPS stimulated DCs. Following cluster explorer plugin, a multi-coloured tSNE plot was made (A) and clusters were separated based on their % (B). Individual marker clusters were produced from tSNE plot (C). Populations between healthy and allergic were analysed for significant differences using multiple Mann-Whitney Tests $p < 0.05$ (D). Significant populations were split between healthy and allergic, and their marker expression was colour coded - Negative (red), Negative/dim (orange), Dim (pale yellow), Dim/moderate (pale green), Moderate (light green), Moderate/bright (green), Bright (dark green) (i., ii.). $n = 4$.

6.3.4.2 Unsupervised clustering analysis of 2nd dose of OVA-stimulated DC-EVs following co-stimulation between healthy and allergic individuals

Analysis was performed as previously stated between 4 healthy and 4 allergic subjects following OVA-stimulated DC-EVs with co-stimulation. By plotting the 23 meta-clusters found by FlowSom a tSNE plot was generated (fig.6.16A) and the percentage in each cluster examined (fig. 6.16B). Marker expression across the clusters was also analysed (fig.6.16C). To find out the

differences between healthy and allergic groups naïve T cells phenotypes, the percentage of naïve T cells present in each cluster was measured and statistical analysis was performed on each cluster, however no statistically significant differences between the groups were observed (fig.6.16D).

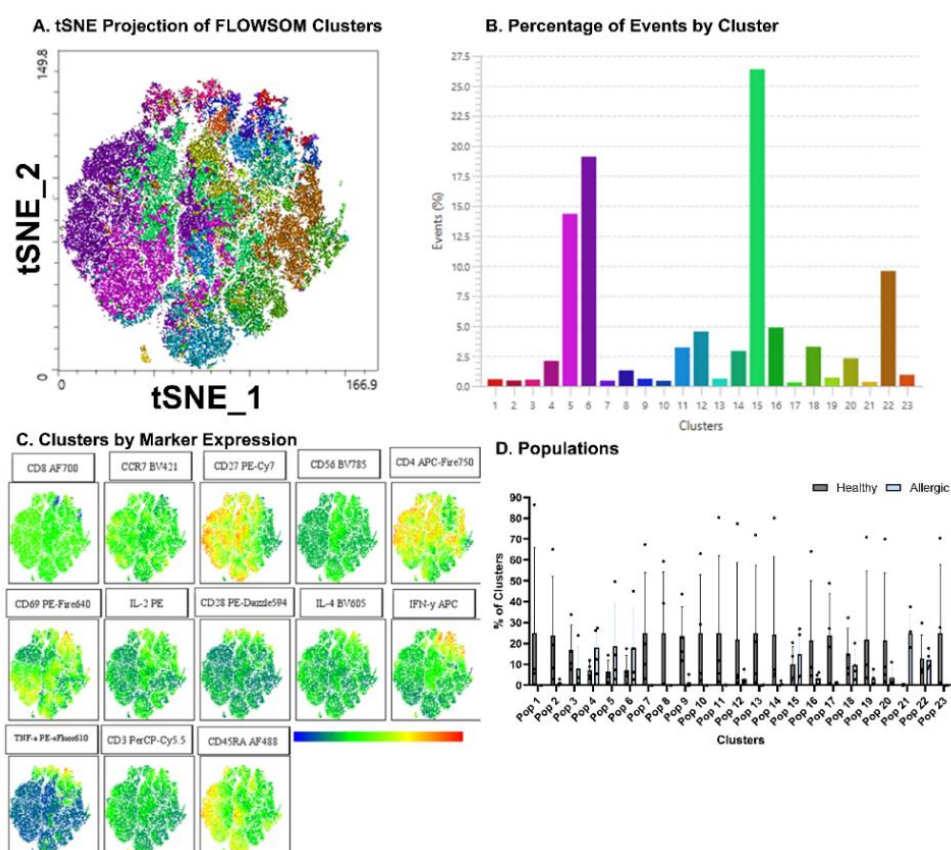


Figure 6.16 - Unsupervised cluster analysis of healthy and allergic naïve T cells exposed to EVs derived from OVA stimulated DCs. Following cluster explorer plugin, a multi-coloured tSNE plot was made (A) and clusters were separated based on their % (B). Individual marker clusters were produced from tSNE plot (C). Populations between healthy and allergic were analysed for significant differences using multiple Mann-Whitney Tests $p < 0.05$ (D). $n = 4$.

6.3.4.3 Unsupervised clustering analysis of 2nd dose of OVA/LPS stimulated DC-EVs following co-stimulation between healthy and allergic individuals

To examine any differences in groups when EVs from OVA/LPS stimulated DCs were added following co-stimulation with anti-CD3/anti-CD28, analysis was performed as previously stated between 4 healthy and 4 allergic subjects. By plotting the 23 meta-clusters found by FlowSom a tSNE plot was generated (fig.6.17A) and the percentage in each cluster examined (fig. 6.17B). Marker expression across the clusters was also analysed (fig.6.17C). To find out the differences between healthy and allergic groups

naïve T cells phenotypes, the percentage of naïve T cells present in each cluster was measured and statistical analysis was performed on each cluster, however, again no statistically significant differences between the groups were observed (fig.6.17D).

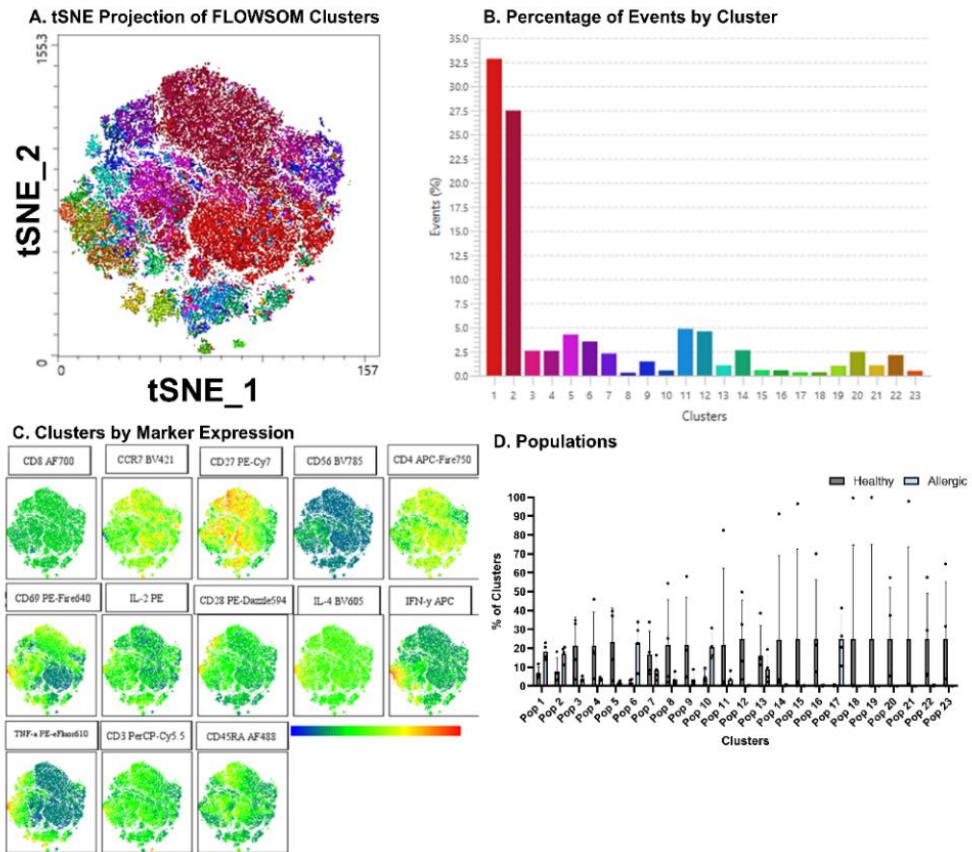


Figure 6.17 - Unsupervised cluster analysis of healthy and allergic naïve T cells exposed to EVs derived from OVALPS stimulated DCs. Following cluster explorer plugin, a multi-coloured tSNE plot was made (A) and clusters were separated based on their % (B). Individual marker clusters were produced from tSNE plot (C). Populations between healthy and allergic were analysed for significant differences using multiple Mann-Whitney Tests $p < 0.05$ (D). $n = 4$.

6.3.4.4 Unsupervised clustering analysis of 2nd dose of unstimulated DC-EVs following co-stimulation between healthy and allergic individuals

To examine any differences in groups when EVs from unstimulated DCs were added with co-stimulation, analysis was performed as previously stated between 4 healthy and 4 allergic subjects. By plotting the 23 meta-clusters found by FlowSom a tSNE plot was generated (fig.6.18A) and the percentage in each cluster examined (fig. 6.18B). Each marker expression across the clusters was analysed (fig.6.18C). To find out the differences between healthy and allergic groups naïve T cells phenotypes, the percentage

of subject naïve T cells present in each cluster was measured and statistical analysis was performed on each cluster, however, no statistically significant differences between the groups were observed (fig.6.18D).

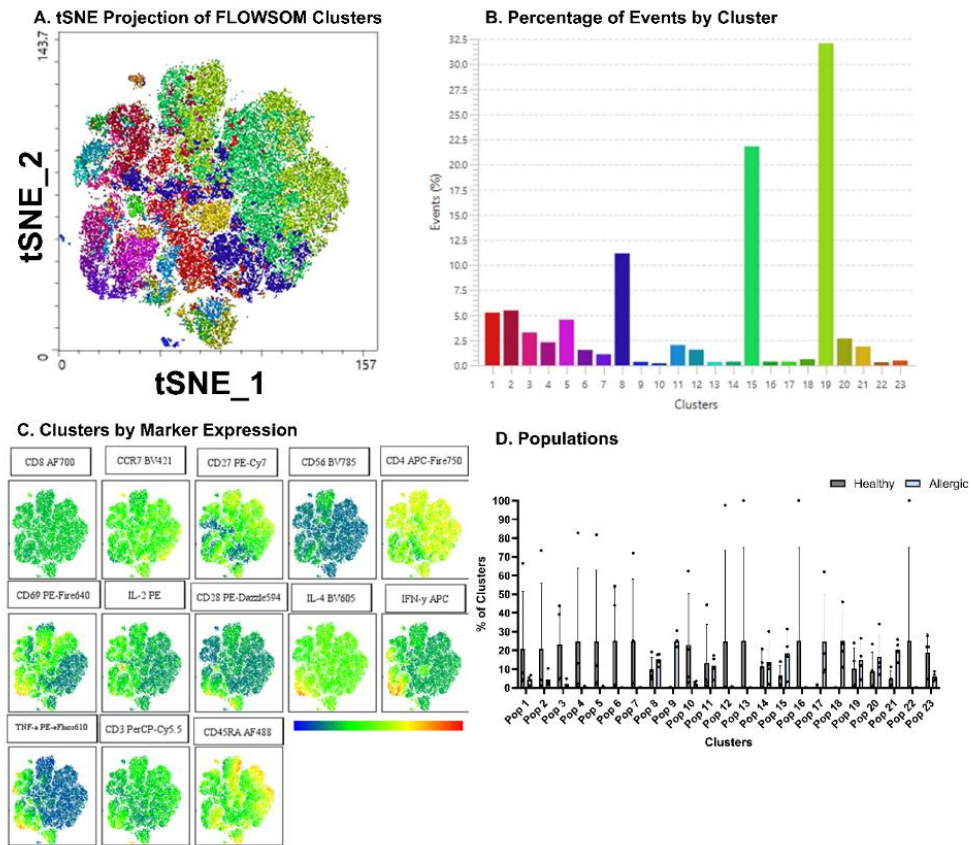


Figure 6.18 - Unsupervised cluster analysis of healthy and allergic naïve T cells exposed to EVs derived from Unstimulated DCs. Following cluster explorer plugin, a multi-coloured tSNE plot was made (A) and clusters were separated based on their % (B). Individual marker clusters were produced from tSNE plot (C). Populations between healthy and allergic were analysed for significant differences using multiple Mann-Whitney Tests $p < 0.05$ (D). $n = 4$.

6.3.4.5 Unsupervised clustering analysis of 2nd dose of PBS following co-stimulation between healthy and allergic individuals

To examine any differences in groups when there was only co-stimulation with anti-CD3/anti-CD28 and no EVs were added, analysis was performed as previously stated between 4 healthy and 4 allergic subjects. By plotting the 23 meta-clusters found by FlowSom a tSNE plot was generated (fig.6.19A) and the percentage in each cluster examined (fig. 6.19B). Each marker expression across the clusters was analysed (fig.6.19C). To find out the differences between healthy and allergic groups naïve T cells phenotypes, the percentage of naïve T cells present in each cluster was measured and statistical analysis

was performed on each cluster, identifying 19 statistically significant different populations between healthy and allergic groups (fig.6.19D). This indicates that there are inherent differences in naïve T cells between healthy and allergic individuals, however, these differences were lost following exposure to EVs derived from OVA, OVA/LPS-stimulated DCs and unstimulated DCs, which could mean that DC-EVs change naïve T cells depending on the stimulus of DCs.

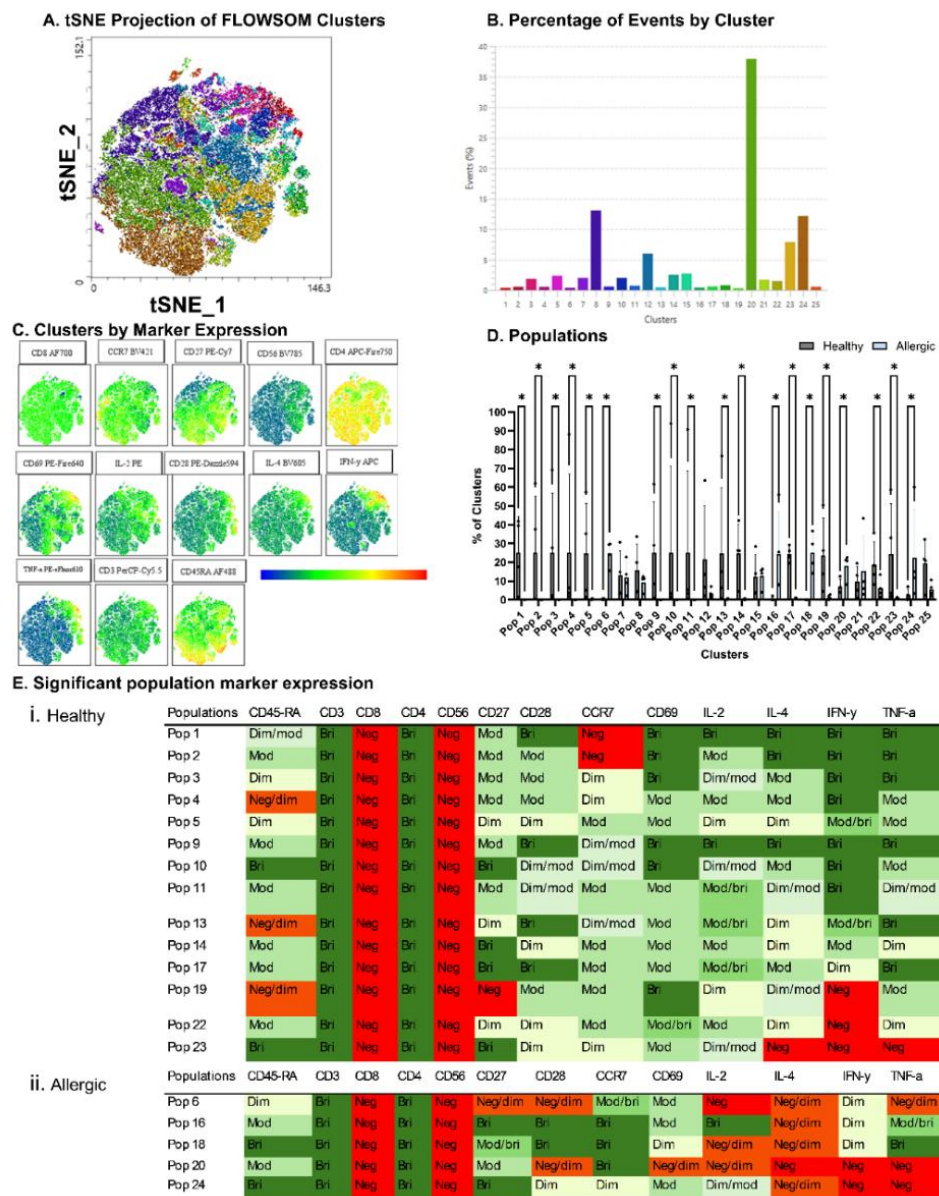


Figure 6.19 - Unsupervised cluster analysis of healthy and allergic naïve T cells with no EVs added. Following cluster explorer plugin, a multi-coloured tSNE plot was made (A) and clusters were separated based on their % (B). Individual marker clusters were produced from tSNE plot (C). Populations between healthy and allergic were analysed for significant differences using multiple Mann-Whitney Tests $p < 0.05$ (D). Significant populations were split between healthy and allergic, and their marker expression was colour coded - Negative (red), Negative/dim (orange), Dim (pale yellow), Dim/moderate (pale green), Moderate (light green), Moderate/bright (green), Bright (dark green) (i., ii.). $n = 4$.

6.3.5. The effect of co-stimulation with DC-EVs and anti-CD3/anti-CD28 on naïve T cells across conditions in healthy and allergic individuals

Previous data presented here suggest that naïve T cells were affected by DC-EVs in a stimulus-dependent manner, so the effects of DC-EVs within the groups were investigated to examine changes in viability, activation and cytokine production depending on the stimulus of DCs.

6.3.5.1 Effect of co-stimulation with DC-EVs and anti-CD3/anti-CD28 on naïve T cell viability in healthy and allergic individuals across conditions

Naïve T cell viability in either allergic or healthy individuals was observed after EVs derived from DCs following different stimuli, plus co-stimulation, were added (fig.6.20). After the 1st and 2nd dose of DC-EVs co-stimulated with CD3/CD28, the naïve T cell viability across all conditions were at similar levels in both the healthy and allergic groups (fig.6.20ABi, ii). After the 3rd exposure of DC-EVs, in healthy individuals, there was a decrease in T cell viability in cells exposed to EVs derived from LPS-stimulated DCs, with statistically significant differences between no EVs and EVs derived from unstimulated DCs (fig.6.20Aiii). In allergic individuals, the naïve T cell viability remained high in naïve T cells across the conditions, with no significant differences observed (fig.6.20Biii). Overall, the naïve T cell viability remained high in both healthy and allergic individuals across conditions and doses.

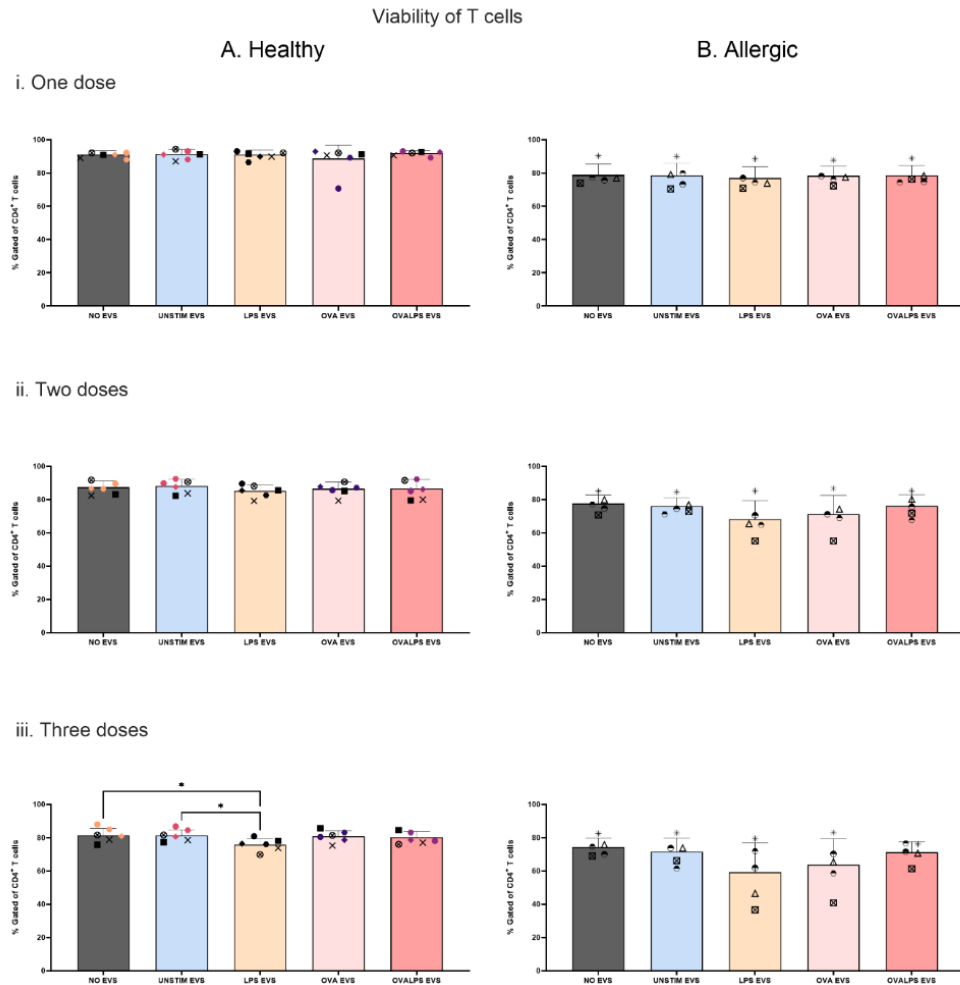


Figure 6.20 - T cell viability in healthy and allergic participants. Naïve T cells were plated in 96-well plate with co-stimulation of coated 2µg/ml CD3 and direct 2µg/ml CD28. Each well afterwards was stimulated with dendritic cell-derived EVs added in 3 doses. DCs were stimulated with 100ng/ml of LPS, 10µg/ml of OVA, 50ng/ml of LPS and 10µg/ml of OVA, and unstimulated, PBS was added as control. T cells were stimulated every 24h for 3 times (i., ii., iii.). Cytokine expression was measured after 42h, following 30h stimulation of EVs and 12h incubation with PTI. Study included 7 healthy and 5 allergic participants. Statistical analysis was performed in GraphPad Prism using one-way ANOVA, $p < 0.05$.

6.3.5.2 Effect of co-stimulation with DC-EVs and CD3/CD28 on naïve T cell activation levels in healthy and allergic individuals across conditions

Next, naïve T cell activation levels were observed following co-stimulation, as previously stated (fig.6.21). After the 1st dose of DC-EVs to naïve T cells co-stimulated with CD3/CD28 the percentage of CD69⁺ naïve T cells was high across all conditions in both healthy and allergic individuals (fig.6.21ABi). The same was observed following the 2nd dose (fig.6.21ABii). After the 3rd dose of DC-EVs, naïve T cells in the healthy group remained activated (fig.6.21Aiii). In allergic individuals the lowest percentage of CD69⁺ T cells was observed following culture with no EVs and EVs derived from

unstimulated DCs. Conversely, naïve T cells exposed to EVs derived from LPS stimulated DCs had the highest percentage of CD69+ naïve T cells at 67%, leading to a statistically significant difference between no EVs and EVs derived from unstimulated DCs (fig.6.21Biii). Overall, CD69 was highly expressed in naïve T cells during the 1st exposure to DC-EVs across all conditions in both healthy and allergic individuals. Naïve T cells in healthy individuals still highly expressed CD69 during the 2nd dose of DC-EVs, while in allergic individuals, when no EVs or EVs from unstimulated DCs were added, naïve T cells had a decreased percentage of CD69+ T cells, which further decreased by the 3rd dose, resulting in a statistically significant difference compared to naïve T cells exposed to EVs derived from LPS-stimulated DCs, suggesting that naïve T cells from allergic individuals required additional stimulation of DC-EVs derived from stimulated DCs to remain activated.

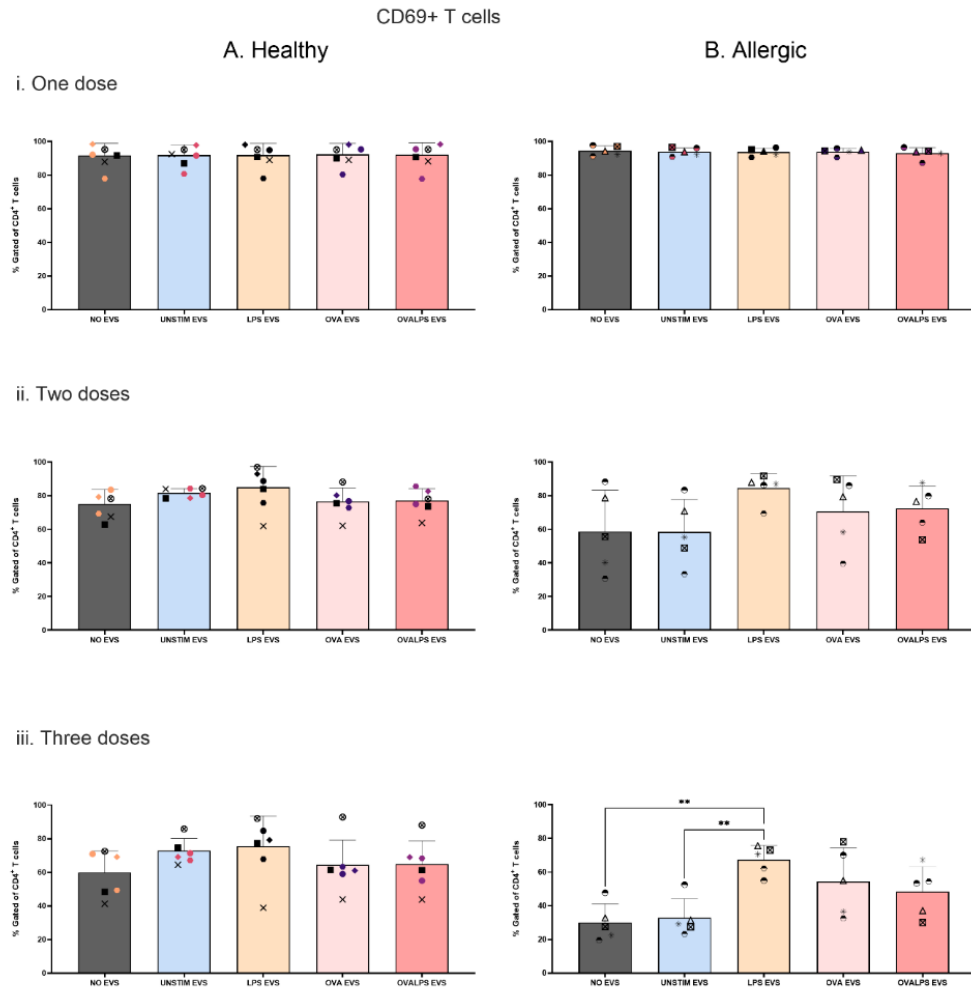


Figure 6.21 - T cell expression of CD69 in healthy and allergic participants. Naïve T cells were plated in 96-well plate with co-stimulation of coated $2\mu\text{g/ml}$ CD3 and direct $2\mu\text{g/ml}$ CD28. Each well afterwards was stimulated with dendritic cell-derived EVs added in 3 doses. DCs were stimulated with 100ng/ml of LPS, $10\mu\text{g/ml}$ of OVA, 50ng/ml of LPS and $10\mu\text{g/ml}$ of OVA, and unstimulated, PBS was added as control. T cells were stimulated every 24h for 3 times (i., ii., iii.). Cytokine expression was measured after 42h, following 30h stimulation of EVs and 12h incubation with PTI. Study included 7 healthy and 5 allergic participants. Statistical analysis was performed in GraphPad Prism using one-way ANOVA, $p < 0.05$.

6.3.5.3 Effect of co-stimulation with DC-EVs and CD3/CD28 on naïve T cell IFN- γ production in healthy and allergic individuals across conditions

Subsequently, IFN- γ production was assessed in naïve T cells following co-stimulation with CD3/CD28 and DC-EV exposure, as previously noted (fig.6.22). After the first dose, IFN- γ + naïve T cells from healthy individuals showed similar levels across all conditions (fig. 6.22Bi), while the second dose increased IFN- γ + T cells to 48-58% in healthy individuals across all conditions, except for those exposed to EVs from LPS-stimulated DCs, which exhibited a significant decrease compared to the no-EV control (fig. 6.22Aii). Similarly, naïve T cells from allergic individuals maintained 16-25% IFN- γ +

levels after the first dose across conditions, with a notable decrease in those exposed to LPS-stimulated DC-EVs compared to the control (fig. 6.22Bii). After the third dose, IFN- γ expression in healthy individuals remained consistent across conditions (fig. 6.22Aiii), and in allergic individuals, it showed no significant variation (fig. 6.22Biii). Overall, while IFN- γ levels were comparable within groups after the first dose, the second dose revealed a significant reduction in IFN- γ + naïve T cells exposed to LPS-stimulated DC-EVs in both healthy and allergic individuals compared to controls, a trend that persisted without statistical significance after the third dose, suggesting that LPS-stimulated DC-EVs may interfere with IFN- γ production.

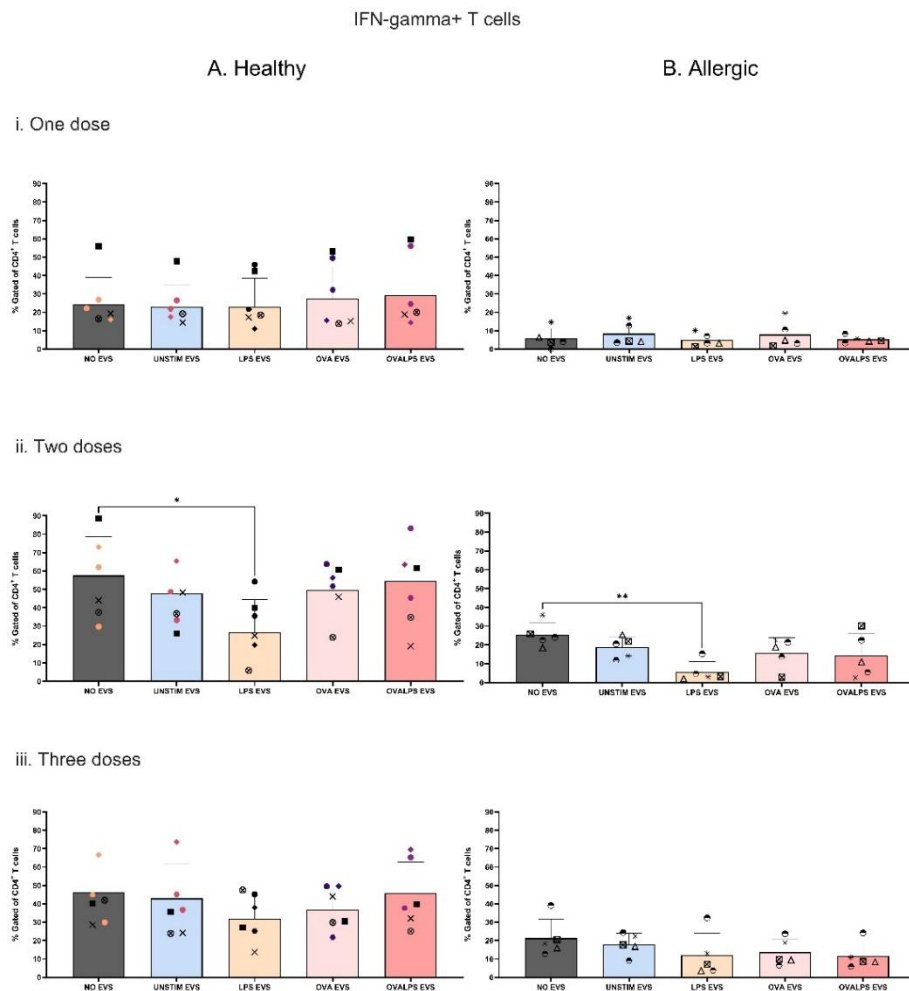


Figure 6.22 - T cell expression of IFN- γ in healthy and allergic participants. Naïve T cells were plated in 96-well plate with co-stimulation of coated $2\mu\text{g/ml}$ CD3 and direct $2\mu\text{g/ml}$ CD28. Each well afterwards was stimulated with dendritic cell-derived EVs added in 3 doses. DCs were stimulated with 100ng/ml of LPS, $10\mu\text{g/ml}$ of OVA, 50ng/ml of LPS and $10\mu\text{g/ml}$ of OVA, and unstimulated, PBS was added as control. T cells were stimulated every 24h for 3 times (i., ii., iii.). Cytokine expression was measured after 42h, following 30h stimulation of EVs and 12h incubation with PTI. Study included 7 healthy and 5 allergic participants. Statistical analysis was performed in GraphPad Prism using one-way ANOVA, $p < 0.05$.

6.3.5.4 Effect of co-stimulation with DC-EVs and CD3/CD28 on naïve T cell TNF- α production in healthy and allergic individuals across conditions

Next, TNF- α production was investigated after co-stimulation, as previously stated (fig.6.23). After the 1st dose of DC-EVs naïve T cells from healthy individuals had similar levels of TNF- α expression across conditions (fig.6.23Ai). Allergic individuals' naïve T cells also showed similar levels of expression of TNF- α (fig.6.23Bi). Following the 2nd dose of DC-EVs the naïve T cells from healthy individuals followed the same trend, with no effect across the conditions (fig.6.23Aii), as did the naïve T cells from allergic individuals (fig.6.23Bii). After the 3rd dose of DC-EVs naïve T cells from healthy individuals had minimal changes between the conditions (fig.6.23Aiii). Naïve T cells from allergic individuals also had little differences between the conditions (fig.6.23Biii). Overall, naïve T cells exposed to EVs from differently stimulated EVs had little effect on the percentage of TNF- α ⁺ T cells in both the healthy and allergic individuals.

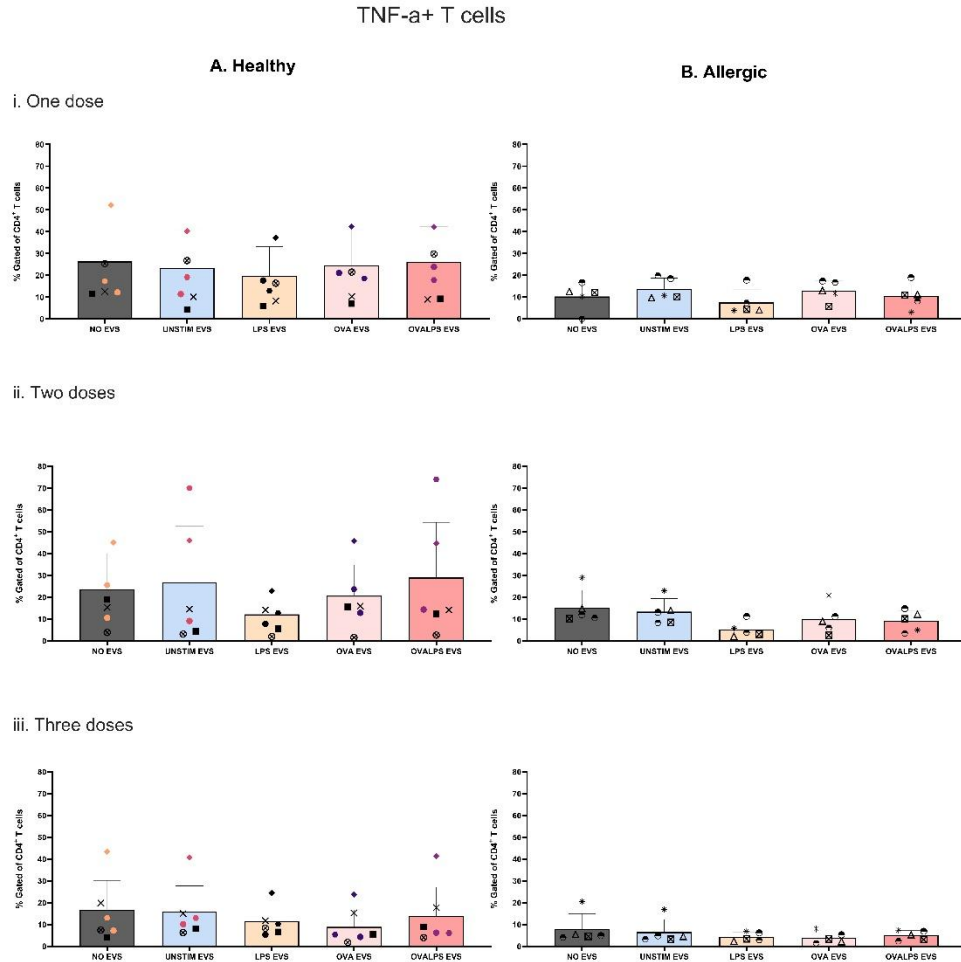


Figure 6.23 - T cell expression of TNF- α in healthy and allergic participants. Naïve T cells were plated in 96-well plate with co-stimulation of coated 2 μ g/ml CD3 and direct 2 μ g/ml CD28. Each well afterwards was stimulated with dendritic cell-derived EVs added in 3 doses. DCs were stimulated with 100ng/ml of LPS, 10 μ g/ml of OVA, 50ng/ml of LPS and 10 μ g/ml of OVA, and unstimulated, PBS was added as control. T cells were stimulated every 24h for 3 times (i., ii., iii.). Cytokine expression was measured after 42h, following 30h stimulation of EVs and 12h incubation with PTI. Study included 7 healthy and 5 allergic participants. Statistical analysis was performed in GraphPad Prism using one-way ANOVA, $p < 0.05$.

6.3.5.5 Effect of co-stimulation with DC-EVs and CD3/CD28 on naïve T cell IL-2 production in healthy and allergic individuals across conditions

IL-2 production was also examined following co-stimulation (fig.6.24). After all doses of DC-EVs, naïve T cells from healthy individuals displayed minimal changes in IL-2 production across the conditions (fig.6.24Ai, ii, iii). The same was also observed in allergic individuals, with no effect across different conditions at all time points (fig.6.24Bi, I, iii). This indicates that DC-EVs from different conditions have minimal influence on IL-2 production in naïve T cells in either healthy or allergic individuals.

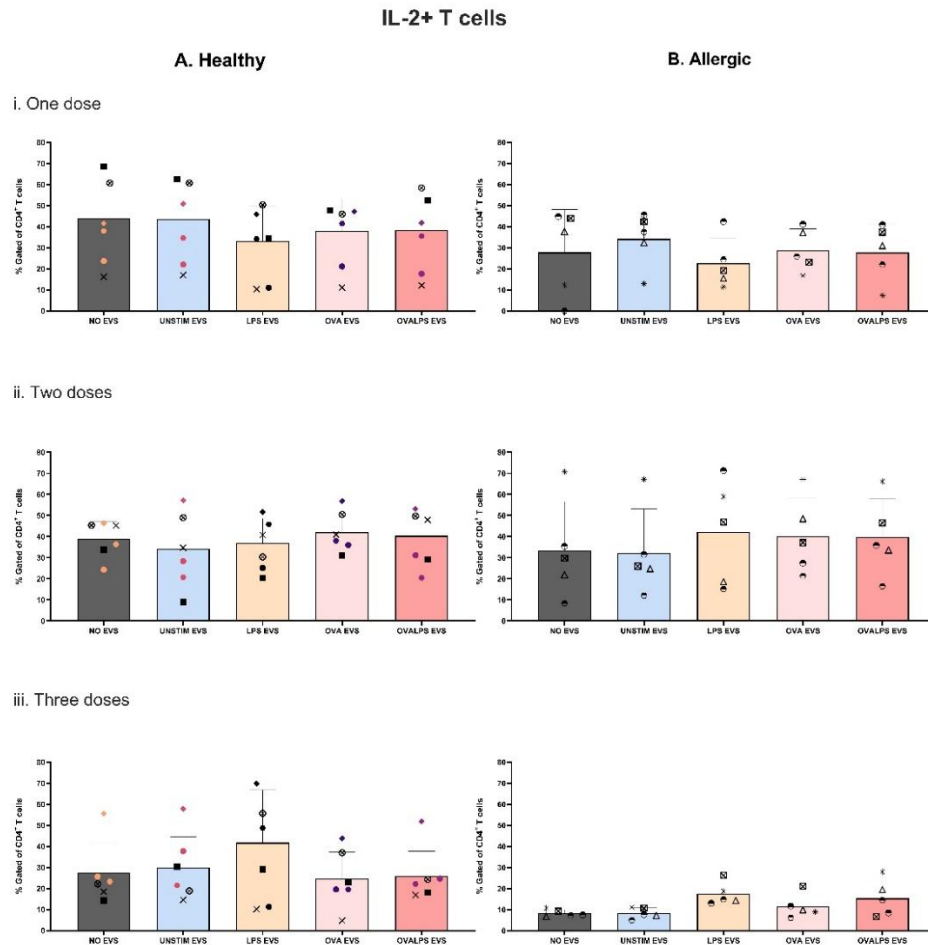


Figure 6.24 - T cell expression of IL-2 in healthy and allergic participants. Naïve T cells were plated in 96-well plate with co-stimulation of coated 2 μ g/ml CD3 and direct 2 μ g/ml CD28. Each well afterwards was stimulated with dendritic cell-derived EVs added in 3 doses. DCs were stimulated with 100ng/ml of LPS, 10 μ g/ml of OVA, 50ng/ml of LPS and 10 μ g/ml of OVA, and unstimulated, PBS was added as control. T cells were stimulated every 24h for 3 times (i., ii., iii.). Cytokine expression was measured after 42h, following 30h stimulation of EVs and 12h incubation with PTI. Study included 7 healthy and 5 allergic participants. Statistical analysis was performed in GraphPad Prism using one-way ANOVA, $p < 0.05$.

6.3.5.6 Effect of co-stimulation with DC-EVs and CD3/CD28 on naïve T cell IL-4 production in healthy and allergic individuals across conditions

Next IL-4 production was explored in naïve T cells following co-stimulation (fig.6.25). After the 1st exposure to DC-EVs the naïve T cells had similar levels of IL-4 expression across the conditions in both healthy and allergic individuals (fig.6.25ABi), and the same was observed following the 2nd exposure to DC-EVs in healthy individuals (fig.6.25Aii) and allergic individuals (fig.6.25Bii). After the 3rd dose of DC-EVs, naïve T cells from healthy individuals had no differences between the conditions (fig.6.25Aiii). However, naïve T cells from allergic individuals had a significantly higher percentage of IL-4+ T cells when exposed to EVs derived from LPS and

OVA/LPS stimulated DCs compared to when no EVs were added (fig.6.25Biii). Overall, naïve T cells from healthy individuals had little differences between the conditions and control groups on the percentage of IL-4⁺ T cells across all doses, however, in allergic individuals naïve T cells had an increased percentage of IL-4⁺ T cells in naïve T cells exposed to EVs derived from LPS, OVA and OVA/LPS stimulated DCs following the 2nd dose, and after the 3rd dose naïve T cells exposed to EVs derived from LPS and OVA/LPS stimulated DCs this difference became significant compared to when no EVs were added. This could potentially suggest that DC-EVs derived from LPS and OVA/LPS stimulated DCs play a role in IL-4 production in allergic individuals.

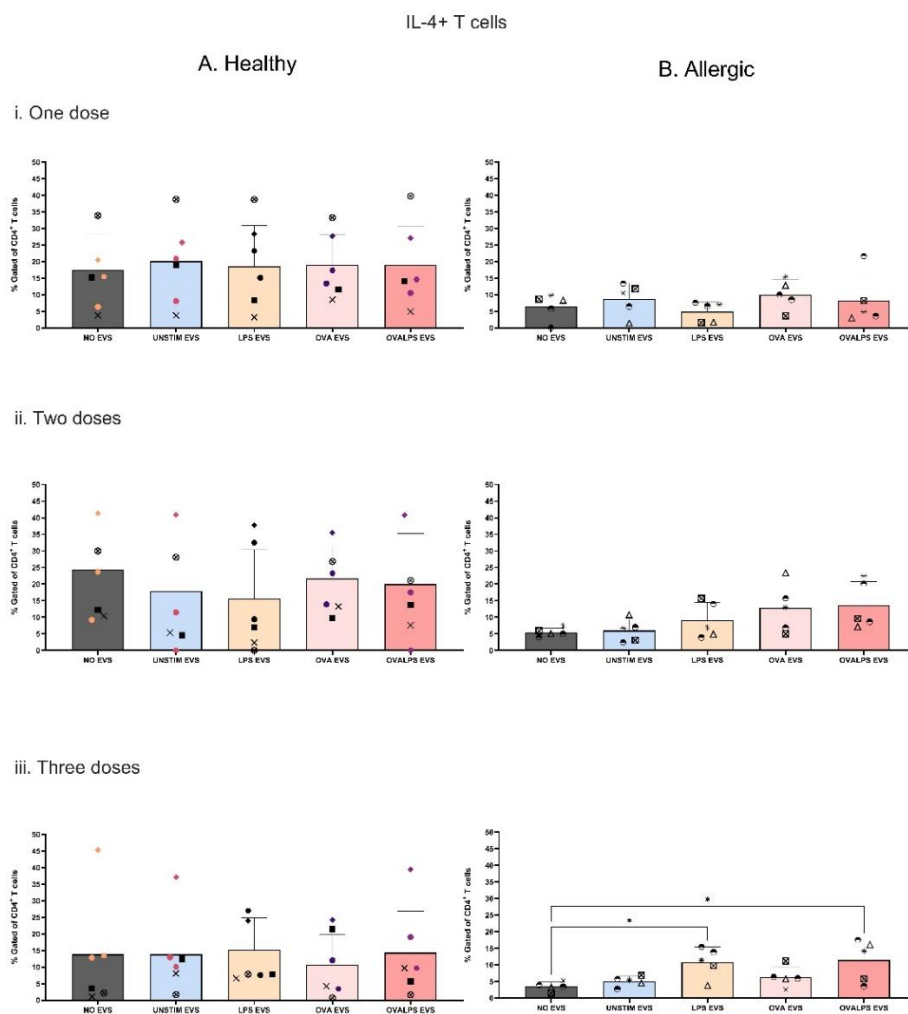


Figure 6.25 - T cell expression of IL-4 in healthy and allergic participants. Naïve T cells were plated in 96-well plate with co-stimulation of coated 2 μ g/ml CD3 and direct 2 μ g/ml CD28. Each well afterwards was stimulated with dendritic cell-derived EVs added in 3 doses. DCs were stimulated with 100ng/ml of LPS, 10 μ g/ml of OVA, 50ng/ml of LPS and 10 μ g/ml of OVA, and unstimulated, PBS was added as control. T cells were stimulated every 24h for 3 times (i., ii., iii.). Cytokine expression was measured after 42h, following 30h stimulation of EVs and 12h incubation with PTI. Study included 7 healthy and 5 allergic participants. Statistical analysis was performed in GraphPad Prism using one-way ANOVA, $p < 0.05$.

6.4 Discussion

Naïve T cells play a critical role in allergic sensitization through their polarisation towards Th2 cells, however, what influences the shift towards Th2 cells in allergy is still unknown. In this research, it was hypothesised that EVs from DCs could drive naïve T cells to polarise towards Th2 cells in allergic individuals. To test this hypothesis, an experimental in vitro assay was developed to investigate the role of EVs in allergic sensitisation. Firstly, the DCs were cultured in the presence of LPS, OVA, OVA/LPS and unstimulated to produce EVs, which were then harvested through SEC. Secondly, naïve T cells were cultured with these EVs in a dose-dependent manner by themselves, or with additional co-stimulation with anti-CD3/anti-CD28 antibodies, to investigate if EVs can act as the only signal or as signal 3 for naïve T cells. Finally, cytokine production was measured to indicate if EVs derived from the DCs could influence allergic sensitisation in naïve T cells. Furthermore, blood from both healthy and allergic individuals was obtained to compare any differences in disease states.

Exposing naïve T cells to DC-EVs alone was undertaken to investigate the potential of DC-EVs to act as the only signal to influence the behaviour of naïve T cells, however by analysing the data, naïve T cells from both healthy and allergic individuals showed minimal effects. Specifically, naïve T cell viability was unaffected by exposure to DC-EVs, regardless of the stimulation. By analysing CD69 expression on naïve T cells exposed to DC-EVs it was possible to monitor activation levels following DC-EVs exposure, however no statistically significant differences were observed. These findings were consistent with previously published data, which found no direct toxicity of DC-EVs towards T cells ([344]). Furthermore, cytokine production of naïve T cells exposed to DC-EVs was minimally affected, as there was little to no change in IFN- γ , IL-2, IL-4 and TNF- α expression in naïve T cells in both healthy and allergic individuals, regardless of stimulation of DCs. Interestingly, when comparing the percentage of IL-2⁺ T cells between healthy and allergic groups after a single exposure of EVs derived from OVA/LPS stimulated DCs, there was a statistically significant difference, where IL-2 was more highly expressed in naïve T cells in the healthy group. This finding could indicate that

naïve T cells from healthy individuals are more likely to respond to external stimuli, as IL-2 production depends on TCR signalling, which is crucial for T cell expansion and differentiation into effector and memory cells ([345]. Interestingly, this was not the case in naïve T cells in the allergic group, which could be explained by the Th2 bias already present in the allergic individuals, characterized by elevated production of IL-4, IL-5 and IL-13, where the usual Th1/Th2 balance is disrupted leading to the diminished IL-2 response upon stimulation when compared to healthy individuals ([346]). Overall, DC-EVs by themselves could not fully activate naïve T cells, either in healthy or allergic groups and thus did not influence polarisation towards a specific phenotype.

After exploring the hypothesis that DC-EVs can act as the only signal for naïve T cells, it was hypothesised that DC-EVs could instead act as signal 3, where signal 1 and signal 2 was provided with the addition of anti-CD3 and anti-CD28 co-stimulation. The data showed statistically significant differences between healthy and allergic groups in CD69, IFN- γ and IL-2 expression, where naïve T cells in the healthy group had higher percentage of these cells in the control groups of no EVs and EVs derived from unstimulated DCs. This indicates that naïve T cells from healthy and allergic groups had inherent differences, where within the healthy group naïve T cells would polarise towards a Th1 phenotype in producing higher amounts of IFN- γ and IL-2. This was further explored by clustering analysis between healthy and allergic individuals following the 2nd dose of DC-EVs after co-stimulation, where it was found that there were statistically significant clusters following exposure to DC-EVs derived from LPS stimulated DCs and with no EVs, indicating that there are indeed inherent differences between healthy and allergic naïve T cells. This finding correlates with previously published literature where naïve T cells from food allergic children had inherent significant differences in gene expression when compared to healthy children's naïve T cells ([341]). However, these differences were lost depending on the source of DC-EVs, which led to the investigation of the source of DC-EV and how this influences T cell phenotypes within the groups. It was observed that within allergic individuals, naïve T cells following the 3rd dose of DC-EVs derived from LPS-stimulated DCs had significantly increased CD69 expression when compared

to the control group, which indicated that DC-EVs were activating naïve T cells. LPS is a known activator of innate immune cells, such as DCs, which leads to increased EV production and altered composition of EVs, making these EVs more immunogenic ([233]). Interestingly, DC-EVs derived from LPS-stimulated DCs lead to decreased production of IFN- γ when compared to the control group, in both healthy and allergic individuals. IFN- γ is a critical cytokine in immune responses, primarily associated with Th1-mediated immunity, where it promotes Th1 polarisation, favouring cellular immunity over humoral responses. However, as the production was decreased, it allows Th2 cytokines, such as IL-4, to predominate, exacerbating allergic sensitization. In a study done by Rudders et al., in 2010 they found that children with persistent food allergies showed reduced IFN- γ production by T cells compared to those who outgrew allergies [342]. This correlates with the observed finding of increased IL-4 production in allergic individuals once exposed to EVs derived from LPS and OVA/LPS stimulated DCs when compared to the control group of no EVs. IL-4 is a key cytokine and the primary driver of Th2-mediated allergic diseases. IL-4 accomplishes the polarisation towards Th2 by inducing naïve T cell differentiation into Th2 cells by activating STAT6 and upregulating GATA3 ([347]), furthermore IL-4 triggers B cell class-switching to produce IgE, which bind to Fc ϵ RI receptors on mast cells and basophils, leading to degranulation and allergic symptoms upon allergen exposure ([348]).

In summary, this study allowed the exploration of the role of DC-EVs in allergic sensitisation, showing that DC-EVs by themselves are unlikely to influence the immune responses, however, with co-stimulation with anti-CD3 and anti-CD28, DC-EVs led to statistically significant differences between healthy and allergic responses that are inherent. However, most notably, EVs derived from LPS-stimulated DCs led to decreased IFN- γ production in both healthy and allergic individuals, and increased IL-4 and CD69 expression in allergic individuals, indicating that DC-EVs potentially can play a role in allergic sensitisation.

Chapter 7: General Discussion

The rising incidence of IgE-mediated allergic conditions highlights a pressing public health concern, yet the processes determining why certain individuals develop sensitivity to allergens remain incompletely understood. Allergic sensitization, the foundational phase of IgE-mediated allergy progression, is initiated when an individual first encounters an allergen, prompting DC presentation of the allergen to naïve T cells via MHC class II complexes. This interaction fosters Th2 cell differentiation and stimulates B cells to produce allergen-specific IgE ([349]). A range of factors has been suggested to influence this sensitization process, including the structural properties and biological functions of protein allergens ([350] [351] [352]). However, the fact that not all individuals react allergically to known allergens reveals a significant knowledge gap in elucidating the underlying mechanisms of allergic sensitization. In recent years, the role of EVs in allergic sensitization has garnered growing interest due to their critical function as mediators of intercellular communication. EVs, secreted by cells into the extracellular milieu, facilitate the transfer of biological signals and information, thereby modulating the function of recipient cells ([353] [274] [224]). These vesicles are instrumental in cell-to-cell interactions, contributing to both physiological homeostasis and the pathological underpinnings of various disorders, notably allergic conditions such as asthma and rhinitis. Despite this, research specifically exploring the role of EVs in allergic sensitization remains limited. A recent systematic review of studies investigating EV involvement in allergic sensitization revealed that these vesicles can influence immune responses by virtue of their surface proteins and cargo, potentially steering the immune system toward a Th2-dominated state ([354]). Given the increasing recognition of EVs' potential significance in this context, despite the scarcity of data, there is a compelling need to develop a robust, high-throughput human-based model to thoroughly investigate the impact of EVs on naïve T-cell development. The research presented in this thesis introduces a novel human in vitro model system designed to probe the role of DC-derived EVs in the context of naïve T-cell involvement in allergic sensitization.. Egg allergy, a prevalent food allergy among children, affects approximately 1.5% of the population in Western

countries and often persists as a chronic condition ([340] [355]). Its symptoms can be markedly severe compared to allergies to milk or fish, occasionally escalating to life-threatening anaphylaxis, which emphasizes the critical need to decipher the mechanisms driving egg allergy to improve therapeutic strategies and mitigate mortality risks. In this study, DC-derived EVs have emerged as pivotal agents, capable of encapsulating OVA, a primary egg allergen, and delivering it to naïve T cells ([356]). This investigation assessed the influence of OVA-carrying DC-derived EVs on the activation and differentiation of naïve T cells, comparing responses between egg-allergic and non-allergic individuals to detect variations in T-cell phenotypes. The analysis explored whether these OVA-enriched EVs could initiate T-cell activation and direct differentiation toward specific immune profiles, offering valuable insights into their potential role in propelling allergic sensitization. Furthermore, this model provides a platform to examine how EV cargo, including allergens like OVA, may interact with DC surface markers (e.g., MHC class II, CD86) to enhance T-cell priming, a process that could be modulated by the inflammatory milieu present in allergic individuals, thus broadening the understanding of EV-mediated immune modulation in allergy development.

7.1. Differences in T cells between healthy and allergic individuals

The investigation into the hypothesis that DC-EVs might serve as the sole signal for naïve T-cell activation initially yielded limited evidence of significant immunomodulatory effects in isolation. This prompted a refined hypothesis positing that DC-EVs could function as signal 3 in the T-cell activation process, complementing signal 1 (provided by anti-CD3) and signal 2 (provided by anti-CD28) through co-stimulation. Analysis of the data revealed statistically significant differences in the expression of CD69, IFN- γ , and IL-2 between healthy and allergic groups. Notably, naïve T cells from the healthy cohort exhibited higher percentages of these markers in both the control conditions (no EVs) and when exposed to EVs derived from unstimulated DCs, suggesting an intrinsic predisposition toward a Th1 phenotype characterized by elevated IFN- γ and IL-2 production. This observation was further substantiated through clustering analysis following the

second dose of DC-EVs with co-stimulation, which identified statistically significant clusters in response to EVs from LPS-stimulated DCs and the no-EV control. These findings underscore inherent immunological distinctions between healthy and allergic naïve T cells, a pattern consistent with prior research demonstrating significant gene expression differences in naïve T cells from food-allergic children compared to their healthy counterparts ([341]). The loss of these differences depending on the source of DC-EVs introduces a layer of complexity to the interpretation of these results. This variability may reflect the diverse cargo and surface marker profiles of EVs, which are modulated by the stimulatory context (e.g., LPS, OVA), potentially altering their capacity to influence T-cell polarization. The higher Th1-associated cytokine production in healthy individuals could indicate a more balanced immune response, whereas the reduced expression in allergic individuals might suggest a pre-existing Th2 bias, a hallmark of allergic sensitization. The significance of these findings lies in their potential to illuminate the underlying mechanisms of immune dysregulation in allergy. Understanding how DC-EVs, particularly those carrying allergens like OVA, interact with naïve T cells under co-stimulatory conditions could reveal critical checkpoints in the sensitization process. This knowledge is particularly important for developing targeted immunotherapies, where modulating EV cargo or co-stimulation signals might restore immune homeostasis or prevent Th2 dominance. Furthermore, the observed inherent differences highlight the need for personalized approaches in allergy research, as genetic or epigenetic variations in naïve T cells may dictate individual responses to EV-mediated signals. Future studies should explore a broader range of EV sources and stimulation conditions, potentially employing longitudinal analyses to track these dynamic shifts, thereby enhancing the therapeutic and diagnostic potential of EV-targeted interventions in allergic diseases.

7.2. EVs influence on naïve T cell activation in healthy and allergic individuals

The observed variations in naïve T-cell responses, which initially distinguished healthy and allergic individuals, were notably diminished depending on the stimulation of DC-EVs, prompting a deeper exploration into how the source of these EVs influences T-cell phenotypes across the study

groups. This investigation revealed that, within allergic individuals, naïve T cells exposed to the third dose of DC-EVs from LPS-stimulated DCs exhibited a significant upregulation of CD69 expression in allergic patients compared to the control group, suggesting a robust activation of these T cells. This finding aligns with the known role of LPS as a potent activator of innate immune cells, including DCs, which enhances EV production and modifies their compositional profile, rendering them more immunogenic ([233]). Of particular interest, DC-EVs derived from LPS-stimulated DCs were associated with a marked reduction in IFN- γ production relative to the control group, a trend observed across both healthy and allergic participants. IFN- γ , a cornerstone cytokine of Th1-mediated immunity, typically promotes cellular immunity and counters humoral responses; its decreased production may thus facilitate a shift toward Th2 dominance, intensifying allergic sensitization. This immunological shift finds support in the literature, where Rudders et al. reported that children with persistent food allergies exhibited lower IFN- γ levels in T cells compared to those who outgrew their allergies, highlighting a potential link between reduced Th1 activity and sustained allergic states ([342]). Consistent with this, the current study observed elevated IL-4 production in allergic individuals following exposure to DC-EVs from LPS- and OVA/LPS-stimulated DCs, relative to the no-EV control. As a pivotal Th2 cytokine, IL-4 drives allergic disease progression by inducing naïve T-cell differentiation into Th2 cells through the activation of STAT6 and upregulation of GATA3 ([347]). Additionally, IL-4 promotes B-cell class-switching to produce IgE, which binds to Fc ϵ RI receptors on mast cells and basophils, triggering degranulation and eliciting allergic symptoms upon allergen exposure ([348]). The interplay between decreased IFN- γ and increased IL-4 suggests that LPS-stimulated DC-EVs may exacerbate a Th2-biased immune environment, a key feature of allergic sensitization. The implications of these findings are multifaceted and underscore their importance in the broader context of allergy research. The loss of inherent T-cell differences based on EV source indicates that the stimulatory context of DCs—such as LPS or allergen exposure—critically shapes EV immunogenicity, potentially overriding baseline immunological predispositions. This variability could reflect adaptive changes in EV cargo (e.g., OVA, cytokines) or surface markers (e.g., MHC

class II), which warrant further investigation to delineate their specific contributions to T-cell activation and polarization. The observed Th2 shift, driven by IL-4 predominance and IFN- γ suppression, may signify a mechanism through which DC-EVs amplify allergic sensitization, offering a novel target for therapeutic intervention. For instance, strategies to modulate EV composition—such as inhibiting pro-Th2 cargo or enhancing Th1-promoting signals—could mitigate allergic responses. Moreover, these insights highlight the necessity for personalized medicine approaches, as individual variations in T-cell responsiveness to EV signals may influence treatment efficacy. Future research should prioritize longitudinal studies and advanced proteomic analyses to unravel the dynamic interplay between EV sources and T-cell phenotypes, thereby paving the way for innovative diagnostics and therapies aimed at curbing the rising tide of allergic diseases.

7.3. General limitations

A significant challenge influencing this research stems from the limited number of egg-allergic and non-allergic participants, which has undermined the statistical robustness of the findings. This issue is particularly pronounced due to considerable variability in results across individual donors. The primary cause was the prolonged delays in finding and recruiting willing participants, which ultimately led to a smaller participant pool.

Another notable limitation of this study was the inability to analyse the cargo carried by EVs prior to their exposure to naïve T cells, a constraint imposed by limitations in time and funding. The cargo of EVs, which may include proteins, lipids, nucleic acids, or allergens like OVA) is widely recognized as a pivotal determinant in shaping the fate of recipient cells, influencing their activation, differentiation, or immune polarization. Gaining insight into whether the cargo composition varied among DC-derived EVs subjected to different stimulations—such as LPS, OVA, or a combination—would have provided valuable clarity. Such an analysis could have illuminated whether changes in cargo or alterations in surface marker expression (e.g., MHC class II, CD86) were the primary drivers behind the activation and differentiation of naïve T cells. This limitation highlights a critical missed opportunity to dissect the mechanistic role of EV cargo versus surface markers,

which could significantly affect the interpretation of EV-mediated immune modulation. Moving forward, securing additional resources to employ advanced techniques—such as mass spectrometry or RNA sequencing—could enable a thorough cargo profiling, thereby enriching the understanding of how DC-derived EVs contribute to allergic sensitization and guiding the development of targeted therapeutic strategies.

The timing of cytokine measurements is a critical factor in immunological studies, as the kinetics of cytokine production can vary significantly between different cytokines, thereby influencing their detectability at a chosen time point. This variability arises because each cytokine exhibits a unique temporal profile, with some, such as IL-2, peaking early due to rapid induction following T-cell activation, while others, like IL-4 or IL-13, may accumulate more gradually as part of a sustained Th2 response. Selecting a single time point to assess cytokine release, as was done in this study at 36 hours for naïve T cells exposed to DC-derived EVs, imposes a notable limitation. This approach risks missing the dynamic peaks or troughs of cytokine expression, potentially underestimating or overestimating the true immunological response. For instance, if a cytokine's peak occurs before or after the 36-hour mark, its levels might be too low to detect or already declining, leading to an incomplete picture of the EV-induced effects on naïve T-cell activation and differentiation. This constraint highlights a general limitation of the study, as the reliance on a single time point may not fully capture the complex temporal dynamics of cytokine release, which could vary between healthy and allergic individuals. Future investigations would benefit from employing a time-course analysis, incorporating multiple time points (e.g., 12, 24, 48, and 72 hours), to better elucidate the kinetics and ensure a comprehensive assessment of the immunomodulatory role of DC-derived EVs in allergic sensitization.

Another significant limitation of this study is the lack of robust confirmation of egg allergy status among the egg-allergic participants, relying solely on general practitioner (GP) records within a database and a self-reported questionnaire. This approach introduces potential inaccuracies, as GP records may not always reflect current or comprehensively verified allergic

conditions, and questionnaires are susceptible to recall bias or misinterpretation by participants. Without confirmatory diagnostic tests, such as skin prick testing, specific IgE serology, or oral food challenges—the gold standard for allergy confirmation—the true allergic status of these individuals remains uncertain. This ambiguity could skew the observed responses of naïve T cells to OVA-carrying DC-derived EVs, potentially confounding the differentiation between allergic and non-allergic phenotypes. Such a limitation underscores the need for more rigorous diagnostic validation in future studies to ensure the reliability of participant classification and the accuracy of EV-mediated immune response data, thereby strengthening the validity of conclusions regarding allergic sensitization.

7.4. Applications

As the prevalence of allergic diseases continues to rise, there is a growing need for innovative treatment options. Current approaches to managing food allergies, such as desensitization therapy, are widely used but often trigger allergic reactions during the sensitization phase. A deeper understanding of the mechanisms driving allergic sensitization could pave the way for novel therapeutic strategies and preventive measures against type 1 hypersensitivity. This research has demonstrated that EVs play a significant role in allergic sensitization, evidenced by their activation and increased production of IL-4 and decreased IFN- γ production in individuals with egg allergy. These findings enhance our comprehension of allergic sensitization and suggest that future investigations could explore EVs as promising therapeutic targets. The findings from this study, demonstrating that OVA-carrying EVs increased IL-4 production and decreased IFN- γ production in egg-allergic individuals, provide a compelling foundation for further exploration. The elevation of IL-4, a hallmark Th2 cytokine, suggests that OVA-EVs may drive a pro-allergic immune shift by promoting naïve T-cell differentiation toward a Th2 phenotype, while the reduction in IFN- γ , a Th1-associated cytokine, may suppress counter-regulatory responses, exacerbating allergic sensitization. This dual effect could inform the design of targeted interventions, such as EV-based immunotherapies that neutralize OVA cargo or modulate cytokine profiles to restore Th1/Th2 balance. Furthermore, these insights could guide the

development of diagnostic tools, where EV cargo analysis might predict individual susceptibility to egg allergy, enabling personalized prevention strategies.

7.5. Concluding remarks

This PhD thesis has provided a comprehensive exploration into the immunomodulatory roles of DC-EVs in allergic sensitization, culminating in the development of a novel human *in vitro* assay designed to elucidate these mechanisms. The assay was meticulously crafted to investigate the influence of EVs on allergic sensitization, involving the stimulation of DCs to generate EVs, followed by their exposure to naïve T cells across three distinct doses. This approach enabled the detailed phenotyping of T-cell responses and the monitoring of cytokine production, offering valuable insights into the dynamic interplay between EVs and immune cell activation. The investigation initially hypothesized that DC-EVs might act as the sole signal for naïve T-cell activation, but subsequent analysis refined this perspective, suggesting that DC-EVs may serve as signal 3 when complemented by anti-CD3 and anti-CD28 co-stimulation. This was evidenced by significant differences in CD69, IFN- γ , and IL-2 expression between healthy and allergic groups, with healthy individuals displaying a Th1-biased profile, while allergic individuals showed a tendency toward Th2 dominance, particularly with LPS-stimulated DC-EVs. Further analysis revealed that the source of DC-EVs plays a critical role in modulating T-cell phenotypes, with EVs from LPS-stimulated DCs inducing heightened CD69 expression in allergic individuals, indicative of T-cell activation. This was accompanied by a notable reduction in IFN- γ production and an increase in IL-4, aligning with a Th2-skewed immune response that exacerbates allergic sensitization. The loss of inherent T-cell differences depending on EV source further underscores the influence of stimulatory context—such as LPS or OVA—on EV cargo and surface markers, which may override baseline immunological predispositions. The implications of this research are profound, enhancing our understanding of how DC-EVs, particularly those carrying allergens like OVA, contribute to the pathogenesis of allergic sensitization. The developed human assay provides a robust platform for future studies, demonstrating the potential of EVs as both

biomarkers and therapeutic targets. The observed Th2 shift, driven by IL-4 predominance and IFN- γ suppression, suggests that modulating EV composition or co-stimulatory signals could offer novel strategies to restore immune balance and mitigate allergic responses. The importance of EVs in allergic sensitization is increasingly evident, as they serve as key mediators of intercellular communication, shaping immune responses and offering a promising avenue for personalized medicine. Continued investigation into the diverse roles of EVs—across varied stimulation conditions, donor populations, and longitudinal frameworks—remains essential to unlock their full therapeutic and diagnostic potential, ultimately advancing the prevention and management of allergic diseases.

References

1. Bendiner, E., *Baron von Pirquet: the aristocrat who discovered and defined allergy*. Hosp Pract (Off Ed), 1981. **16**(10): p. 137, 141, 144 passim.
2. Alexander, H., et al., *The role of bacterial skin infections in atopic dermatitis: expert statement and review from the International Eczema Council Skin Infection Group*. Br J Dermatol, 2020. **182**(6): p. 1331-1342.
3. Novak, N., T. Bieber, and D.Y. Leung, *Immune mechanisms leading to atopic dermatitis*. J Allergy Clin Immunol, 2003. **112**(6 Suppl): p. S128-39.
4. Pawanker R, C.G., Holgate S, Lockey R., *WAO White Book on Allergy 2011-2012*. 2011: World Allergy Organization.
5. Asher, M.I., et al., *Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC Phases One and Three repeat multicountry cross-sectional surveys*. The Lancet, 2006. **368**(9537): p. 733-743.
6. Prescott, S. and K.J. Allen, *Food allergy: riding the second wave of the allergy epidemic*. Pediatr Allergy Immunol, 2011. **22**(2): p. 155-60.
7. Feng, H., et al., *Prevalence of parent-reported food allergy among children in China: A population-based cross-sectional survey*. Frontiers in Immunology, 2022. **Volume 13 - 2022**.
8. Warren, C., et al., *The epidemiology of food allergy in adults*. Ann Allergy Asthma Immunol, 2023. **130**(3): p. 276-287.
9. Gupta, R., et al., *GLOBAL PREVALENCE OF PEDIATRIC AND ADULT IGE-MEDIATED FOOD ALLERGIES: Results: FROM THE ASSESS FA STUDY*. Annals of Allergy, Asthma & Immunology, 2023. **131**(5): p. S7-S8.
10. Turner, P.J., et al., *Time trends in the epidemiology of food allergy in England: an observational analysis of Clinical Practice Research Datalink data*. Lancet Public Health, 2024. **9**(9): p. e664-e673.
11. Gupta, R.S., et al., *Prevalence and Severity of Food Allergies Among US Adults*. JAMA Netw Open, 2019. **2**(1): p. e185630.
12. Sampson, H.A., et al., *Mechanisms of food allergy*. J Allergy Clin Immunol, 2018. **141**(1): p. 11-19.
13. Lambrecht, B.N. and H. Hammad, *The immunology of the allergy epidemic and the hygiene hypothesis*. Nat Immunol, 2017. **18**(10): p. 1076-1083.
14. Platts-Mills, T.A., *The allergy epidemics: 1870-2010*. J Allergy Clin Immunol, 2015. **136**(1): p. 3-13.
15. Cooke, R.A. and A.V. Veer, Jr., *Human Sensitization*. The Journal of Immunology, 1916. **1**(3): p. 201-305.
16. Ober, C. and T.-C. Yao, *The genetics of asthma and allergic disease: a 21st century perspective*. Immunological Reviews, 2011. **242**(1): p. 10-30.
17. Portelli, M.A., E. Hodge, and I. Sayers, *Genetic risk factors for the development of allergic disease identified by genome-wide association*. Clin Exp Allergy, 2015. **45**(1): p. 21-31.
18. Kabesch, M. and J. Tost, *Recent findings in the genetics and epigenetics of asthma and allergy*. Semin Immunopathol, 2020. **42**(1): p. 43-60.
19. Cereta, A.D., et al., *Early Life Microbial Exposure and Immunity Training Effects on Asthma Development and Progression*. Front Med (Lausanne), 2021. **8**: p. 662262.
20. Haahtela, T., et al., *The biodiversity hypothesis and allergic disease: world allergy organization position statement*. World Allergy Organ J, 2013. **6**(1): p. 3.

21. Wang, R., et al., *The interaction of innate immune and adaptive immune system*. MedComm (2020), 2024. **5**(10): p. e714.
22. Yamamoto-Hanada, K., et al., *Cumulative inactivated vaccine exposure and allergy development among children: a birth cohort from Japan*. Environ Health Prev Med, 2020. **25**(1): p. 27.
23. Krauss, S.R., et al., *Aluminium adjuvants versus placebo or no intervention in vaccine randomised clinical trials: a systematic review with meta-analysis and Trial Sequential Analysis*. BMJ Open, 2022. **12**(6): p. e058795.
24. Li, P.H., et al., *Epidemiology, Management, and Treatment Access of Hereditary Angioedema in the Asia Pacific Region: Outcomes From an International Survey*. J Allergy Clin Immunol Pract, 2023. **11**(4): p. 1253-1260.
25. Vercelli, D., *From Amish farm dust to bacterial lysates: The long and winding road to protection from allergic disease*. Seminars in Immunology, 2023. **68**: p. 101779.
26. Wang, W., et al., *Gradient disparities in allergy and the gut microbiome among rural, migrant, and urban populations across China*. World Allergy Organ J, 2025. **18**(1): p. 101018.
27. Food Allergy Commission, S., Anania, C., Cuomo, B., D'Auria, E. ., Decimo, F., Indirli, G. C., Manca, E. ., Mondì, F. ., Pendezza, E. ., Sartorio, M. U. A. and Calvani, M. , *ntestinal barrier dysfunction and food allergy*. Italian Journal of Pediatric Allergy and Immunology, 38(2). doi: 10.53151/2531-3916/2024-352., 2024.
28. Maintz, L., et al., *From Skin Barrier Dysfunction to Systemic Impact of Atopic Dermatitis: Implications for a Precision Approach in Dermocosmetics and Medicine*. J Pers Med, 2022. **12**(6).
29. Marenholz, I., et al., *New insights from genetic studies of eczema*. Med Genet, 2023. **35**(1): p. 33-45.
30. Sampson, H.A., *The riddle of response to cutaneous allergen exposure in patients with atopic dermatitis*. Ann Allergy Asthma Immunol, 2024. **133**(3): p. 244-251.
31. Tordesillas, L. and M.C. Berin, *Mechanisms of Oral Tolerance*. Clin Rev Allergy Immunol, 2018. **55**(2): p. 107-117.
32. Ulfman, L., et al., *Relevance of Early Introduction of Cow's Milk Proteins for Prevention of Cow's Milk Allergy*. Nutrients, 2022. **14**(13).
33. Lv, Y., et al., *Associations between diet diversity during infancy and atopic disease in later life: Systematic review*. J Allergy Clin Immunol Glob, 2024. **3**(2): p. 100221.
34. Galand, C., et al., *IL-33 promotes food anaphylaxis in epicutaneously sensitized mice by targeting mast cells*. J Allergy Clin Immunol, 2016. **138**(5): p. 1356-1366.
35. Macchiaverni, P., et al., *Respiratory allergen from house dust mite is present in human milk and primes for allergic sensitization in a mouse model of asthma*. Allergy, 2014. **69**(3): p. 395-8.
36. Hund, S.K., et al., *Scientific developments in understanding food allergy prevention, diagnosis, and treatment*. Frontiers in Immunology, 2025. **Volume 16 - 2025**.
37. Wang, J., et al., *Pathogenesis of allergic diseases and implications for therapeutic interventions*. Signal Transduction and Targeted Therapy, 2023. **8**(1): p. 138.

38. Shokouhi Shoormasti, R., et al., *The Most Common Cow's Milk Allergenic Proteins with Respect to Allergic Symptoms in Iranian Patients*. Iran J Allergy Asthma Immunol, 2016. **15**(2): p. 161-5.
39. Ogulur, I., et al., *Type 2 immunity in allergic diseases*. Cell Mol Immunol, 2025. **22**(3): p. 211-242.
40. Tong, P. and D.R. Wesemann, *Molecular Mechanisms of IgE Class Switch Recombination*. Curr Top Microbiol Immunol, 2015. **388**: p. 21-37.
41. Sutanto, H., *Mechanobiology of Type 1 hypersensitivity: Elucidating the impacts of mechanical forces in allergic reactions*. Mechanobiology in Medicine, 2024. **2**(1): p. 100041.
42. Berin, M.C. and H.A. Sampson, *Mucosal immunology of food allergy*. Curr Biol, 2013. **23**(9): p. R389-400.
43. Ouerdani, Y., et al., *Molecular allergen sensitization profile and casein threshold determination predicting the persistence of cow's milk protein allergy in Tunisia (North Africa)*. Frontiers in Allergy, 2025. **Volume 6 - 2025**.
44. Gilles, S., et al., *The role of environmental factors in allergy: A critical reappraisal*. Experimental Dermatology, 2018. **27**.
45. Abbas M, G.J., *Type I Hypersensitivity Reaction*. 2025: StatPearls Publishing.
46. Espinosa-Riquer, Z.P., et al., *Signal Transduction Pathways Activated by Innate Immunity in Mast Cells: Translating Sensing of Changes into Specific Responses*. Cells, 2020. **9**(11).
47. Charles, N. and U. Blank, *IgE-Mediated Activation of Mast Cells and Basophils in Health and Disease*. Immunological Reviews, 2025. **331**(1): p. e70024.
48. Jo-Watanabe, A., T. Okuno, and T. Yokomizo, *The Role of Leukotrienes as Potential Therapeutic Targets in Allergic Disorders*. International Journal of Molecular Sciences, 2019. **20**(14): p. 3580.
49. Olivarria, G. and T.E. Lane, *Evaluating the role of chemokines and chemokine receptors involved in coronavirus infection*. Expert Rev Clin Immunol, 2022. **18**(1): p. 57-66.
50. Chigbu, D.I., et al., *Cytokines in Allergic Conjunctivitis: Unraveling Their Pathophysiological Roles*. Life (Basel), 2024. **14**(3).
51. Zielen, S., et al., *The Late Asthmatic Reaction Is in Part Independent from the Early Asthmatic Reactions*. International Journal of Molecular Sciences, 2025. **26**(5): p. 2088.
52. Domingo, C., et al., *The Direct and Indirect Role of IgE on Airway Epithelium in Asthma*. Allergy, 2025. **80**(4): p. 919-931.
53. Eggel, A., L.F. Pennington, and T.S. Jardetzky, *Therapeutic monoclonal antibodies in allergy: Targeting IgE, cytokine, and alarmin pathways*. Immunological Reviews, 2024. **328**(1): p. 387-411.
54. Pecoraro, L., et al., *Nutritional and Psychosocial Impact of Food Allergy in Pediatric Age*. Life (Basel), 2024. **14**(6).
55. Garcia-Carmona, Y. and M.A. Curotto de Lafaille, *Advances in Food Allergy Immunotherapy: Current Strategies and Role of Antibodies Isotypes*. Cells, 2025. **14**(12).
56. Mori, F., et al., *Oral Immunotherapy for Food-Allergic Children: A Pro-Con Debate*. Front Immunol, 2021. **12**: p. 636612.
57. Kucuksezer, U.C., et al., *Mechanisms of allergen-specific immunotherapy and allergen tolerance*. Allergology International, 2020. **69**(4): p. 549-560.
58. Matsuda, M., et al., *Roles of type 1 regulatory T (Tr1) cells in allergen-specific immunotherapy*. Frontiers in Allergy, 2022. **Volume 3 - 2022**.

59. Sato, S., et al., *Current perspective on allergen immunotherapy for food allergies*. Allergology International, 2024. **73**(4): p. 501-514.
60. Domingo, C., et al., *The Incredible Adventure of Omalizumab*. International Journal of Molecular Sciences, 2024. **25**(5): p. 3056.
61. Corren, J., et al., *Lebrikizumab in Uncontrolled Asthma: Reanalysis in a Well-Defined Type 2 Population*. The Journal of Allergy and Clinical Immunology: In Practice, 2024. **12**(5): p. 1215-1224.e3.
62. Chen, Y.L., et al., *Proof-of-concept clinical trial of etokimab shows a key role for IL-33 in atopic dermatitis pathogenesis*. Sci Transl Med, 2019. **11**(515).
63. Harb, H. and T.A. Chatila, *Mechanisms of Dupilumab*. Clin Exp Allergy, 2020. **50**(1): p. 5-14.
64. Antosz, K., et al., *Insight into IL-5 as a Potential Target for the Treatment of Allergic Diseases*. Biomedicines, 2024. **12**(7).
65. Manti, S., et al., *Monoclonal Antibodies in Treating Food Allergy: A New Therapeutic Horizon*. Nutrients, 2021. **13**(7).
66. Liang TZ, C.J., *Inhaled Corticosteroids*. 2023, Treasure Island (FL): StatPearls Publishing.
67. Linton, S., L. Hossenbaccus, and A.K. Ellis, *Evidence-based use of antihistamines for treatment of allergic conditions*. Annals of Allergy, Asthma & Immunology, 2023. **131**(4): p. 412-420.
68. Olivieri, B., et al., *The combination of allergen immunotherapy and biologics for inhalant allergies: Exploring the synergy*. Annals of Allergy, Asthma & Immunology, 2025. **134**(4): p. 385-395.
69. Lechuga, S., et al., *Regulation of Epithelial and Endothelial Barriers by Molecular Chaperones*. Cells, 2024. **13**(5).
70. Chen, R., et al., *Pattern recognition receptors: function, regulation and therapeutic potential*. Signal Transduct Target Ther, 2025. **10**(1): p. 216.
71. Sun, N., et al., *The epithelial barrier theory and its associated diseases*. Allergy, 2024. **79**(12): p. 3192-3237.
72. Plewa, P., et al., *The Role of Alarmins in the Pathogenesis of Asthma*. Biomolecules, 2025. **15**(7): p. 996.
73. Willart, M. and H. Hammad, *Lung dendritic cell-epithelial cell crosstalk in Th2 responses to allergens*. Curr Opin Immunol, 2011. **23**(6): p. 772-7.
74. Çelebi Sözüner, Z., et al., *Global warming and implications for epithelial barrier disruption and respiratory and dermatologic allergic diseases*. Journal of Allergy and Clinical Immunology, 2023. **152**(5): p. 1033-1046.
75. Ouyang, C., et al., *Dendritic cell immunometabolism - a potential therapeutic target for allergic diseases*. Int J Med Sci, 2025. **22**(2): p. 417-431.
76. Liu, J., et al., *Dendritic cell migration in inflammation and immunity*. Cellular & Molecular Immunology, 2021. **18**(11): p. 2461-2471.
77. León, B. and F.E. Lund, *Compartmentalization of dendritic cell and T-cell interactions in the lymph node: Anatomy of T-cell fate decisions*. Immunol Rev, 2019. **289**(1): p. 84-100.
78. Worbs, T., S.I. Hammerschmidt, and R. Förster, *Dendritic cell migration in health and disease*. Nat Rev Immunol, 2017. **17**(1): p. 30-48.
79. Angelina, A., et al., *C-Type Lectin Receptor Mediated Modulation of T2 Immune Responses to Allergens*. Curr Allergy Asthma Rep, 2023. **23**(3): p. 141-151.
80. Magee, C.N., O. Boenisch, and N. Najafian, *The role of costimulatory molecules in directing the functional differentiation of alloreactive T helper cells*. Am J Transplant, 2012. **12**(10): p. 2588-600.

81. Onoé, K., et al., *Th1 or Th2 balance regulated by interaction between dendritic cells and NKT cells*. Immunol Res, 2007. **38**(1-3): p. 319-32.
82. Reschner, A., et al., *Innate lymphocyte and dendritic cell cross-talk: a key factor in the regulation of the immune response*. Clin Exp Immunol, 2008. **152**(2): p. 219-26.
83. Abbaszadeh, M., et al., *Overview of dendritic cells subsets and their involvement in immune-related pathological disease*. Bioimpacts, 2025. **15**: p. 30671.
84. Xu, J., et al., *Batf3-dependent orchestration of the robust Th1 responses and fungal control during cryptococcal infection, the role of cDC1*. mBio, 2024. **15**(3): p. e0285323.
85. Lamiable, O., et al., *Dendritic cells in Th2 immune responses and allergic sensitization*. Immunology & Cell Biology, 2020. **98**(10): p. 807-818.
86. van Ree, R., et al., *Allergic sensitization: host-immune factors*. Clin Transl Allergy, 2014. **4**(1): p. 12.
87. Ubags, N.D., et al., *Microbiome-induced antigen-presenting cell recruitment coordinates skin and lung allergic inflammation*. Journal of Allergy and Clinical Immunology, 2021. **147**(3): p. 1049-1062.e7.
88. Kim, R., et al., *Potential functional role of plasmacytoid dendritic cells in cancer immunity*. Immunology, 2007. **121**(2): p. 149-57.
89. Bencze, D., T. Fekete, and K. Pázmándi, *Type I Interferon Production of Plasmacytoid Dendritic Cells under Control*. Int J Mol Sci, 2021. **22**(8).
90. Oh, N.A., et al., *Plasmacytoid Dendritic Cell-driven Induction of Treg Is Strain Specific and Correlates With Spontaneous Acceptance of Kidney Allografts*. Transplantation, 2020. **104**(1): p. 39-53.
91. Uto, T., et al., *Critical role of plasmacytoid dendritic cells in induction of oral tolerance*. J Allergy Clin Immunol, 2018. **141**(6): p. 2156-2167.e9.
92. Swiecki, M. and M. Colonna, *The multifaceted biology of plasmacytoid dendritic cells*. Nat Rev Immunol, 2015. **15**(8): p. 471-85.
93. Coutant, F., J.J. Pin, and P. Miossec, *Extensive Phenotype of Human Inflammatory Monocyte-Derived Dendritic Cells*. Cells, 2021. **10**(7).
94. Plantinga, M., et al., *Conventional and monocyte-derived CD11b(+) dendritic cells initiate and maintain T helper 2 cell-mediated immunity to house dust mite allergen*. Immunity, 2013. **38**(2): p. 322-35.
95. Ashjaei, K., et al., *Atopic donor status does not influence the uptake of the major grass pollen allergen, Phl p 5, by dendritic cells*. Journal of Immunological Methods, 2015. **424**: p. 120-130.
96. Lian, J. and A.D. Luster, *Chemokine-guided cell positioning in the lymph node orchestrates the generation of adaptive immune responses*. Curr Opin Cell Biol, 2015. **36**: p. 1-6.
97. Ashjaei, K., et al., *Differential T-helper cell polarization after allergen-specific stimulation of autologous dendritic cells in polysensitized allergic patients*. Int Arch Allergy Immunol, 2015. **166**(2): p. 97-106.
98. Kinnebrew, Melissa A., et al., *Interleukin 23 Production by Intestinal CD103+CD11b+ Dendritic Cells in Response to Bacterial Flagellin Enhances Mucosal Innate Immune Defense*. Immunity, 2012. **36**(2): p. 276-287.
99. Rimoldi, M., et al., *Monocyte-derived dendritic cells activated by bacteria or by bacteria-stimulated epithelial cells are functionally different*. Blood, 2005. **106**(8): p. 2818-26.
100. Shvets, Y., et al., *Microbiota substances modulate dendritic cells activity: A critical view*. Heliyon, 2024. **10**(5): p. e27125.

101. Pletinckx, K., et al., *Role of dendritic cell maturity/costimulation for generation, homeostasis, and suppressive activity of regulatory T cells*. Front Immunol, 2011. **2**: p. 39.
102. de Greef, P.C., et al., *The TCR assigns naive T cells to a preferred lymph node*. Sci Adv, 2024. **10**(30): p. eadl0796.
103. Zheng, C., Y. Shi, and Y. Zou, *T cell co-stimulatory and co-inhibitory pathways in atopic dermatitis*. Frontiers in Immunology, 2023. **Volume 14 - 2023**.
104. Silva-Filho, J.L., C. Caruso-Neves, and A.A.S. Pinheiro, *IL-4: an important cytokine in determining the fate of T cells*. Biophys Rev, 2014. **6**(1): p. 111-118.
105. Deo, S.S., et al., *Role played by Th2 type cytokines in IgE mediated allergy and asthma*. Lung India, 2010. **27**(2): p. 66-71.
106. Dutta, A., H. Venkataganesh, and P.E. Love, *New Insights into Epigenetic Regulation of T Cell Differentiation*. Cells, 2021. **10**(12).
107. Berghi, N.O., et al., *Relationship between chemokines and T lymphocytes in the context of respiratory allergies (Review)*. Exp Ther Med, 2020. **20**(3): p. 2352-2360.
108. Agache, I., et al., *Immune-mediated disease caused by climate change-associated environmental hazards: mitigation and adaptation*. Frontiers in Science, 2024. **Volume 2 - 2024**.
109. Lamiable, O., et al., *Dendritic cells in Th2 immune responses and allergic sensitization*. Immunol Cell Biol, 2020. **98**(10): p. 807-818.
110. Lee, G.R., *Molecular Mechanisms of T Helper Cell Differentiation and Functional Specialization*. Immune Netw, 2023. **23**(1): p. e4.
111. Shi, Y., H. Zhang, and C. Miao, *Metabolic reprogram and T cell differentiation in inflammation: current evidence and future perspectives*. Cell Death Discov, 2025. **11**(1): p. 123.
112. Athie-Morales, V., et al., *Sustained IL-12 signaling is required for Th1 development*. J Immunol, 2004. **172**(1): p. 61-9.
113. Yoshimoto, T., *The Hunt for the Source of Primary Interleukin-4: How We Discovered That Natural Killer T Cells and Basophils Determine T Helper Type 2 Cell Differentiation In Vivo*. Frontiers in Immunology, 2018. **Volume 9 - 2018**.
114. Gao, Y.-d., et al., *The Evolution, Immunopathogenesis and Biomarkers of Type 2 Inflammation in Common Allergic Disorders*. Allergy, 2025. **80**(7): p. 1848-1877.
115. Pawankar, R., et al., *The paradigm of cytokine networks in allergic airway inflammation*. Current opinion in allergy and clinical immunology, 2015. **15**(1): p. 41-48.
116. Muraille, E., O. Leo, and M. Moser, *TH1/TH2 paradigm extended: macrophage polarization as an unappreciated pathogen-driven escape mechanism?* Front Immunol, 2014. **5**: p. 603.
117. Conrad, M.L., et al., *Regulatory T cells and their role in allergic disease*. Allergy, 2025. **80**(1): p. 77-93.
118. Soyer, O.U., et al., *Mechanisms of peripheral tolerance to allergens*. Allergy, 2013. **68**(2): p. 161-70.
119. Leonard, J.D., et al., *Identification of Natural Regulatory T Cell Epitopes Reveals Convergence on a Dominant Autoantigen*. Immunity, 2017. **47**(1): p. 107-117.e8.

120. Calzada, D., et al., *Immunological Mechanisms in Allergic Diseases and Allergen Tolerance: The Role of Treg Cells*. J Immunol Res, 2018. **2018**: p. 6012053.
121. Bellinghausen, I., R. Khatri, and J. Saloga, *Current Strategies to Modulate Regulatory T Cell Activity in Allergic Inflammation*. Front Immunol, 2022. **13**: p. 912529.
122. Wawrzyniak, M., L. O'Mahony, and M. Akdis, *Role of Regulatory Cells in Oral Tolerance*. Allergy Asthma Immunol Res, 2017. **9**(2): p. 107-115.
123. Sharma, A., G. Sharma, and S.H. Im, *Gut microbiota in regulatory T cell generation and function: mechanisms and health implications*. Gut Microbes, 2025. **17**(1): p. 2516702.
124. Bertolini, T.B., et al., *Role of orally induced regulatory T cells in immunotherapy and tolerance*. Cell Immunol, 2021. **359**: p. 104251.
125. Allen, C.D.C., *Features of B Cell Responses Relevant to Allergic Disease*. J Immunol, 2022. **208**(2): p. 257-266.
126. Lin, Y.-J., A. Goretzki, and S. Schülke, *Immune Metabolism of IL-4-Activated B Cells and Th2 Cells in the Context of Allergic Diseases*. Frontiers in Immunology, 2021. **Volume 12 - 2021**.
127. van de Veen, W., et al., *Role of regulatory B cells in immune tolerance to allergens and beyond*. J Allergy Clin Immunol, 2016. **138**(3): p. 654-665.
128. Sugimoto, M., et al., *Differential response in allergen-specific IgE, IgGs, and IgA levels for predicting outcome of oral immunotherapy*. Pediatr Allergy Immunol, 2016. **27**(3): p. 276-82.
129. Boldison, J., et al., *Dendritic cells license regulatory B cells to produce IL-10 and mediate suppression of antigen-specific CD8 T cells*. Cell Mol Immunol, 2020. **17**(8): p. 843-855.
130. Campbell, E., L.A. Hesser, and C.R. Nagler, *B cells and the microbiota: a missing connection in food allergy*. Mucosal Immunology, 2021. **14**(1): p. 4-13.
131. Min, K.Y., et al., *Entinostat, a histone deacetylase inhibitor, increases the population of IL-10(+) regulatory B cells to suppress contact hypersensitivity*. BMB Rep, 2021. **54**(10): p. 534-539.
132. Artis, D. and H. Spits, *The biology of innate lymphoid cells*. Nature, 2015. **517**(7534): p. 293-301.
133. Halim, T.Y., et al., *Group 2 innate lymphoid cells are critical for the initiation of adaptive T helper 2 cell-mediated allergic lung inflammation*. Immunity, 2014. **40**(3): p. 425-35.
134. Mjösberg, J. and H. Spits, *Human innate lymphoid cells*. J Allergy Clin Immunol, 2016. **138**(5): p. 1265-1276.
135. Barlow, J.L. and A.N. McKenzie, *Type-2 innate lymphoid cells in human allergic disease*. Curr Opin Allergy Clin Immunol, 2014. **14**(5): p. 397-403.
136. Schneider, C., et al., *A Metabolite-Triggered Tuft Cell-ILC2 Circuit Drives Small Intestinal Remodeling*. Cell, 2018. **174**(2): p. 271-284.e14.
137. Teng, F. and M. Fussenegger, *Shedding Light on Extracellular Vesicle Biogenesis and Bioengineering*. Adv Sci (Weinh), 2020. **8**(1): p. 2003505.
138. Giri, K.R., et al., *Molecular and Functional Diversity of Distinct Subpopulations of the Stressed Insulin-Secreting Cell's Vesiculome*. Front Immunol, 2020. **11**: p. 1814.
139. Liu, Y.-J. and C. Wang, *A review of the regulatory mechanisms of extracellular vesicles-mediated intercellular communication*. Cell Communication and Signaling, 2023. **21**(1): p. 77.

140. Yu, L., et al., *Apoptotic bodies: bioactive treasure left behind by the dying cells with robust diagnostic and therapeutic application potentials*. J Nanobiotechnology, 2023. **21**(1): p. 218.
141. Li, S., et al., *Immune cell-derived extracellular vesicles for precision therapy of inflammatory-related diseases*. Journal of Controlled Release, 2024. **368**: p. 533-547.
142. Wu, J., et al., *Interplay between extracellular vesicles and allergic diseases: Current insights and future perspectives*. Interdisciplinary Medicine, 2025. **3**(1): p. e20240049.
143. Buzas, E.I., *The roles of extracellular vesicles in the immune system*. Nature Reviews Immunology, 2023. **23**(4): p. 236-250.
144. Marie, P.P., et al., *Accessory ESCRT-III proteins are conserved and selective regulators of Rab11a-exosome formation*. J Extracell Vesicles, 2023. **12**(3): p. e12311.
145. Gurung, S., et al., *The exosome journey: from biogenesis to uptake and intracellular signalling*. Cell Communication and Signaling, 2021. **19**(1): p. 47.
146. Record, M., et al., *Extracellular vesicles: lipids as key components of their biogenesis and functions*. Journal of Lipid Research, 2018. **59**(8): p. 1316-1324.
147. Jin, H., et al., *Rab GTPases: Central Coordinators of Membrane Trafficking in Cancer*. Front Cell Dev Biol, 2021. **9**: p. 648384.
148. Liu, C., et al., *Identification of the SNARE complex that mediates the fusion of multivesicular bodies with the plasma membrane in exosome secretion*. J Extracell Vesicles, 2023. **12**(9): p. e12356.
149. Wang, Y., et al., *The Regulation of Exosome Generation and Function in Physiological and Pathological Processes*. Int J Mol Sci, 2023. **25**(1).
150. Lee, Y.J., K.J. Shin, and Y.C. Chae, *Regulation of cargo selection in exosome biogenesis and its biomedical applications in cancer*. Exp Mol Med, 2024. **56**(4): p. 877-889.
151. Kang, C., et al., *Role of dendritic cell-derived exosomes in allergic rhinitis (Review)*. Int J Mol Med, 2023. **52**(6): p. 117.
152. Han, Q.-F., et al., *Exosome biogenesis: machinery, regulation, and therapeutic implications in cancer*. Molecular Cancer, 2022. **21**(1): p. 207.
153. Jiang, H., et al., *Hypoxia Induced Changes of Exosome Cargo and Subsequent Biological Effects*. Front Immunol, 2022. **13**: p. 824188.
154. Sun, M., et al., *Ectosome biogenesis and release processes observed by using live-cell dynamic imaging in mammalian glial cells*. Quant Imaging Med Surg, 2021. **11**(11): p. 4604-4616.
155. Clancy, J.W., M. Schmidtman, and C. D'Souza-Schorey, *The ins and outs of microvesicles*. FASEB Bioadv, 2021. **3**(6): p. 399-406.
156. Best, J.T., P. Xu, and T.R. Graham, *Phospholipid flippases in membrane remodeling and transport carrier biogenesis*. Curr Opin Cell Biol, 2019. **59**: p. 8-15.
157. Meldolesi, J., *Exosomes and Ectosomes in Intercellular Communication*. Current Biology, 2018. **28**(8): p. R435-R444.
158. Xu, M., et al., *The biogenesis and secretion of exosomes and multivesicular bodies (MVBs): Intercellular shuttles and implications in human diseases*. Genes & Diseases, 2023. **10**(5): p. 1894-1907.
159. Alam, M.R., M.M. Rahman, and Z. Li, *The link between intracellular calcium signaling and exosomal PD-L1 in cancer progression and immunotherapy*. Genes Dis, 2024. **11**(1): p. 321-334.

160. Jankovičová, J., et al., *Tetraspanins, More than Markers of Extracellular Vesicles in Reproduction*. Int J Mol Sci, 2020. **21**(20).
161. Gurung, S., et al., *The exosome journey: from biogenesis to uptake and intracellular signalling*. Cell Commun Signal, 2021. **19**(1): p. 47.
162. Jin, Y., et al., *Extracellular signals regulate the biogenesis of extracellular vesicles*. Biological Research, 2022. **55**(1): p. 35.
163. Bunn, K.E., B.G. Giese-Byrne, and H.H. Pua, *Th2 cell extracellular vesicles promote eosinophil survival through the cytokine cargo IL-3 and prolong airway eosinophilia*. bioRxiv, 2024: p. 2024.07.23.600647.
164. Yang, J., et al., *A new horizon of precision medicine: combination of the microbiome and extracellular vesicles*. Experimental & Molecular Medicine, 2022. **54**(4): p. 466-482.
165. Mannavola, F., et al., *Extracellular Vesicles and Epigenetic Modifications Are Hallmarks of Melanoma Progression*. Int J Mol Sci, 2019. **21**(1).
166. Herranz, G., et al., *Protein Kinase C δ Regulates the Depletion of Actin at the Immunological Synapse Required for Polarized Exosome Secretion by T Cells*. Frontiers in Immunology, 2019. **Volume 10 - 2019**.
167. Heckman, C.A., et al., *Activated Protein Kinase C (PKC) Is Persistently Trafficked with Epidermal Growth Factor (EGF) Receptor*. Biomolecules, 2020. **10**(9).
168. Hovhannisyan, L., E. Czechowska, and D. Gutowska-Owsiak, *The Role of Non-Immune Cell-Derived Extracellular Vesicles in Allergy*. Front Immunol, 2021. **12**: p. 702381.
169. Umeda, R., et al., *Structural insights into tetraspanin CD9 function*. Nat Commun, 2020. **11**(1): p. 1606.
170. Yang, Y., et al., *Open conformation of tetraspanins shapes interaction partner networks on cell membranes*. The EMBO Journal, 2020. **39**(18): p. e105246.
171. Dunlock, V.E., *Tetraspanin CD53: an overlooked regulator of immune cell function*. Med Microbiol Immunol, 2020. **209**(4): p. 545-552.
172. Tognoli, M.L., et al., *Lack of involvement of CD63 and CD9 tetraspanins in the extracellular vesicle content delivery process*. Communications Biology, 2023. **6**(1): p. 532.
173. Fagúndez, P., et al., *Tetraspanin positivity as a function of extracellular vesicle size measured by a modified immuno-TEM protocol*. bioRxiv, 2025: p. 2025.03.13.643132.
174. Duke, L.C., et al., *Tetraspanin CD9 alters cellular trafficking and endocytosis of tetraspanin CD63, affecting CD63 packaging into small extracellular vesicles*. Journal of Biological Chemistry, 2025. **301**(3): p. 108255.
175. Toribio, V. and M. Yáñez-Mó, *Tetraspanins interweave EV secretion, endosomal network dynamics and cellular metabolism*. European Journal of Cell Biology, 2022. **101**(3): p. 151229.
176. van Niel, G., et al., *The tetraspanin CD63 regulates ESCRT-independent and -dependent endosomal sorting during melanogenesis*. Dev Cell, 2011. **21**(4): p. 708-21.
177. Duke, L.C., et al., *Tetraspanin CD9 alters cellular trafficking and endocytosis of tetraspanin CD63, affecting CD63 packaging into small extracellular vesicles*. J Biol Chem, 2025. **301**(3): p. 108255.
178. Perez-Hernandez, D., et al., *The Intracellular Interactome of Tetraspanin-enriched Microdomains Reveals Their Function as Sorting Machineries toward Exosomes**. Journal of Biological Chemistry, 2013. **288**(17): p. 11649-11661.

179. Padbury, E.H., et al., *TRPC3 signalling contributes to the biogenesis of extracellular vesicles*. Journal of Extracellular Biology, 2024. **3**(1): p. e132.
180. Thuault, S., et al., *A Journey on Extracellular Vesicles for Matrix Metalloproteinases: A Mechanistic Perspective*. Front Cell Dev Biol, 2022. **10**: p. 886381.
181. Mazurov, D., L. Barbashova, and A. Filatov, *Tetraspanin protein CD9 interacts with metalloprotease CD10 and enhances its release via exosomes*. Febs j, 2013. **280**(5): p. 1200-13.
182. Zeng, F. and A.E. Morelli, *Extracellular vesicle-mediated MHC cross-dressing in immune homeostasis, transplantation, infectious diseases, and cancer*. Semin Immunopathol, 2018. **40**(5): p. 477-490.
183. Synowsky, S.A., et al., *The major histocompatibility complex class I immunopeptidome of extracellular vesicles*. J Biol Chem, 2017. **292**(41): p. 17084-17092.
184. Rocha-Perugini, V., et al., *Tetraspanins CD9 and CD151 at the immune synapse support T-cell integrin signaling*. European Journal of Immunology, 2014. **44**(7): p. 1967-1975.
185. Morelli, A.E., et al., *Endocytosis, intracellular sorting, and processing of exosomes by dendritic cells*. Blood, 2004. **104**(10): p. 3257-3266.
186. Buschow, S.I., et al., *MHC II in dendritic cells is targeted to lysosomes or T cell-induced exosomes via distinct multivesicular body pathways*. Traffic, 2009. **10**(10): p. 1528-42.
187. Hallal, S., et al., *Understanding the extracellular vesicle surface for clinical molecular biology*. J Extracell Vesicles, 2022. **11**(10): p. e12260.
188. Jankovicová, J., et al., *Tetraspanins, More than Markers of Extracellular Vesicles in Reproduction*. International Journal of Molecular Sciences, 2020. **21**: p. 7568.
189. Chairoungdua, A., et al., *Exosome release of β -catenin: a novel mechanism that antagonizes Wnt signaling*. J Cell Biol, 2010. **190**(6): p. 1079-91.
190. Toribio Serrano, V. and M. Yáñez-Mó, *Tetraspanins interweave EV secretion, endosomal network dynamics and cellular metabolism*. European Journal of Cell Biology, 2022. **101**: p. 151229.
191. Kumar, M.A., et al., *Extracellular vesicles as tools and targets in therapy for diseases*. Signal Transduction and Targeted Therapy, 2024. **9**(1): p. 27.
192. Vallhov, H., et al., *Dendritic cell-derived exosomes carry the major cat allergen Fel d 1 and induce an allergic immune response*. Allergy, 2015. **70**(12): p. 1651-5.
193. Tinè, M., et al., *Extracellular Vesicles (EVs) as Crucial Mediators of Cell-Cell Interaction in Asthma*. Int J Mol Sci, 2023. **24**(5).
194. Shefler, I., P. Salamon, and Y.A. Mekori, *Extracellular Vesicles as Emerging Players in Intercellular Communication: Relevance in Mast Cell-Mediated Pathophysiology*. Int J Mol Sci, 2021. **22**(17).
195. Fyfe, J., et al., *Role of lipid signalling in extracellular vesicles-mediated cell-to-cell communication*. Cytokine & Growth Factor Reviews, 2023. **73**: p. 20-26.
196. Liu, X., et al., *Roles of Exosomal miRNAs in Asthma: Mechanisms and Applications*. J Asthma Allergy, 2024. **17**: p. 935-947.
197. Peregrino, E.S., et al., *The Role of Bacterial Extracellular Vesicles in the Immune Response to Pathogens, and Therapeutic Opportunities*. Int J Mol Sci, 2024. **25**(11).
198. Nagano, T., et al., *Crucial Role of Extracellular Vesicles in Bronchial Asthma*. Int J Mol Sci, 2019. **20**(10).

199. Zeng, H.-T., et al., *Exosomes carry IL-10 and antigen/MHC II complexes to induce antigen-specific oral tolerance*. Cytokine, 2020. **133**: p. 155176.
200. Eckhardt, C.M., A.A. Baccarelli, and H. Wu, *Environmental Exposures and Extracellular Vesicles: Indicators of Systemic Effects and Human Disease*. Curr Environ Health Rep, 2022. **9**(3): p. 465-476.
201. Demkow, U. and A. Stelmaszczyk-Emmel, *Extracellular Vesicles in Allergic Rhinitis and Asthma and Laboratory Possibilities for Their Assessment*. Int J Mol Sci, 2021. **22**(5).
202. Hazrati, A., et al., *Immune cells-derived exosomes function as a double-edged sword: role in disease progression and their therapeutic applications*. Biomarker Research, 2022. **10**(1): p. 30.
203. Tan, J., et al., *Your Regulatory T Cells Are What You Eat: How Diet and Gut Microbiota Affect Regulatory T Cell Development*. Frontiers in Nutrition, 2022. **Volume 9 - 2022**.
204. Möbs, C. and A.L. Jung, *Extracellular vesicles: Messengers of allergic immune responses and novel therapeutic strategy*. Eur J Immunol, 2024. **54**(5): p. e2350392.
205. Chiang, T.Y., et al., *Microbiome profiling of nasal extracellular vesicles in patients with allergic rhinitis*. World Allergy Organ J, 2022. **15**(8): p. 100674.
206. Berumen Sánchez, G., et al., *Extracellular vesicles: mediators of intercellular communication in tissue injury and disease*. Cell Communication and Signaling, 2021. **19**(1): p. 104.
207. Ghanam, J., et al., *DNA in extracellular vesicles: from evolution to its current application in health and disease*. Cell Biosci, 2022. **12**(1): p. 37.
208. Rädler, J., et al., *Exploiting the biogenesis of extracellular vesicles for bioengineering and therapeutic cargo loading*. Molecular Therapy, 2023. **31**(5): p. 1231-1250.
209. Srisomboon, Y., et al., *Allergen-induced DNA release by the airway epithelium amplifies type 2 immunity*. J Allergy Clin Immunol, 2023. **151**(2): p. 494-508.e6.
210. Gargano, D., et al., *Food Allergy and Intolerance: A Narrative Review on Nutritional Concerns*. Nutrients, 2021. **13**(5).
211. Atarashi, N., M. Morishita, and S. Matsuda, *Activation of innate immune receptor TLR9 by mitochondrial DNA plays essential roles in the chemical long-term depression of hippocampal neurons*. J Biol Chem, 2024. **300**(3): p. 105744.
212. Zhang, Y., et al., *Extracellular vesicles derived from ODN-stimulated macrophages transfer and activate Cdc42 in recipient cells and thereby increase cellular permissiveness to EV uptake*. Sci Adv, 2019. **5**(7): p. eaav1564.
213. Thomas, S.Y., et al., *MyD88-dependent dendritic and epithelial cell crosstalk orchestrates immune responses to allergens*. Mucosal Immunol, 2018. **11**(3): p. 796-810.
214. Park, K.-S., et al., *Targeting Myd88 using peptide-loaded mesenchymal stem cell membrane-derived synthetic vesicles to treat systemic inflammation*. Journal of Nanobiotechnology, 2022. **20**(1): p. 451.
215. Papareddy, P., et al., *The role of extracellular vesicle fusion with target cells in triggering systemic inflammation*. Nature Communications, 2024. **15**(1): p. 1150.

216. Yuan, H., et al., *A model to predict a risk of allergic rhinitis based on mitochondrial DNA copy number*. Eur Arch Otorhinolaryngol, 2022. **279**(10): p. 4997-5008.
217. Joshi, B.S., et al., *Endocytosis of Extracellular Vesicles and Release of Their Cargo from Endosomes*. ACS Nano, 2020. **14**(4): p. 4444-4455.
218. Wu, Q., et al., *Insights into the unique roles of extracellular vesicles for gut health modulation: Mechanisms, challenges, and perspectives*. Current Research in Microbial Sciences, 2024. **7**: p. 100301.
219. Liu, W., et al., *Development of allergic rhinitis immunotherapy using antigen-loaded small extracellular vesicles*. Journal of Controlled Release, 2022. **345**: p. 433-442.
220. Hough, K.P., et al., *Exosomal transfer of mitochondria from airway myeloid-derived regulatory cells to T cells*. Redox Biol, 2018. **18**: p. 54-64.
221. van der Pol, E., et al., *Classification, functions, and clinical relevance of extracellular vesicles*. Pharmacol Rev, 2012. **64**(3): p. 676-705.
222. Stoorvogel, W., et al., *The Biogenesis and Functions of Exosomes*. Traffic, 2002. **3**(5): p. 321-330.
223. Gon, Y., et al., *Selective release of miRNAs via extracellular vesicles is associated with house-dust mite allergen-induced airway inflammation*. Clin Exp Allergy, 2017. **47**(12): p. 1586-1598.
224. Valadi, H., et al., *Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells*. Nat Cell Biol, 2007. **9**(6): p. 654-9.
225. Okoye, I.S., et al., *MicroRNA-containing T-regulatory-cell-derived exosomes suppress pathogenic T helper 1 cells*. Immunity, 2014. **41**(1): p. 89-103.
226. Levänen, B., et al., *Altered microRNA profiles in bronchoalveolar lavage fluid exosomes in asthmatic patients*. J Allergy Clin Immunol, 2013. **131**(3): p. 894-903.
227. Banerjee, S., W.E. Thompson, and I. Chowdhury, *Emerging roles of microRNAs in the regulation of Toll-like receptor (TLR)-signaling*. Front Biosci (Landmark Ed), 2021. **26**(4): p. 771-796.
228. Fabbri, M., et al., *MicroRNAs bind to Toll-like receptors to induce prometastatic inflammatory response*. Proc Natl Acad Sci U S A, 2012. **109**(31): p. E2110-6.
229. Wang, T., et al., *Exosomal lncRNA Nuclear Paraspeckle Assembly Transcript 1 (NEAT1) contributes to the progression of allergic rhinitis via modulating microRNA-511/Nuclear Receptor Subfamily 4 Group A Member 2 (NR4A2) axis*. Bioengineered, 2021. **12**(1): p. 8067-8079.
230. Zhu, T., et al., *Plasma Exosomes from Children with Atopic Dermatitis May Promote Apoptosis of Keratinocytes and Secretion of Inflammatory Factors in vitro*. Clin Cosmet Investig Dermatol, 2022. **15**: p. 1909-1917.
231. Hong, S.W., et al., *Extracellular vesicles derived from Staphylococcus aureus induce atopic dermatitis-like skin inflammation*. Allergy, 2011. **66**(3): p. 351-9.
232. Lässer, C., et al., *Immune-Associated Proteins Are Enriched in Lung Tissue-Derived Extracellular Vesicles during Allergen-Induced Eosinophilic Airway Inflammation*. Int J Mol Sci, 2021. **22**(9).
233. Tkach, M., et al., *Qualitative differences in T-cell activation by dendritic cell-derived extracellular vesicle subtypes*. Embo j, 2017. **36**(20): p. 3012-3028.
234. Fischer, S.F., et al., *Proapoptotic BH3-only protein Bim is essential for developmentally programmed death of germinal center-derived memory B cells and antibody-forming cells*. Blood, 2007. **110**(12): p. 3978-84.

235. Hong, S.W., et al., *An important role of α -hemolysin in extracellular vesicles on the development of atopic dermatitis induced by Staphylococcus aureus*. PLoS One, 2014. **9**(7): p. e100499.
236. Yang, J., et al., *Diagnostic Models for Atopic Dermatitis Based on Serum Microbial Extracellular Vesicle Metagenomic Analysis: A Pilot Study*. Allergy Asthma Immunol Res, 2020. **12**(5): p. 792-805.
237. Lässer, C., et al., *RNA-containing exosomes in human nasal secretions*. Am J Rhinol Allergy, 2011. **25**(2): p. 89-93.
238. Wagner, N., et al., *Plasma-derived extracellular vesicles discriminate type-1 allergy subjects from non-allergic controls*. World Allergy Organization Journal, 2021. **14**(9): p. 100583.
239. Wu, G., et al., *Altered microRNA Expression Profiles of Extracellular Vesicles in Nasal Mucus From Patients With Allergic Rhinitis*. Allergy Asthma Immunol Res, 2015. **7**(5): p. 449-57.
240. Meizhen, Z., et al., *Expression Levels of MicroRNA-125b in Serum Exosomes of Patients with Asthma of Different Severity and its Diagnostic Significance*. Current Drug Metabolism, 2019. **20**(10): p. 781-784.
241. Meizhen, Z., et al., *Expression Level of MiRNA-126 in Serum Exosomes of Allergic Asthma Patients and Lung Tissues of Asthmatic Mice*. Current Drug Metabolism, 2019. **20**(10): p. 799-803.
242. Fang, S.-B., et al., *Small extracellular vesicles derived from human MSCs prevent allergic airway inflammation via immunomodulation on pulmonary macrophages*. Cell Death & Disease, 2020. **11**(6): p. 409.
243. Huanjin, L., et al., *Intranasal Immunotherapy with Allergen-Loaded Extracellular Vesicles from Mast Cells Alleviates Allergic Asthma Inflammation in a Murine Model*. Inflammation, 2025.
244. Zhou, H., et al., *Extracellular Vesicles of Commensal Skin Microbiota Alleviate Cutaneous Inflammation in Atopic Dermatitis Mouse Model by Re-Establishing Skin Homeostasis*. Journal of Investigative Dermatology, 2025. **145**(2): p. 312-322.e9.
245. Asadirad, A., et al., *Sublingual prophylactic administration of OVA-loaded MSC-derived exosomes to prevent allergic sensitization*. International Immunopharmacology, 2023. **120**: p. 110405.
246. Kim, M.H., et al., *Lactobacillus plantarum-derived Extracellular Vesicles Protect Atopic Dermatitis Induced by Staphylococcus aureus-derived Extracellular Vesicles*. Allergy Asthma Immunol Res, 2018. **10**(5): p. 516-532.
247. Kim, J.-H., et al., *Extracellular vesicle-derived protein from *Bifidobacterium longum* alleviates food allergy through mast cell suppression*. Journal of Allergy and Clinical Immunology, 2016. **137**(2): p. 507-516.e8.
248. Ghaemmaghami, A.M., et al., *The proteolytic activity of the major dust mite allergen Der p 1 conditions dendritic cells to produce less interleukin-12: allergen-induced Th2 bias determined at the dendritic cell level*. Clin Exp Allergy, 2002. **32**(10): p. 1468-75.
249. Alkhattabi, N., et al., *Tobacco smoke and nicotine suppress expression of activating signaling molecules in human dendritic cells*. Toxicol Lett, 2018. **299**: p. 40-46.
250. Pawankar, R., *Allergic diseases and asthma: a global public health concern and a call to action*. World Allergy Organ J, 2014. **7**(1): p. 12.
251. Gupta, R., et al., *Burden of allergic disease in the UK: secondary analyses of national databases*. Clinical & Experimental Allergy, 2004. **34**(4): p. 520-526.

252. Rennie, G.H., et al., *Influence of Lifestyle and Dietary Habits on the Prevalence of Food Allergies: A Scoping Review*. *Foods*, 2023. **12**(17): p. 3290.
253. Collin, M. and V. Bigley, *Human dendritic cell subsets: an update*. *Immunology*, 2018. **154**(1): p. 3-20.
254. Hopkins, G.V., et al., *The Role of Lipids in Allergic Sensitization: A Systematic Review*. *Front Mol Biosci*, 2022. **9**: p. 832330.
255. Hopkins, G.V., et al., *Invariant NKT cells are more abundant in peanut-allergic adults and a subset of CD8(+) iNKT cells are depleted after peanut oil exposure*. *Front Immunol*, 2023. **14**: p. 1293158.
256. Keumatio Doungstop, B.C., et al., *Carbohydrates in allergy: from disease to novel immunotherapies*. *Trends in Immunology*, 2021. **42**(7): p. 635-648.
257. Demkow, U. and A. Stelmazczyk-Emmel, *Extracellular Vesicles in Allergic Rhinitis and Asthma and Laboratory Possibilities for Their Assessment*. *International Journal of Molecular Sciences*, 2021. **22**(5): p. 2273.
258. Mathieu, M., et al., *Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication*. *Nature Cell Biology*, 2019. **21**(1): p. 9-17.
259. Johnson, S.M., et al., *Large Extracellular Vesicles Can be Characterised by Multiplex Labelling Using Imaging Flow Cytometry*. *International Journal of Molecular Sciences*, 2020. **21**(22): p. 8723.
260. Alipoor, S.D., et al., *Exosomes and Exosomal miRNA in Respiratory Diseases*. *Mediators Inflamm*, 2016. **2016**: p. 5628404.
261. Schorey, J.S., et al., *Exosomes and other extracellular vesicles in host–pathogen interactions*. *EMBO reports*, 2015. **16**(1): p. 24-43.
262. Welsh, J.A., et al., *Minimal information for studies of extracellular vesicles (MISEV2023): From basic to advanced approaches*. *Journal of Extracellular Vesicles*, 2024. **13**(2): p. e12404.
263. De Toro, J., et al., *Emerging roles of exosomes in normal and pathological conditions: new insights for diagnosis and therapeutic applications*. *Front Immunol*, 2015. **6**: p. 203.
264. Sangaphuchai, P., I. Todd, and L.C. Fairclough, *Extracellular vesicles and asthma: A review of the literature*. *Clin Exp Allergy*, 2020. **50**(3): p. 291-307.
265. Admyre, C., et al., *B cell-derived exosomes can present allergen peptides and activate allergen-specific T cells to proliferate and produce T_H2-like cytokines*. *Journal of Allergy and Clinical Immunology*, 2007. **120**(6): p. 1418-1424.
266. Fang, S.-B., et al., *Plasma EVs Display Antigen-Presenting Characteristics in Patients With Allergic Rhinitis and Promote Differentiation of Th2 Cells*. *Frontiers in Immunology*, 2021. **Volume 12 - 2021**.
267. Huang, L., et al., *Exosomes from Thymic Stromal Lymphopoietin-Activated Dendritic Cells Promote Th2 Differentiation through the OX40 Ligand*. *Pathobiology*, 2018. **86**(2-3): p. 111-117.
268. Teng ZX, Z.X., Xu RT, Zhu FY, Bing X, Guo N, Shi L, Qi WW, Liu CC, Xia M., *Tfh Exosomes Derived from Allergic Rhinitis Promote DC Maturation Through miR-142-5p/CDK5/STAT3 Pathway*. *J Inflamm Res*, 2022. **3187-3205**.
269. Zhu, X., et al., *Exosomal long non-coding RNA GAS5 suppresses Th1 differentiation and promotes Th2 differentiation via downregulating EZH2 and T-bet in allergic rhinitis*. *Mol Immunol*, 2020. **118**: p. 30-39.
270. Fang, S.-B., et al., *Plasma EVs Display Antigen-Presenting Characteristics in Patients With Allergic Rhinitis and Promote Differentiation of Th2 Cells*. *Frontiers in Immunology*, 2021. **12**.

271. Chen, X., et al., *Intestinal epithelial cell-derived integrin α 86 plays an important role in the induction of regulatory T cells and inhibits an antigen-specific Th2 response*. Journal of Leukocyte Biology, 2011. **90**(4): p. 751-759.
272. Peng, Y.Q., et al., *Mesenchymal stromal cells-derived small extracellular vesicles modulate DC function to suppress Th2 responses via IL-10 in patients with allergic rhinitis*. Eur J Immunol, 2022. **52**(7): p. 1129-1140.
273. Prado, N., et al., *Bystander suppression to unrelated allergen sensitization through intranasal administration of tolerogenic exosomes in mouse*. Mol Immunol, 2010. **47**(11-12): p. 2148-51.
274. Prado, N., et al., *Exosomes from bronchoalveolar fluid of tolerized mice prevent allergic reaction*. J Immunol, 2008. **181**(2): p. 1519-25.
275. Wahlund, C.J.E., et al., *Exosomes from antigen-pulsed dendritic cells induce stronger antigen-specific immune responses than microvesicles in vivo*. Sci Rep, 2017. **7**(1): p. 17095.
276. Yu, D., et al., *Specific antigen-guiding exosomes inhibit food allergies by inducing regulatory T cells*. Immunology & Cell Biology, 2020. **98**(8): p. 639-649.
277. Zeng, H.T., et al., *Exosomes carry IL-10 and antigen/MHC II complexes to induce antigen-specific oral tolerance*. Cytokine, 2020. **133**: p. 155176.
278. Zhou, J., et al., *HMSC-Derived Exosome Inhibited Th2 Cell Differentiation via Regulating miR-146a-5p/SERPINE2 Pathway*. Journal of Immunology Research, 2021. **2021**(1): p. 6696525.
279. Asadirad, A., et al., *Sublingual prophylactic administration of OVA-loaded MSC-derived exosomes to prevent allergic sensitization*. Int Immunopharmacol, 2023. **120**: p. 110405.
280. Asano, K., et al., *Extracellular vesicles from methicillin resistant Staphylococcus aureus stimulate proinflammatory cytokine production and trigger IgE-mediated hypersensitivity*. Emerging Microbes & Infections, 2021. **10**(1): p. 2000-2009.
281. Hong, S.-W., et al., *An Important Role of α -Hemolysin in Extracellular Vesicles on the Development of Atopic Dermatitis Induced by Staphylococcus aureus*. PLOS ONE, 2014. **9**(7): p. e100499.
282. Staudenmaier, L., et al., *Bacterial membrane vesicles shape Staphylococcus aureus skin colonization and induction of innate immune responses*. Experimental Dermatology, 2022. **31**(3): p. 349-361.
283. Akbar, A., et al., *Methodologies to Isolate and Purify Clinical Grade Extracellular Vesicles for Medical Applications*. Cells, 2022. **11**(2): p. 186.
284. Effah, C.Y., et al., *Bacteria-derived extracellular vesicles: endogenous roles, therapeutic potentials and their biomimetics for the treatment and prevention of sepsis*. Frontiers in Immunology, 2024. **Volume 15 - 2024**.
285. Kim, Y.-S., et al., *Extracellular vesicles, especially derived from Gram-negative bacteria, in indoor dust induce neutrophilic pulmonary inflammation associated with both Th1 and Th17 cell responses*. Clinical & Experimental Allergy, 2013. **43**(4): p. 443-454.
286. Gutowska-Ślesik, J., B. Samoliński, and E. Krzych-Fałta, *The increase in allergic conditions based on a review of literature*. Postepy Dermatol Alergol, 2023. **40**(1): p. 1-7.
287. Della-Torre, E., et al., *IgG4-related disease and allergen-specific immunotherapy*. Ann Allergy Asthma Immunol, 2020. **124**(6): p. 631-633.

288. Shamji, M.H. and S.R. Durham, *Mechanisms of allergen immunotherapy for inhaled allergens and predictive biomarkers*. Journal of Allergy and Clinical Immunology, 2017. **140**(6): p. 1485-1498.
289. Engeroff, P. and M. Vogel, *The Potential of Exosomes in Allergy Immunotherapy*. Vaccines (Basel), 2022. **10**(1).
290. Gon, Y., et al., *Selective release of miRNAs via extracellular vesicles is associated with house-dust mite allergen-induced airway inflammation*. Clinical & Experimental Allergy, 2017. **47**(12): p. 1586-1598.
291. Park, J.A., et al., *Tissue factor-bearing exosome secretion from human mechanically stimulated bronchial epithelial cells in vitro and in vivo*. J Allergy Clin Immunol, 2012. **130**(6): p. 1375-83.
292. Ginini, L., et al., *Insight into Extracellular Vesicle-Cell Communication: From Cell Recognition to Intracellular Fate*. Cells, 2022. **11**(9).
293. Haj-Salem, I., et al., *Fibroblast-derived exosomes promote epithelial cell proliferation through TGF- β 2 signalling pathway in severe asthma*. Allergy, 2018. **73**(1): p. 178-186.
294. Lambring, C.B., et al., *Impact of the Microbiome on the Immune System*. 2019. **39**(5): p. 313-328.
295. Nedorost, S. and M. Hammond, *Art of prevention: Allergic sensitization through damaged skin: Atopic, occupational, and stasis dermatitis*. International Journal of Women's Dermatology, 2020. **6**(5): p. 381-383.
296. Mestas, J. and C.C.W. Hughes, *Of Mice and Not Men: Differences between Mouse and Human Immunology*. The Journal of Immunology, 2004. **172**(5): p. 2731-2738.
297. Rayamajhi, S., et al., *Extracellular vesicles production and proteomic cargo varies with incubation time and temperature*. Experimental Cell Research, 2023. **422**(2): p. 113454.
298. Long, L., et al., *Extracellular Vesicles Are Prevalent and Effective Carriers of Environmental Allergens in Indoor Dust*. Environmental Science & Technology, 2025. **59**(4): p. 1969-1983.
299. Zhao, C., et al., *Mesenchymal stem cell derived extracellular vesicles loaded thermosensitive chitosan-based hydrogel alleviates allergic rhinitis in mouse model*. Materials & Design, 2023. **233**: p. 112271.
300. von Bubnoff, D., E. Geiger, and T. Bieber, *Antigen-presenting cells in allergy*. J Allergy Clin Immunol, 2001. **108**(3): p. 329-39.
301. Lambrecht, B.N. and H. Hammad, *Allergens and the airway epithelium response: gateway to allergic sensitization*. J Allergy Clin Immunol, 2014. **134**(3): p. 499-507.
302. Rincón, M., et al., *Interleukin (IL)-6 directs the differentiation of IL-4-producing CD4⁺ T cells*. J Exp Med, 1997. **185**(3): p. 461-9.
303. Strickland, D.H., J.W. Upham, and P.G. Holt, *Epithelial-dendritic cell interactions in allergic disorders*. Curr Opin Immunol, 2010. **22**(6): p. 789-94.
304. Tucus, D., et al., *Dendritic Cell-Derived Extracellular Vesicles Mediate Inflammation in Egg Allergy Patients*. International Journal of Molecular Sciences, 2026. **27**(2): p. 1042.
305. Montecalvo, A., et al., *Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes*. Blood, 2012. **119**(3): p. 756-66.
306. Segura, E., et al., *ICAM-1 on exosomes from mature dendritic cells is critical for efficient naive T-cell priming*. Blood, 2005. **106**(1): p. 216-23.

307. Kozbial, A., L. Bhandary, and S.K. Murthy, *Effect of Monocyte Seeding Density on Dendritic Cell Generation in an Automated Perfusion-Based Culture System*. Biochem Eng J, 2019. **150**.
308. Calmeiro, J., et al., *In-Depth Analysis of the Impact of Different Serum-Free Media on the Production of Clinical Grade Dendritic Cells for Cancer Immunotherapy*. Front Immunol, 2020. **11**: p. 593363.
309. Napoletano, C., et al., *A comparative analysis of serum and serum-free media for generation of clinical grade DCs*. J Immunother, 2007. **30**(5): p. 567-76.
310. Wang Y, Y.Z., Zhou Y, Zhu Y, Wang J, Fu J, Yuan Y, Chen S, Wang Y, Yu W, Gao P, Zhu W, Cheng Q, et al, *Different doses of ovalbumin exposure on dendritic cells determine their genetic/epigenetic regulation and T cell differentiation*. Aging, 2020(12:25432-25451).
311. Zeng, Y., et al., *Biological Features of Extracellular Vesicles and Challenges*. Front Cell Dev Biol, 2022. **10**: p. 816698.
312. Welsh, J.A., et al., *Minimal information for studies of extracellular vesicles (MISEV2023): From basic to advanced approaches*. J Extracell Vesicles, 2024. **13**(2): p. e12404.
313. Witwer, K.W., et al., *Standardization of sample collection, isolation and analysis methods in extracellular vesicle research*. J Extracell Vesicles, 2013. **2**.
314. Livshits, M.A., et al., *Isolation of exosomes by differential centrifugation: Theoretical analysis of a commonly used protocol*. Sci Rep, 2015. **5**: p. 17319.
315. Vergauwen, G., et al., *Confounding factors of ultrafiltration and protein analysis in extracellular vesicle research*. Scientific Reports, 2017. **7**(1): p. 2704.
316. Konoshenko, M.Y., et al., *Isolation of Extracellular Vesicles: General Methodologies and Latest Trends*. Biomed Res Int, 2018. **2018**: p. 8545347.
317. Rikkert, L.G., et al., *Quality of extracellular vesicle images by transmission electron microscopy is operator and protocol dependent*. J Extracell Vesicles, 2019. **8**(1): p. 1555419.
318. van der Pol, E., et al., *Single vs. swarm detection of microparticles and exosomes by flow cytometry*. J Thromb Haemost, 2012. **10**(5): p. 919-30.
319. Dragovic, R.A., et al., *Sizing and phenotyping of cellular vesicles using Nanoparticle Tracking Analysis*. Nanomedicine, 2011. **7**(6): p. 780-8.
320. Szatanek, R., et al., *The Methods of Choice for Extracellular Vesicles (EVs) Characterization*. Int J Mol Sci, 2017. **18**(6).
321. Nolan, J.P., et al., *Single cell analysis using surface enhanced Raman scattering (SERS) tags*. Methods, 2012. **57**(3): p. 272-9.
322. Gardiner, C., et al., *Extracellular vesicle sizing and enumeration by nanoparticle tracking analysis*. J Extracell Vesicles, 2013. **2**.
323. Malenica, M., et al., *Perspectives of Microscopy Methods for Morphology Characterisation of Extracellular Vesicles from Human Biofluids*. Biomedicines, 2021. **9**(6).
324. Filipe, V., A. Hawe, and W. Jiskoot, *Critical evaluation of Nanoparticle Tracking Analysis (NTA) by NanoSight for the measurement of nanoparticles and protein aggregates*. Pharm Res, 2010. **27**(5): p. 796-810.
325. Anderson, W., et al., *A comparative study of submicron particle sizing platforms: accuracy, precision and resolution analysis of polydisperse particle size distributions*. J Colloid Interface Sci, 2013. **405**: p. 322-30.
326. Lannigan, J. and U. Erdbruegger, *Imaging flow cytometry for the characterization of extracellular vesicles*. Methods, 2017. **112**: p. 55-67.

327. Erdbrügger, U., et al., *Imaging flow cytometry elucidates limitations of microparticle analysis by conventional flow cytometry*. Cytometry A, 2014. **85**(9): p. 756-70.
328. Headland, S.E., et al., *Neutrophil-derived microvesicles enter cartilage and protect the joint in inflammatory arthritis*. Sci Transl Med, 2015. **7**(315): p. 315ra190.
329. Ricklefs, F.L., et al., *Imaging flow cytometry facilitates multiparametric characterization of extracellular vesicles in malignant brain tumours*. J Extracell Vesicles, 2019. **8**(1): p. 1588555.
330. Mastoridis, S., et al., *Multiparametric Analysis of Circulating Exosomes and Other Small Extracellular Vesicles by Advanced Imaging Flow Cytometry*. Front Immunol, 2018. **9**: p. 1583.
331. Gray, W.D., A.J. Mitchell, and C.D. Searles, *An accurate, precise method for general labeling of extracellular vesicles*. MethodsX, 2015. **2**: p. 360-7.
332. Calderón-Peláez, M.A., J.E. Castellanos, and M.L. Velandia-Romero, *A protocol for loading Calcein-AM into extracellular vesicles from mammalian cells for clear visualization with a fluorescence microscope coupled to a deconvolution system*. PLoS One, 2025. **20**(1): p. e0317689.
333. Sokolova, V., et al., *Characterisation of exosomes derived from human cells by nanoparticle tracking analysis and scanning electron microscopy*. Colloids Surf B Biointerfaces, 2011. **87**(1): p. 146-50.
334. Lucchetti, D., et al., *Measuring Extracellular Vesicles by Conventional Flow Cytometry: Dream or Reality?* Int J Mol Sci, 2020. **21**(17).
335. Benjamin-Davalos, S., et al., *Co-Isolation of Cytokines and Exosomes: Implications for Immunomodulation Studies*. Frontiers in Immunology, 2021. **12**.
336. Banchereau, J. and R.M. Steinman, *Dendritic cells and the control of immunity*. Nature, 1998. **392**(6673): p. 245-52.
337. Hammad, H. and B.N. Lambrecht, *Barrier Epithelial Cells and the Control of Type 2 Immunity*. Immunity, 2015. **43**(1): p. 29-40.
338. Willart, M.A., et al., *Interleukin-1 α controls allergic sensitization to inhaled house dust mite via the epithelial release of GM-CSF and IL-33*. J Exp Med, 2012. **209**(8): p. 1505-17.
339. Akdis, C.A. and M. Akdis, *Mechanisms of immune tolerance to allergens: role of IL-10 and Tregs*. The Journal of Clinical Investigation, 2014. **124**(11): p. 4678-4680.
340. Sicherer, S.H. and H.A. Sampson, *Food allergy: A review and update on epidemiology, pathogenesis, diagnosis, prevention, and management*. J Allergy Clin Immunol, 2018. **141**(1): p. 41-58.
341. Martino, D., et al., *Epigenetic dysregulation of naive CD4⁺ T-cell activation genes in childhood food allergy*. Nature Communications, 2018. **9**(1): p. 3308.
342. Rudders, S.A., et al., *Trends in pediatric emergency department visits for food-induced anaphylaxis*. Journal of Allergy and Clinical Immunology, 2010. **126**(2): p. 385-388.
343. Harvey-Seutcheu, C., G. Hopkins, and L.C. Fairclough, *The Role of Extracellular Vesicles in Atopic Dermatitis*. Int J Mol Sci, 2024. **25**(6).
344. Lindenbergh, M.F.S., et al., *Bystander T-Cells Support Clonal T-Cell Activation by Controlling the Release of Dendritic Cell-Derived Immune-Stimulatory Extracellular Vesicles*. Frontiers in Immunology, 2019. **Volume 10 - 2019**.

345. McKarns, S.C. and R.H. Schwartz, *Biphasic regulation of IL2 transcription in CD4+ T cells: roles for TNF-alpha receptor signaling and chromatin structure*. J Immunol, 2008. **181**(2): p. 1272-81.
346. Woodfolk, J.A., *Cytokines as a therapeutic target for allergic diseases: a complex picture*. Curr Pharm Des, 2006. **12**(19): p. 2349-63.
347. Maier, E., A. Duschl, and J. Horejs-Hoeck, *STAT6-dependent and -independent mechanisms in Th2 polarization*. Eur J Immunol, 2012. **42**(11): p. 2827-33.
348. Chakma, C.R. and K.L. Good-Jacobson, *Requirements of IL-4 during the Generation of B Cell Memory*. J Immunol, 2023. **210**(12): p. 1853-1860.
349. Holgate, S.T. and R. Polosa, *The mechanisms, diagnosis, and management of severe asthma in adults*. Lancet, 2006. **368**(9537): p. 780-93.
350. Ridolo, E., et al., *The Present and Future of Allergen Immunotherapy in Personalized Medicine*. J Pers Med, 2022. **12**(5).
351. Callery, E.L., et al., *Component-resolved diagnostics in the clinical and laboratory investigation of allergy*. Annals of Clinical Biochemistry, 2020. **57**(1): p. 26-35.
352. Brozek, J.L., et al., *Allergic Rhinitis and its Impact on Asthma (ARIA) guidelines: 2010 revision*. J Allergy Clin Immunol, 2010. **126**(3): p. 466-76.
353. Admyre, C., et al., *Exosomes with immune modulatory features are present in human breast milk*. J Immunol, 2007. **179**(3): p. 1969-78.
354. Tucis, D., et al., *The Role of Extracellular Vesicles in Allergic Sensitization: A Systematic Review*. Int J Mol Sci, 2024. **25**(8).
355. Burks, A.W., et al., *ICON: food allergy*. J Allergy Clin Immunol, 2012. **129**(4): p. 906-20.
356. Dehnavi, S., et al., *Loading Ovalbumin into Mesenchymal Stem Cell-Derived Exosomes as a Nanoscale Carrier with Immunomodulatory Potential for Allergen-Specific Immunotherapy*. Rep Biochem Mol Biol, 2023. **11**(4): p. 626-634.