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**Roles and Actions of Resolvin D1 on Pulmonary and Extra-pulmonary
Complications of Cystic Fibrosis**

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Acronyms

- **AA:** Arachidonic Acid
- **ACN:** Acetonitrile
- **ACS:** American Chemical Society
- **ALX:** Lipoxin A4 Receptor
- **ANOVA:** Analysis of Variance
- **ARDS:** Acute Respiratory Distress Syndrome
- **ASA:** Aspirin
- **ASN:** Airway Supernatant
- **ATP:** Adenosine Triphosphate
- **AUC:** Area Under the Curve
- **BAL:** Bronchoalveolar Lavage
- **BLT1:** Leukotriene B4 Receptor 1
- **CCL2:** C-C Motif Chemokine Ligand 2
- **CF:** Cystic Fibrosis
- **CFBEC:** Cystic Fibrosis Bronchial Epithelial Cells
- **CFRD:** Cystic Fibrosis-Related Diabetes
- **CFTR:** Cystic Fibrosis Transmembrane Conductance Regulator
- **CFU:** Colony-Forming Units
- **CGM:** Continuous Glucose Monitoring
- **COPD:** Chronic Obstructive Pulmonary Disease
- **COVID:** Coronavirus Disease
- **COX:** Cyclooxygenase
- **CRP:** C-Reactive Protein
- **CXCL8:** C-X-C Motif Chemokine Ligand 8
- **DHA:** Docosahexaenoic Acid
- **DNA:** Deoxyribonucleic Acid
- **DPA:** Docosapentaenoic Acid
- **DRV2:** Resolvin D2 Receptor
- **EE:** Energy Expenditure
- **ELISA:** Enzyme-Linked Immunosorbent Assay
- **ENaC:** Epithelial Sodium Channel
- **EPA:** Eicosapentaenoic Acid
- **ETI:** Elexacaftor-Tezacaftor-Ivacaftor
- **FACS:** Fluorescence-Activated Cell Sorting
- **FEV1:** Forced Expiratory Volume in One Second
- **FPR2:** Formyl Peptide Receptor 2
- **GTT:** Glucose Tolerance Test
- **HPLC:** High-Performance Liquid Chromatography
- **HS:** Hypertonic Saline
- **IFN:** Interferon
- **IL:** Interleukin
- **I.P.:** intra-peritoneal
- **IPA:** Ingenuity Pathway Analysis
- **IR:** Insulin Resistance
- **IRT:** Immunoreactive Trypsinogen
- **IV:** Intravenous
- **KO:** Knockout
- **LC:** Liquid Chromatography
- **LGR6:** Leucine-Rich Repeat Containing G Protein-Coupled Receptor 6

- **LO:** Lipoxygenase
- **LPS:** Lipopolysaccharide
- **LTA4:** Leukotriene A4
- **LTB4:** Leukotriene B4
- **LX:** Lipoxin
- **LXA4:** Lipoxin A4
- **MCP:** Monocyte Chemoattractant Protein
- **MΦ:** Macrophage
- **MHC:** Major Histocompatibility Complex
- **MS:** Mass Spectrometry
- **NSAIDs:** Non-Steroidal Anti-Inflammatory Drugs
- **OGTT:** Oral Glucose Tolerance Test
- **PCR:** Polymerase Chain Reaction
- **PD1:** Protectin D1
- **PG:** Prostaglandin
- **PGD2:** Prostaglandin D2
- **PMN:** Polymorphonuclear Neutrophil
- **pwCF:** people with Cystic Fibrosis
- **RNA:** Ribonucleic Acid
- **RQ:** Respiratory Quotient
- **RvD1:** Resolvin D1
- **SARS:** Severe Acute Respiratory Syndrome
- **SPM:** Specialized Pro-Resolving Mediators
- **TGF:** Transforming Growth Factor
- **THP-1:** Human Monocytic Cell Line
- **TNF:** Tumor Necrosis Factor
- **TXB2:** Thromboxane B2
- **UV:** Ultraviolet
- **VEGF:** Vascular Endothelial Growth Factor
- **WT:** Wild Type

Abstract:

Aberrant inflammation is a key driver of the progression of cystic fibrosis (CF) disease, particularly of lung damage. This thesis explores the therapeutic potential of Resolvin D1, a specialized pro-resolving lipid mediator, in targeting inflammation and metabolic dysregulation associated with CF. In the β ENaC mouse model, which mimics human CF lung pathology, Resolvin D1 effectively reduced lung inflammation by limiting immune cell infiltration into lung tissue. Recognizing the high incidence of cystic fibrosis-related diabetes (CFRD) as a significant comorbidity, we also evaluated the impact of Resolvin D1 on glucose homeostasis in in vivo and in vitro experimental settings. We induced diabetes in C57BL/6 mice by administering high glucose load and assessed glucose tolerance with the glucose tolerance test (GTT). Notably, Resolvin D1 treatment normalized glucose levels, indicating its potential benefit in the management of CFRD. Further, we investigated the cellular response of THP-1 macrophage-like cells exposed to high-glucose medium and made CF-like by blocking Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) using the selective inhibitor CFTRinh-172. In this setting, CFTR blockade exacerbated molecular events typically triggered by hyperglycemia, whereas Resolvin D1 restored cellular functionality and reduced inflammation.

These findings collectively highlight Resolvin D1 as a promising therapeutic agent, not only for mitigation of CF chronic lung inflammation, but also to counteract metabolic imbalance associated with CFRD.

Aim:

The specific objectives of the research proposed in this thesis were to:

- 1) Determine the impact of resolvin D1 on cystic fibrosis (CF) lung inflammation.
- 2) Try to mimic the association CF and diabetes in a mouse model.
- 3) Understand the interactions between cystic fibrosis-related diabetes (CFRD) and macrophage (M Φ) inflammatory responses.
- 4) Investigate the actions of resolvin D1 on these interactions.

Chapter 1

INFLAMMATION and SPECIALIZED PRORESOLVING LIPID MEDIATORS (SPM)

1.1 Inflammation:

Inflammation is an essential reaction of the immune system against pathogens, injured cells, or irritants (Chen et al., 2018). This process is necessary to protect our body from infections and promote the repair and regeneration of damaged tissues. However, when inflammation becomes dysregulated or chronic, it can lead to the development of various diseases, including autoimmune disorders, cardiovascular disease, and cancer (Medzhitov, 2008).

The inflammatory response is driven by a series of molecular and cellular responses involving various immune cells, signaling molecules, and tissue-resident cells. When a threat is detected, resident immune cells such as macrophages and mast cells release pro-inflammatory substances, such as cytokines, chemokines, and lipid mediators (Nathan, 2002). To eliminate pathogens and damaged tissue, these mediators attract circulating immune cells, such as neutrophils and monocytes, to the site of injury or infection (Chen et al., 2018).

A key role in the pathogenesis of many diseases is due to the dysfunction of the inflammatory process, especially when it becomes chronic, making inflammation an interesting target for a better understanding of inflammatory response pathways and molecular mechanisms undoubtedly contributing to improving the prevention and treatment of inflammatory diseases (Chen et al., 2018). Commonly used to relieve inflammation and its associated symptoms are anti-inflammatory drugs, such as nonsteroidal anti-inflammatory drugs (NSAIDs) (Ricciotti and FitzGerald, 2011). However, the development of innovative therapies targeting specific inflammatory pathways or mediators offers the prospect of more targeted and effective treatments with fewer side effects (Medzhitov, 2008).

This intricate balance between activation and resolution of inflammation highlights the delicate interplay between a beneficial and a harmful immune response. Therefore, a greater understanding of the molecular pathways involved in dysfunctional inflammation may help open new avenues for therapeutic interventions and understand the underlying causes of inflammatory diseases.

1.2 Inflammation dual role: Guardian and Aggressor

Inflammation can be seen as a double-edged sword: it is essential for survival, but it is potentially harmful if not properly regulated. The protective aspect of inflammation is most evident in acute inflammation, a rapid, localized response that occurs immediately after injury or infection. This process is meant to eliminate pathogens, clear away dead cells, and initiate tissue repair. Key

players in this rapid response are neutrophils, which are among the first immune cells to arrive at the site of injury, where they engulf pathogens and release enzymes that help clear debris (Medzhitov, 2010). Acute inflammation is a protective host response that is activated in response to tissue injury and infection, characterized by two phases: initiation and resolution; in fact, once the threat is neutralized, the inflammatory response is attenuated through the release of anti-inflammatory signals and the recruitment of cells involved in tissue repair (Sansbury and Spite, 2016).

However, if the resolution phase of inflammation is not adequately completed, acute inflammation can progress to a chronic state, with signs of acute inflammation, such as neutrophil accumulation, potentially resurfacing at later stages (Perretti and Dalli, 2023). It doesn't matter how many times it occurs, but how long the inflammation persists in not going down. Unlike acute inflammation, which is temporary, chronic inflammation is a long-lasting and dysregulated process that can persist for months or even years. This type of inflammation is typically marked by the continued presence of immune cells, such as macrophages and T lymphocytes, which may result in ongoing tissue damage and fibrosis. Unresolved inflammation is a major disease trigger (Nathan and Ding, 2010). Chronic inflammation is not "positive" and therefore protective; rather, it contributes to the progression of various diseases by promoting cellular dysfunction and oxidative stress. Atherosclerosis, for example, is a chronic disease that causes arterial plaque to spread, increasing the risk of heart attack and stroke (Libby et al., 2011). Another example is cystic fibrosis (CF), a genetic disease characterized by unresolved chronic inflammation of the lungs driven by persistent bacterial infections. This leads to progressive lung damage and fibrosis that cause permanent structural complications of the airways and, if uncontrolled, eventually causes respiratory failure and death (Cantin et al., 2015).

Additionally, chronic inflammation has been implicated in the pathogenesis of autoimmune diseases, in which the immune system mistakenly targets the body's own tissues. In conditions such as rheumatoid arthritis, chronic inflammation leads to the continual recruitment of inflammatory cells that contribute to local destruction (Firestein, 2003). Similarly, even in cancer, chronic inflammation can contribute to the creation of an environment favorable to tumor growth and metastasis. In fact, inflammatory cells and cytokines can promote genetic mutations, angiogenesis and suppress immune responses against tumor cells (Mantovani et al., 2008)

The dual nature of inflammation is easier to understand when we look at bacterial or viral infections. In these cases, inflammation can either protect or harm the body. For example, in a bacterial infection like pneumonia, the body uses inflammation to fight off bacteria, helping to stop the spread of the infection. But if the inflammation lasts too long, like in sepsis, it can cause serious damage to tissues and organs. Similarly, in viral infections such as COVID-19, inflammation helps the body fight the virus first. However, if it becomes too strong, it can lead to dangerous conditions like a cytokine storm, where the body harms itself by attacking healthy tissues. These examples clearly show how

inflammation can help at first but become harmful if it goes on for too long (Hotchkiss et al., 2016; Mehta et al., 2020).

The dual nature of inflammation highlights the critical need for careful regulation of the inflammatory response. For this reason, therapeutic approaches must preserve the protective aspects of acute inflammation, avoiding the adverse effects of chronic inflammation. This balance highlights the complexity involved in designing effective and safe anti-inflammatory treatments, particularly for chronic inflammatory conditions that require long-term control of inflammation without compromising the immune system's response to sudden threats.

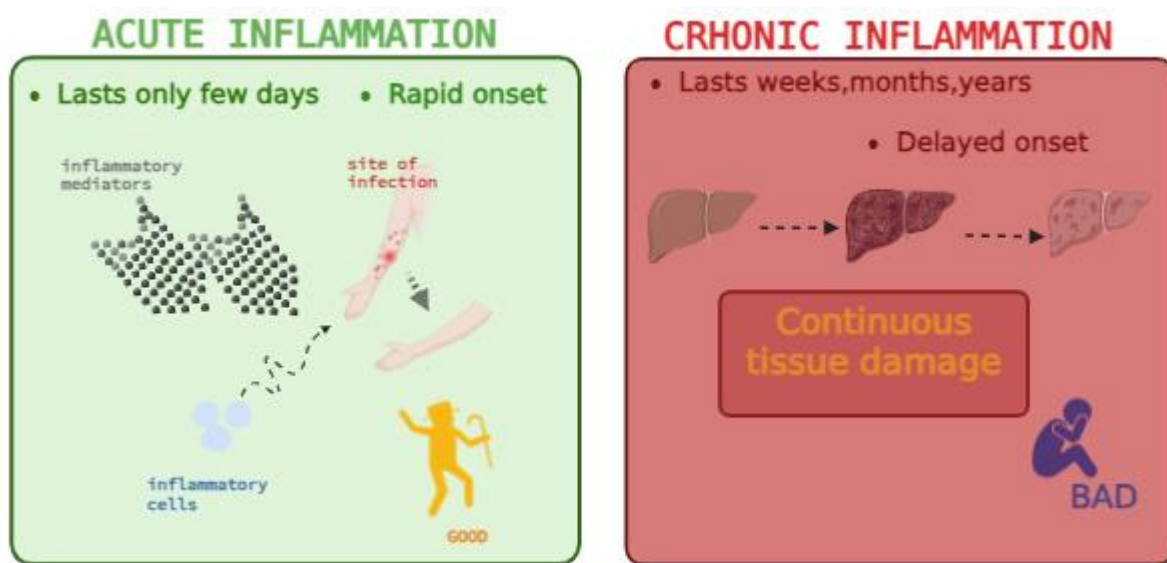


Fig1. Differences between acute and chronic inflammation.

On the left, acute inflammation is depicted as having a rapid onset and lasting only a few days. It is triggered quickly in response to infection or injury, involving inflammatory mediators and cells. This type of inflammation is considered "good" because it helps fight infection and repair tissue. On the right, chronic inflammation is characterized by prolonged duration, lasting weeks, months, or even years, with a delayed onset. Unlike acute inflammation, chronic inflammation leads to continuous tissue damage, as shown by the progressive deterioration of organs (e.g., the liver), and it is associated with harmful long-term effects labeled as "bad." Image generated using Biorender.

1.3 Mechanisms of inflammatory response and the key role of macrophages

To have an inflammatory response, there must be a rapid recruitment of immune cells to the site of infection, to neutralize the harmful agents and initiate tissue repair (Chen et al., 2018). A key process in this is setting chemotaxis, the direct migration of cells along a chemical gradient. Chemotactic signals are typically generated by damaged tissue, pathogens, or immune cells, which release chemokines such as CCL2 (MCP-1) and CXCL8 (IL-8) (Hughes and Nibbs, 2018). These molecules create a gradient that guides immune cells, particularly neutrophils and monocytes, toward the inflamed tissue. Once in the tissues, monocytes differentiate into macrophages, which are the main players in both the inflammatory and resolution phases. Macrophages initially exist in a quiescent, nonpolarized state known as M0 macrophages (Mendoza-Coronel and Ortega, 2017). In response to environmental stimuli, such as microbial products or signals from damaged tissues, these M0 macrophages can undergo either “classical” or “alternative” polarization (Strizova et al., 2023). Macrophages of the classical pathway are designated M1, and those of the alternative pathway are designated M2 (Peng et al., 2023). Classical pathway macrophages, in response to both extracellular and intracellular pathogens, increase inducible nitric oxide synthases and secrete proinflammatory chemokines and cytokines. They expose antigens using MHC class II, causing inflammation, granulocyte cell recruitment and activation of the Th1 response (Chiu and Bharat, 2016). Differently, M2 macrophages secrete histamine under the stimulation of IL-4 and IL-13 in case of allergies or infections, contributing to the destruction and encapsulation of parasites. These macrophages also facilitate the resolution of inflammation, tissue regeneration and the initiation of fibrosis (Chiu and Bharat, 2016). M2, as a type of immune cells, can secrete anti-inflammatory cytokines, in fact they secrete IL-10, which can influence the effect of IL-4 and IL-13 on macrophages and reduce the expression of MHC class II molecules (Wang et al., 2014). TGF- β is another distinctive protein of M2 macrophages (Zhang et al., 2016). M2 macrophages, through the secretion of IL-6, TGF- β and IL-10, contribute to healing, fibroblast proliferation and collagen production. In addition, TNF- α and IFN- γ can regulate M2 macrophages, promoting angiogenesis, which is a fundamental step in the wound healing process (Bi et al., 2019).

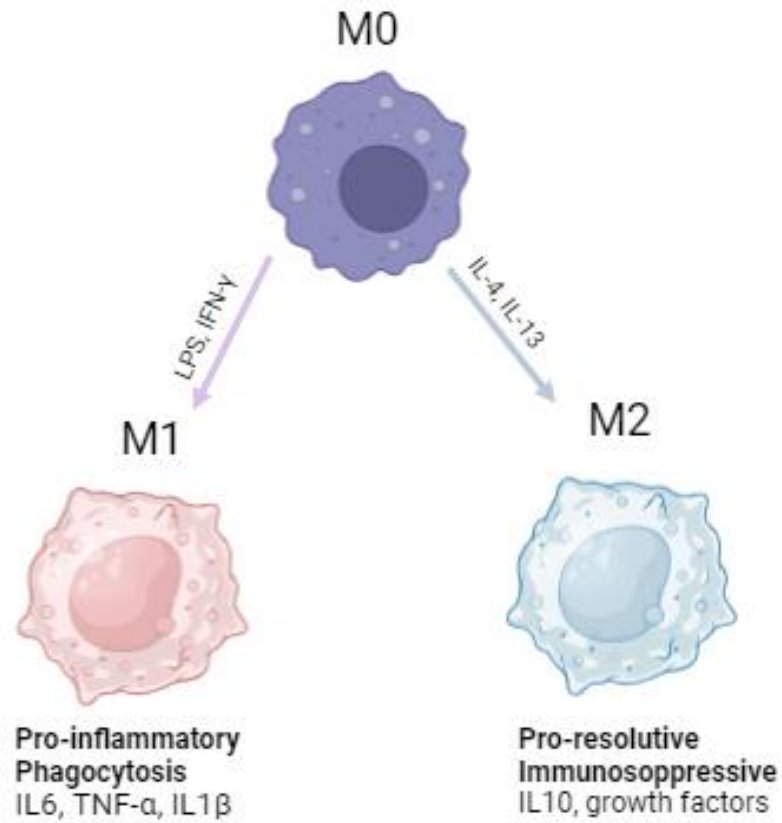


Fig.2 Macrophage Differentiation.

This diagram illustrates the polarization of macrophages. Starting from the non-polarized state (M0), macrophages can differentiate into either the pro-inflammatory M1 or the pro-resolving M2 phenotype. M1 macrophages are activated by signals such as LPS and IFN- γ and are involved in pro-inflammatory activities like phagocytosis, secretion of IL-6, TNF- α , and IL-1 β cytokines. M2 macrophages, activated by IL-4 and IL-13, are associated with anti-inflammatory and immunosuppressive functions, producing IL-10 and growth factors that promote tissue repair.

In this transition from inflammation to resolution, TGF- β , a pleiotropic cytokine produced by macrophages, platelets, and fibroblasts, is a key mediator that functions both as an anti-inflammatory agent and as a regulator of tissue remodeling (Sanjabi et al., 2017). In the early stages of inflammation, TGF- β dampens the immune response by inhibiting the proliferation of effector T cells and promoting the expansion of regulatory T cells (Treg), thereby mitigate tissue damage caused by excessive inflammation (Wan and Flavell, 2008). Furthermore, TGF- β plays a central role in fibrogenesis by stimulating fibroblasts to produce extracellular matrix components such as collagen and fibronectin, which are essential for tissue repair and scar formation. Although TGF- β is crucial for the conversion of fibroblasts to myofibroblasts in fibrotic tissues, overactivation of TGF- β signaling can lead to pathological conditions such as fibrosis, in which excessive collagen deposition causes tissue stiffening and organ dysfunction (Frangogiannis, 2020). Chronic inflammation, often characterized by persistent macrophage activation and TGF- β production, creates a microenvironment that promotes angiogenesis and immune evasion. In the course of chronic diseases, TGF- β has emerged as a crucial factor to control inflammation and promote healing, its dysregulation contributes to the progression of these causing fibrosis (Sisto et al., 2021). The balance between its immunosuppressive and reparative functions is therefore critical for maintaining tissue homeostasis and preventing the transition from acute inflammation to chronic pathology.

1.4 Mechanisms of inflammation resolution

Resolution of inflammation is described as an active and controlled endogenous process that promotes the restoration of balance in the immune microenvironment and tissue repair after an immune response (Zhang et al., 2024). Unlike previous studies that viewed resolution as simply fading away of the inflammatory response, we now understand that resolution is a structured and dynamic phase. This process not only eliminates the initial threat, such as pathogens or damaged tissue, but also ensures that immune cells involved in the response are safely removed to prevent further tissue damage. When the resolution phase fails, it can lead to chronic inflammatory diseases, such as cystic fibrosis, in which ongoing inflammation causes progressive tissue destruction (Serhan et al., 2008a).

During the resolution phase, several key events occur. The recruitment of neutrophils, immune cells that can cause collateral damage in inflamed tissues, is interrupted by macrophages derived from recruited monocytes (Oliveira-Costa et al., 2022). During resolution we also see a change in macrophages: the traditional M1, recognized as pro-inflammatory, acquire M2 phenotype, which is anti-inflammatory effects, contributing to tissue the regeneration (Krzyszczuk et al., 2018; Strizova et al., 2023).

Central to this process are Specialized Pro-resolving Mediators (SPM), a group of bioactive lipid compounds that include lipoxins, resolvins, protectins, and maresins. These mediators, derived from

omega-3 and omega-6 fatty acids, actively drive the resolution of inflammation by stopping the recruitment of inflammatory cells, enhancing macrophage ability to clear debris, and by promoting tissue healing. Unlike traditional anti-inflammatory agents that suppress the immune response, SPM resolve inflammation in a way that avoids long-term damage, minimizing the risk of fibrosis or ongoing inflammation (Serhan et al., 2015; Serhan and Savill, 2005).

In summary, the resolution phase of inflammation ensures that the inflammatory response is contained and does not become chronic. When resolution fails, it can lead to chronic conditions in which persistent inflammation continues to damage tissue (Serhan et al., 2015).

SPM offers a promising therapeutic avenue, as their role in resolving inflammation could help manage diseases caused by chronic inflammation.

1.5 Specialized Pro-resolving Mediators (SPM)

SPM are a class of bioactive lipid mediators that play a crucial role in the active resolution of inflammation. Unlike traditional anti-inflammatory agents, which aim to inhibit or block the inflammatory response, SPM promote effective resolution of inflammation by transducing anti-inflammatory and pro-resolving signals that disrupt leukocyte recruitment into inflamed tissues and increase the clearance of apoptotic polymorphonuclear neutrophils (PMN) (Lavy et al., 2021). They are short-lived molecules that are further metabolized and inactivated by various enzymatic pathways involving β -oxidation, ω -oxidation, dehydrogenation, and reduction (Arita et al., 2006). SPMs are generated from omega-3 (n-3) and omega-6 (n-6) fatty acids, which are classified as essential polyunsaturated fatty acids (PUFAs) (Recchiuti and Serhan, 2012). These mediators are enzymatically generated during the later stages of inflammation and play a pivotal role in shifting the immune response from a pro-inflammatory to a pro-resolving state (Ferri et al., 2023a).

SPM are divided into four main families: lipoxins, resolvins, protectins, and maresins, each of which contributes uniquely to the resolution of inflammation.

Derivatives of arachidonic acid (AA), an omega-6 fatty acid, **Lipoxins** are metabolites known for their unique anti-inflammatory and immunoregulatory properties. The main elements of this series are LXA₄ (5S,6R,15S-trihydroxy-7,9,13-trans-11-cis-eicosatetraenoic acid) and its positional isomer LXB₄ (5S,14R,15S-trihydroxy-6,10,12-trans-8-cis-eicosatetraenoic acid) (Serhan et al., 1984).

They play a key role in regulating the immune response by promoting monocyte recruitment in a non-inflammatory manner, aiding in the clearance of apoptotic neutrophils via efferocytosis. Lipoxins also limit neutrophil infiltration and suppress the production of pro-inflammatory cytokines, such as TNF- α and IL-1 β , while balancing inflammatory mediators. This ensures that tissue repair can occur without an overactive immune response (Serhan et al., 2015).

Resolvins, which are derived from omega-3 fatty acids, are stereospecific small molecules divided into two main families: E-series resolvins, which originate from eicosapentaenoic acid (EPA), and D-series resolvins, which are synthesized from docosahexaenoic acid (DHA). In addition, aspirin-activated epimers of these compounds have also been identified (Levy, 2010). Among the most extensively studied are resolvin E1 (RvE1) from the E-series and resolvins D1 (RvD1), D2 (RvD2), D3 (RvD3), and D5 (RvD5) from the D-series. These molecules are crucial in the resolution phase of inflammation, regulating various immune processes. RvE1, the first discovered in the E-series, significantly decreases neutrophil infiltration into inflamed tissues, enhances macrophage-mediated clearance of apoptotic cells, and reduces the production of pro-inflammatory cytokines such as IL-1 β and TNF- α . It has also been shown to facilitate the restoration of tissue homeostasis in multiple inflammation models (Celik et al., 2023; Serhan et al., 2015).

In addition to lipid mediators, some proteins and peptides exhibit pro-resolving properties that are crucial in the resolution of inflammation. For example, Annexin A1 (AnxA1) is a glucocorticoid-inducible protein that promotes the cessation of inflammatory responses and facilitates tissue repair

(Chen et al., 2014). Similarly, Galectin-1 is a lectin that modulates immune cell activity, contributing to the resolution of inflammation (Seropian et al., 2018). Melanocortin peptides, such as α -MSH, have also demonstrated significant anti-inflammatory and pro-resolving effects (Delgado et al., 1998). Therefore, the resolution phase can be targeted with specific pharmacology approaches.

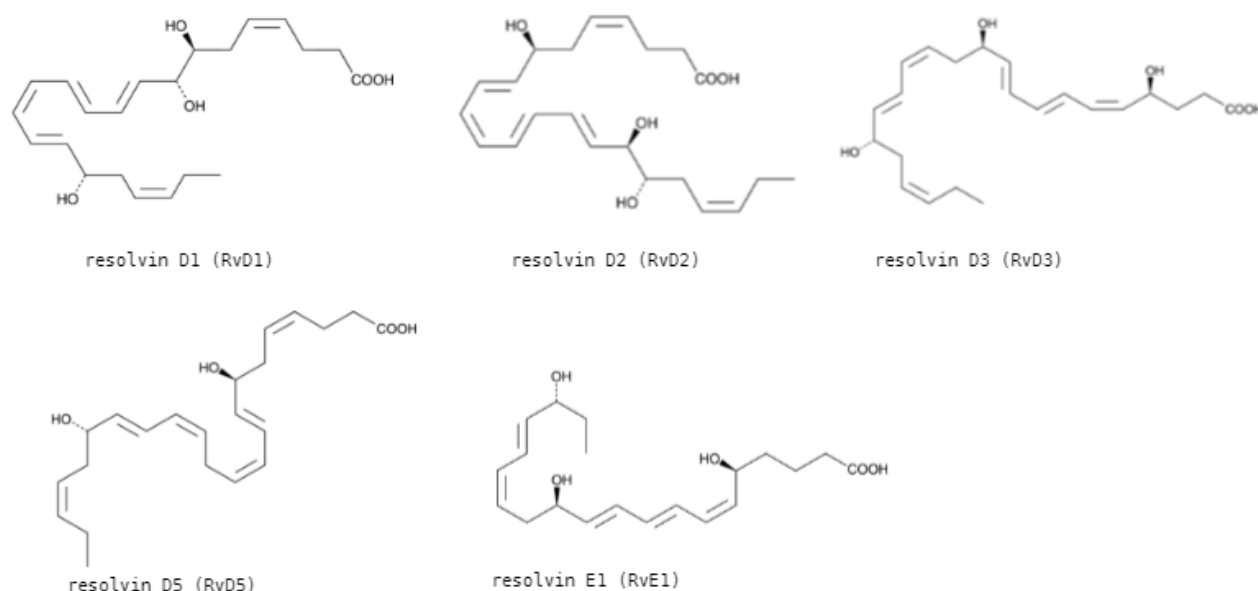


Fig.3: Structure of Resolvins

RvD1, part of the D-series resolvins, plays a key role in reducing the recruitment of inflammatory cells by decreasing neutrophil migration and lowering the expression of adhesion molecules on endothelial cells (Ferri et al., 2023b; Isopi et al., 2020; Recchiuti et al., 2021).

Meanwhile, RvD2 not only enhances the process of macrophages engulfing apoptotic cells but also aids in clearing cellular debris, which is essential for tissue recovery. Furthermore, RvD2 has been found to influence both innate and adaptive immune responses by promoting the removal of microbial pathogens and curbing excessive inflammation (Chiang et al., 2017).

Among within the D-series-resolvins, the recently discovered RvD3 and RvD5 have emerged as important mediators. RvD3 is particularly active at the later stages of inflammation, where it aids in resolving the inflammatory response by decreasing neutrophil levels and enhancing macrophage efficiency in clearing apoptotic cells (<https://cdn.caymanchem.com/cdn/seawolf/insert/13834.pdf>). Research indicates that RvD3 is vital for sustaining long-term tissue health once inflammation has subsided, playing a key role in preventing the development of chronic inflammation or fibrosis (Dalli et al., 2013).

RvD5 has been recognized for its ability to alleviate inflammation in both acute and chronic models, acting via similar mechanisms to other D-series resolvins, but with a focus on reducing the production

of pro-inflammatory cytokines and increasing macrophage efferocytosis (Ganesan et al., 2020). These resolvins work in concert to shift the immune response from an inflammatory to a pro-resolving state, ensuring that inflammation is controlled and resolved properly. By limiting neutrophil infiltration, enhancing the clearance of apoptotic cells by macrophages, and supporting tissue repair, resolvins help prevent excessive immune reactions that could otherwise lead to tissue damage or chronic inflammation. Furthermore, their ability to modulate immune activity without compromising the body's defenses against pathogens makes them an attractive target for therapeutic strategies in disorders characterized by unresolved inflammation (Dona et al., 2008).

Protectins, such as protectin D1 (PD1), primarily originate from DHA and are essential for protecting tissues from damage during inflammation, especially within the nervous system and lungs (Levy, 2010). These molecules help limit neutrophil infiltration while promoting tissue regeneration and wound healing. PD1 has been found to reduce the production of inflammatory cytokines and contribute to the resolution phase by aiding in the removal of apoptotic cells and cellular debris. Additionally, protectins have demonstrated neuroprotective effects, playing a key role in preventing inflammation-induced neuronal damage (Nijmeh and Levy, 2021; Serhan et al., 2015).

Maresins (Macrophage Mediators in Resolving Inflammation), a group of specialized pro-resolving mediators derived from DHA, are primarily produced by macrophages (Sun et al., 2024). Among them, Maresin 1 (MaR1) is crucial for resolving acute inflammation, facilitating tissue regeneration and wound healing. Maresins enhance the process of efferocytosis by macrophages, which helps clear apoptotic cells and reduce neutrophil recruitment. This action minimizes tissue damage and speeds up recovery. Additionally, maresins support tissue repair by stimulating the production of growth factors that encourage the proliferation of epithelial and endothelial cells, further aiding in the restoration of damaged tissues (Serhan et al., 2015).

1.6 Biosynthesis and Receptors:

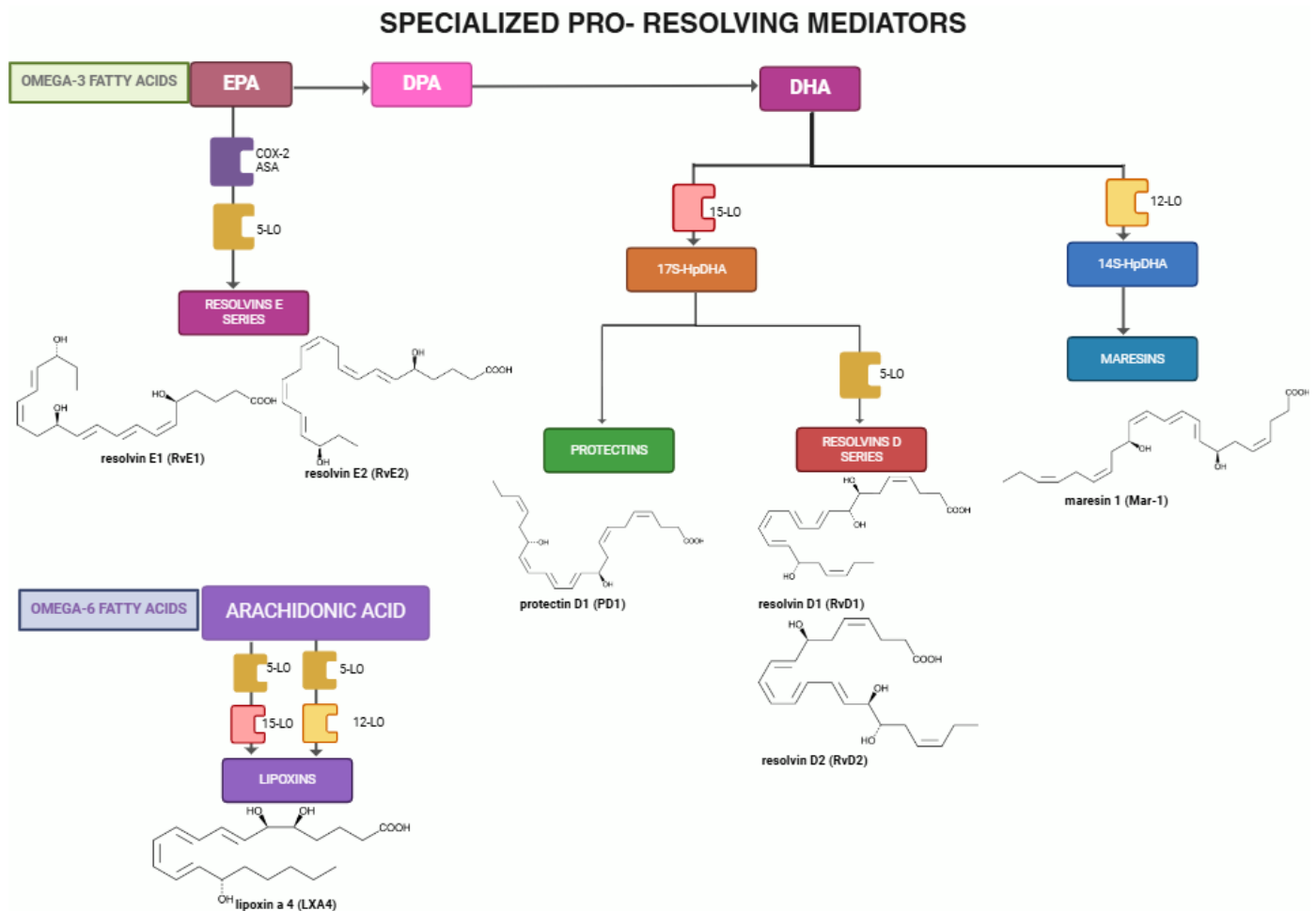


Fig.4 Specialized Pro-Resolving Mediators (SPMs).

The graph shows the biosynthetic pathways of specialized pro-resolving mediators (SPM), derived from polyunsaturated fatty acids such as EPA, DPA, DHA and AA. Enzymes such as COX and LO convert these precursors into pro-resolving molecules such as lipoxins, resolvins, protectins, and maresins which play crucial roles in the resolution of inflammation. Image generated using Biorender.

The synthesis of these mediators is a tightly regulated process involving specific enzymes, mainly cyclooxygenases (COX) and lipoxygenases (LO) (Serhan et al., 2008b). These enzymes, particularly COX-2, 5-LO and 15-LO, act on their respective fatty acid precursors to produce bioactive lipid mediators. Depending on the type of precursor involved, such as AA, DHA, EPA or DPA, different families of pro-resolving mediators are formed, each with a unique role in the resolution of inflammation (Serhan et al., 2000).

Resolvin biosynthesis occurs mainly through a transcellular pathway involving neutrophils, macrophages and endothelial cells. Specialized pro-resolving mediators in the resolvin family are divided into D-series and E-series resolvins, derived from DHA and EPA, respectively.

D-Series Resolvins:

- **RvD1** synthesis can occur within a single cell (PMN or macrophage) or during cell-cell interaction (leukocytes-endothelial cells). 15-LO initiates the conversion of DHA to a 17-HDHA intermediate, which can be converted to D-series resolvins by 5-LO. In particular, the peroxide formed by the action of 5-LO can undergo epoxidation, generating a 7S,8S epoxide, which is subsequently enzymatically hydrolyzed to produce RvD1 (Sansbury and Spite, 2016). RvD1 binds to two receptors, Formyl peptide receptor 2 (ALX/FPR2) and G protein-coupled receptor 32 (GPR32), both expressed on immune cells. RvD1 binds to ALX/FPR2 expressed on both monocytes and neutrophils (Krishnamoorthy et al., 2010a). ALX/FPR2 activation correlates with the transition of macrophages towards a pro-resolving phenotype with consequent reduction in neutrophil chemotaxis (Centanni et al., 2023). RvD1 interacts with GPR32, leading to the suppression of inflammatory chemo attractants released by monocytes, neutrophils, and macrophages. Activation of GPR32 also promotes the shift of macrophages from a pro-inflammatory state to a pro-resolution phenotype. Unlike ALX/FPR2, which is predominantly found on neutrophils and monocytes, GPR32 is highly expressed on macrophages (Centanni et al., 2023).
- **RvD2** biosynthesis begins with the 17-lipoxygenation of DHA, resulting in the formation of 17S-hydroperoxy-DHA. This compound is subsequently converted by leukocytes, via 5-LO, to an intermediate with a 7(8) epoxide group. Finally, an enzymatic hydrolysis process completes the conversion to RvD2. The RvD2 receptor, known as DRV2 (previously identified as GPR18), is a G-coupled protein expressed on neutrophils, monocytes, and macrophages. RvD2 binds to the DRV2 receptor, activating it to reduce neutrophil infiltration, enhance bacterial phagocytic capacity, and promote resolution of tissue inflammation (Chiang et al., 2017)

E-Series Resolvins:

- **RvE1** is synthesized through transcellular biosynthesis, involving the interaction between PMN and vascular endothelial cells under hypoxic conditions. These cells release 18-HEPE, which is subsequently converted to bioactive RvE1. The released 18-HEPE is converted by PMN 5-LO to a 5(6)-epoxide-containing intermediate, which is further converted to RvE1 by the action of the enzyme LTA₄ hydrolase (LTA₄H) in neutrophils (Serhan et al., 2022). RvE1 is formed through a transcellular biosynthetic process, involving human neutrophils and the action of enzymes, such as acetylated cyclooxygenase-2 (COX-2) or cytochrome P450. In summary, the biosynthesis of RvE1 (5S,12R,18R-trihydroxy-eicosapentaenoic acid, 6Z,8E,10E,14Z,16E) from EPA requires the cooperation of multiple enzymes, including COX-2, cytochrome P450, and 5-LO (Ji et al., 2011). ChemR23 has been identified as an RvE1 receptor, it is abundantly expressed in macrophages and dendritic cells (DCs), but apparently not so much in PMN, where it plays a regulatory role to control migration and cytokine production. In addition to ChemR23, RvE1 interacts specifically with the LTB₄ receptor BLT1. BLT1 is highly expressed in PMN, and RvE1-BLT1 interactions regulate leukocyte migration in acute inflammation. Additionally, RvE1 attenuates LTB₄-dependent proinflammatory signals such as intracellular calcium mobilization and NF-κB activation (Arita et al., 2007).
- Endothelial cells play an important role in **RvE2** biosynthesis by oxygenating EPA to 18-HEPE, which is further metabolized by PMN or macrophages. These interactions highlight the cooperative nature of transcellular biosynthesis, ensuring efficient production of resolvins in inflamed tissues. Additionally, epithelial cells are involved in the production of resolvins in mucosal tissues, enhancing localized anti-inflammatory responses (Serhan et al., 2022; Yamaguchi et al., 2022).

Lipoxins:

Lipoxins are derived from arachidonic acid (AA), an omega-6 fatty acid, rather than omega-3 sources such as DHA or EPA (Spite et al., 2014). One of the most studied lipoxins is: **Lipoxin A₄** (LXA₄), which is mainly produced via a collaboration between the AA-acting enzyme 5-LO that generates LTA₄ in PMN, which is converted by platelet 12-LO to LXA₄ and LXB₄ (Romano and Serhan, 1992; Romano et al., 1993). Another pathway of lipoxin generation includes the conversion of AA by 5-LO into an intermediate 5S,6S,15S-epoxytetraene, which is enzymatically converted by 15-LO into LXA₄ and LXB₄ (Serhan et al., 1986). Generation of LX by this pathway also occurs in isolated human PMN and alveolar macrophage (Romano, 2010).

An alternative pathway generates the epimer 15-epi-lipoxin A₄ (15-epi- LXA₄) through aspirin's (ASA) action. This mechanism involves the interaction between endothelial cells (ECs) and PMNs, where ASA acetylates COX-2, also known as prostaglandin H synthase-2 (PGHS-2), in ECs. This acetylation leads to the formation of 15R-HETE, an intermediate subsequently converted by 5-LO in PMNs into 15-epi- LXA₄ (Clària and Serhan, 1995).

LXA₄ is essential for reducing neutrophil activity and enhancing clearance of apoptotic cells, thus ensuring that inflammation does not become chronic (Fu et al., 2020). It binds to the ALX/FPR2 receptor, which is essential for the execution of its anti-inflammatory and pro-resolution functions (Fredman and Spite, 2017).

Protectins:

Another SPM that plays a critical role in resolving inflammation is protectin D1 (**PD1**), which is the product of the dual lipoxygenase 10,17-diHDHA obtained via two sequential steps with reduction of the hydroperoxide intermediate to 10S,17S-diHETE named PDX, an isomer of PD1 (Serhan et al., 2006). PD1 is produced enzymatically by both PMN and macrophages and eosinophils (Perretti, 2015; Serhan and Levy, 2018). When produced in neural systems, PD1 is termed neuroprotectin D1 (NPD1/PD1) and displays powerful protective effects on the nervous system and pain modulation (Serhan and Levy, 2018). In addition to its neuroprotective effects, PD1 also aids in resolving inflammation in other tissues by modulating the activity of immune cells. Its actions are and tissue repair (Bang et al., 2018).

Maresins:

Finally, Maresin 1 (**Mar1**) is another key pro-resolving mediator derived from DHA, a member of the omega-3 fatty acid family (Serhan et al., 2009). The synthesis of Mar1 begins with the activity of 12-LO, which acts on DHA to produce intermediates that are then converted to Maresin 1 (Serhan et al., 2009; Marcon et al., 2013). Mar1, was shown to be a potent mediator, blocking PMN infiltration and stimulating MΦ phagocytosis, thus it may play a key role in tissues to transition from inflammatory state to homeostasis (Serhan et al., 2012).

For this reason, Mar1 has been indicated as a significant mediator in promoting the resolution of inflammation by enhancing macrophage activity, specifically helping these cells to phagocytose and remove cellular debris. This action is critical for restoring tissue function after injury. Mar1 acts through the stereospecific binding to the leucine-rich repeat containing G protein-coupled receptor 6 (LGR6), Mar-1 activates phagocytosis and stops PMN chemotaxis (Chiang et al., 2019).

Overall, SPM and their respective cognate receptors represent an important therapeutic target for an innovative strategy to tackle inflammatory diseases.

Chapter 2

CYSTIC FIBROSIS

2.1 Cystic fibrosis

Cystic fibrosis (CF) is a genetic disorder caused by mutations in the Cystic Fibrosis Transmembrane Regulator (*CFTR*) gene, which lead to dysfunction of the CFTR protein (Gregory et al., 1991).

This protein regulates anion, i.e. chloride and bicarbonate, transportation across cellular membranes. Moreover, it indirectly regulates sodium transport by controlling the epithelial sodium channel (ENaC) (Mall et al., 2004). Thus, CFTR activity is essential to maintain fluid and electrolytic balance in mucus and sweat. In people with CF, CFTR loss-of-function results in the presence of thick, sticky mucus in the lungs, digestive tract, and other secretory organs. This leads to obstruction, tissue damage, inflammation and frequent infection (Ratjen et al., 2015).

CFTR is widely expressed in body's tissue, with high protein amounts present in airway and intestinal epithelial cells, as well as immune cells (e.g., neutrophils, monocytes, macrophages, lymphocytes), and platelets. The broad distribution of CFTR justifies its multiple physiological roles and accounts for the diverse symptoms of CF, which include pulmonary inflammation, as well as gastrointestinal inflammation, and pancreatic disease (Bell et al., 2020; Mall et al., 2024).

The biophysical properties of mucus are regulated by macromolecules called mucins. In the respiratory system, the secretion of gel-forming mucins occurs from both goblet cells and submucosal glands. Goblet cells produce primarily MUC5B and MUC5AC, which create a thin layer of mucus to coat and protect epithelial surfaces. Submucosal glands, however, release exclusively MUC5B, excreting it through glandular ducts in the form of filaments that traverse the large airways to eliminate inhaled pathogens. The biochemical and biophysical properties of mucus produced in these two compartments may be altered differently by CFTR dysfunction, even though both sources participate in mucus secretion. Consequently, the two compartments could have separate functions in the advancement of CF-related lung disease (Morrison et al., 2019).

In diseases such as CF, the use of treatments with the presence of osmotic agents, that is, agents that attract water to airway surfaces, appear to be promising. These agents work by counteracting the effects of epithelial sodium channel (ENaC)-induced water hyperabsorption. Inhaled hypertonic saline has shown clinical efficacy, although its exact mechanism of action remains unclear. Once water enters the airway lumen, it hydrates and expands the saline. The degree of swelling is influenced by mucus concentration: higher mucus concentrations prolong the duration of swelling. This suggests that hyper concentrated mucus in CF provides an additional osmotic driving force, potentially mediated by counterions on mucin glycans. Blocking of ENaC amplifies the response to hypertonic saline, suggesting that sodium reabsorption diminishes the efficacy of aerosolized saline. This highlights ENaC as a promising therapeutic target in CF (Morrison et al., 2019).

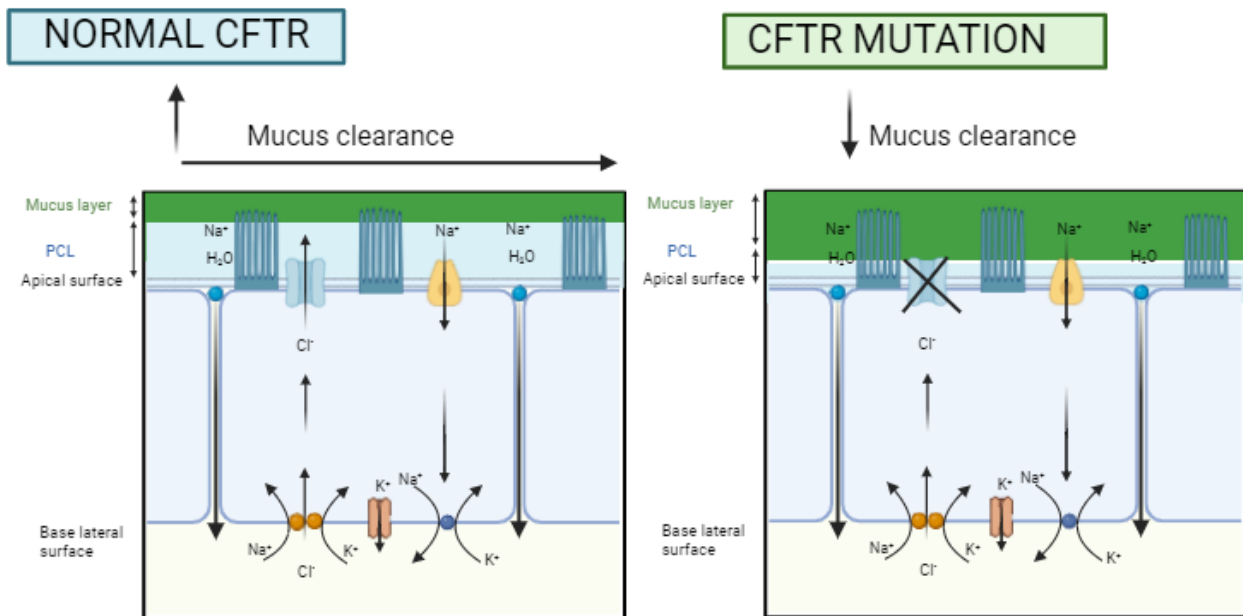


Fig.5 The comparison between "normal CFTR" and "mutated CFTR". (Kumar et al., 2014)

On the left side, with normal CFTR (Cystic Fibrosis Transmembrane Conductance Regulator) activity, chloride ions (Cl^-) are transported out of the cell, leading to proper hydration of the mucus layer and the periciliary liquid (PCL). This promotes efficient mucus clearance, allowing the removal of pathogens and debris from the airways. On the right side, with a mutated CFTR, chloride transport is impaired (indicated by the X). As a result, water and salt balance is disrupted, leading to a thicker mucus layer and reduced PCL height. This condition promotes inflammation, infection and progressive organ damage.

While CF was once largely fatal in childhood, advances in medical care, including early detection through newborn screening, has significantly extended life expectancy of pwCF and children born in recent years have an average life expectancy of over 50 years https://www.ecfs.eu/sites/default/files/publications-free-access-articles/01_ECFS%202018_Yearbook_MAI.pdf.

CF primarily affects the respiratory tract, causing chronic coughing, difficult breathing, and frequent lung infections. However, it can also impact other systems and apparatus, causing delayed growth, infertility, pancreatitis, diabetes and salty skin (Dickinson and Collaco, 2021).

CF is an autosomal recessive disease, meaning that a person must receive two copies of the defective CFTR gene from each parent to develop the disease. Carriers who have only one mutated copy generally do not develop the disease, although they may experience mild symptoms. More than 2,000 mutations in the CFTR gene have been identified, but many of these have not been studied for their impact on the function of the protein, resulting in a wide range of disease severity (Ferec and Cutting, 2012).

Depending on the type of defect, CFTR mutations have been organized in six different classes (see Fig. 6 for details). Class I-III mutations are generally associated with severe forms of the disease,

while class IV, V, and VI mutations leave some residual CFTR function, causing milder symptoms (Dickinson and Collaco, 2021).

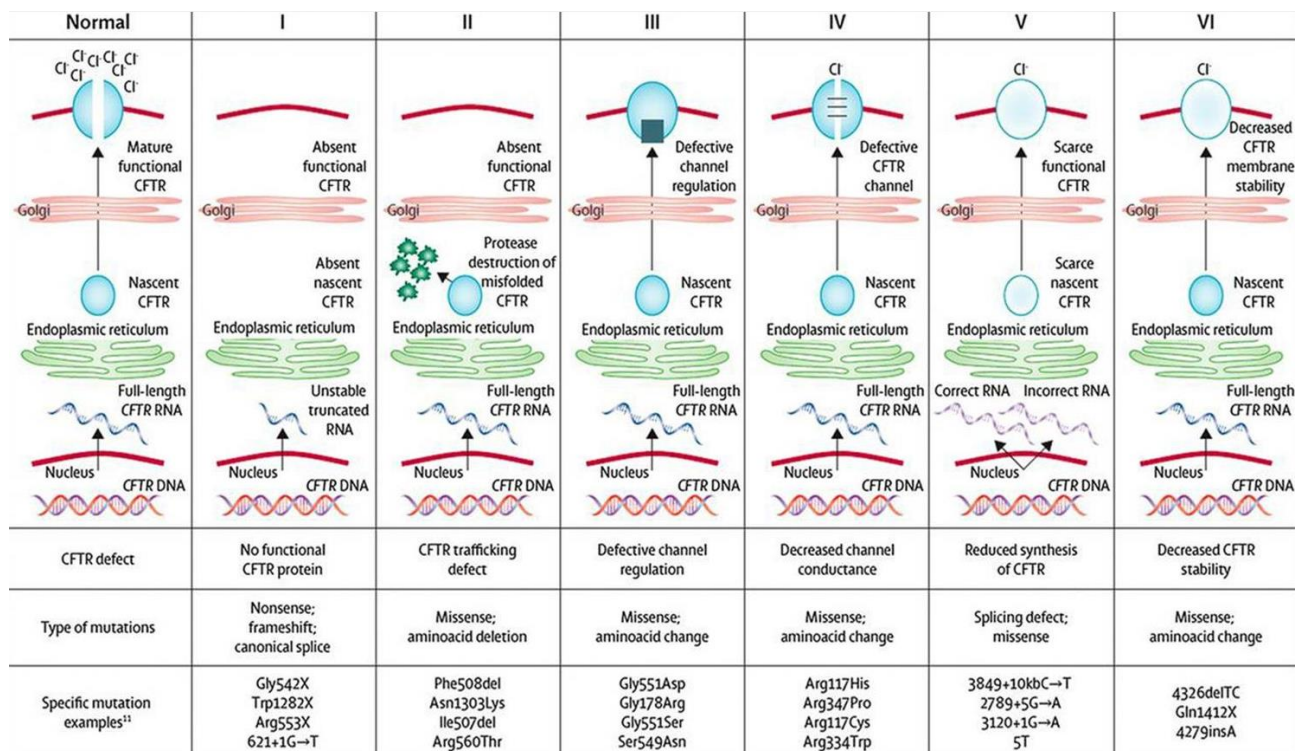


Fig. 6 Classification of CFTR mutations, effects on protein quantity or function, and main human mutations in each class. (Dickinson and Collaco, 2021)

Class I mutations (e.g. G542X) represent approximately 10% of cases (Jia and Taylor-Cousar, 2023). These mutations interrupt protein translation due to the presence of premature stop codons, which result in the synthesis of truncated proteins and the absence of functional CFTR on the cell membrane. In the specific case of G542X, the codon encoding glycine at position 542 (GGA) is mutated into a stop codon (TGA).

The class II F508del-CFTR mutation (delta-F508 or Δ F508) is present in approximately 80% of pwCF. It involves the loss of three nucleotides that code for phenylalanine at position 508 of the polypeptide chain. Although the protein is fully translated, the absence of this phenylalanine causes misfolding in the endoplasmic reticulum, leading to degradation of the protein. Normal CFTR also undergoes proteasomal degradation, but enough amount reaches the plasma membrane to ensure normal chloride transport, which does not occur in individuals homozygous for Δ F508-CFTR. A key therapeutic goal has been to develop drugs that promote the correct folding of Δ F508-CFTR, bypassing cellular quality control mechanisms and restoring protein function (Fraser-Pitt and O'Neil, 2015).

Class III mutations, such as G551D, cause structural and functional changes in the CFTR protein (Jia and Taylor-Cousar, 2023). This missense mutation replaces the glycine (G) at position 551 with

an aspartic acid residue (D), introducing a negatively charged amino acid in place of a nonpolar one. Although the mutated CFTR protein reaches the plasma membrane, the mutation abolishes ATP-dependent gating of the ion channel, maintaining the channel in a predominantly closed conformation. This results in a significant reduction in anion conduction and a severe clinical phenotype (Fraser-Pitt and O'Neil, 2015).

Class IV mutations, such as R117H, result in reduced conductance mutations, thus altering the conductance of the channel by impeding the ion conduction pore (De Boeck et al., 2014; Veit et al., 2016)

Class V mutations do not cause the loss of protein conformation but rather lead to a reduction in normal CFTR levels because of splicing abnormalities (Santos et al., 2023; Veit et al., 2016)

Class VI mutations, including Q1412X and 4326delTC, destabilize the protein, leading to its premature removal from the membrane. They are characterized by defects that lead to reduced stability of the CFTR protein on the cell surface. These mutations result in increased degradation of the protein from the plasma membrane (Veit et al., 2016; Yeh et al., 2019).

Management of CF involves integrated physical and pharmacological treatments to clear thick mucus from the lungs, control infections, and maintain good nutritional health. This approach has considerably extended life expectancy of pwCF. Further progress is expected with the introduction of a new class of drugs comprehensively termed CFTR modulators, which aim to restore the function of the defective protein (see below). Lung transplantation remains the ultimate option when pharmacology fails.

People with CF can lead now more active and fulfilling lives, although they remain at risk of serious complications, such as respiratory failure, pancreatitis, diabetes, and malnutrition.

Early diagnosis is essential to effective management of CF. Newborn screening, which is standard practice in many countries, can identify CF before symptoms appear. The initial test typically measures levels of immunoreactive trypsinogen (IRT) in the blood, followed by a genetic test to confirm the diagnosis. Early intervention with treatments that improve lung function and nutritional status can dramatically improve long-term outcomes, including life expectancy.

Gene therapy is a growing area of interest in CF treatment research. This approach aims to correct the defective CFTR gene by introducing a working copy into the affected cells. Although still in the experimental stages, gene therapy has the potential to address the root cause of CF, leading to a cure in the future.

2.2 The innovation of CFTR modulator therapy

The introduction of CFTR modulators has significantly improved the health status of pwCF. These drugs have been specifically designed to rescue the function of the defective CFTR protein. They do this in many ways, depending on the nature of the mutation that causes the defect.

One of the first major breakthroughs in CF treatment came with the introduction of ivacaftor, a type of CFTR modulator, also termed potentiator, that improves the opening of the CFTR channel, allowing chloride ions to flow more effectively. Ivacaftor is indicated for class III CFTR mutations, such as the G551D, and significantly improved lung function and overall health of pwCF carrying this type of mutations (Jia and Taylor-Cousar, 2023).

These molecules, such as lumacaftor and tezacaftor, facilitate CFTR protein correct folding and trafficking to the plasma membrane(<https://www.trikaftahcp.com/mechanism-of-action>). The association of these molecules with the potentiator ivacaftor has generated the drugs Orkambi and Symdeko, for a better recovery of the function of $\Delta F508$ -CFTR (Jia and Taylor-Cousar, 2023). More recently, the association of two correctors (Elexacaftor and Tezacaftor) and one potentiator (Ivacaftor) (ETI) has led to the development of Trikafta (Ridley and Condren, 2020). Accumulating data indicate that this drug may represent a remarkable game-changer for pwCF, since it significantly improves lung function, reduces pulmonary exacerbations, with a significant overall improvement in quality of life (Bacalhau et al., 2023). In addition to improving lung function, CFTR modulators have also been shown to improve nutritional health in pwCF. by normalizing mucus production in the digestive tract, thereby alleviating the block of the release of pancreatic enzymes caused by the thick mucus, resulting in better nutrient absorption and improved growth (López Cárdenes et al., 2024). A recent study investigated the effects of ETI on innate immune responses and clinical outcomes in individuals with CF. CFTR expression and function, phagocytosis, cytokine production in macrophages, and clinical parameters post-ETI treatment were analyzed. Variable effects on macrophage function, persistent phagocytic deficits, and improvements in clinical outcomes, such as reduced hospitalizations were reported (Zhang et al., 2023).

However, despite these advances, it is important to recognize that CFTR modulators are not a definitive cure for CF. They improve the function of the defective CFTR protein, but do not fully restore it to normal levels. Additionally, these therapies are most effective in patients with specific mutations, meaning that not all individuals with CF may benefit from them. For those with rare mutations who do not respond to current modulators, treatment options remain limited, although ongoing research continues to explore new strategies(Bulloch et al., 2017).

2.3 ENaC channel: revealing its impact on Cystic Fibrosis

In addition to advances in CFTR modulators, CF research has also focus on the role of the epithelial sodium channel and its beta subunit (ENaC). The ENaC plays a critical role in regulating sodium transport in epithelial tissues, including the lung. Composed of several subunits, including the beta

subunit, ENaC is essential for maintaining sodium balance and fluid homeostasis (Aufy et al., 2023). In individuals with CF, CFTR impairment can cause ENaC hyperactivation, resulting in excessive sodium absorption. This hyperactivity contributes to dehydration of airway surfaces and the production of thick, sticky mucus, creating significant challenges to lung function and increasing susceptibility to infection (Mall, 2020). Given this pathophysiological context, targeting ENaC has emerged as a therapeutic target to alleviate dehydration and enhance mucus clearance in CF (Shei et al., 2018). In the airways of pwCF, the absence of CFTR and excessive ENaC activity cause mucus dehydration, leading to obstruction, neutrophil infiltration, and chronic bacterial infections. Therefore, blocking the action of ENaC could improve airway hydration by reducing sodium absorption, thus promoting a more adequate mucus consistency (Moore and Tarran, 2018). The less viscous and more elastic mucus is moved efficiently by ciliary activity and can be eliminated more easily, in fact improving mucociliary clearance reduces the risk of bacterial infections and respiratory exacerbations (Fahy and Dickey, 2010). This is especially important for pwCF, who often have difficulty with mucus clearance and related complications. Furthermore, the combined use of ENaC inhibitors with CFTR modulators could offer a synergistic approach to treatment. In patients, inhibition of hyperactive ENaC in the apical membrane could have two effects. On the one hand, it would block ENaC-mediated sodium and fluid uptake. On the other hand, it would hyperpolarize the apical membrane, increasing the driving force for chloride and fluid secretion. This process may take place via mutated CFTR channels, rescued and inserted into the membrane by CFTR modulators (Mall, 2020). Research into ENaC inhibition is actively ongoing, with clinical trials investigating the safety and efficacy of various ENaC inhibitors. Early results have shown promise, indicating potential benefits in lung function and overall health (Goss et al., 2020). For example, compounds such as amiloride, which is known to inhibit ENaC, are being evaluated for their ability to improve airway hydration and reduce mucus viscosity (Lemmens-Gruber and Tzotzos, 2023). The potential benefits of targeting ENaC go beyond lung function; improved mucus clearance could also improve the deficit in absorption of nutrients, a complication seen in children with CF.

2.4 Actions of RvD1 in Cystic Fibrosis

The pro-resolving properties of Resolvin D1 (RvD1) have been the focus of several recent studies, particularly in CF models related to chronic inflammation. In my PhD program, I demonstrated that RvD1 not only alleviates inflammatory responses in the airways of CF patients, but also significantly improves exercise and respiratory capacity in mouse models of CF-associated lung disease. For this, I used β ENaC-Tg mice, which develop a mucus layer similar to that observed in CF patients, to evaluate the effects of RvD1 on inflammation resolution and improvement in lung function and exercise. RvD1 was shown to significantly reduce the presence of neutrophils and macrophages in the lungs of these mice, helping to attenuate chronic inflammation related to the accumulation of thick mucus. Furthermore, RvD1 promoted the resolution of pulmonary exacerbations caused by

infections with *Pseudomonas aeruginosa*, a bacterium commonly associated with the progression of lung disease in CF patients (Codagnone et al., 2018; Isopi et al., 2020).

This study also demonstrated improvements in exercise capacity in β ENaC mice treated with RvD1. Specifically, mice treated with RvD1 demonstrated increased exercise tolerance and improved energy expenditure, reducing disease-induced metabolic stress. These results suggest that RvD1 not only reduces airway inflammation but also improves the quality of life of pwCF by promoting tissue healing without compromising immune defenses.

2.5 Results:

2.5.1. Resolvin D1 reduces airway mucus-driven inflammatory responses of neutrophils and airway epithelial cells.

Mucus that accumulates in the airways of people with CF disrupts airway homeostasis and produces pro-inflammatory effects on epithelial cells (Fritzsche et al., 2015; Ribeiro et al., 2009; Zhou-Suckow et al., 2017) and leukocytes (Ford et al., 2021; Gehrig et al., 2014; Hey et al., 2021; Margaroli et al., 2021; Trojanek et al., 2014). To determine whether RvD1 modified responses of CF leukocytes and epithelial cells to ASN containing stimulatory factors derived from sputum of patients with CF, as it occurs following noxious (e.g., bacterial infection) stimuli (Isopi et al., 2020; Recchiuti et al., 2021), we measured loss of L-selectin (CD62L) and of the phagocytic receptor CD16 from cell surface of blood PMN migrating across CFBEc as a readout of their acquisition of a pathogenic phenotype in CF airways. Indeed, CD62L is important for mediating PMN migration and CD16 is important for PMN phagocytosis. As shown (Fig. 7), ASN was sufficient to induce a significant CD62L and CD16 membrane loss in transmigrated PMN, confirming its dominant role in priming their pathological condition. RvD1 (10 nM) significantly diminished L-selectin shedding and CD16 downregulation. Of note, when we assessed chemical integrity of RvD1 in CF ASN (which may contain oxidant species and enzymes from airway cells) we found that ~ 80% [median = 80.43, 71.82 (25th percentile), 86.46 (75th percentile)] of RvD1 input could be recovered intact after 4 h of incubation as determined with HPLC-UV spectrum analysis and matching of retention time with authentic RvD1 standard (not shown). A major RvD1 further metabolism product with anticipated retention time and conserved UV absorbance was identified following incubation with CF ASN, which is likely to correspond to an “all-trans” RvD1.

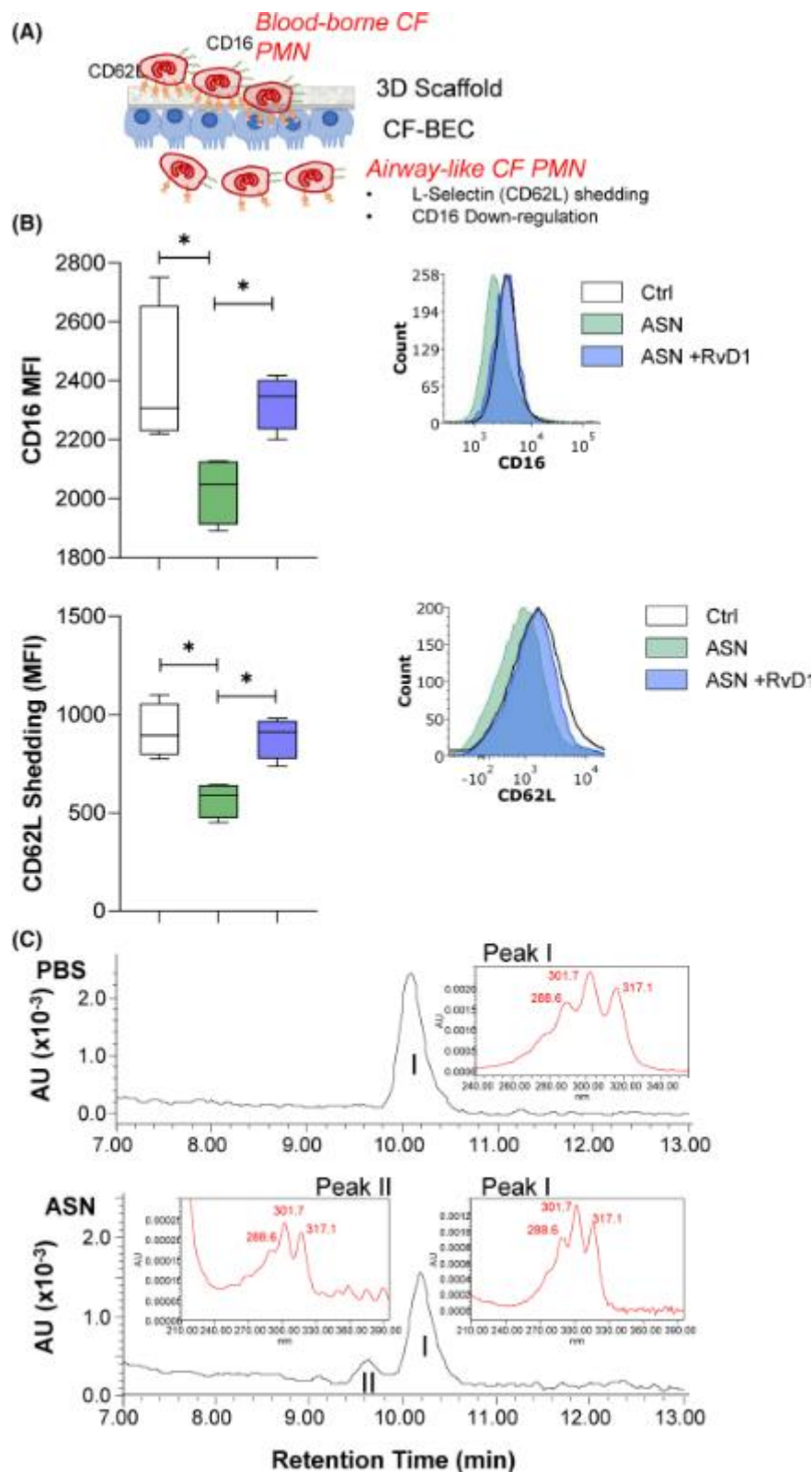
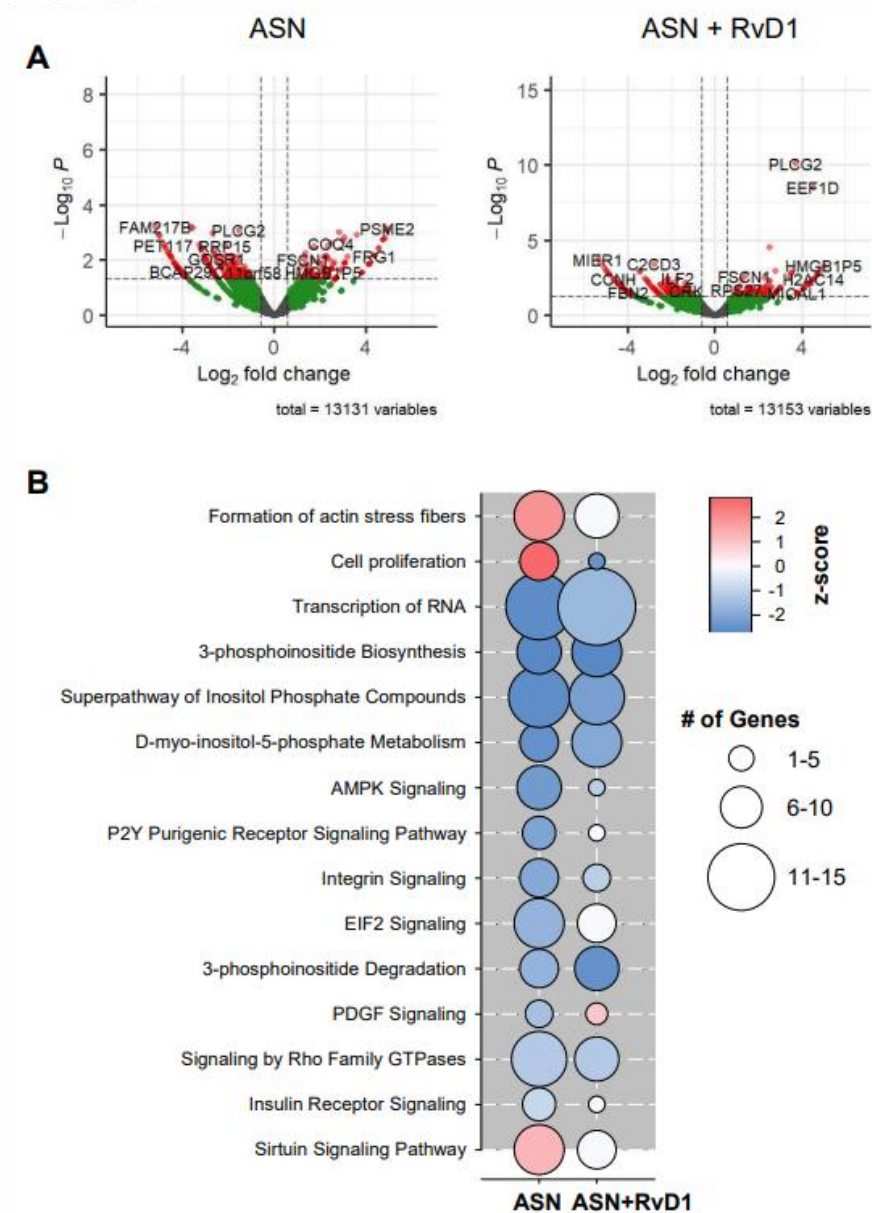


Fig.7 RvD1 regulates CF PMN pathological conditioning.

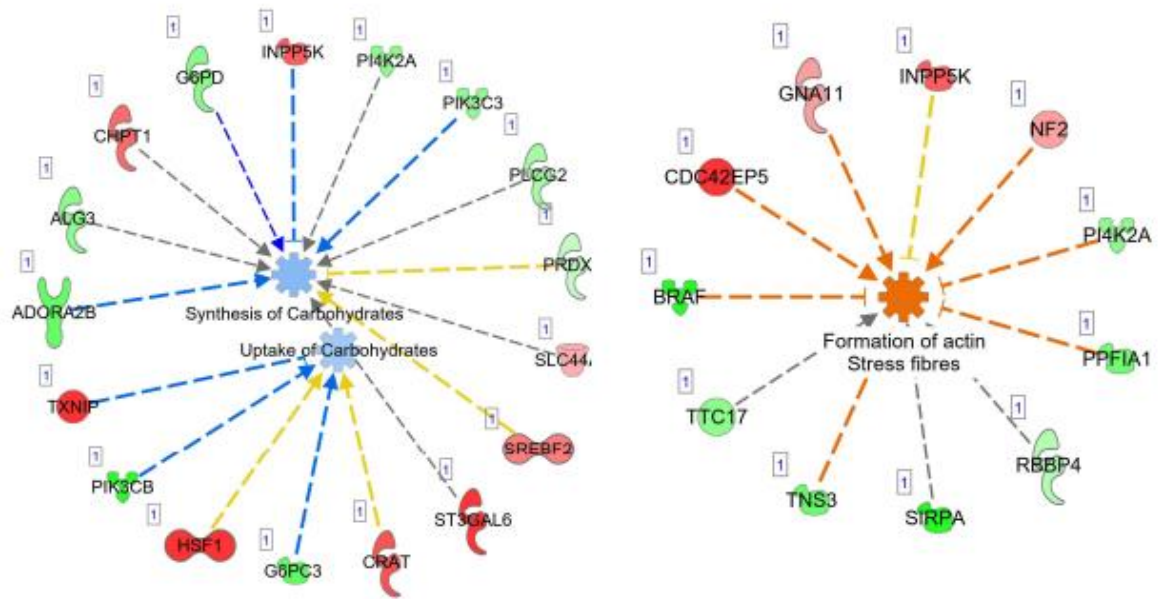
(A) In vitro model mimicking PMN pathological changes (CD62L shedding and CD16 downregulation) in CF airways based of transepithelial transmigration (see Ref.(Forrest et al., 2018)). Primary CFBEK grown (2–4 weeks) as in Ref.(Isopi et al., 2020) at air-liquid interface on porous 3D scaffolds (Alvetex) were placed with the apical side exposed to medium (M) alone or mixed with airway supernatants (ASN) from CF volunteers (1:30) and/or RvD1 (10 nM). Blood PMN (1×10^6 /well) from volunteers with CF were loaded on the upper compartment in medium or RvD1 (10 nM). (B) Surface expression of

CD62L and CD16 determined with FACS analysis of CF PMN transmigrated (3 h) across CFBEC exposed to ASN. Results are median and interquartile range ($n = 4$ experiments with cells from different volunteers). $*p < .05$ (Kruskal-Wallis test). Representative flow cytometry histograms are in right inserts. (C) HPLC analysis of RvD1 after incubation in ASN obtained from CF donors. Spontaneous sputum (~5 mL) collected from volunteers with stable CF (FEV1 > 70%) was gently dissociated after addition of 10 mL of PBS and centrifuged (300 g, 7 min; 3000 g, 10 min) to generate a cell/bacteria free airway supernatant. RvD1 (50 ng) was incubated in 0.5 mL ASN at 37°C for 4 h, after which 1 mL of MeOH was added and samples were taken to dryness prior injection into a C18-HPLC system for determining RvD1 isomerization. RvD1 Representative HPLC chromatograms are shown in the inserts displaying UV-spectra of the corresponding peaks.

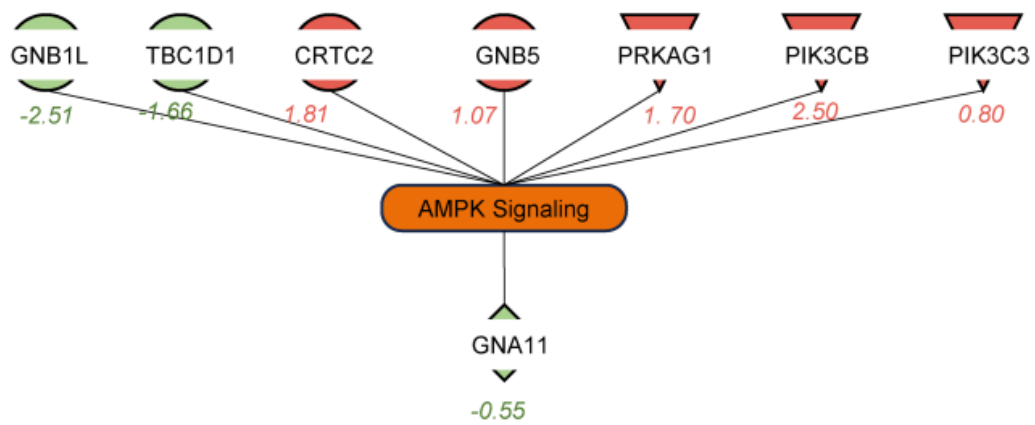
Based on these findings, we carried out RNASeq analyses to evaluate global changes in transcriptome of CFBEC exposed to ASN and if RvD1 had effects on these changes. Among the 13,131 mRNA that were mapped and showed variances in ASN-treated CFBEC vs control (CFBEC exposed to buffer devoid of ASN), we identified 142 and 245 genes significantly (adjusted P-value < 0.05) up- or downregulated ($\log_2$ fold changes > |0.58|) in response to ASN. RvD1 treatment resulted in a significant upregulation of 122 genes and downregulation of 245 transcripts (out of 13,152 mapped variables) compared to ASN (Fig. 8A). IPA-based bioinformatics analysis revealed that biochemical pathways and functions associated to sugar metabolism, biosynthesis and degradation of inositol phosphate and 3-phosphoinositide, insulin signaling, and RNA transcription were significantly inhibited by ASN (IPA z-score < -1.5). In contrast, cell proliferation, sirtuin signaling, and formation of actin stress fibers were amongst the pathways activated by ASN (Fig. 8B). For instance, ASN significantly altered expression of glucose-6-phosphatase C3 (G6PC3), glucose-6-phosphate dehydrogenase (G6PD), and several phosphatidylinositol 3-kinases (PIK3) leading to the inactivation of sugar uptake and biosynthesis and sirtuin, while it activated stress fiber formation by modifying (among other genes) the kinases BRAF and phosphatidylinositol 4-kinase (PI4K2A) and the phosphates signal regulatory protein alpha (SIRPA) and inositol polyphosphate-5-phosphatase (INPP5K) (Figure 8C). In contrast, IPA analysis demonstrated that RvD1 determined global shifts of pathways activated or inhibited in CFBEC exposed to ASN. For example, RvD1 blunted formation of stress fibers, cell proliferation, and sirtuin signaling pathways (Figure 8B) and restored the AMPK by regulating several kinases, including PIK3CB, PIK3C3, and protein kinase AMP-activated (PRKAG1) (Figure 8D). Moreover, RvD1 treatment resulted in inactivation of pathways involved in inflammation, including NF- κ B, interferons, engulfment of leukocytes, and immune responses (Fig. 8E). Overall, these results indicate that RvD1 abates inflammatory responses induced by CF mucus-derived factors on neutrophils and epithelial cells.



C



(D)



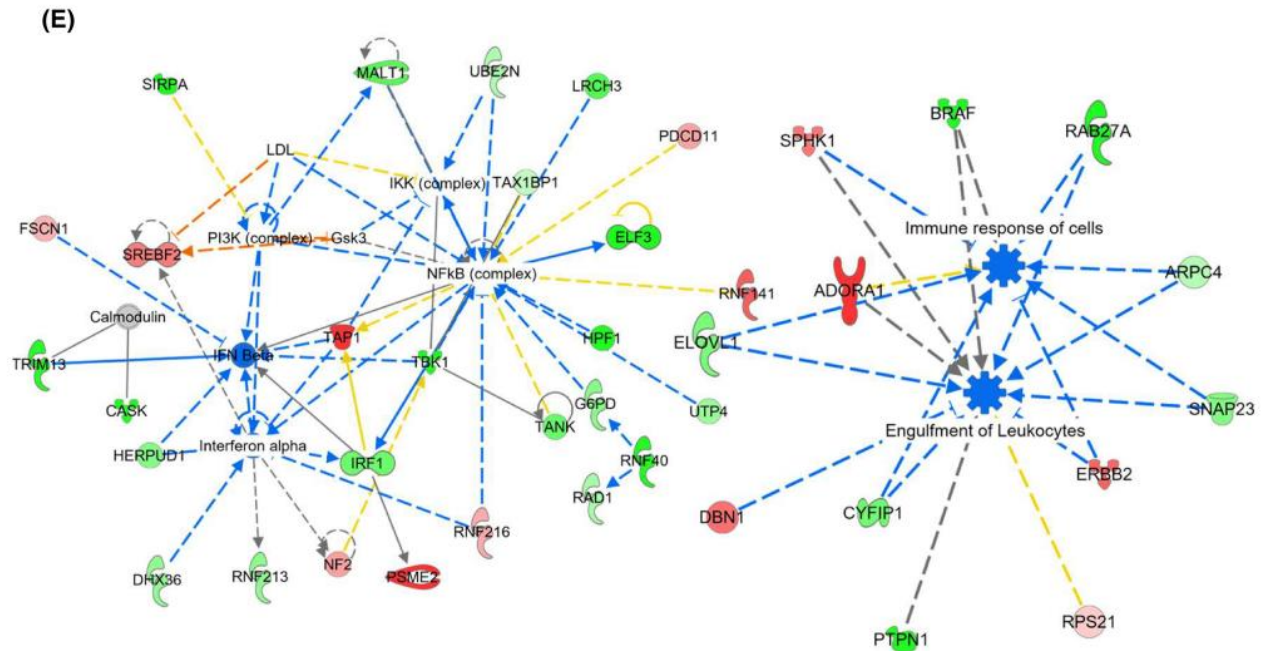


Fig. 8 RvD1 restores CF bronchial epithelial cell homeostasis disrupted by ASN.

(A) Volcano plots of differentially expressed genes identified with transcriptomic analysis alone or with RvD1 compared to CFBEC exposed to cell culture medium devoid of ASN. Genes below the $\log_2$ fold change $> |0.58|$ and $p < .05$ (with false discovery rate < 0.1) cut-off are shown in red. (B) Pathway analysis of differentially expressed genes derived from the IPA bioinformatic analysis tools. Pathways are color-coded and sized based on the IPA z-score and number of beneath genes. (C–E) Functional roles and interactions of differentially expressed genes in CFBEC exposed to ASN (C) or ASN + RvD1 (D and E) identified using the IPA analysis tools. Symbols are colored based on differential expression (red = upregulated genes; green = downregulated genes; blue lines and symbols = blocked pathways and functions; yellow-orange lines and symbols = activated pathways and functions). Results are mean from analyses carried out 3 times with samples derived from cells different donors treated in separate experiments.

2.5.2. Resolvin D1 reduces airway inflammation due to mucus obstruction in vivo

Given these findings, we sought to investigate RvD1 actions on lung inflammation driven by mucus accumulation in vivo. To this end, we treated with RvD1 specific pathogen-free β ENaC-Tg mice, which have dehydrated airway surface liquid, impaired mucus transport, and spontaneous mucus obstruction with lung disease that shared key features with people suffering from CF including chronic neutrophilic airway inflammation (Mall et al., 2004). As expected from previous studies (Mall et al., 2004), adult (10 week-old) β ENaC-Tg mice had significantly higher numbers of leukocytes in bronchoalveolar lavage (BAL) compared to age- and sex-matched WT littermates, with increased airway neutrophilia and M Φ (Fig. 9).

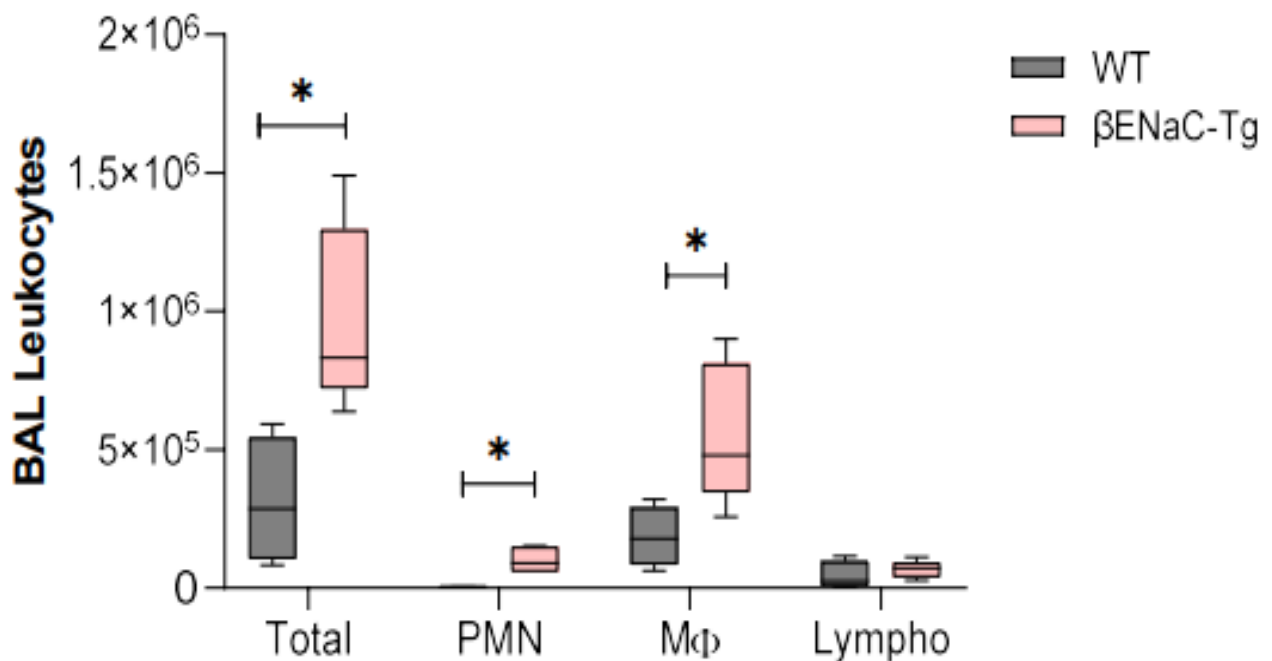


Fig.9 Characterization of lung inflammation in β ENaC Tg mice (BAL leukocyte total and differential counts.

Total PMN number, Macrophages and Lymphocytes of total BAL leukocytes quantified at 10 weeks in β ENaC mice compared with WT mice. Box-whisker plots show 25th and 75th percentile range (box) with 95% confidence interval (whiskers) and median values (transverse lines in box) from $n = 5$ mice for group). * $P < 0.05$ (Mann Whitney test).

To determine if RvD1 could stop the development of CF-like lung disease triggered by mucus obstruction, β ENaC were given RvD1 (100 ng every 2 days by I.P.) or vehicle from 6 to 8 weeks of age. Treatments of β ENaC mice with RvD1 resulted in a significantly lower number of infiltrated total leukocytes and PMN at the end of the two weeks of administration. RvD1 also significantly decreased Siglec- BAL M Φ (Fig.10), whereas total BAL M Φ were not significantly different when comparing RvD1-treated vs veh-treated mice (Fig.11).

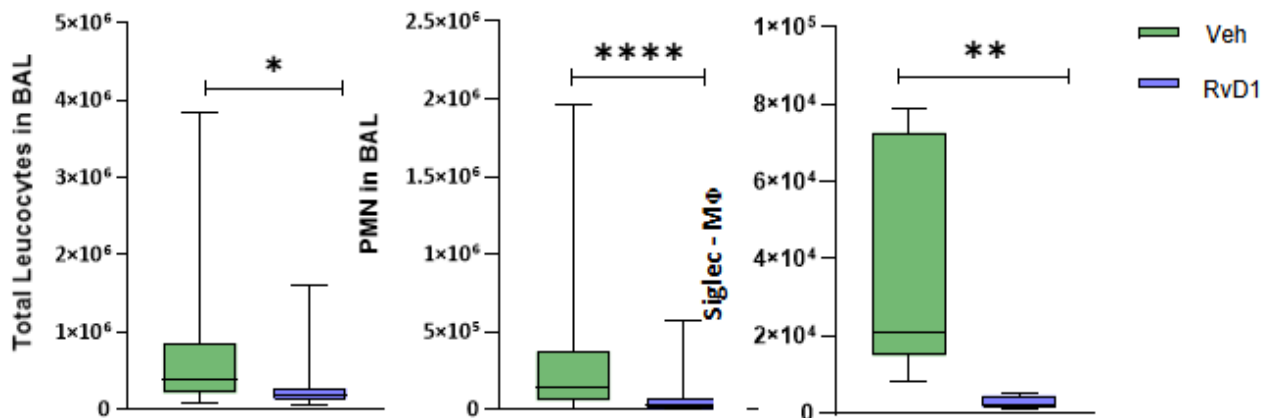


Fig.10 Effects of RvD1 treatment on lung disease development in β ENaC mice.

Total leukocyte count, PMN number, and M Φ in BAL from 6-week-old β ENaC mice treated with vehicle (0.5% EtOH) or RvD1 (100 ng). Mice received treatments q.a.d for 2 weeks via I.P. Box-whisker plots show 25th and 75th percentile range (box) with 95% confidence interval (whiskers) and median values (transverse lines in box) from $n = 15$ mice/group. * $p < .05$; ** $p < .01$; **** $p < .0001$ (Mann-Whitney test).

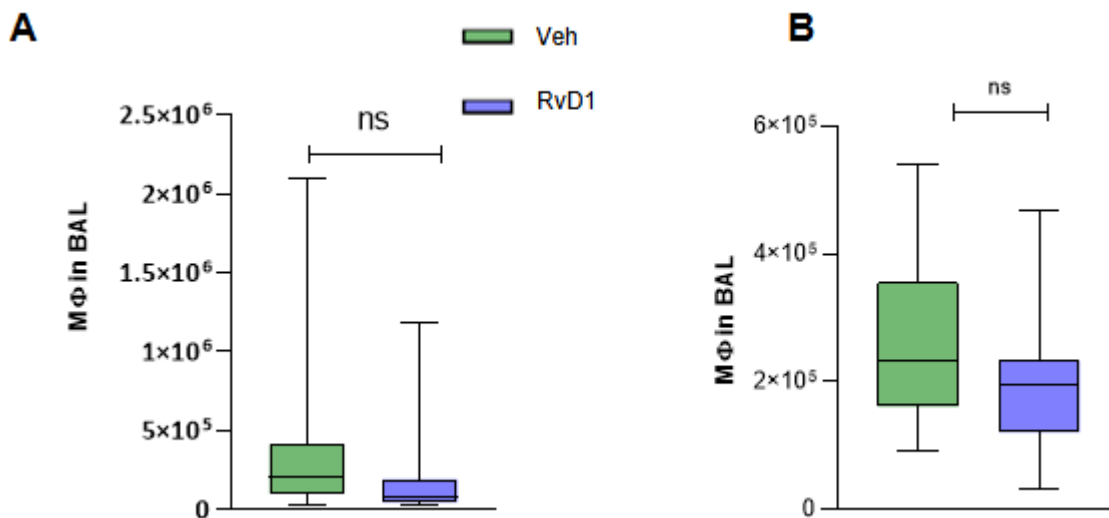


Fig.11 Numbers of total MΦ in BAL from RvD1- or vehicle treated βENaC mice.

Total MΦ (F4/80+) BAL leukocytes were determined in 6-week-old (**panel A**) or 10-week-old (**panel B**) βENaC mice receiving RvD1 (100 ng) or vehicle (0.5 % EtOH) q.a.d, I.P using flow cytometry. Box–whisker plots show 25th and 75th percentile range (box) with 95% confidence interval (whiskers) and median values (transverse lines in box) from n = 15 mice for group). * P = ns (Mann Whitney test).

Subsequently, based on these indications of homeostatic roles of RvD1 in early phases of lung disease in β ENaC mice, we sought to determine if RvD1 could revert this lung dysfunction in older mice. To this end, we treated β ENaC-Tg mice at 10-12 weeks of age with RvD1. In these mice, we still observed reduction in total leukocytes, PMN, and Siglec-M Φ compared to vehicle (Fig.12A). Moreover, RvD1 significantly decreased BAL concentrations of KC (the main PMN attractant chemokine), TNF- α , G-CSF (main M Φ -derived pro-inflammatory cytokines), and VEGF (key pro-angiogenic factor), which were higher in β ENaC-Tg mice treated with vehicle compared to WT, while it increased the anti-inflammatory protein IL-10 (Fig.12B).

Hence, these results indicate that RvD1 reduces cellular and soluble components of mucus-induced airway inflammation in β ENaC-Tg mice at different ages.

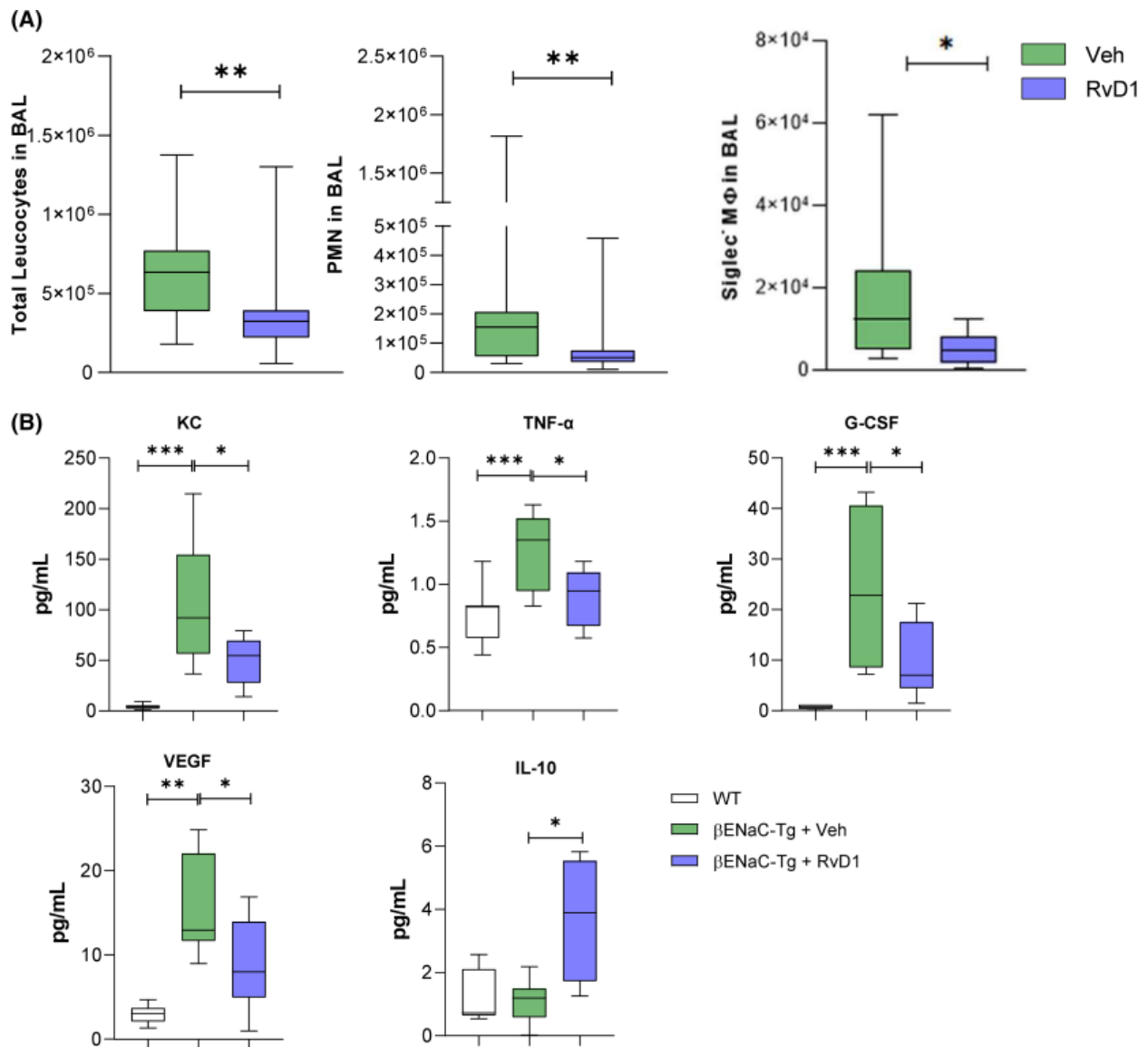


Fig.12 RvD1 corrects lung disease in old βENaC mice.

(A) Total leukocyte count, PMN number, MΦ in BAL from 10-week-old in WT, or βENaC mice with vehicle (0.5% EtOH) or RvD1 (100 ng). (B) soluble mediators from 10-week-old in WT, or βENaC mice with vehicle (0.5% EtOH) or RvD1 (100 ng). Mice were treated q.a.d. for 2 weeks via I.P. Box-whisker plots show 25th and 75th percentile range (box) with 95% confidence interval (whiskers) and median values (transverse lines in box) from $n = 15$ mice/group. * $p < .05$; ** $p < .01$, *** $p < .001$ (Mann-Whitney test or ANOVA on Ranks).

2.5.3. Resolvin D1 reduces *P. aeruginosa* infection in β ENaC tg mice

Given the essential role of *P. aeruginosa* infections in pulmonary exacerbations and the progression of CF lung disease (Konstan et al., 2012), we tested β ENaC mice with a infection with the RP73, a clinical strain isolated from a patient with CF at a late stage of infection (Codagnone et al., 2018) . As shown in Figure 13, following inoculum with RP73, β ENaC mice had a significantly higher loss of body weight, bacterial load, and inflammatory cells in BAL compared to WT, confirming that in β ENaC mice, like in people with CF, inflammation that predates infection is incapable to clear pathogens.

Administration of RvD1 (100 ng, I.P. once a day starting 3 h post infection) significantly improved weight recovery following infection and diminished bacterial load, total leukocyte, PMN number in BAL. Moreover, both total and interstitial M Φ were significantly decreased in β ENaC mice treated with RvD1. Interestingly, the effect of RvD1 on macrophages was distinct in this infectious condition compared to sterile inflammation in β ENaC mice. It is possible that this opposite effect could arise from different expression of RvD1 receptor in these cells depending on the inflammatory condition. This hypothesis warrants further investigation. Thus, RvD1 reduces mucus-driven lung inflammation exacerbated by *P. aeruginosa* superinfection in β ENaC mice.

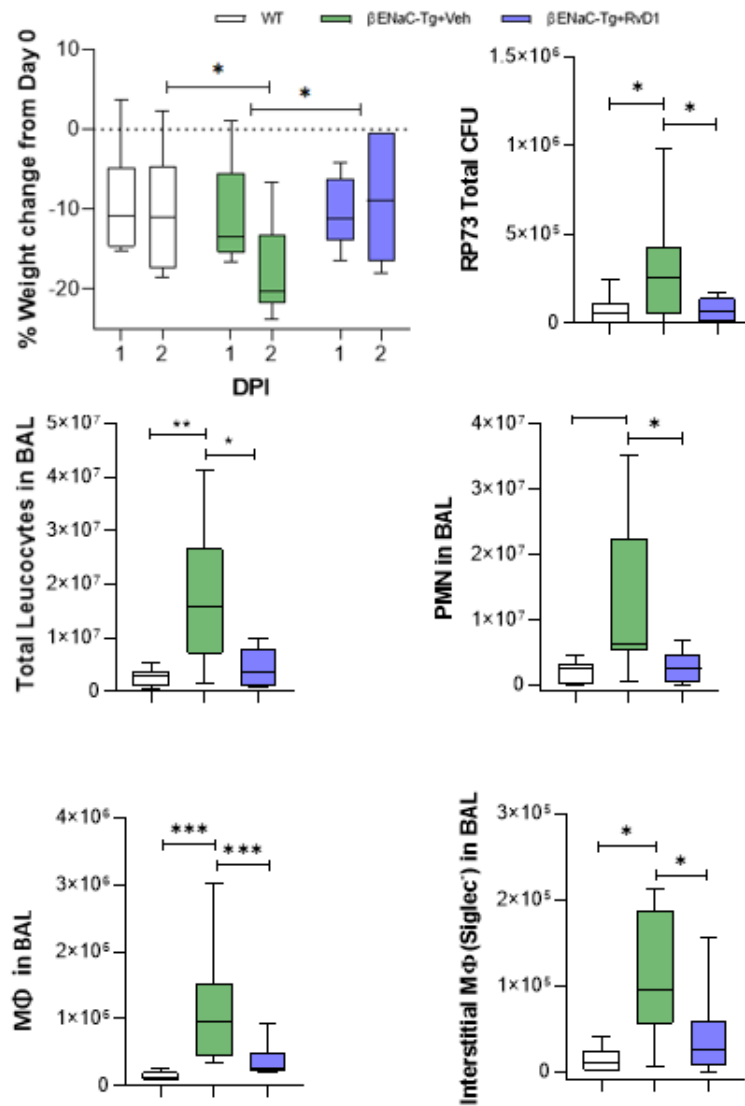


Fig.13 RvD1 resolves exacerbation of lung inflammation in β ENaC mice following *P. aeruginosa* infection.

Body weight, bacterial counts, BAL total and differential cell numbers from WT or β ENaC mice infected with RP73. Mice were treated with RvD1 (100 ng) or vehicle (0.5% EtOH) I.P. once a day starting 3 h post infection. Bacteria and leukocytes were quantified 2 days post infection (DPI). Box-whisker plots show 25th and 75th percentile range (box) with 95% confidence interval (whiskers) and median values (transverse lines in box) from $n = 7$ mice/group. * $p < .05$; ** $p < .01$; *** $p < .001$ (Mann-Whitney test).

2.5.4. Resolvin D1 improves physical activity of β ENaC mice

Since people with CF have a marked intolerance to physical exercise related to both pulmonary function and infection (Burtin and Hebestreit, 2015), we determined energy expenditure (EE) and physical activity of β ENaC compared to mice that do not have lung disease. Results from indirect calorimetry indicated that β ENaC mice have increased VO_2 consumption (Fig. 14A and D) compared to WT [area under the curve (AUC) VO_2 603.70 ± 28.88 mL/min/Kg $^{0.75}$ vs. 508.80 ± 13.18 mL/min/Kg $^{0.75}$]. This increase was maintained all day (dark and light cycle), suggesting a higher O_2 demand of β ENaC to produce energy even in resting conditions. VCO_2 was significantly higher in β ENaC mice than WT (Fig. 14B and E) (AUC CO_2 444.10 ± 20.42 mL/min/Kg $^{0.75}$ vs. 383.80 ± 13.72 mL/min/Kg $^{0.75}$). Respiratory quotient (RQ), which is the ratio between VCO_2 produced and VO_2 consumed, is indicative of the principal energetic substrate utilized by mitochondria to produce energy (Even and Nadkarni, 2012). RQ values shift depending to the energetic substrate metabolized, because the $\text{VCO}_2 / \text{VO}_2$ ratio is higher in glucose than in lipids: a ratio between 0.7 and 0.8 indicates the prevalent use of lipids and proteins as substrates, whereas a ratio above 0.8 indicates increasing use of carbohydrates as main source of energy. Here, no differences were found in RQ between β ENaC-Tg and WT mice (Fig. 14C and F), 0.73 ± 0.01 vs. 0.75 ± 0.02 . These results were reinforced by EE data (Figure 14G), showing an average increase by 40% in β ENaC mice during all circadian cycle when compared to WT (178.40 ± 7.29 Kcal/day/kg $^{0.75}$ vs. 152.40 ± 4.31 Kcal/day/kg $^{0.75}$). The data collected indicates that mitochondria from β ENaC mice require more O_2 and effort to provide to the basal energetic demands of the body. However, absence of RQ value shift toward a more lipid-oriented metabolism to supply the increased energetic demand, suggesting that a balance in the energetic substrate use is still maintained.

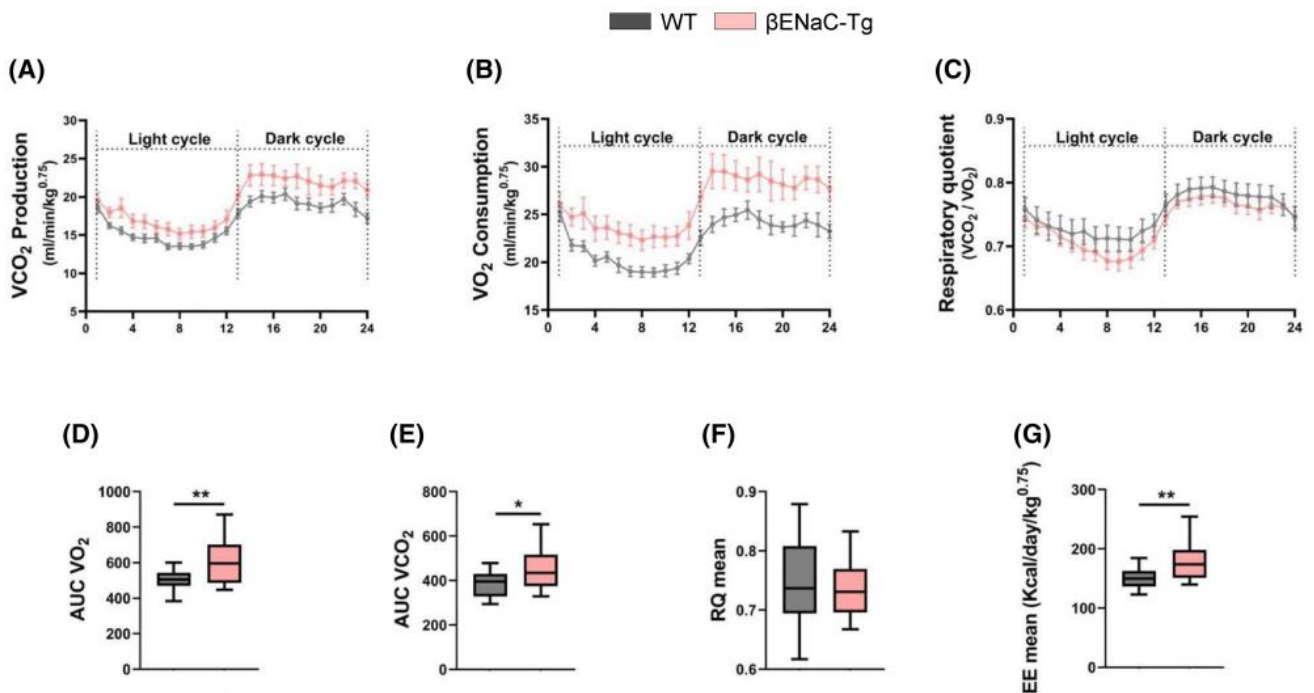


Fig.14 Altered VO₂ consumption, CO₂ production, respiratory quotient, and energy expenditure in β ENaC mice.

(A and B) Oxygen consumption and CO₂ production expressed as mL/min/kg^{0.75}. (C) Respiratory quotient (RQ) expressed as VCO₂/VO₂ during 24 h. (D and E) Area under the curve (AUC) of O₂ consumption (panel D) and CO₂ production (panel E). (F and G) Mean values of Respiratory Quotient (RQ) and Energy expenditure (EE). Legend: Light cycle (8 AM to 7:59 PM); dark cycle (8 PM to 7:59 AM). Data are shown as mean $\pm$ SEM (WT $n = 16$; β ENaC $n = 19$) of data recorded for 24 h. * $p < .05$ as evaluated by Student's t test followed by Tukey's post-hoc test. n = number of mice.

Next, we assessed tolerance to aerobic exercise of β ENaC mice, subjecting them to an incremental speed test during which O₂ consumption and CO₂ production were finely registered to assess respiratory metabolism during physical activity. As shown in Fig. 15A, β ENaC-Tg mice displayed lower tolerance to exercise than WT. Indeed, the distance run by β ENaC mice was lower (379.20 ± 15.23 m vs. 507.8 ± 33.55 m) than WT. Moreover, the maximal speed reached during the test by β ENaC was inferior to that reached by WT mice (Fig.15B) (25.00 ± 2.17 m/min vs. 29.4 ± 3.41 m/min). On the contrary, β ENaC-Tg mice displayed enhanced EE per meters run than WT (Fig.15C) (7.22 ± 0.24 (Kcal/day/kg^{0.75})/m vs. 6.27 ± 0.41 (Kcal/day/kg^{0.75})/m). Therefore, results from incremental speed tests confirmed and reinforced those obtained in standard housing conditions, demonstrating that β ENaC mice clearly show an excessive O₂ demand and EE during the running, enhancing their intolerance to exercise in comparison to WT mice, mirroring the clinical hallmarks of people with CF.

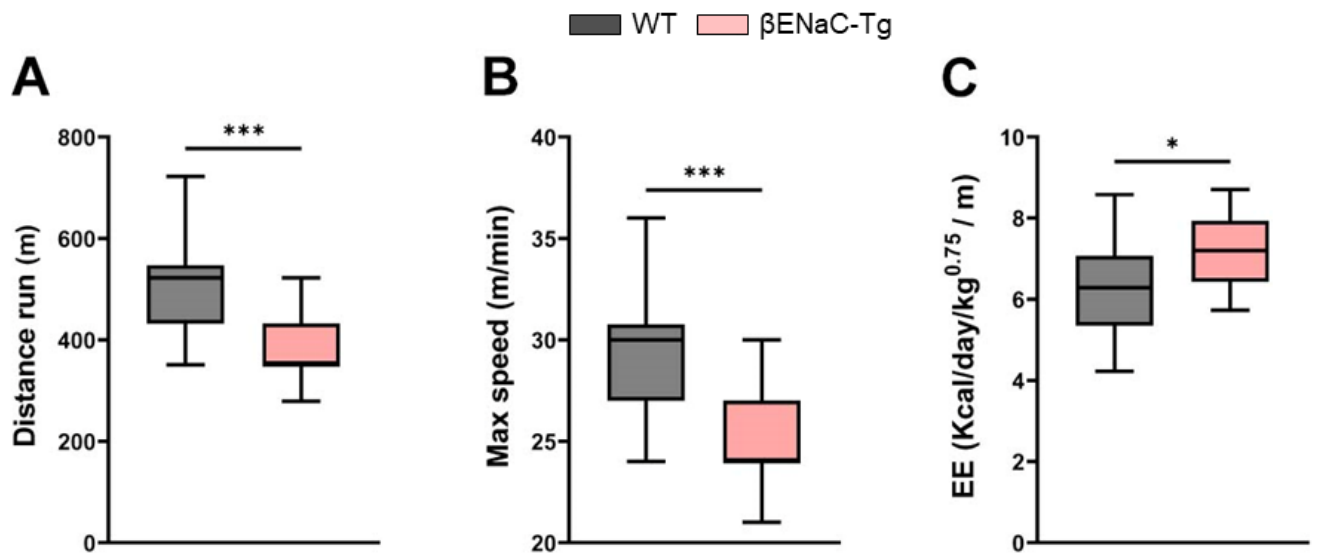


Fig.15 Impaired physical activity of β ENaC mice during incremental speed test.

(A) Distance run by WT or β ENaC-Tg mice during the test. (B) Maximal speed reached by each mouse during the test. (C) Energy expenditure (EE) per meters run expressed as (Kcal/day/kg^{0.75})/m. Data are shown as mean $\pm$ SEM (WT $n = 16$; β ENaC $n = 19$). * $p < .05$ as evaluated by Student's t test followed by Tukey's post-hoc test.

The same incremental speed test was performed in β ENaC-Tg mice (10-week-old) after a 2 week treatment with RvD1 (100 ng q.a.d, I.P.). As shown in Fig. 8, RvD1 treatment ameliorated exercise tolerance of β ENaC mice. In details, RvD1 treated mice covered a distance 17% higher than no-treated mice (Fig.16A) (446.10 ± 22.55 m vs. 379.20 ± 15.23 m). Moreover, the maximal speed was increased by about 10% (Fig.16B) (27.43 ± 0.78 m/min vs. 25.00 ± 0.56 m/min) by RvD1, which also rectified the augmented EE in β ENaC-Tg mice (5.42 ± 0.28 Kcal/day/kg^{0.75}/m vs. 7.22 ± 0.24 Kcal/day/kg^{0.75}/m) (Fig. 16C). Therefore, RvD1 positively modifies EE reducing the energy demand required to sustain the running task, improves physical exercise, and lowers airway inflammation in β ENaC-Tg mice.

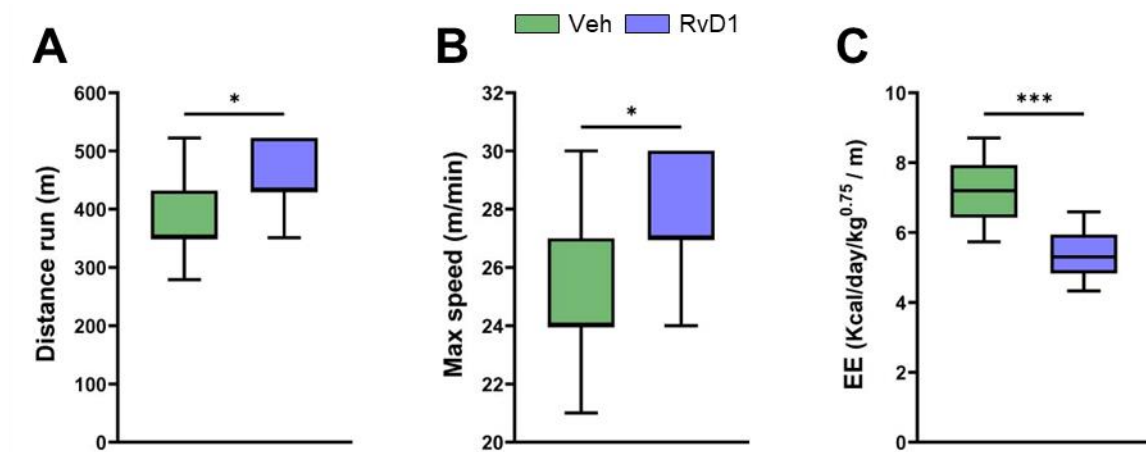


Fig.16 Impaired physical activity of β ENaC mice during incremental speed test is improved by RvD1.

(A) Distance run by β ENaC mice treated with vehicle (0.5% EtOH) or RvD1 (100 ng q.a.d. for 2 weeks via I.P). during the test. (DB) Maximal speed reached by each mouse during the test. C) Energy expenditure (EE) per meters run expressed as (Kcal/day/kg^{0.75})/m. Data are shown as mean $\pm$ SEM (WT $n = 16$; β ENaC $n = 19$). * $p < .05$ as evaluated by Student's t test followed by Tukey's post-hoc test.

2.6 Discussion

In the present study we report that RvD1 dampens mucus-driven inflammatory responses in human cells from people with CF and in β ENaC mice with CF-like muco-obstructive lung disease. RvD1 also improves exacerbation of lung inflammation during *P. aeruginosa* infection and restores physical activity of β ENaC mice.

The classical model of CF pathophysiology points towards defects in CFTR activity as the underlying cause of altered Cl⁻ and water homeostasis in the airway. This is believed to lead to the thickening and stasis of the mucus that entraps bacteria and impairs mucociliary clearance contributing to a perpetual self-feeding cycle of infections, secondary inflammation, and collateral tissue damage. However, a series of observations have demonstrated that inflammation and mucus plugging are already evident prior to detectable bacterial colonization in young children with CF (Balough et al., 1995; Khan et al., 1995; Sly et al., 2009), in tracheal grafts from CF fetuses (Tirouvanziam et al., 2000), and in mice with CF-like airway surface dehydration (Fritzsche et al., 2015; Livraghi-Butrico et al., 2012; Mall et al., 2004, 2008), indicating that sterile CF-mucus per se constitutes a sufficient environmental inciting of factor airway inflammation that precedes pathogen colonization (reviewed in (Balázs and Mall, 2019)). Although PMN are terminally differentiated leukocytes, several studies (Forrest et al., 2018; Laval et al., 2013; Margaroli et al., 2021) indicate that CF mucus modify phenotypes of neutrophils recruited into the airways via miRNA and protein synthesis, including increase of anabolic pathway and downregulation of antimicrobial genes. Using CFBE4-2C exposed to CF ASN we found that the molecular components of mucus alter epithelial cellular and biochemical pathways that impact their structure (e.g., induction of stress fibers), proliferation, and metabolism (e.g., reduction in carbohydrate synthesis and uptake) converging towards inflammatory mechanisms. Hence, these results contribute towards a better understanding of basic pathophysiological mechanisms of CF associated with mucus accumulation.

Here, we present that RvD1 is chemically stable in CF ASN and rescues loss of L-selectin and CD16 by CF neutrophils while dampening their degranulation and transmigration across CF epithelia (Fig. 7), which are key determinants of PMN-mediated, relentless, and tissue damaging lung inflammation in people with CF. RvD1 originally identified in vivo in resolving murine exudates, is present in human plasma and airway exudates from patients with CF as demonstrated by two independent studies (Eickmeier et al., 2017; Serhan et al., 2002). In their work, Eickmeier and colleagues hypothesized that sputum RvD1, rather than a bystander, could identify patients with CF having better control of neutrophil accumulation in their lungs and respiratory function. RvD1 reduces leukocyte infiltration in the airways in COPD (Hsiao et al., 2015), stops PMN transepithelial migration (Sun et al., 2007) and dampens upregulation of CD11b (integrin α M) in human neutrophils as a mechanism regulating their recruitment in inflammatory loci (Krishnamoorthy et al., 2010b). We also show here that RvD1 blunts inflammatory signaling responses of CFBE4-2C that include alteration of cell growth, cellular

stress, and NF- κ B activation (Fig. 8) given by ASN. RvD1 carries many biological actions on epithelial tissues. In CF airway epithelial cells RvD1 lowers cytokine secretion and inflammatory genes downstream *P. aeruginosa* infection (Isopi et al., 2020; Ringholz et al., 2018) and reduces bronchial hyperplasia in vivo in mice bearing chronic bacterial infection (Codagnone et al., 2018). Of interest, Ringholz and coworkers demonstrated that RvD1 enhances Cl⁻ secretion in CFBEc while diminishing ENaC-mediated Na⁺ absorption, thus determining a marked improvement in airway surface liquid height, a key mechanism of airway mucus clearance (Ringholz et al., 2018). Thus, results shown here provide a functional role and action for RvD1, which is produced (Eickmeier et al., 2017; Isopi et al., 2020) and remain active in airway secretions, in regulating airway inflammation in CF.

Here we also demonstrate beneficial effects of RvD1 on mucus-derived lung inflammation were also demonstrated here in vivo with β ENaC-Tg mice (Fig. 10-11-12). The mouse colony utilized in this study is suitable for preclinical studies, as it permits for in vivo studies with aseptic lung infection and inflammation exhibiting cellular, biochemical, and histological characteristics (including mucus plugging, heightened inflammatory reactions, and defective bacterial clearance) similar to that seen in patients with CF (Mall et al., 2004). Previous studies carried out with these mice demonstrated that therapies with hypertonic (3 or 6 %) saline (which is thought to enhance mucus hydration and kinetics) reduce airway mucus accumulation, a finding reproduced in children with CF (Ratjen et al., 2019), but have limited effects on inflammation (Graeber et al., 2013), suggesting that improvement in mucus clearance provides limited improvements to people with CF. In addition, targeting the β ENaC channel with amiloride proved to dampen airway inflammation as well as mucus obstruction if administered to 5-day-old Tg mice but not at a later age (i.e., 4 weeks) (Zhou et al., 2008), which may indicate that β ENaC is not the only contributor to mucus dehydration and stagnation in CF. More recently, Addante and coworkers showed the synthetic thiol-saccharide MUC-031, which acts by disrupting disulfide crosslinks between mucins, decreases total airway leukocytes and M Φ in adult β ENaC-Tg mice following chronic (twice-a-day, 14 days) local treatment (Addante et al., 2023), supporting the hypothesis that mucus-driven chronic inflammation can be targeted pharmacologically. We report that administration of nanogram doses of RvD1 to β ENaC-Tg mice alleviates pulmonary symptoms in vivo, markedly reducing total leukocytes, PMN and M Φ . These results are consistent with data from Hsiao and coworkers, showing that 17R-RvD1 reduces airway leukocyte infiltration and oxidative stress in mice with smoke-induced emphysema and chronic bronchitis (Hsiao et al., 2015). Thus, the present results provide evidence for RvD1 as a pro-resolution therapeutic strategy to reduce the burden of inflammation derived from mucus obstruction. It is known that airway clearance following infections is impaired in CF (Shteinberg et al., 2021). Current strategies generally rely on mucociliary clearance and aggressive treatment of infections to improve life expectancy for people with CF. Hence, we exacerbated the pulmonary status of β ENaC mice by infection with the clinical strain RP73 *P. aeruginosa* and found that the pulmonary

exacerbation was reduced after RvD1 treatment (Fig. 13) with an improved weight recovery and reduced *P. aeruginosa* titer and BAL leukocyte numbers. Previous works from this laboratory were the first to show the pro-resolving effects of RvD1 treatment in chronic lung inflammation associated with persistent infection in COPD and CF (Codagnone et al., 2018; Isopi et al., 2020). Along with RvD1, other SPM proved multipronged, anti-inflammatory, organ-protective, and antimicrobial actions in the lungs (Krishnamoorthy et al., 2023; Walker et al., 2021; Wang et al., 2017) that are important to preserve this organ essential for life.

An important finding here is that RvD1 had distinct effects on lung MΦ of βENaC on sterile conditions versus under infection. Indeed, in specific pathogen-free mice RvD1 selectively reduced Siglec – MΦ, which are primarily responsible for driving continuous PMN infiltration and lung remodeling in chronic respiratory disease like CF (Öz et al., 2022). On the contrary, in *P. aeruginosa*-infected mice RvD1 reduced both Siglec – and total MΦ, which are effector of innate immunity during infections. Hence, RvD1 modifies MΦ-dependent lung immune responses in a stimulus specific manner: reducing tissue-damaging lung leukocytes while improving antimicrobial responses of the airways as demonstrated by the reduction in *P. aeruginosa* colonies in βENaC mice.

CF is a multisystem disease and patients develop a progressive exercise intolerance that has been identified as an independent predictor of mortality (Moorcroft et al., 1997). Chronic exposure to pro-inflammatory mediators accumulating locally in inflammatory loci or circulating in blood, such as TNF-α and other chemokines, long-term infections, and reduced FEV1% have long been known as a determinant of reduced exercise capacity in people, mainly children, with CF (Van De Weert-van Leeuwen et al., 2012). In this study, we provide the first evidence that βENaC mice bearing a chronic obstructive lung pathology have the same impaired physical tolerance seen in people with CF, corroborating the pathophysiological link between reduced lung function and chronic inflammation and exercise intolerance. Moreover, we demonstrate the beneficial effects of a short treatment with RvD1 in reverting this pathological reduction exercise capacity associated with lung obstruction (Fig. 14-15-16). A recent study conducted by Markworth et al. reports that RvD1 increases skeletal muscle maximal force and regeneration following injury (Markworth et al., 2020). Along with them, our present findings suggest that RvD1 could be beneficial for supporting physical resistance in people suffering from debilitating diseases like CF. They also prompt further investigations to ascertain direct functions and actions on skeletal muscles.

In summary, here we demonstrate roles and therapeutic effects of RvD1 in resolving chronic airway inflammation and physical impairment associated with airway mucus obstruction that is highly clinically relevant in CF and other muco-obstructive lung diseases.

2.7 Materials and methods:

2.7.1. Study Participants, Sample Collection, and Analyses

Blood samples anticoagulated with K3EDTA and airway sputum were obtained from five adult volunteers (>18 years) with a confirmed diagnosis of cystic fibrosis (CF) and mild lung disease (FEV1% between 70% and 90%). All participants provided written informed consent in accordance with the approved clinical protocol (Protocol RECCHI19, approved on September 24, 2020), following the principles of the Declaration of Helsinki.

Organotypic airway chips were generated using CF bronchial epithelial cells (CFBECs) cultured on Alvetex scaffolds. The cells were initially submerged in Ham's F12 medium supplemented with 2% Ultrosor G (Pall Corp., New York) for 7–10 days, followed by air-liquid interface culture for up to 2 weeks. The airway chips were treated apically with 3.3% CF airway surface liquid (a mixture of sputum from three different donors). Additionally, resolvin D1 (RvD1) at 10 nM or ethanol (0.01%) as a vehicle control was added to both the apical and basolateral compartments. RvD1 was sourced from Cayman Chemical (Ann Arbor, MI, Cat. #10012554) and stored at -80°C, shielded from light. The concentration of stock solutions was determined using ultraviolet spectroscopy in methanol, applying the Lambert-Beer law (molar extinction coefficient $\epsilon = 50,000 \text{ M}^{-1} \text{ cm}^{-1}$).

Blood PMN (1×10^6 /well) from volunteers with CF were isolated with Dextran sedimentation and loaded on the upper compartment of CFBEC on Alvetex scaffolds in the presence of medium or RvD1 (10 nM). Surface expression of CD62L and CD16 on CF PMN transmigrated after 3 h across CFBEC exposed to ASN was measured with flow cytometry.

Total RNA was extracted from CFBECs and processed following the Nanopore protocol (SQKDCS 109, with EXP-NBD 104 and EXP-NBD 114). Barcoded cDNA libraries were prepared and sequenced using a MinION flow cell (Nanopore). The raw data are publicly available on the GEO database (GSE234839). All analyses were repeated three times, using RNA from different donors.

2.7.2. Animal Models

Hemizygous C57Bl6/N mice overexpressing a transgenic murine β ENaC (Scnn1b) allele and wild-type (WT) littermates were bred and genotyped through PCR analysis of tail DNA. Mice, matched by age and gender, were treated intraperitoneally (I.P.) every other day for two weeks with RvD1 (100 ng/mouse) or ethanol (0.5% vol/vol) as a vehicle control. Mice were monitored daily for signs of illness. After the two-week treatment period, the animals were euthanized, and bronchoalveolar lavage (BAL) samples were collected by injecting and aspirating three 1 mL aliquots of sterile DPBS intratracheally using a catheter (0.9 × 25 mm) connected to a 1 mL syringe. Total leukocyte counts

in BAL were assessed using a FACS Canto II flow cytometer and FACS Diva software (BD Bioscience). Samples were stained for 30 minutes at 4°C with fluorochrome-conjugated antibodies targeting CD45, CD11b, Siglec, F4/80, and Ly6G antigens (Sony Biotechnology or Biolegend).

The concentrations of keratinocyte chemoattractant (KC), tumor necrosis factor (TNF)- α , interleukin (IL)-10, granulocyte colony-stimulating factor (G-CSF), and vascular endothelial growth factor (VEGF) in BAL fluid were quantified using a Milliplex multianalyte magnetic bead array. All analyses were performed in a blinded manner at Transmed Research (<https://transmedresearch.com>).

2.7.3. Bacterial Infection Experiments

For experiments involving bacterial exacerbation, mice were infected with the clinical strain of *Pseudomonas aeruginosa* RP73. In the case of acute infections with planktonic bacteria, RP7341 was grown in tryptic soy broth (TSB) to the mid-log phase ($OD_{600\text{ nm}} = 0.45 \pm 0.05$, $\sim 2 \times 10^8$ CFU/mL). The bacteria were then washed, suspended in DPBS to a final concentration of approximately 2×10^7 CFU/mL, and injected intratracheally into the mice. Following the RP73 inoculation, the animals were monitored daily and treated via oral gavage with RvD1 (100 ng/mouse) or ethanol (0.5% vol/vol). The animal protocols were approved by the Ministry of Health (Protocol No. 852/2019-PR).

2.7.4. Metabolic Analysis

Mice were individually placed in the calorimetry system's registration chamber (Pan Lab/Harvard Instruments, Spain), where they had free access to food and water for 48 consecutive hours. After a 24-hour adaptation period, carbon dioxide production (VCO_2) and oxygen consumption (VO_2) were measured over the subsequent 24 hours using the OxyletPro calorimetry system (Pan Lab/Harvard Instruments, Spain). These measurements were used to calculate the respiratory quotient (RQ) and energy expenditure (EE) using the formulas: $RQ = VCO_2/VO_2$ and $EE = (3.815 + (1.32 \times RQ)) \times VO_2 \times 1.44$.

The incremental speed test was conducted on a treadmill apparatus (LE8708TS, OxyletPro system, Pan Lab/Harvard Instruments, Spain) connected to the calorimetry system, enabling integration with respiratory metabolism data (O_2 consumption and CO_2 production).

2.7.5. Statistical Analysis

Data were presented as median with interquartile range (25th/75th percentiles) or as mean $\pm$ standard error, depending on data normality assessed by the Shapiro-Wilk test. Statistical comparisons were made using the Mann-Whitney U test, ANOVA on ranks, or parametric t-tests

followed by Tukey's post-hoc test, as appropriate. A p-value of <0.05 was considered statistically significant.

Chapter 3

CYSTIC FIBROSIS RELATED DIABETES

3.1 Diabetes

Diabetes is a persistent metabolic disease characterized by hyperglycemia due to defects in insulin secretion, its action, or a combination of the two (American Diabetes Association, 2011). The disease is classified into type 1 diabetes, caused by the suppression in insulin production due to autoimmune destruction of the pancreatic β -cells (Popoviciu et al., 2023), and type 2 diabetes, which is associated with dysfunctional insulin release from pancreatic β -cells and the insulin resistance (IR) (Galicia-Garcia et al., 2020). The prevalence of diabetes is increasing globally, largely due to an increase in sedentary lifestyle and poor dietary habits, contributing to the growing burden of this condition on healthcare systems (Khan et al., 2019). Long-term hyperglycemia in diabetes contributes to serious complications, including cardiovascular problems, neuropathy, and retinopathy, which negatively impact quality of life and increase the risk of death. The pathogenesis of these complications involves complex mechanisms, including oxidative stress, inflammation, and endothelial dysfunction, which damage both the microvascular and macrovascular systems (Giri et al., 2018).

Diabetes treatment involves a complex approach that includes lifestyle changes, drug therapy for glycemic control, and regular monitoring for complications. Consistent glycemic control has been shown to reduce the risk of microvascular complications, while blood pressure and plasma lipid control help prevent macrovascular complications (Davies et al., 2018).

3.2 Diabetes and Inflammation

Chronic inflammation plays a critical role in the pathogenesis and progression of type 1 and type 2 diabetes (Tsalamandris et al., 2019). In type 2 diabetes, the turn on of the system is closely correlated with disease progression. Both the innate and adaptive immune systems contribute to inflammation in adipose tissue. The change in macrophage phenotype from M2, which is primarily anti-inflammatory, to a greater proportion of M1 macrophages, which are proinflammatory, is crucial for the onset and intensification of islet inflammation (Tsalamandris et al., 2019). Adipose tissue, particularly visceral fat, acts as an active endocrine organ, releasing pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF- α), interleukin-6 (IL-6), and C-reactive protein (CRP), which interfere with insulin signaling pathways and exacerbate hyperglycemia. The inflammatory NF- κ B pathway is also activated in obesity. In type 2 diabetes, there is also overexpression of IL-1 β in pancreatic β cells, which reduces the expression of glucose transporter type 4 (GLUT4) and inhibits the translocation of GLUT4 to the plasma membrane (Zatterale et al., 2020).

In type 1 diabetes, inflammation is mediated primarily by an autoimmune response, in which pancreatic beta cells are destroyed by T cells (Burrack et al., 2017). This autoimmune process

involves a more complex interaction between different immunological actors of adaptive and innate immunity, leading to macrophage activation and the release of pro-inflammatory cytokines, which play a role in β -cells apoptosis (Citro et al., 2021).

3.3 Diabetes and Cystic fibrosis

Diabetes is the most common comorbidity in pwCF with a prevalence that increases with age and affects approximately 50% of adults with CF (Moran et al., 2009). This distinct condition is termed "double diabetes" (DD) which combines aspects of type 1 and type 2 diabetes, due to a dual profile of insulin deficiency and insulin resistance, making both diagnostic and therapeutic approaches extremely difficult (Khawandanah, 2019).

Cystic fibrosis related diabetes (CFRD) is primarily caused by a reduced capacity for insulin production, accompanied by a variable level of IR, as evidenced by the presence of fasting hyperinsulinemia. This condition is also associated with impaired exocrine and endocrine function of the pancreas, which in turn appears to be linked to mutations in the CFTR gene. CFRD is thought to result from damage to the exocrine pancreas, which impairs the proper functioning of the endocrine islet cells (Ntimbane et al., 2009) (Fig.17). However, epidemiological data indicate that diabetes is present also in patients with normal exocrine pancreatic function, suggesting a more complex scenario in CFRD.

CFRD differs from other forms of diabetes in its clinical presentation because it is often asymptomatic and begins insidiously, remaining silent for a long time. Glucose levels in CFRD are highly variable, and the disease can often be diagnosed in the phases of acute lung infection or corticosteroid therapy, but ketoacidosis remains a rare event (Iafusco et al., 2021).

However, both insulin insufficiency and hyperglycemia adversely affect CF lung disease by compromising lung function and nutritional status, while increasing the rate of pulmonary complications and mortality compared to pwCF without diabetes (Moran et al., 2010). Early diagnosis of CFRD is difficult; because the classic symptoms of diabetes (such as polyuria and polydipsia) are often not evident, thus patients with early glucose abnormalities benefit from routine screening and the oral glucose tolerance test (OGTT) (Khare et al., 2022). Insulin therapy is usually required to maintain glycemic control and improve in lung function and nutrition (Owens, 2013). Unlike type 2 diabetes, oral hypoglycemics are rarely effective due to the predominant insulin deficiency (Zammitt and Frier, 2005).

The pathophysiology of CFRD is complex and multifaceted. CFTR is highly expressed in the epithelial cells of the small pancreatic ducts that empty the pancreatic acini, where it regulates the secretion of chloride, water, and bicarbonate into the ducts. When it is dysfunctional, these small ducts dilate and become plugged with thick secretions, resulting in widespread destruction of the acini, with extensive fibrosis and cystic dilatation of the larger ducts (Norris et al., 2019). Patients with severe CFTR mutations (classes I and II) are at increased risk of developing diabetes compared

to those with milder mutations. However, not all patients with the same mutations develop CFRD, suggesting that additional factors influence the onset and severity of the disease (Coderre et al., 2021). Additionally, the CFTR genetic defect, genetic factors related to type 2 diabetes, inflammatory processes, and alterations in the incretin system likely play a role in compromising the function of the remaining pancreatic islets (Malik et al., 2023).

The main problem with CFRD is a deficiency in insulin production; almost all individuals with pancreatic exocrine insufficiency, regardless of whether they have diabetes or not, show signs of β -cell impairment, which ultimately results in a more pronounced reduction in insulin response to glucose (Ode et al., 2022). Early glucose abnormalities can be detected by continuous glucose monitoring (CGM), with over half of pwCF showing intermittent post-meal glucose levels above 200 mg/dL (11.1 mmol/L) even when OGTT results are normal (Ode et al., 2022). Over time, glucose tolerance generally worsens, progressing through phases of indeterminate glucose tolerance to CFRD, with or without fasting hyperglycemia, largely influenced by infection status (Kelly and Moran, 2013).

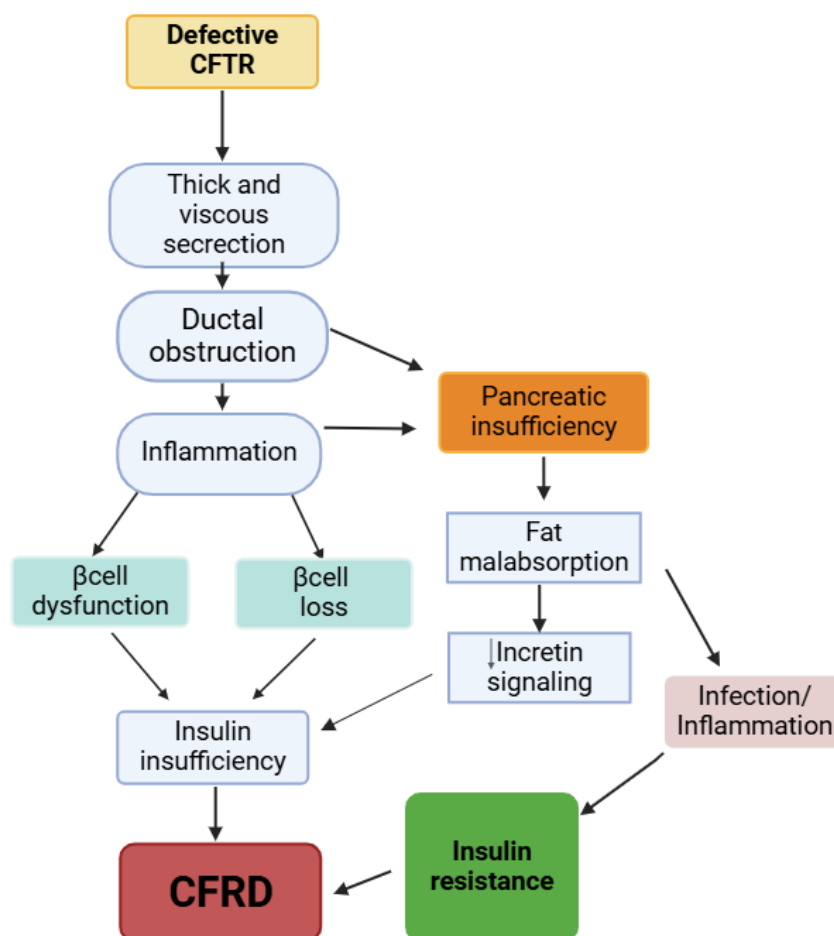


Fig 17. Diagram illustrating the intricate mechanisms underlying the development of CFRD.

Image generated using Biorender.

3.4 SPM and CFRD

Recent research has highlighted the potential role of SPM, such as resolvins, protectins, and maresins, in modulating inflammation in CFRD. SPM, as mentioned above, actively promotes resolution of inflammation and tissue repair. For example, in the work of Isopi et al, we demonstrated that RvD1 in CF mice subjected to chronic *P. aeruginosa* lung infection exerts protective effects in vivo, limiting neutrophil infiltration, lung inflammation and bacterial load, while enhancing phagocytosis and reducing organ damage (Isopi et al., 2020). Furthermore, we have shown that RvD1 attenuates mucus-induced inflammatory responses in human cells from people with CF and in β ENaC mice with muco-obstructive lung disease similar to CF (Ferri et al., 2023b). Since chronic inflammation plays a key role in the pathogenesis of both CF and diabetes, improving the resolution phase of inflammation may highly benefit people with CFRD (Fredman and Tabas, 2017). Studies indicate that SPM can improve insulin sensitivity, reduce levels of pro-inflammatory cytokines, and promote beta-cell survival, potentially alleviating some of the metabolic dysfunction associated with CFRD (Spite et al., 2014).

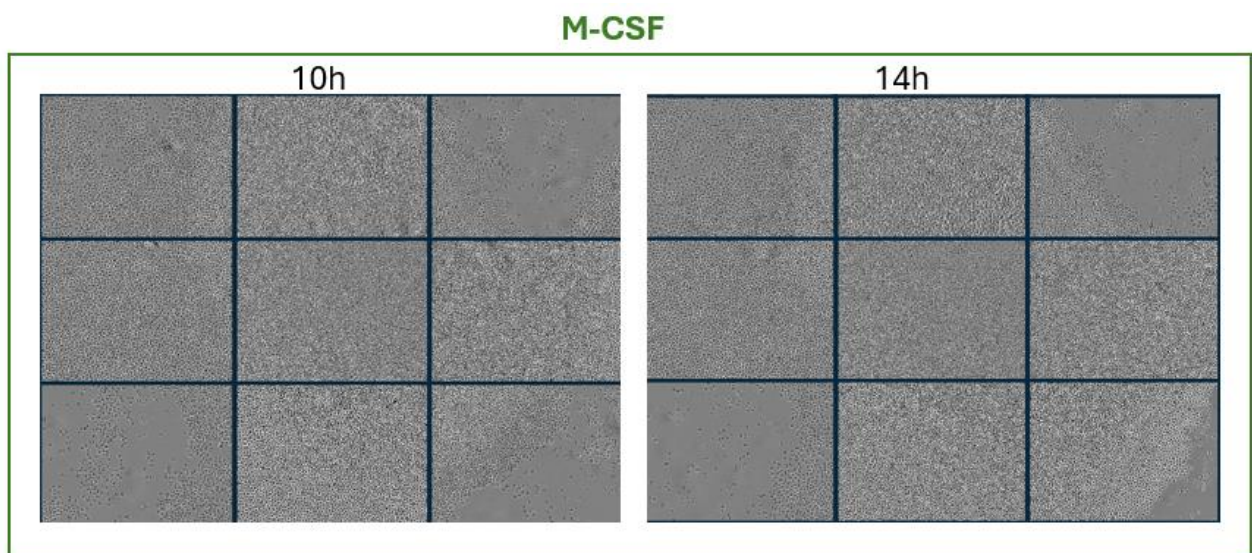
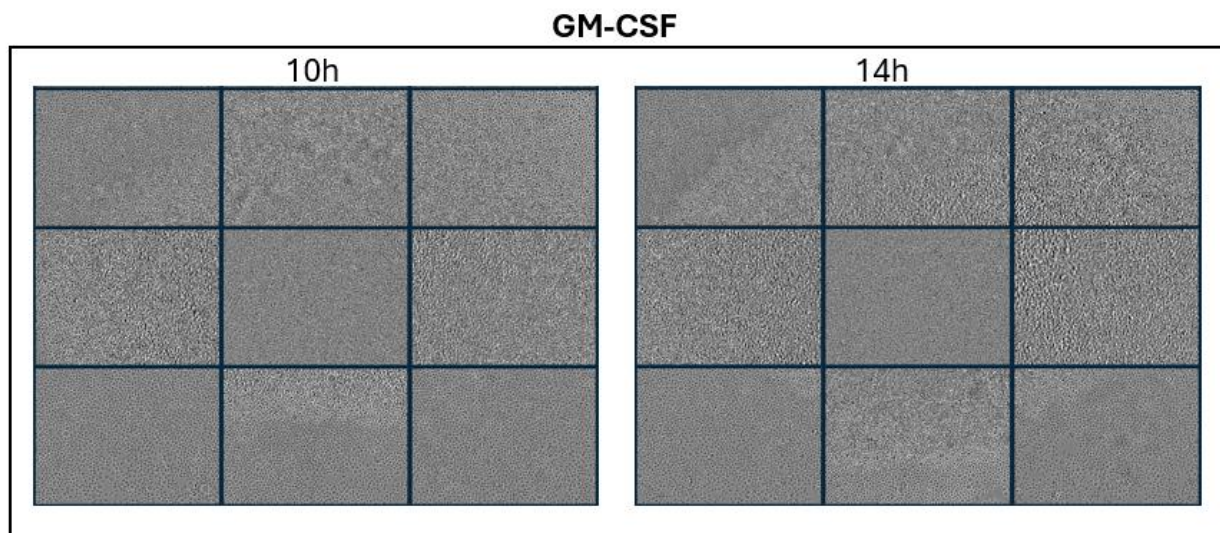
Therefore, during my PhD I sought to investigate the actions of RvD1 on CFRD in mice as well as to determine the interplay of high glucose and CFTR on monocyte/macrophage activation and functions.

The following results should be considered preliminary and, while not yet significant, they lay a promising foundation for future research in a field of great interest and potential development.

3.5 Results:

3.5.1. Effects of CFTR inhibition on the proliferation and spatial organization of THP-1 cells cultured with GM-CSF and M-CSF

To understand the effects of CFTR loss function on monocyte/macrophage biology, we cultured THP-1 monocytes in the presence of GM-CSF or M-CSF for 72 h to induce differentiation into macrophages and polarization into M1- or M2-like phenotypes, before assessing the impact of CFTR blockade by a selective CFTR inhibitor (CFTRinh-172, 10 μ M). The initial differentiation step was critical, as GM-CSF drives polarization toward M1-like macrophages, that release pro-inflammatory cytokines such as TNF- α and IL-6, while M-CSF promotes M2-like macrophages with anti-inflammatory properties, including the production of IL-10 (Mohd Idrus et al., 2021; Wang et al., 2014). Following differentiation, we monitored cells for 24 h using the IncuCyte system to examine spatial organization (3D confluence). Under control conditions, both GM-CSF and M-CSF (Fig. 18) consistently reduced confluence that stabilized after 10 h. The introduction of CFTRinh-172 (Fig. 19), however, altered these mechanisms. Indeed, in cells exposed to CFTRinh-172, following a decrease in confluence from 2 to 10 h, an increase in cell confluence between 10 and 24 h was observed, indicating that CFTR activity influences macrophage spatial organization. These results indicate that CFTR is essential to maintain the adhesive and structural properties of macrophages.



3D Spatial organization (cell confluency)

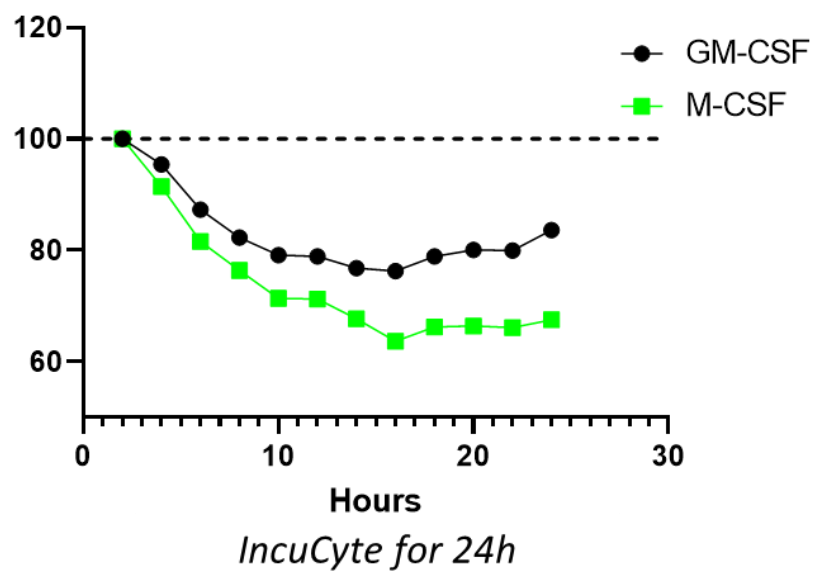
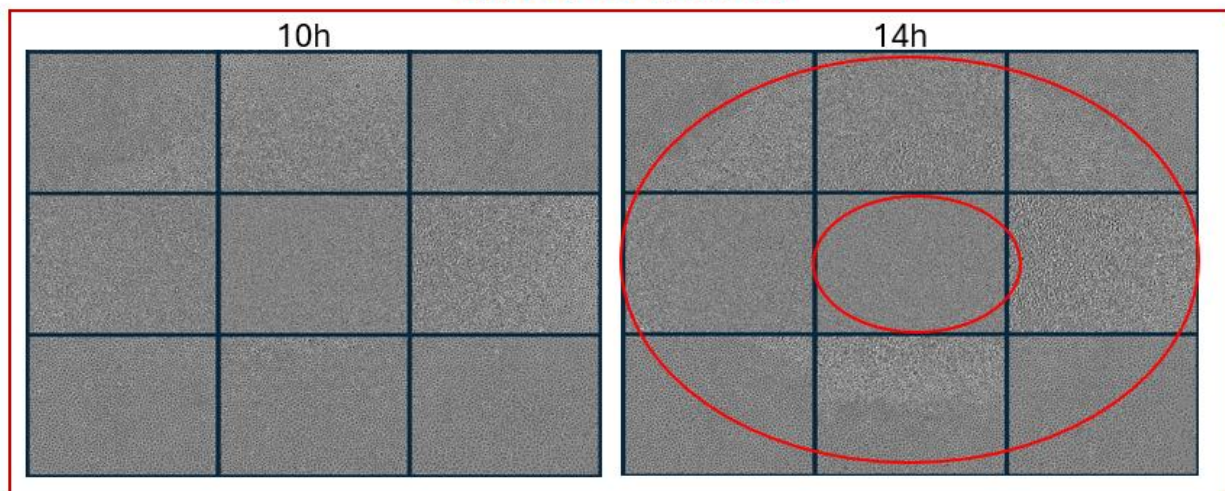


Fig.18. Effects of GM-CSF and M-CSF on THP-1 cell confluence and density:

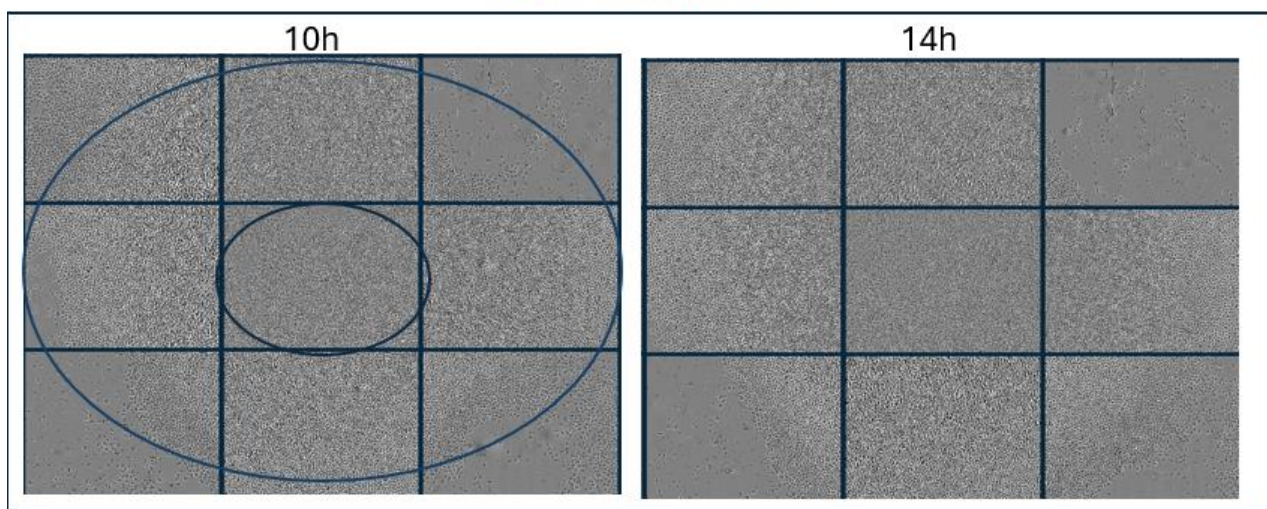
(Top) Representative images of THP-1 cells cultured with GM-CSF (left) or M-CSF (right) at 10 and 14 hours, obtained using the IncuCyte live-cell imaging system. The grid highlights areas used for spatial organization analysis.

(Bottom) Graphs display % confluence over 24 hours, analyzed via the IncuCyte software. Results are representative of 3 separate experiments.

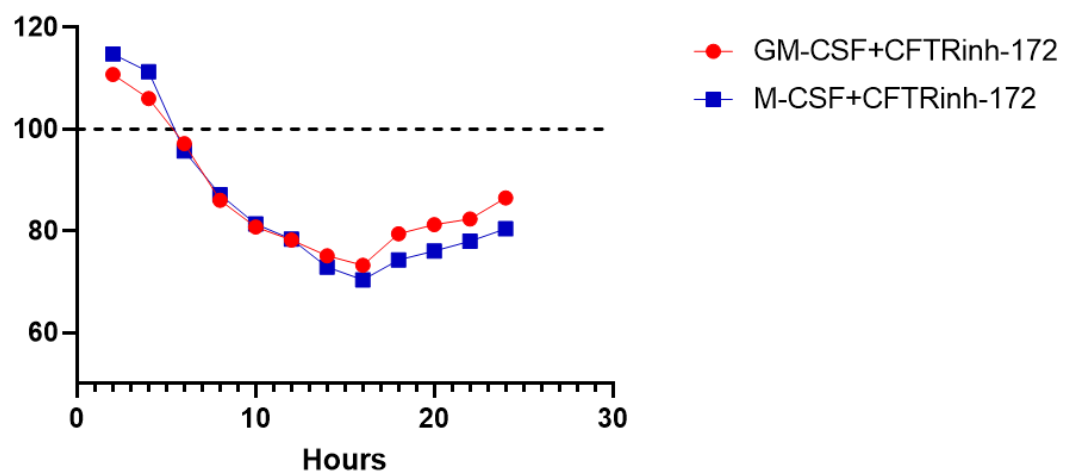
GM-CSF+CFTRinh172



M-CSF+CFTRinh-172



3D Spatial organization (cell confluency)



IncuCyte for 24h

Fig.19: Impact of CFTR inhibition on GM-CSF and M-CSF-treated THP-1 cells

(Top) Representative images of THP-1 cells treated with the CFTR inhibitor [CFTRinh-172:10 μ M) after differentiation with GM-CSF (left) or M-CSF (right) at 10 and 14 hours, captured using the IncuCyte live-cell imaging system. Disruptions in confluence are outlined (red ovals for GM-CSF+CFTRinh-172 and blue ovals for M-CSF+CFTRinh-172).

(Bottom) Graphs show % confluence for GM-CSF+CFTRinh-172 (red) and M-CSF+CFTRinh-172 (blue) groups, analyzed using the IncuCyte software.

Results are representative of 3 separate experiments. For quantitative analyses, cells kept with GM-CSF or M-CSF were taken as a control for their respective groups (i.e., 100 % confluence measured 2 h after cells were placed in the IncuCyte instrument).

3.5.2. Analysis of Gene Expression and Cytokine Secretion in THP-1 Differentiated Macrophages

To further investigate the effects of GM-CSF- and M-CSF-induced differentiation on THP-1-derived macrophages, both under normal conditions and in the presence of CFTRinh-172, we used an integrated approach. This included real-time PCR to analyze gene expression levels (Fig. 20) and Meso Scale Discovery (MSD) ELISA to quantify cytokine secretion (Fig. 21). These complementary methods allowed us to characterize both the polarization markers and functional pathways activated in macrophages, as well as their secretory profiles under these experimental conditions.

Real-time PCR data (Fig. 20) revealed distinct transcriptional profiles, confirming the differentiation of THP-1 monocytes into macrophages. Compared to naïve monocytes, the expression of CD14, a marker of monocyte activation, and CD68, a pan-marker of macrophage activation, was consistently increased by GM-CSF and M-CSF, which, on the contrary, caused a slight decrease in CD163, a marker associated with anti-inflammatory M2 polarization, although these changes did not attain significance due to variability in cell response.

The addition of CFTRinh-172 (10 μ M) reduced the expression of CD14 and increased CD163 in response to MCS but not to GM-CSF, indicating a critical role of CFTR in macrophage differentiation and polarization processes. Furthermore, the apoptosis regulator TRAIL was significantly downregulated by CFTRinh-172 in macrophages treated with GM-CSF, whereas it was increased in M-CSF-treated cells, suggesting that CFTR also influences apoptotic signaling in opposite manner depending on the cell microenvironment.

On the inflammatory front, cells treated with GM-CSF+CFTRinh72 showed high levels of TNF- α and IL-1 β , pro-inflammatory mediators involved in the NF- κ B and NLRP3 inflammasome pathways, compared to GM-CSF alone, though the increase did not reach significance. CFTR inhibition in M-CSF-treated cells led to a reduction of these markers.

Finally, CFTR inhibition led to a reduction in MCP-1 in both GM-CSF and M-CSF-treated macrophages, indicating an impairment of chemotactic functionality.

The results obtained by MSD ELISA analysis (Fig. 21) showed no substantial changes in cytokine production by macrophages in the presence of CFTRinh-172, except for TRAIL, which was increased by CFTRinh-172 in M-CSF-differentiated THP-1 cells.

Overall, these results, although preliminary, show that CFTR influences macrophage activation and chemotaxis, and prompt to assess the effects of long-term CFTR inhibition on these cytokines and chemokines in more complex scenarios, e.g., in vivo.

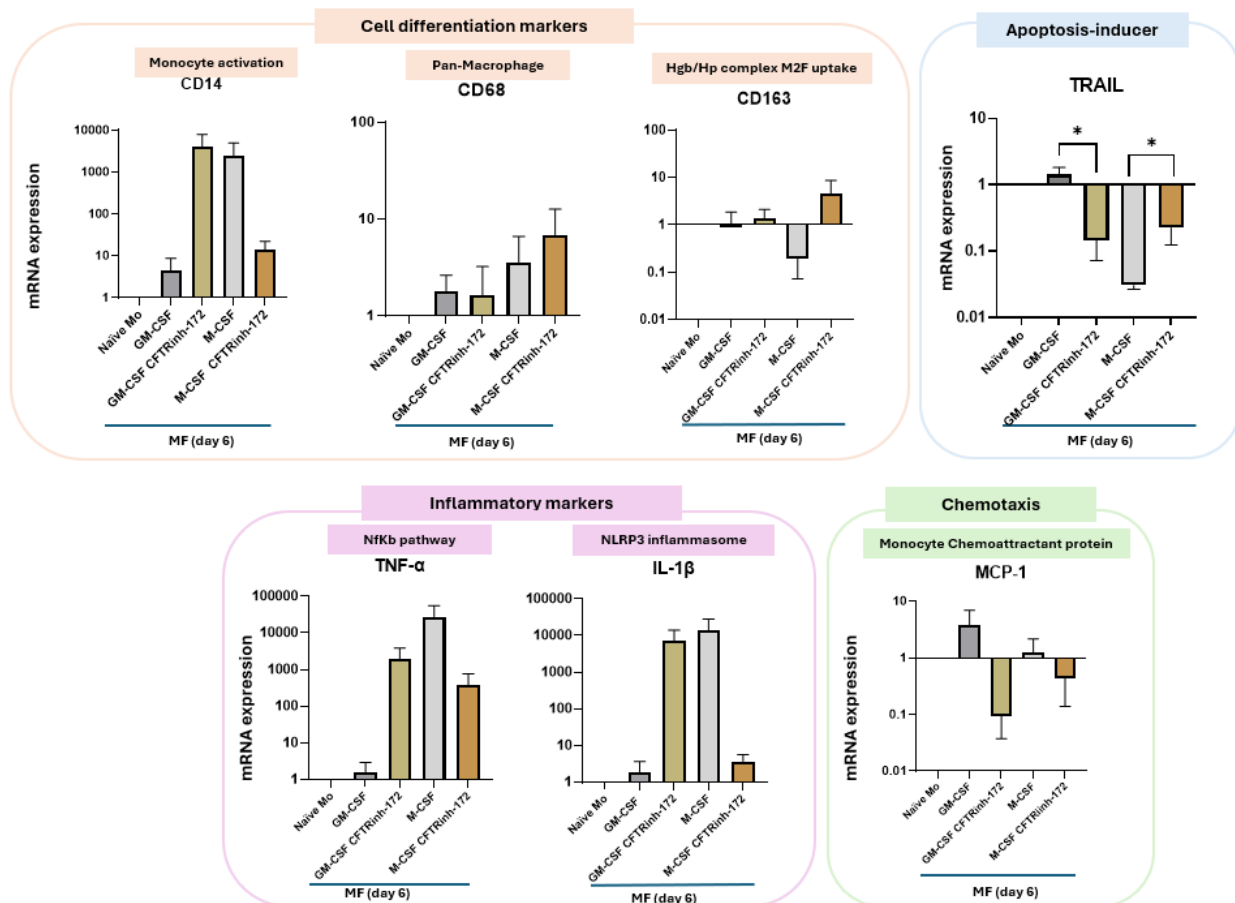


Fig.20. Gene expression analysis of macrophage differentiation and inflammatory markers via RT-PCR.

THP-1 monocytes were differentiated into macrophages by 72-hour treatment with GM-CSF or M-CSF (day 6) in control conditions or in the presence of the CFTR inhibitor (CFTRinh-172). The expression levels of markers associated with differentiation (CD14, CD68, and CD163), inflammatory activation (TNF- α , IL-1 β), chemotaxis (MCP-1), and apoptosis induction (TRAIL) were quantified. Data are presented as mean $\pm$ SEM (n=3).

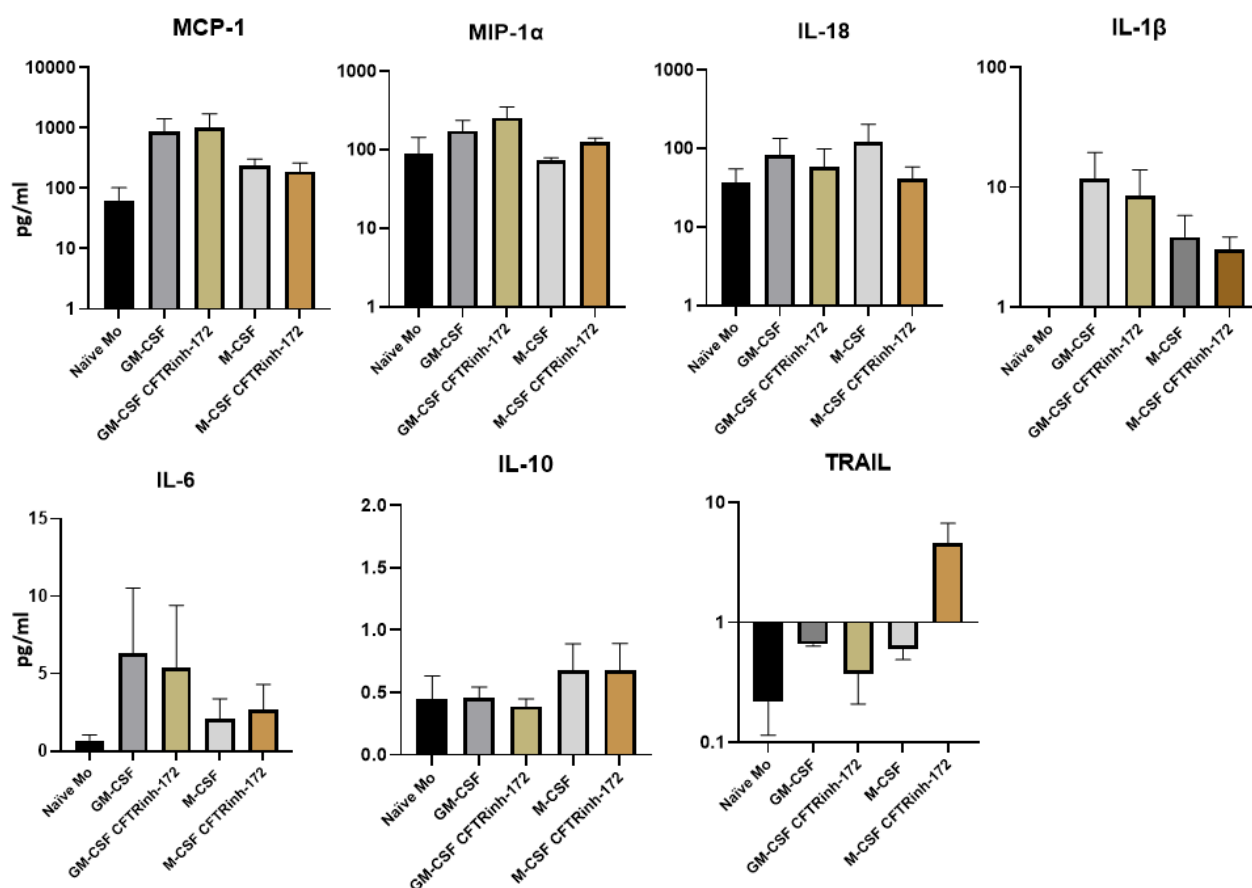


Fig.21. Cytokine secretion profiles of GM-CSF- and M-CSF-differentiated macrophages via MSD ELISA.

THP-1-derived macrophages, treated with GM-CSF or M-CSF for 72 hours, were analyzed for cytokine production at day 6 under control and CFTR-inhibited conditions. Pro-inflammatory cytokines (MIP-1α, IL-1β, IL-18), anti-inflammatory cytokines (IL-10), and apoptotic signals (TRAIL) were measured. Results are expressed in pg/ml as mean ± SEM.

3.5.3. Impact of CFTR inhibition and hyperglycemia on macrophage differentiation and spatial organization:

Data revealed a complex interplay between differentiation signals triggered by M-CSF or GM-CSF and CFTRinh-172. Initial experiments confirmed that CFTR inhibition effectively mimics cystic fibrosis-like conditions in THP-1 cells, disrupting both cell differentiation and adhesion dynamics. These effects were particularly pronounced in the presence of M-CSF and GM-CSF, which are critical for driving macrophage differentiation.

Based on these findings, we explored how hyperglycemia affects THP-1 cells under the same differentiation conditions and CFTR inhibition.

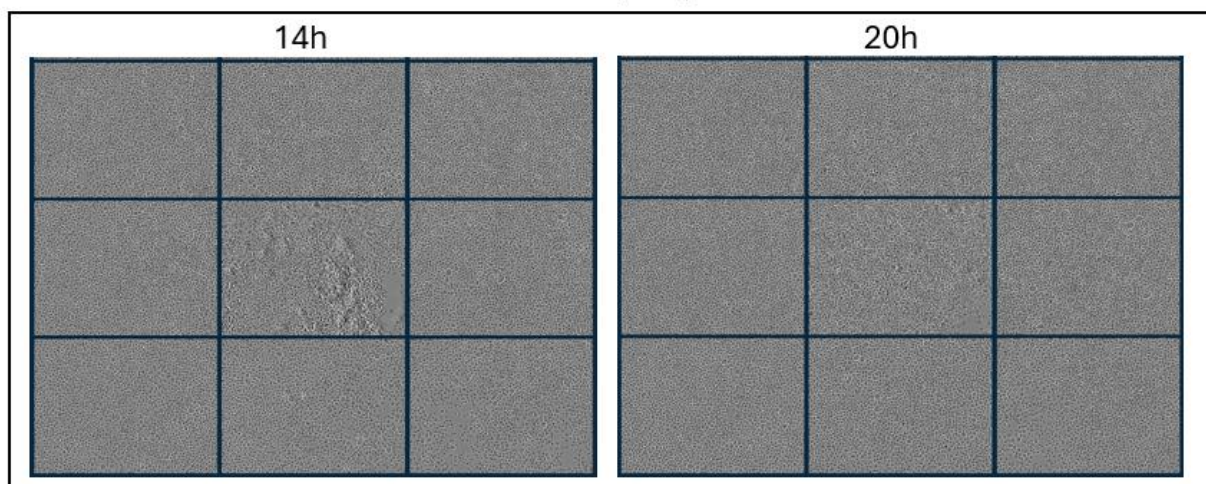
Cells, initially treated with M-CSF for 72 hours to induce adhesion and differentiation, were exposed to glucose at normal (5 mM) or high (25 mM) concentrations. To monitor in real time the changes in 3D spatial organization and cell density, the IncuCyte system was used, over a period of 48 hours. The images acquired (Fig. 22A) at 14 and 20 hours revealed a stable and adherent configuration in cells treated with M-CSF or cultured in RPMI, under normal glucose conditions. M-CSF gave a time-dependent increase in THP-1 confluence compared to RPMI. In contrast, CFTRinh-72 (10 μ M) inhibited confluence of THP-1 cells treated with M-CSF, while increased their confluence when cells were maintained in RPMI medium alone (Fig. 22B).

When glucose levels were high, a significant spatial disorganization was observed, particularly evident in samples treated with M-CSF. This alteration was characterized by a reduced and inhomogeneous cellular distribution and suggests that hyperglycemia compromises the spatial organization and the differentiation process. Furthermore, the addition of CFTRinh-172 blunted both M-CSF effects and cell behavior under RPMI (Fig. 22C), suggesting interactions of the chloride channel inhibition with the cell response to metabolic stress.

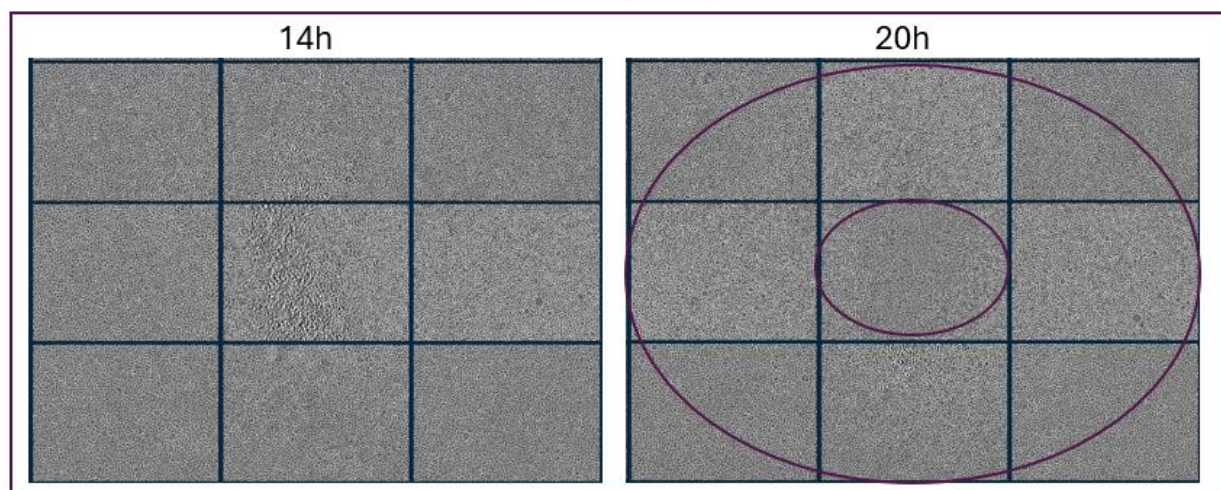
Of interest, these effects were not associated with changes in cell counts for any of the measured outcomes (not shown), suggesting a limited impact of CFTR on cell survival under these conditions. Taken together, these results highlight the importance of the cellular microenvironment, including glucose level and CFTR activity, in regulating macrophage organization.

A

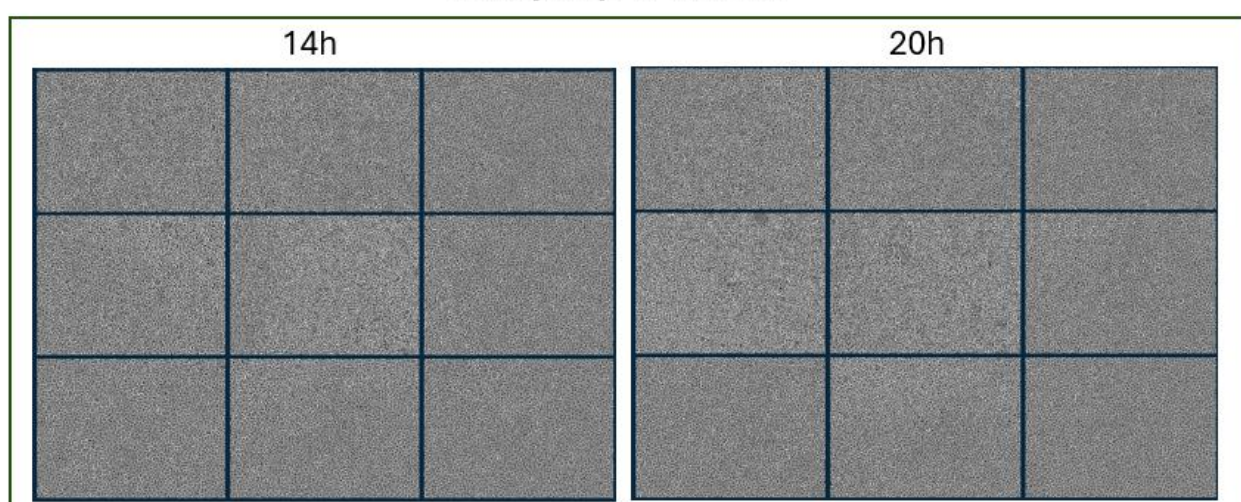
M-CSF [5mM]



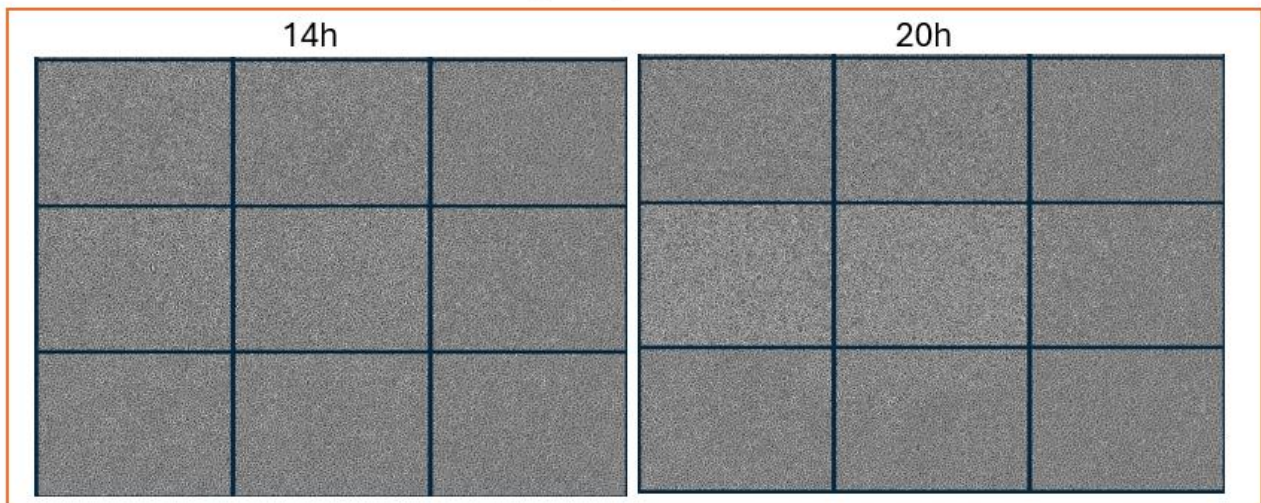
M-CSF [25mM]



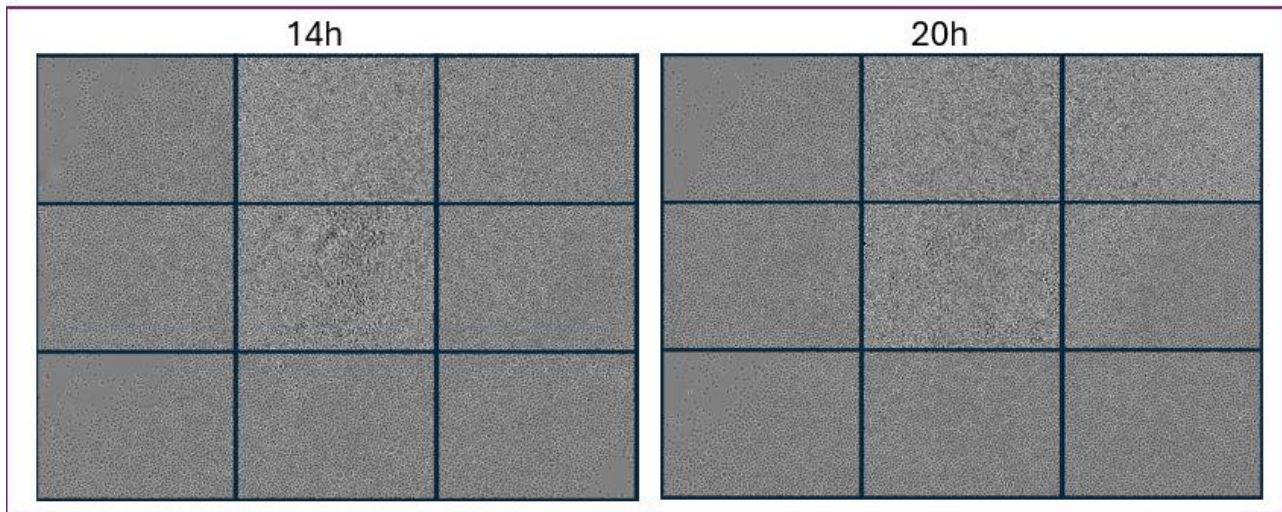
M-CSF [5mM] + CFTRinh-172



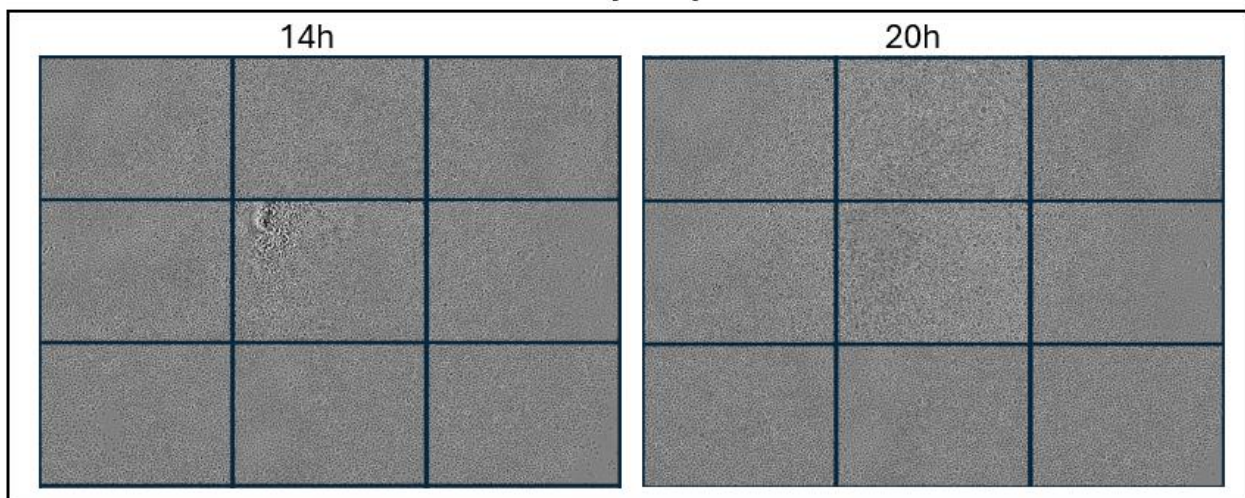
M-CSF [25mM] + CFTRinh-172



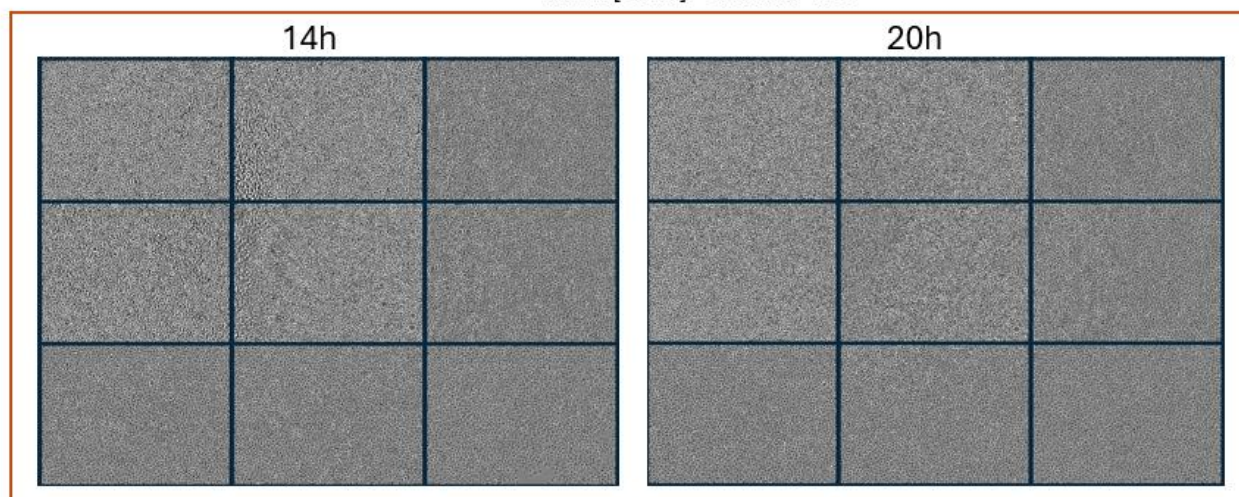
RPMI [5mM]



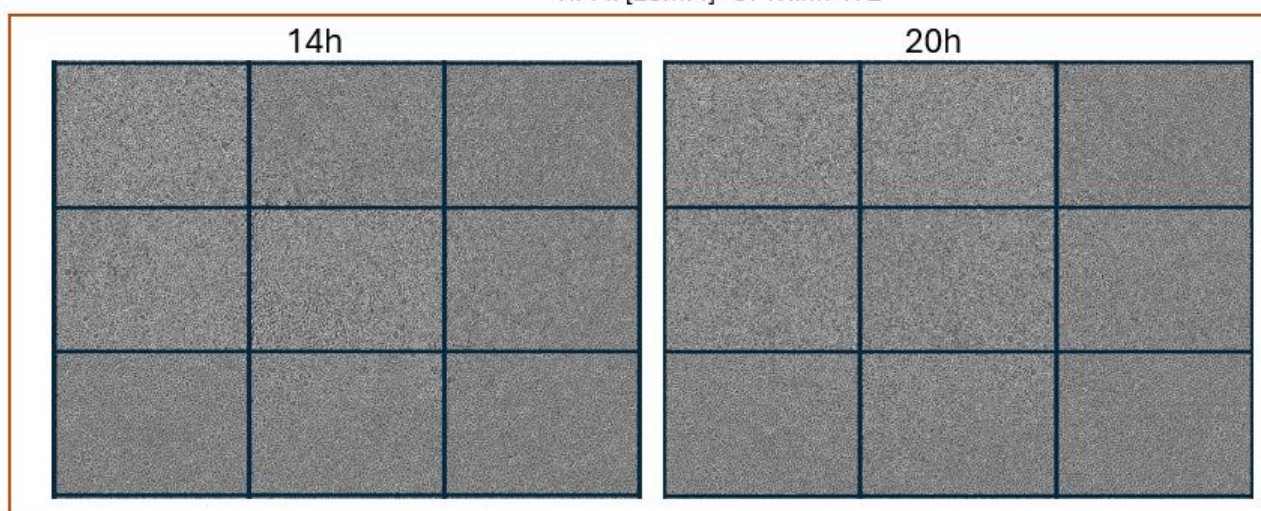
RPMI [25mM]



RPMI [5mM]+CFTRinh-172



RPMI [25mM]+CFTRinh-172



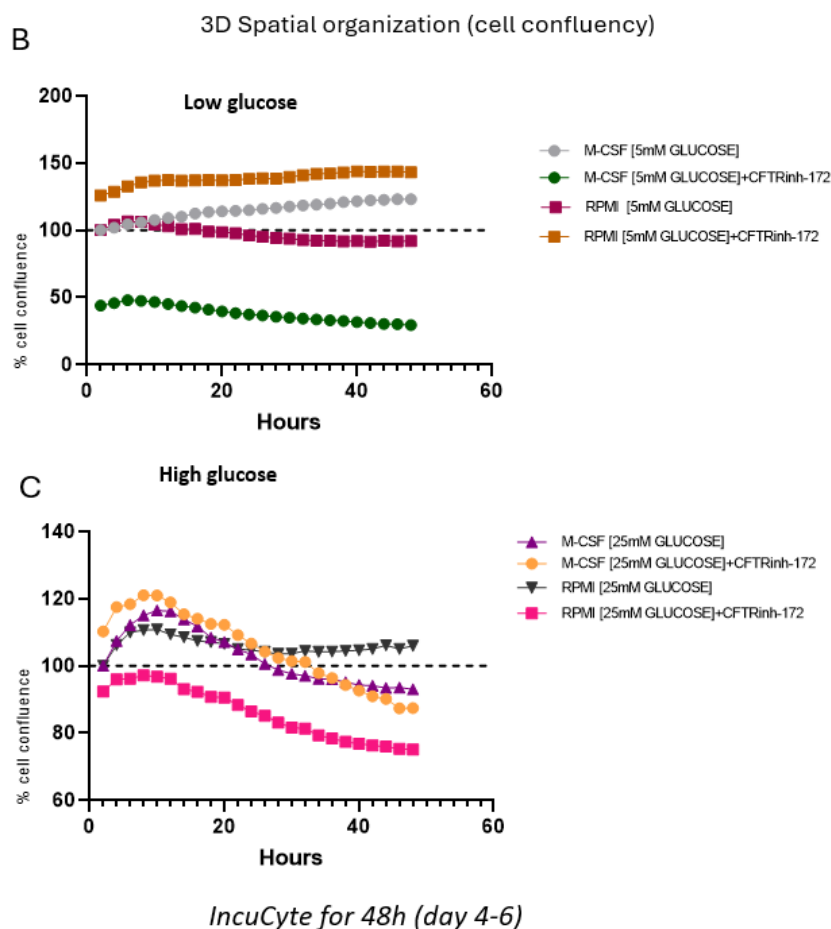


Fig. 22 Combined effects of M-CSF, glucose and CFTR inhibition on spatial organization and adhesion of THP-1 cells.

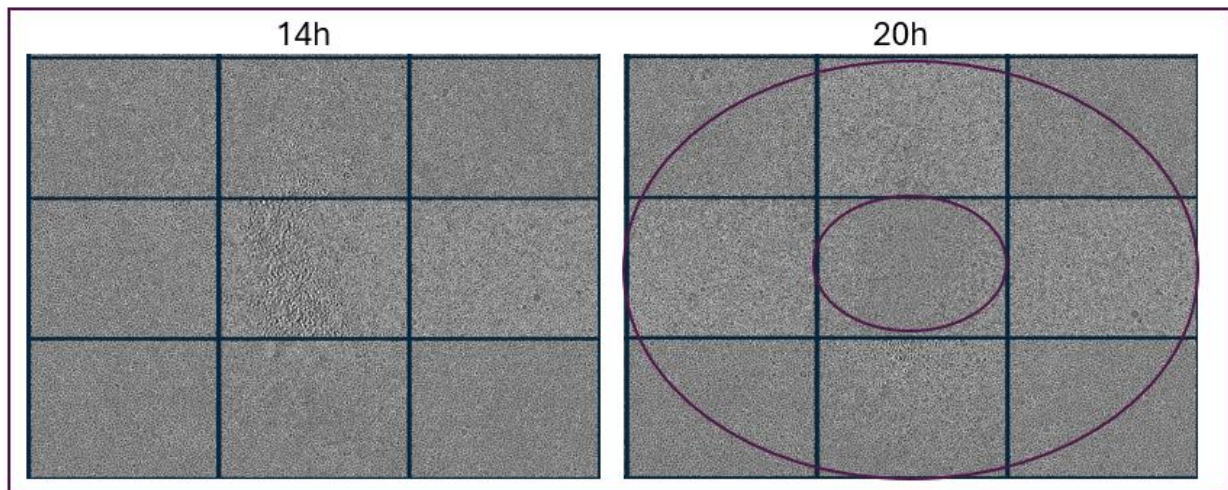
A) Representative snapshots of THP-1 cells grown under the indicated conditions taken at 14 or 20 h (on day 4) to evaluate spatial organization and adhesion. **B-C)** Representative quantitative analyses performed with IncuCyte software on THP-1 cells pre-treated for 72 hours with M-CSF or RPMI, in the presence of low (5 mM) or high (25 mM) glucose concentrations, with or without the CFTRinh-172 (10 μ M). Cells were observed from day 4 to 6 to evaluate spatial organization and adhesion. Results are representative of 3 separate experiments. For quantitative analyses, cells kept with RPMI or M-CSF (either in 5 or 25 mM glucose) were taken as a control for their respective groups (i.e., 100 % confluency measured 2 h after cells were placed in the IncuCyte instrument).

3.5.4. RvD1 mitigates the combined effects of hyperglycemia and CFTRinh-172 on macrophage differentiation and organization

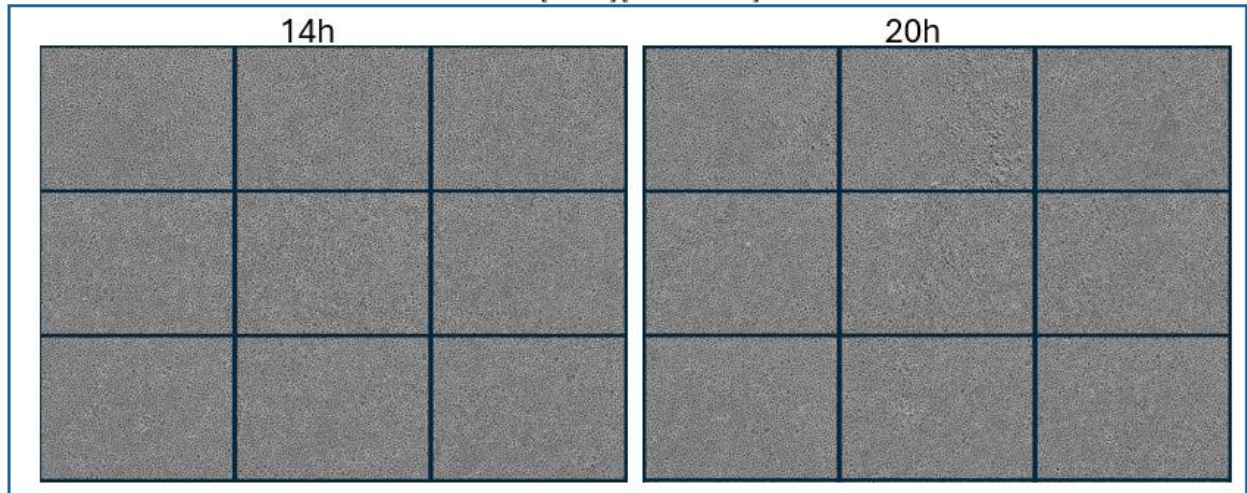
To further explore potential therapeutic strategies to counteract the effects of hyperglycemia and CFTR inhibition on macrophage differentiation and spatial organization, we explored the role of RvD1, a specialized pro-resolving lipid mediator known for its anti-inflammatory properties. THP-1 cells were pretreated with M-CSF for 72 h to induce differentiation and adhesion, followed by exposure to high glucose (25 mM) with or without CFTRinh-172 (10 μ M) and addition of 100 nM RvD1. Observations were performed using IncuCyte imaging over a period of 48 h after addition of the treatments (CFTRinh-172, glucose, and RvD1). Under conditions with M-CSF alone and hyperglucose, an increase in 3D spatial organization was observed, consistent with previous findings (Fig. 23). In terms of cell density, high glucose conditions combined with CFTRinh-172 resulted in modest reductions in cell counts (not shown).

Addition of RvD1 reduced the time-dependent increase in cell confluence induced by M-CSF. These effects were also more evident in the presence of CFTRinh-172, suggesting that RvD1 may counteract the destabilizing effects of hyperglycemia on cell morphology (Fig. 23).

M-CSF [25mM]



M-CSF [25mM] [100nM RvD1]



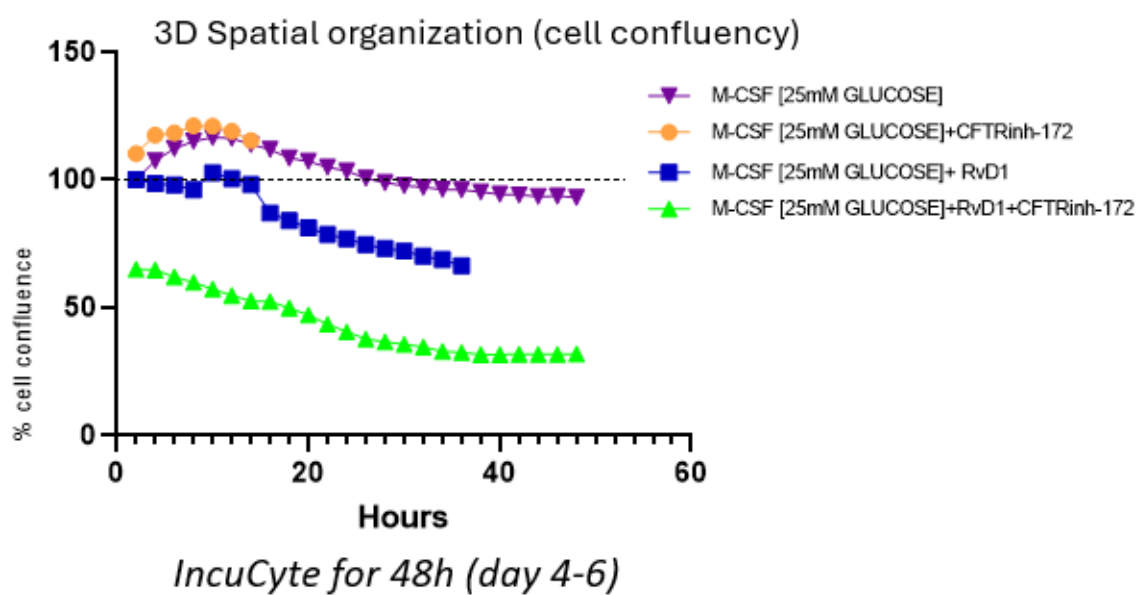
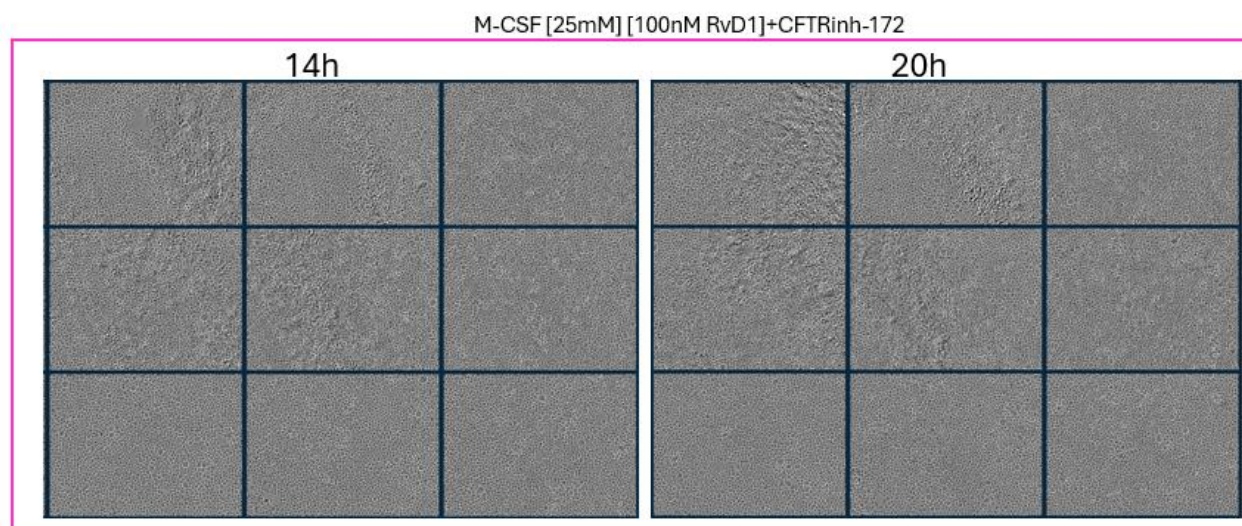
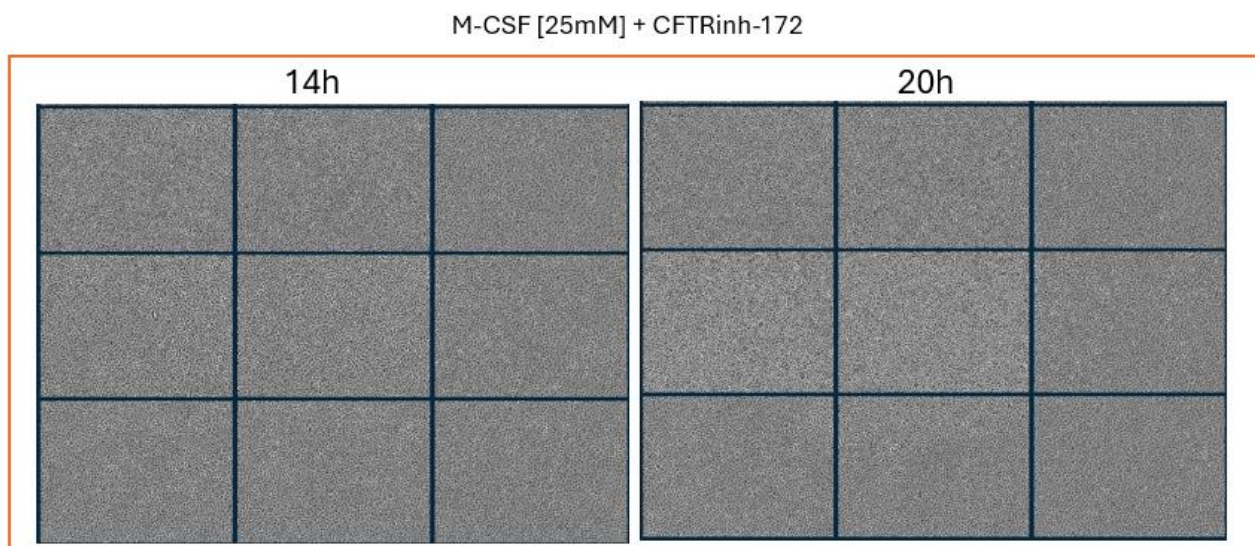


Fig. 23 Effects of RvD1 on THP-1 macrophages under hyper-glucose conditions with CFTRinh-172.

Effects of RvD1 (100 nM) on THP-1 macrophages exposed to high glucose conditions (25 mM) with or without CFTR-172 (10 μ M) during M-CSF-driven differentiation. The **top** panel shows representative IncuCyte images, captured at 14 and 20 hours from day 4, to evaluate spatial organization and adhesion. The **bottom** graph shows a representative quantitative analysis of 3D spatial organization measured from day 4 to 6. Results are representative of 3 separate experiments. For quantitative analyses, cells kept with M-CSF alone or plus RvD1 (in 25 mM glucose) were taken as a control for their respective groups (i.e., 100 % confluence measured 2 h after cells were placed in the IncuCyte instrument).

3.5.5. Effects of hyperglycemia and RvD1 on macrophage polarization and inflammatory responses:

To further explore the effects of metabolic stress and pro-resolution mediators, THP-1-derived macrophages were cultured under high glucose conditions (25 mM), both in the presence and absence of RvD1(100 nM) and CFTRinh-172. Their responses were examined at the transcriptional and protein levels. Gene expressions of differentiation, inflammation, and chemotaxis markers were analyzed by quantitative real-time PCR (Fig. 24), while cytokine production in supernatants was measured using MSD ELISA (Fig. 25). RvD1 (10 nM) treatment gave consistent increases in the expression of CD14, which was suppressed by the high-glucose challenge, both in the presence and in the absence of CFTRinh-172; also, RvD1 gave increase or reduction in CD68 depending on the presence of CFTRinh-172, although these changes did not attain significance due to the variability in cell responses. On the contrary, there was a significant reduction in CD163 with RvD1 in cells exposed to M-CSF and CFTRinh-172 (Fig. 24). Moreover, RvD1 increased the expression of TNF- α in THP-1 exposed to high glucose and treated with CFTRinh-172, while it enhanced IL-1 β in cells exposed to high glucose alone and reduced this cytokine in the presence of CFTRinh-172 (Fig. 24). It appears that the expression of TNF- α and IL-1 β in THP-1 macrophages under high glucose is impaired compared to cells differentiated with M-CSF in normal growth medium. In addition, a reduction in MCP-1 was observed in THP-1 under high glucose conditions, which was significantly enhanced by RvD1 treatment. On the contrary, RvD1 gave a trend towards diminished MCP-1 when CFTR was inhibited by CFTRinh-172.

At the protein level (Fig. 25), the amounts of MCP-1, MIP-1 α , IL-18, IL-1 β , and TRAIL released by THP-1 cells differentiated with M-CSF under high glucose were generally lower, while IL-6 was increased, compared with amounts released by cells differentiated with M-CSF in standard growth medium. No major changes were observed in protein concentrations following RvD1 treatment, except for IL-6 that was significantly diminished only in THP-1 differentiated with M-CSF under high glucose. Of interest, previous studies by Börgeson et al., demonstrate that LXA₄ (which shares RvD1 receptors on human macrophages (Krishnamoorthy et al., 2010b)) treatment also reduces IL-6 in macrophages co-cultured with adipocytes. This indicates common effects of LXA₄ and RvD1 on macrophages inflammatory cytokines in setting of diabetes and obesity. In addition, LXA₄ caused an Annexin-A1 release by macrophages. Further studies will be required to determine if RvD1 also modifies Annexin-A1 and other chemical mediators by THP-1 cells in the setting of CFRD(Börgeson et al., 2015).

These results suggest that hyperglycemia can hamper normal macrophage responses and that RvD1 can restore cell functionality under these conditions. Furthermore, the reduction in the release of TRAIL by macrophages challenged with high glucose and slightly further inhibited in CFTRinh-172-treated cells indicates an increase in cell survival under stress conditions that can be potentiated

when CFTR is inactive. However, a larger number of experiments will be required to corroborate these findings.

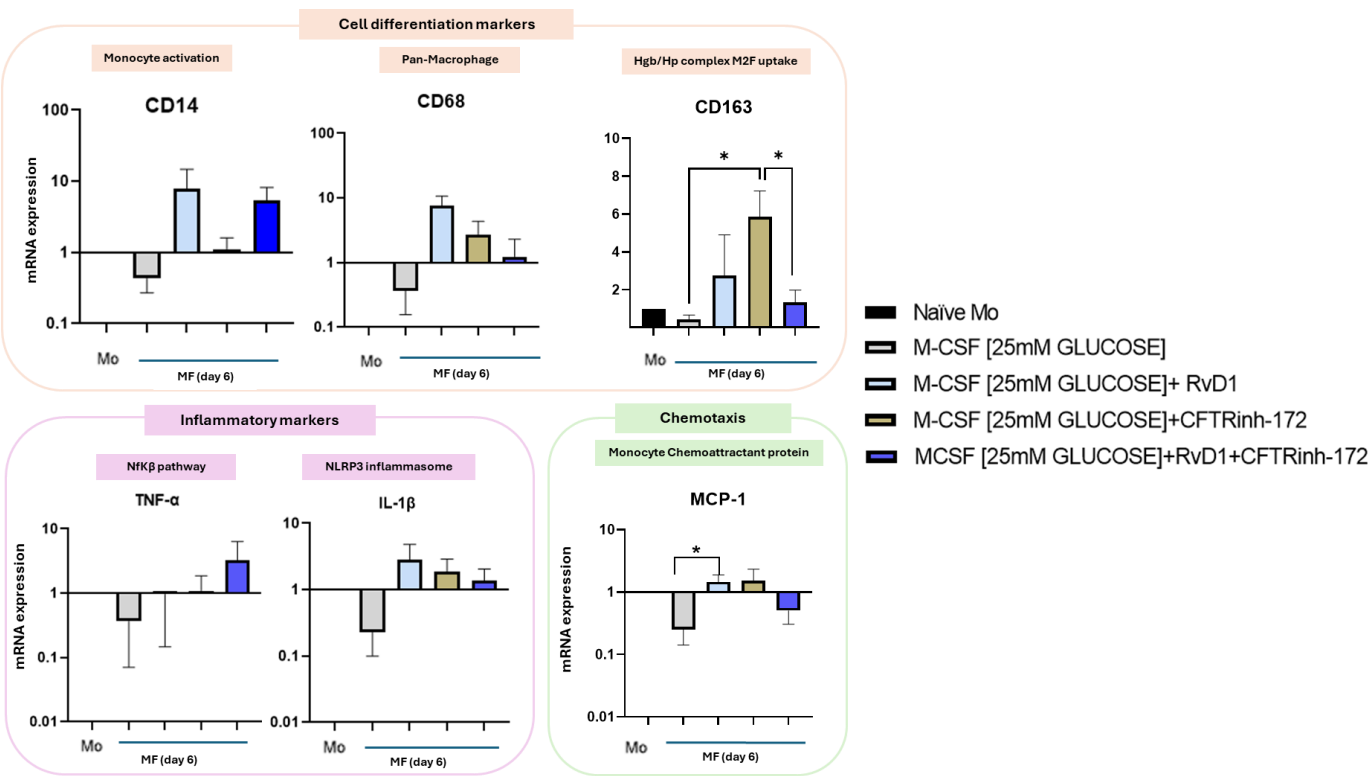


Fig.24.Expression of differentiation, inflammatory, and chemotactic markers in macrophages exposed to high glucose, RvD1, and CFTR-172.

Bar graphs show transcriptional levels of CD14, CD68, CD163 (differentiation markers), TNF-α, IL-1β (inflammatory markers), and MCP-1 (chemotaxis marker) in THP1-derived macrophages under naïve or iper-glucose (25 mM) conditions, with or without RvD1(100 nM) and CFTRinh-172 (10 μM). Data are presented as mean ± SEM (n=3).

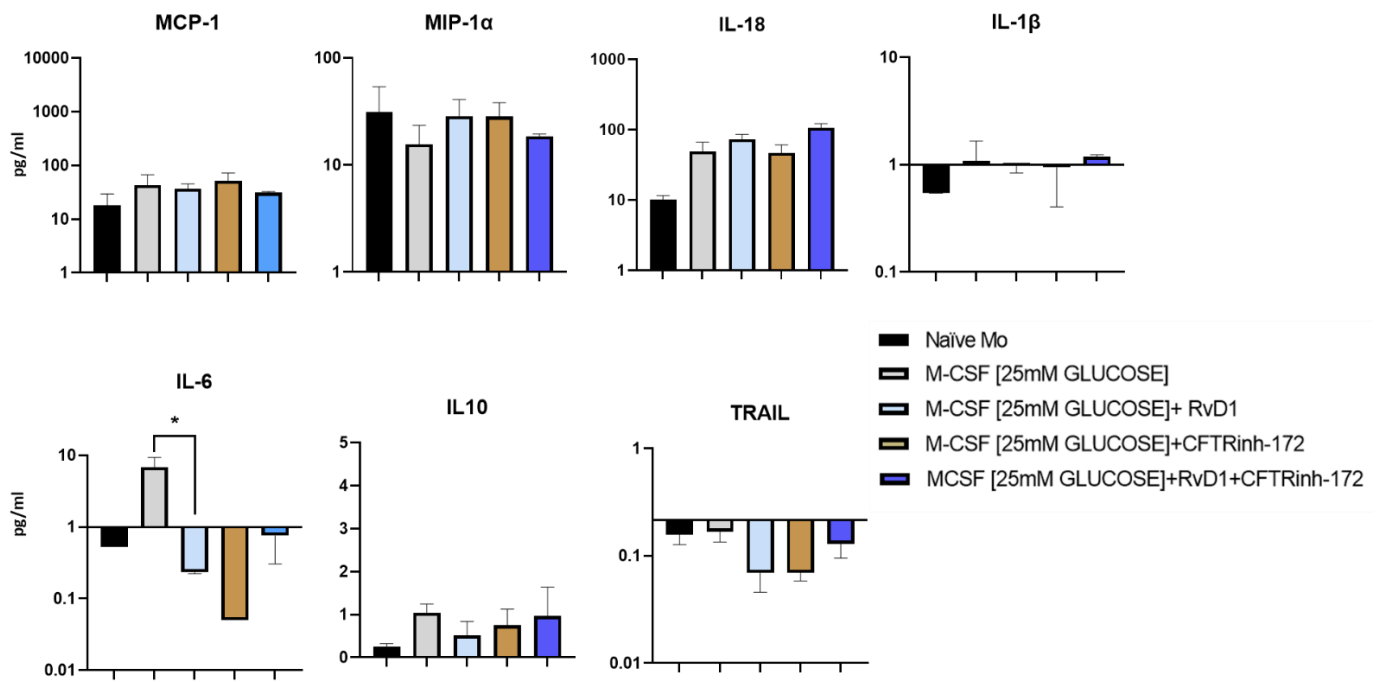


Fig.25 Cytokine secretion profiles of macrophages under high glucose, RvD1, and CFTR-172, as quantified by MSD ELISA

Graphs show protein concentrations (pg/mL) of MCP-1, MIP-1α, IL-18, IL-1β, IL-6, β0, and TRAIL in cell culture supernatants. Data are presented as mean ± SEM (n=3).

3.5.6. CFTR knock-out (KO) mice spontaneously develop cystic fibrosis-related diabetes (CFRD).

Establishing an appropriate experimental model to study CFRD pathophysiology and the role of inflammation is crucial. In vitro systems are unsuitable due to complex cellular interactions. Organ-on-chip models show promise but have limitations. Large animal models closely mimic human pathology but are resource intensive. Small CF animal models, such as mice, are preferred for preclinical studies. Uncertainty persists regarding diabetes susceptibility in CF mice. Therefore, during my PhD I characterized glucose homeostasis and signs of diabetes in CFTR knock-out (KO) mice.

Under fasting conditions, CFTR KO mice exhibited lower body weight, hyperglycemia, and insulin resistance, demonstrated by increased fasting insulin levels and HOMA-IR index, compared to wild-type (WT) littermates, consistent with CFRD (Fig.26). Moreover, when challenged with an intra peritoneal glucose tolerance test (IPGTT), which measures the body's response to glucose overload, CFTR-KO had an impaired capacity to regulate blood glucose levels, a hallmark of diabetes. The area under the curve (AUC) indicated that CFTR-KO had a significantly higher global exposure to glucose compared to WT mice.

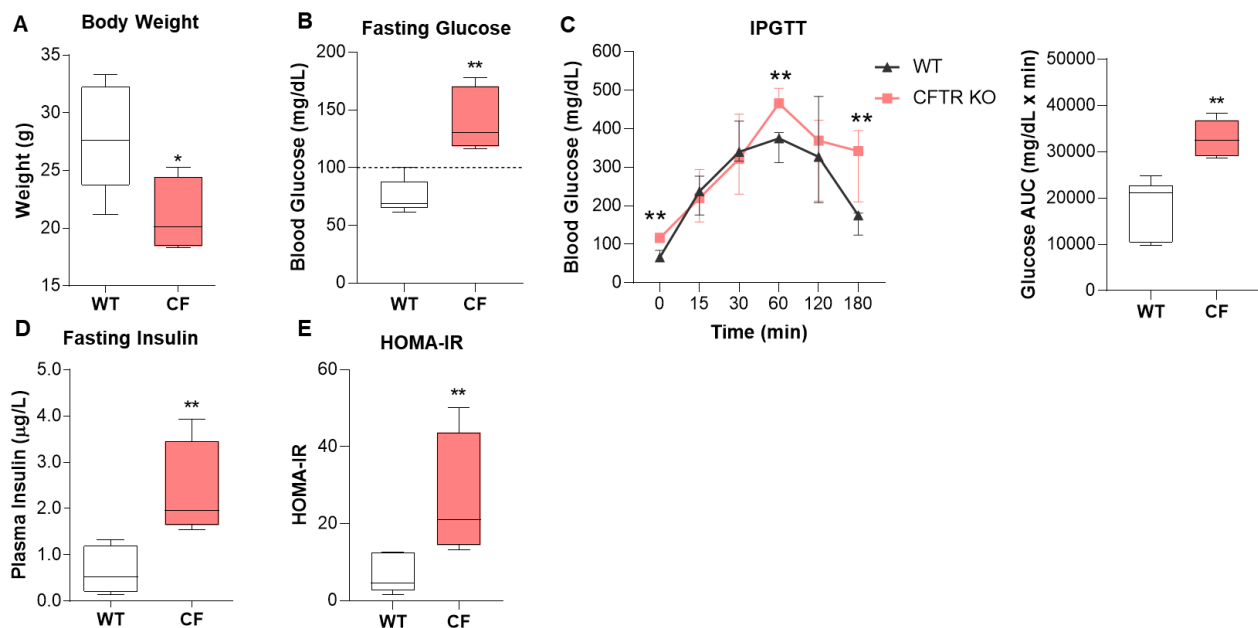


Fig.26 CFTR KO mice develop CFRD.

Body weight, fasting and post-IPGTT blood glucose, plasma insulin, and HOMA-IR determined in S489X-CFTR KO mice and age/sex-matched WT littermates (n = 5-6 mice/group) at 18-20 weeks of age. Mice were starved from 8 am to 2 pm prior the experiment. Blood glucose and plasma insulin were measured with a portable glucose meter or ELISA respectively. HOMA-IR were calculated according to the formula: $[\text{glucose (mM)} \times \text{insulin (pM)}]/135$. Box-whisker plots show the 25th and 75th percentile range (box) with a 95% confidence interval (whiskers) and median values (transverse lines in a box). P values were calculated with the SigmaPlot 14.5 software using nonparametric Mann-Whitney or Kruskal Wallis. (*, $P < 0.05$; **, $P < 0.01$ compared to WT at the same time-point). CF, CFTR KO mice.

3.5.7. Mice with CFRD have increased inflammation and decreased resolution.

Analysis of leukocyte populations in pancreases revealed significant infiltration compared to non-diabetic wild-type (WT) littermates, particularly with a trend towards increased polymorphonuclear cells (PMN) and M1 (CD80⁺) macrophages (Fig.27). Lipid mediator profiling using LC-MS/MS showed elevated pro-inflammatory eicosanoids such as TXB₂ and PGD₂, alongside decreased SPM, including RvD1, RvE2, and RvE4 in CFRD pancreases.

Interestingly, the increase in MaR2 could be an adaptive response or a signal of a specific lipid imbalance in CF (Fig.28). The concomitant reduction of RvE1 and RvD1 suggests that the metabolic pathways of SPM are differentially altered. Therefore, to give more support and provide clearer information to these data, it would be necessary to investigate the enzyme levels (such as 12-LO and 15-LO involved in the synthesis of Mar2) and lipid levels (such as DHA or other precursor levels).

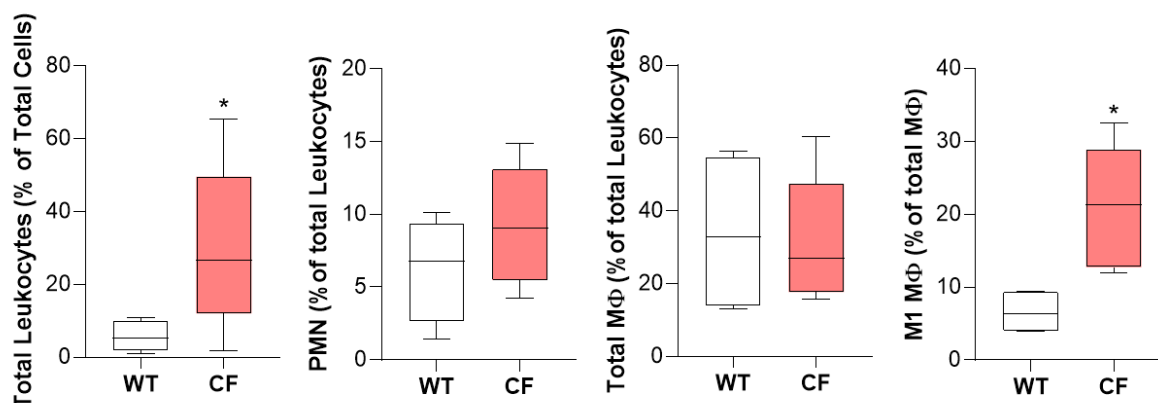


Fig.27 Leukocyte infiltration and activation status in pancreatic tissue from mice with CFRD.

Flow cytometry analysis of total leukocytes (CD45⁺), PMN (CD11b+Ly6G⁺), total MΦ (F4/80⁺) and M1-polarized MΦ in pancreases from mice with CFRD (CF, n= 6) or WT non-diabetic littermates (n= 5). Box-whisker plots show the 25th and 75th percentile range (box) with a 95% confidence interval (whiskers) and median values (transverse lines in a box). P values were calculated with the SigmaPlot 14.5 software using non-parametric Mann-Whitney test.

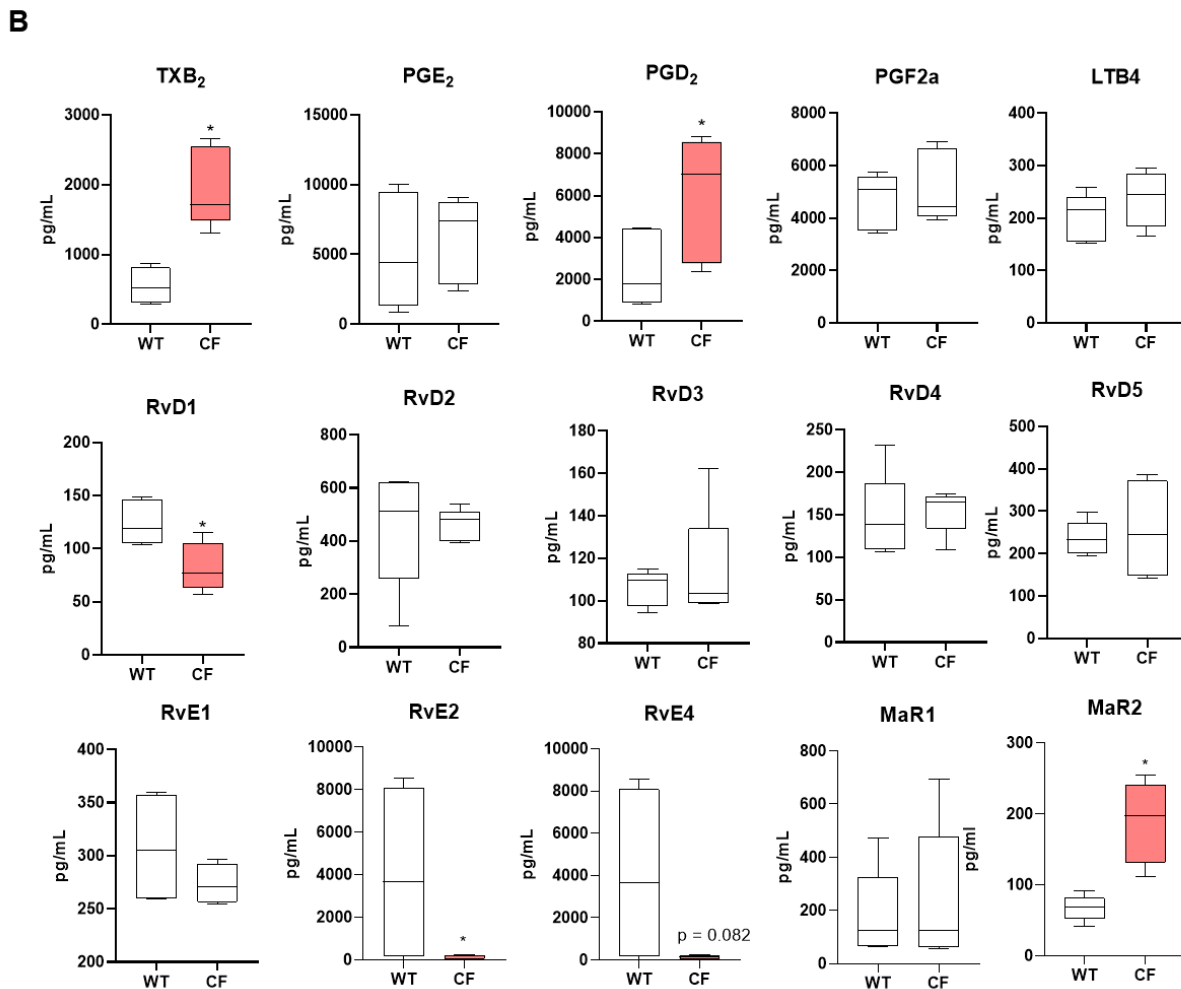
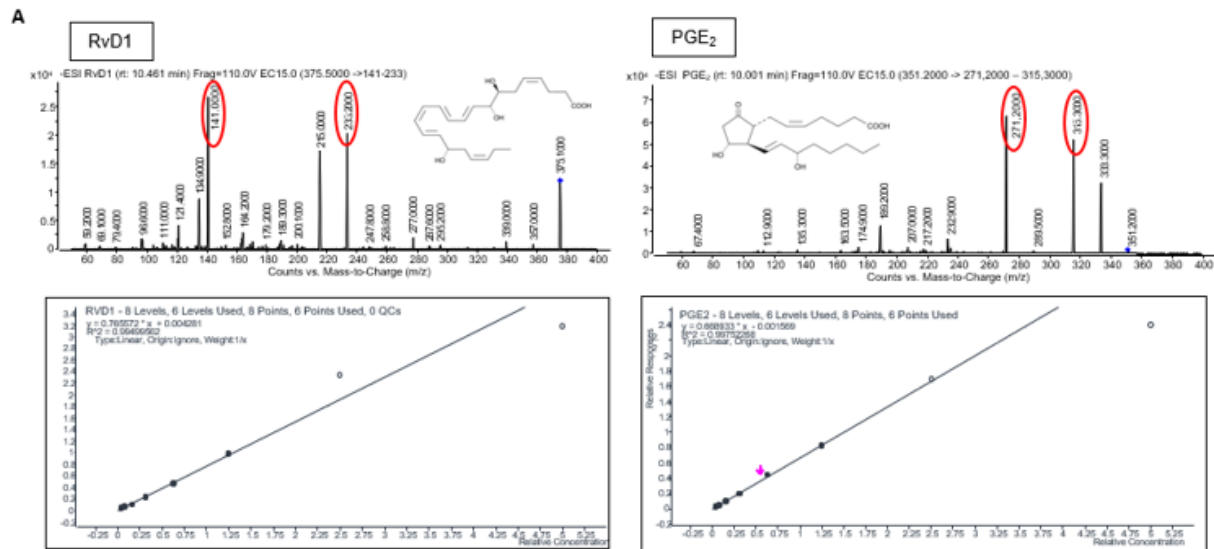


Fig.28 Lipid mediator profiling in pancreas from mice with CFRD and non-diabetic WT mice.

A) Representative MS/MS spectra and standard curve of select eicosanoids and SPM identified and quantified in pancreatic tissues collected from age/sex-matched CFTR KO mice with CFRD and non-diabetic WT littermates. Standard curves were calculated plotting the relative response vs concentration of deuterium-labeled standards. Each data point was run as n = 3 replicates. **B)** Quantification of identified PG, TX, LTB₄, D- and E-series-Rv, and MaR in pancreases of WT non-diabetic mice (n = 5) vs CFTR KO (CF) diabetic littermates (n = 5). Box-whisker plots show the 25th and 75th percentile range (box) with a 95% confidence interval (whiskers) and median values (transverse lines in a box). *, P < 0.05; P values were calculated with the SigmaPlot 14.5 software using non-parametric Mann-Whitney test.

3.5.8. RvD1 improves glucose homeostasis, reduces inflammation, and increases SPM in mice with CFRD

Treatment with RvD1 (100 ng, I.P., every 2 days) for 15 days resulted in basal fasting glycemia that was significantly lower compared to vehicle-treated mice, in an improved glucose tolerance, as evidenced by a sharper decrease in blood glucose from 60 to 180 min post IPGTT, and a significant reduction in glucose AUC (Fig.29).

Overall, these results highlight the potential beneficial *in vivo* actions of RvD1 in diminishing CF-associated diabetes.

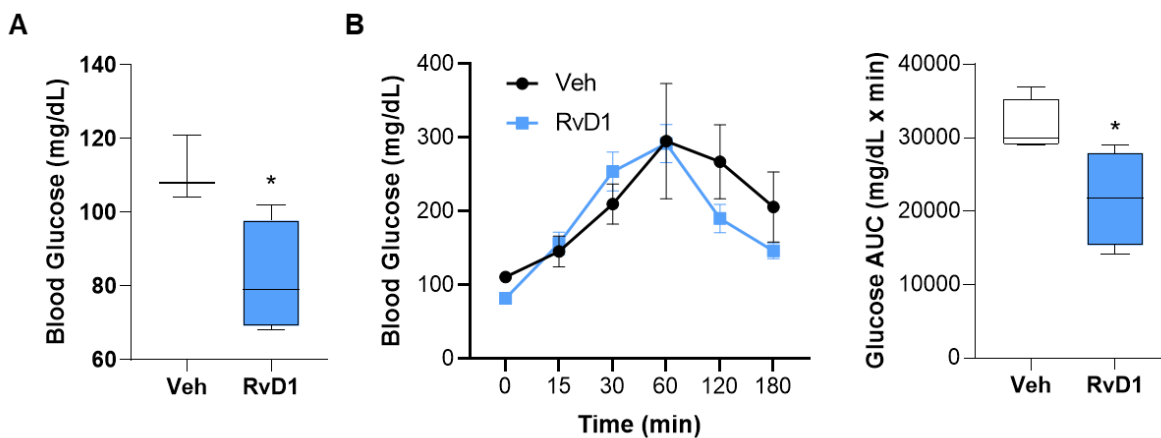


Fig.29 RvD1 improves glycemia and glucose tolerance in mice with CFRD.

A) Fasting glycemia and B) IPGTT in CFTR KO mice at day 15 of treatment with RvD1 (100 ng) or vehicle (0.5 % EtOH in PBS) by I.P. every 2 days. Glucose AUC was calculated from blood glucose measured from 0 to 180 min during IPGTT. Box-whisker plots show the 25th and 75th percentile range (box) with a 95% confidence interval (whiskers) and median values (transverse lines in a box) from $n = 4$ mice/group. *, $P < 0.05$. Statistical analyses were carried out with the SigmaPlot 14.5 software using non-parametric Mann-Whitney test.

3.6. Discussion:

Macrophages play a critical role in immune regulation, balancing inflammatory and reparative processes through their ability to polarize into different subtypes, originally described as pro-inflammatory (M1) or anti-inflammatory (M2) phenotypes, depending on growth factors and other microenvironmental signals. Dysregulation of macrophage function has been implicated in various chronic inflammatory conditions, including cystic fibrosis-related diabetes (CFRD) and type 2 diabetes (T2D) (Coderre et al., 2021). This study, using THP-1-derived macrophages and CFTR-KO mouse models, sheds light on the impact of CFTR loss-of-function and hyperglycemia on macrophages and CFRD, as well as on the regulatory actions of the specialized pro-resolving mediator RvD1,

CFTR expression in macrophages was demonstrated by Yoshimura et al., several years ago (Yoshimura et al., 1991). In addition to the cell membrane, CFTR is present intracellularly in macrophages, which indicates a more complex role of this protein in these immune cells. Consequently, mutations in CFTR protein and/or reduction of its function can affect several cellular pathways and responses in macrophages.

It is well established that macrophages derived from people with CF have abnormalities in their polarization and activities. For instance, Meyer et al. demonstrated that macrophages, in both M1 and M2 profiles, exhibit hyperactivation. In the case of M1 macrophages, they found a high expression of IL-8, IL-6 and TNF- α and low expression of IL-10, which can lead to reduced phagocytic activity, while an increase in M2 profile may result in a reduced ability to perform endocytosis and a propensity to induce remodeling and fibrosis of the inflamed tissue (Meyer et al., 2009). Bruscia et al., demonstrated the direct contribution of macrophages carrying a defective CFTR in the establishment and perpetuation of inflammation in CF. Specifically, they found that lack of CFTR results in persistent activation of NF- κ B that leads to sustain production of cytokines and other inflammatory proteins (Bruscia et al., 2009). Nonetheless, in our study, we uncover previously unknown influences of CFTR on macrophage activities. Indeed, we found that, in THP-1 differentiated with both GM-CSF or M-CSF, CFTR inhibition interferes with normal macrophage 3D structural organization and adhesion, which are essential steps for the defensive activities of these cells. These observations suggest that CFTR is indispensable not only for macrophage adhesion and spatial organization, but also for their functional plasticity, in line with previous studies (Meoli et al., 2022).

Moreover, we uncovered the interplay of CFTR inhibition and high glucose challenge on macrophages, revealing that hyperglycemia compromises their spatial organization and the differentiation process, which is further blunted when CFTR is inhibited.

The interaction between macrophages and the altered metabolic environment is well established for other metabolic disease, such as T2D, in which high glucose levels shift macrophage towards a pro-inflammatory profile. On the contrary, the role of altered metabolism in the context of CF and CFRD

remains not fully understood. Our results indicate a synergy between metabolic and ionic stresses in impairing macrophage function, highlighting the need to investigate these interactions both in the setting of CFRD and other types of diabetes. Along these lines, diabetes, directly deriving from CFTR mutations or due to changes in the lifestyle of people with CF (e.g., type 2-like diabetes) are emerging as new medical priorities (Coderre et al., 2021).

It is possible that macrophage function could be affected by the mutation in the CFTR gene, with different effects also due to the type of mutation and the phase of differentiation in which they are located. While the current project was carried out using a macrophage cell line in which CFTR was chemically blocked, our results prompt to investigate in future studies on the interactions between CFTR and hyperglycemia in primary macrophages derived from patients with CF.

In vivo studies on CFTR-KO mice have strengthened these findings. CFTR-KO mice spontaneously developed CFRD, characterized by hyperglycemia, insulin resistance, and increased pancreatic inflammation (Hart et al., 2018). To our knowledge, our data represent the first demonstration that CFTR KO mice have alterations in the glucose pathway comparable to patients with diabetes, which can be useful in future studies aimed at better understanding the pathophysiology of CFRD and testing potential drugs.

Our results also demonstrate that RvD1 has the potential to counteract these pathological effects. In vitro, RvD1 restored macrophage adhesion and spatial organization disrupted by hyperglycemia and CFTR inhibition. In vitro, we obtained clues that RvD1 can reduce the secretion of inflammatory cytokines, particularly IL-6, while mitigating the pro-inflammatory shift induced by CFTR-172. Furthermore, RvD1 enhanced macrophage chemotactic function by restoring MCP-1 expression, which was significantly suppressed under hyperglycemic conditions. These findings prompt to test the direct effects of RvD1 on macrophage chemotactic responses under hyperglycemia.

In vivo, we found that RvD1 treatment, as short as 15 days, was able to improve glucose homeostasis in CFTR KO mice. Previous studies have proved that RvD1 reduces inflammation and ameliorates the metabolic status associated with obesity, T2D, and T1D (Bathina and Das, 2021; Clària et al., 2012; Gemperle et al., 2021; Hellmann et al., 2011; Rius et al., 2014).

Therefore, results from this study are consistent with the well-established role of RvD1 in promoting resolution of inflammation and maintaining homeostasis when the metabolism is altered. Hence, RvD1 represents a promising therapeutic agent, capable of restoring macrophage homeostasis and resolving inflammation in CFRD conditions.

Conclusion and future studies

This thesis reports on PhD research aimed at exploring the therapeutic potential of Resolvin D1 in targeting aberrant inflammation and metabolic dysregulation associated with CF.

Results with different *in vivo* and *in vitro* approaches demonstrate that RvD1 effectively reduces lung inflammation and bacterial infection, restoring immune and epithelial cell functions. Furthermore, RvD1 normalized glucose balance in mice with CFRD and acted on macrophage to correct cellular functionality and inflammatory factors.

Collectively, these findings highlight multiple beneficial actions of resolvin D1 on pulmonary and extra-pulmonary complications of CF, pointing it as a promising therapeutic agent for mitigating both chronic lung inflammation and metabolic abnormalities associated with this genetic disease.

Upon completion of this 3-year project, several avenues for further research could be pursued:

1. Evaluate the efficacy of other pro-resolving mediators or anti-inflammatory agents, alone or in combination with CFTR modulators, to refine potential therapeutic strategies for the management of lung disease and cystic fibrosis-related diabetes (CFRD).
2. Investigate the role of the gut microbiota and its metabolites in modulating inflammation and glucose metabolism in CF patients, considering that gut dysbiosis is a common feature in CF. Understanding these interactions could provide insights into novel therapeutic targets.
3. Conduct long-term *in vivo* studies in mouse models of CF to assess the progression of CFRD and the sustained effects of treatments such as RvD1 or CFTR modulators on metabolic complications, including dyslipidemia and atherosclerosis, and inflammation. These studies would provide valuable data on the chronic impacts of such therapies.

3.7. Materials and methods:

3.7.1 Sample Collection and Analyses:

THP-1 cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% L-glutamine. A total of 500,000 cells were seeded in each well of a 24-well plate. Each well received a different treatment: M-CSF and GM-CSF were used at a concentration of 100 ng/ml, while 4-[[4-Oxo-2-thioxo-3-[3-(trifluoromethyl)phenyl]-5-thiazolidinylidene]methyl]benzoic acid (CFTRinh-172, CAS 307510-92-5) was used at 10 μ M. The cells were maintained in differentiation medium for 72 hours with M-CSF or GM-CSF. After 3 days, once the cells had adhered, the medium was refreshed, and CFTRinh-172 was added. The plate was immediately placed in the IncuCyte incubator for a 24-hour treatment evaluation. When mimicking the high glucose condition, 25 mM of glucose was added at the time of cell seeding along with M-CSF, and the cells were maintained for 72 hours. Subsequently, the medium was refreshed, and CFTRinh-172, glucose, and RvD1 at 100 nM were added. In this case, the plate was also transferred to the IncuCyte for a 48-hour observation. For all experiments, the supernatant was collected after 24 hours in the IncuCyte (thus after 4 or 5 days when glucose and resolvin were present) for MSD multiplex ELISA analysis. Cells were detached and lysed to extract RNA and study macrophage differentiation markers, inflammation, and chemotaxis with RealTime QuantStudio 5Pro. The protocol for the MSD ELISA can be found at the following link: [MSD ELISA Protocol](#).

3.7.2. Animal Models:

CFTR knockout (CFTR KO) mice and wild-type (WT) C57BL/6 mice were administered glucose via intraperitoneal (I.P.) injection, and insulin levels were monitored for up to 180 minutes. Body weight, glucose tolerance, and the presence of lipid mediators were assessed using mass spectrometry. The experiment was then repeated using only CFTR KO mice, which were treated with RvD1 (10 nM via I.P.) or Vehicle (0.01% ethanol via I.P.) immediately after glucose administration, followed by the same analysis. Mice used in the study were 8 weeks old.

The analyses were performed using a FACS Canto II flow cytometer and FACS Diva software (BD Bioscience). Samples were stained for 30 minutes at 4°C with fluorochrome-conjugated antibodies targeting CD45, CD11b, Siglec, F4/80, and Ly6G antigens (Sony Biotechnology or Biolegend). Additionally, the Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) was evaluated. HOMA-IR is an index used to estimate insulin resistance. In mice, it is calculated using fasting glucose and insulin levels.

The commonly used formula is:
$$\text{HOMA-IR} = \frac{\text{Fasting Glucose (mg/dL)} \times \text{Fasting Insulin (}\mu\text{U/mL)}}{405}$$

This index helps assess insulin sensitivity in the context of metabolic studies.

3.7.3. Mass spectrometry

Lipidomic analysis was performed using LC-MS/MS. Biological samples, such as plasma, tissues, or biological fluids from WT and CF mice, were collected and processed for lipid extraction. Extraction of lipid mediators was performed using organic solvents, such as methanol and ethyl acetate, in the presence of deuterated internal standards for absolute quantification.

Supernatants were mixed with 2 volumes of ice-cold MeOH containing a mixture of deuterium labeled internal standards (10 ppb each).

Following protein precipitation and solid phase extraction, eluted fractions were taken to dryness, suspended in mobile, and injected into an Agilent 1260 Infinity II LC coupled to a Ultivo Triple Quadrupole mass spectrometer, equipped with a Jet Stream electrospray (ESI) ionization source. The chromatographic separation was achieved using a Luna Omega C18 column (100x2.1mm, 1.6µm, Phenomenex) and a gradient elution with a mobile phase consisting of 0.1% aqueous formic acid and 50:50 v/v MeOH:ACN.

The MS operated in negative ionization mode and performed dynamic multiple reaction monitoring (MRM). Ions from reported MRM methods were used for identification and quantification. Target analytes were quantified by calibration curves plotting the analyte to an internal standard (quantifier transition) vs the corresponding concentration ratio.

3.7.4. Statistical Analysis

Data were presented as median with interquartile range (25th/75th percentiles) or as mean $\pm$ standard error, depending on data normality assessed by the Shapiro-Wilk test. Statistical comparisons were made using the Mann-Whitney U test, ANOVA on ranks, or parametric t-tests followed by Tukey's post-hoc test, as appropriate. A p-value of <0.05 was considered statistically significant.

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