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Early interferon- λ production by human nasal epithelial cells

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By

Valeriia Korovanenko

MD

School of Medicine

Trinity College Dublin

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Supervisor: Cliona O'Farrelly, PhD FTCD, Professor of
Comparative Immunology, Trinity College Dublin

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Abstract

Nasal epithelial cells serve as a first line of defence against respiratory pathogens, playing a particularly crucial role in the detection and response to viruses that enter the body via the nose. Despite their importance, current research largely overlooks early antiviral responses, particularly within the first hours post-infection. This study aims to address this gap by optimising extraction methods, cell preparation and characterisation of the early immune response in nasal epithelial cells.

To achieve this, various techniques for cell isolation, including mechanical dissociation and the use of animal component-free kit, were evaluated for their efficiency and effectiveness. We measured cell yield, viability, and RNA quality across different extraction methods. Our results indicated that while both methods yielded comparable cell counts, the mechanical dissociation was more efficient, cells had less debris and when cultured grew better. They also provided higher RNA concentrations and purity, highlighting its suitability for downstream applications such as gene expression analysis.

This study also explores the natural antiviral immune response in nasal epithelial cells by examining differences in immune cell composition and interferon expression in healthy individuals. Participants were recruited through an institutional campaign and ethical approval for the study was obtained from the TCD Research Ethics Committee. Nasal swabs were collected to measure baseline levels of type I and III interferons (IFNs) as well as interferon-stimulated genes (ISGs), followed by stimulation with Poly IC. A novel approach was used in which nasal swab samples were directly stimulated, preserving the cells' physiological state.

Our findings reveal significant variability in interferon baseline expression and responses across individuals. Type III IFNs responses were detected as early as 4 hours post-stimulation, with correlations observed between the epithelial cell marker EpCAM and *IFNL1* expression upon stimulation. Additionally, a negative correlation was found between IFN- ϵ (*IFNE*) and IFN- λ (*IFNL2/3*), suggesting a potential regulatory mechanism or competition between these interferons in controlling inflammation and antiviral responses in mucosal tissues.

The direct swab stimulation method proved to be an efficient and non-invasive technique for quickly assessing immune responses in vivo, offering a practical alternative to time-consuming culture-based methods.

Abbreviations

ALI	Air-liquid interphase
AMPs	Antimicrobial peptides
APCs	Antigen-presenting cells
DC	Dendritic cells
EpCAM	Epithelial Cell Adhesion Molecule
HNEC	Human nasal epithelial cell
IFN	Interferon
IRF	Interferon regulatory factor
ISGs	Interferon-stimulated genes
MDA-5	Melanoma differentiation-associated gene-5
MHC	Major histocompatibility complex
NETs	Neutrophil extracellular traps
NP	Nasopharyngeal
PAMPs	Pathogen-associated molecular patterns
PRR	Pattern-recognition receptor
RIG-I	Retinoic acid-inducible gene-1
RLR	RIG-I-like receptor
RNA	Ribonucleic Acid

RT	Room temperature			
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction			
SARS-CoV-2	Severe	Acute	Respiratory	Syndrome
	Coronavirus 2			
TLR	Toll-like receptor			

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1. Chapter 1. General introduction

1.1 Innate antiviral immunity

1.1.1 Overview of innate antiviral immunity

The innate immune system is the first line of protection against invading pathogens in the body. This response is non-specific and rapid, limiting viral replication and spread before the adaptive immune response is fully mobilised. A key feature of innate immunity is the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs). PRRs are known to be expressed by innate immune cells, such as macrophages, dendritic cells, natural killer (NK) cells and neutrophils, but recent studies have showed that also nonimmune cells, such as epithelial cells do express PRRs and are able to induce an innate immune response (1–3).

PAMPs are highly conserved molecular structures, such as nucleic acids, lipids, carbohydrates, and proteins, shared by the same type of pathogens. PAMPs are essential for pathogen survival and have molecular structures not found in host cells. PAMPs absence in the host is at the basics for recognising the “self” from “non-self” by PRRs. Viral nucleic acids, both DNA and RNA, present molecular and biochemical features not shared by eukaryotic cells, and because of these characteristics they are recognized by different PRRs as “non-self” and trigger the activation of the innate antiviral response. Viral replication intermediates, such as double stranded RNAs (dsRNA), which are absent in eukaryotic cells, are recognized by PRRs. Different viruses, among others SARS-CoV-2 and Influenza A virus, produce dsRNA intermediates during viral infection, activating an innate antiviral response in the host cell (4).

Recognition of viral PAMPs by PRRs triggers signalling cascades resulting in the production of interferons (IFNs) and pro-inflammatory cytokines, which activate a variety of antiviral mechanisms (5,6).

Upon viral detection, type I interferons, particularly IFN- β , are produced by infected cells and neighbouring immune cells, which activate interferon-stimulated genes (ISGs) that inhibit various stages of viral replication (7). IFNs create an antiviral state within the cell, restricting viral replication and spread. Additionally, type III interferons, including IFN- λ , play a crucial role in the epithelial tissues, particularly in the respiratory tract, by inducing antiviral responses without causing excessive inflammation (8). These IFNs activate the JAK-STAT signalling pathway, leading to the upregulation of ISGs that directly inhibit viral replication, such as CXCL10 and ISG15 (Figure 1.1) (9).

NK cells, recognise and eliminate virus-infected cells through the release of cytotoxic molecules, including perforin and granzyme, which induce apoptosis in the infected cells (10). NK cells also produce IFN- γ , which activates macrophages, enhancing their ability to phagocytose and destroy virus-infected cells (11). Neutrophils, another critical component of the innate immune response, contribute by producing neutrophil extracellular traps (NETs), which capture and neutralize viral particles, limiting their spread and facilitating clearance from infected tissues (12). The macrophages and other immune cells produce cytokines such as IL-6 and TNF- α in order to recruit additional immune cells to the site of infection, thus amplifying the immune response.(13).

Another important player in the innate immune response is the complement system, a group of plasma proteins that enhances pathogen

clearance through processes like opsonization and the formation of the membrane attack complex (MAC), which lyses infected cells(14). The complement system amplifies the inflammatory response and aids in recruiting immune cells to the site of infection, boosting the antiviral defence(15).

Additionally, antimicrobial peptides (AMPs), such as defensins and cathelicidins, are secreted by epithelial cells and play a direct role in disrupting viral envelopes, adding another layer of defence at mucosal surfaces (16).

As well as controlling viral replication, innate immunity plays a crucial role in shaping the adaptive immune response. The production of cytokines and chemokines by innate immune cells creates a pro-inflammatory environment that supports the activation and recruitment of adaptive immune cells, such as T cells and B cells, which are necessary for long-term immunity. Dendritic cells (DCs) and macrophages serve as main antigen-presenting cells (APCs), processing and presenting viral antigens via major histocompatibility complex (MHC) molecules, which is vital for activating naive T cells (5). Therefore, all the described components of innate immune system not only contain viral infections but also prime the adaptive immune response for more targeted and sustained immunity.

1.1.2 Pattern recognition receptors of innate antiviral immunity

PRRs are specialised proteins that serve as sentinels of the innate immune system, enabling the early detection of bacterial and viral particles by recognising unique PAMPs (1). These receptors are distributed throughout various cellular compartments, including the plasma membrane, endosome and the cytoplasm, positioning them to effectively monitor and detect viral

invaders at different stages of the infection cycle (17). Upon engagement with viral PAMPs, PRRs initiate a cascade of intracellular signalling events, leading to the production of interferons (IFNs) and pro-inflammatory cytokines, which play a critical role in halting viral replication and recruiting additional immune components (13).

Key families of PRRs are the Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), and NOD-like receptors (NLRs) (1,17). TLRs, such as TLR3, TLR7, and TLR8, are located in endosomal compartments where they recognize viral RNA(17). TLR3 specifically binds double-stranded RNA (dsRNA), an intermediate of viral replication, while TLR7 and TLR8 detect single-stranded RNA (ssRNA), a hallmark of RNA viruses (18–20).

In contrast, RLRs, including RIG-I and MDA5, detect viral RNA within the cytoplasm, allowing them to identify a wide range of RNA viruses. RIG-I primarily detects short dsRNA with 5'-triphosphates, whereas MDA5 senses long dsRNA (21,22).

Upon detection of viral components, these PRRs activate downstream signalling pathways that activate transcription factors such as nuclear factor- κ B (NF- κ B) and interferon regulatory factors (IRFs), driving the expression of IFNs and other antiviral molecules (23). In RIG-I activation, mitochondrial antiviral signalling protein (MAVS) is critical to amplifying the signal via IRF3 and IRF7, which leads to robust IFN production(24).

NOD-like receptors (NLRs) are another important class of PRRs that detect intracellular alterations caused by viral infections. One of the most well-studied NLRs is NLRP3, which forms the NLRP3 inflammasome in response to viral infection or cellular stress. This inflammasome activates caspase-1, which in turn releases pro-inflammatory cytokines such as

interleukin-1 β (IL-1 β), thereby contributing to the inflammatory response (25). NLRP3 has been shown to play a role in the immune response to RNA viruses, including influenza and respiratory syncytial virus (RSV), highlighting its role in antiviral immunity (26)

The essential role of PRRs in defending against viral infections is further emphasised by research showing that mice deficient in TLR3 or RIG-I exhibit increased susceptibility to viral pathogens, such as West Nile virus and Sendai virus, respectively (22,27). In humans, mutations in PRR-related genes have been linked to heightened vulnerability to viral diseases including herpes simplex virus (HSV) encephalitis (28). It is clear from these findings that PRRs, such as TLRs, RLRs, and NLRs, play a critical role in mounting an effective immune response against viral pathogens(1). Some viruses, such as hepatitis C virus (HCV) and SARS-CoV-2, can delay or inhibit this response to evade early immune detection, leading to uncontrolled viral replication(29–31).

1.1.3 Interferons and ISGs

IFNs (interferons) are essential cytokines in antiviral defence, inhibiting viral replication and activating immune cells. IFNs are categorised into three types based on their structure and function, as well as the specific receptors they bind to on the cell surface: type I, type II, and type III interferons (32,33).

Type I interferons include several subtypes such as IFN- α , IFN- β , IFN- ϵ , IFN- κ , and IFN- ω , and they signal through the IFN- α/β receptor (IFNAR). These interferons are primarily responsible for initiating early antiviral responses in cells (34,35).

Type II interferon, consisting solely of IFN- γ , interacts with the IFN- γ receptor (IFNGR) and plays a significant role in immune regulation and the activation of macrophages (36,37).

Finally, type III interferons, which include IFN- λ 1, IFN- λ 2, IFN- λ 3, and IFN- λ 4, signal through the IL-28 receptor and are particularly important in antiviral defences at mucosal surfaces, such as in the respiratory and gastrointestinal tracts (8,38).

IFN- ϵ , a less studied type I interferon, is constitutively expressed, particularly at mucosal surfaces, such as those found in the reproductive tract, where it plays a key role in maintaining baseline immune defence even in the absence of infection (39,40). Its constitutive expression sets it apart from other type I interferons, such IFN- α and IFN- β , which are typically produced only upon infection. Recent research suggests that while IFN- ϵ plays a role primarily in localized mucosal immunity, its contribution to systemic immunity is limited. Unlike IFN- α and IFN- β , which are rapidly produced in response to viral infections and trigger broad systemic responses, IFN- ϵ is constitutively expressed and has a more subdued pro-inflammatory effect(39,41,42)

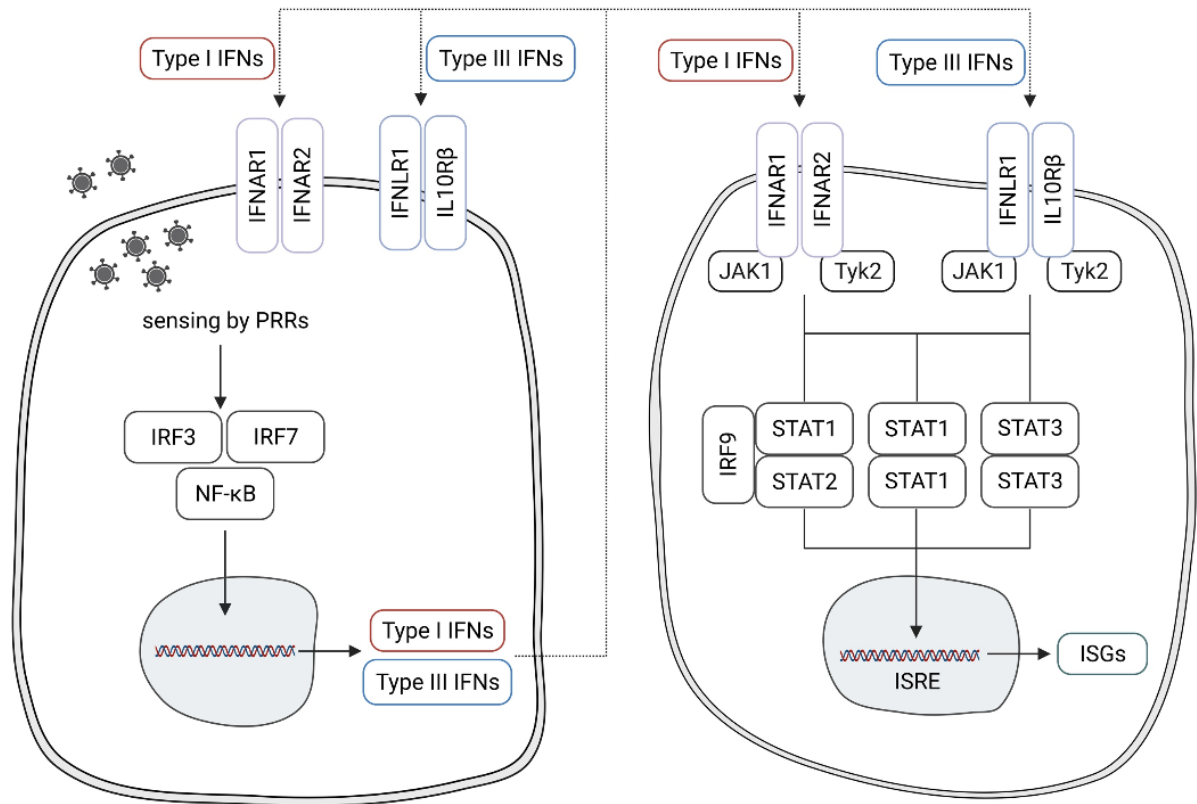


Figure 1.1 Overview of Type I and III IFNs signalling. Viral infections activate cytoplasmic and endosomal pattern recognition receptors (PRRs), which in turn activate transcription factors such as NF-κB, IRF3, and IRF7. This leads to the production of type I interferons (IFN-I) and type III interferons (IFN-III). IFN-I signals through the IFNAR1/IFNAR2 receptor, while IFN-III signals through IFNLR1/IL10Rb heterodimers. Both engage the JAK/STAT signalling pathway, which upregulates interferon-stimulated genes (ISGs) that protect the host by inhibiting viral replication and promoting immune responses. Figure from (43)

The downstream effects of interferons are mediated by hundreds of ISGs, which provide direct antiviral activities(7). For instance, the ubiquitin-like molecule known as ISG15 disrupts viral replication by altering viral and host proteins, thereby inhibiting infectious virus assembly (44). OAS1 activates RNase L, which degrades viral RNA, effectively blocking the replication of many RNA viruses (45).

In addition to their antiviral function, ISGs also play a role in modulating immune responses. Promoted by interferon signalling, chemokines like CXCL10 and CCL5 (RANTES) attract immune cells to the site of infection. A key function of CXCL10 is to attract T cells and NK cells to infected tissues, amplifying the immune response (46). By recruiting additional immune cells, CCL5 helps clear infected cells and control viral spread(47) .

Recent studies, particularly those focused on SARS-CoV-2, have shown that effective interferon signalling is critical for controlling viral load early in infection. In patients with impaired IFN responses, the severity of disease is greater, demonstrating the importance of these cytokines in shaping the immune response (48–52)

1.2 Nasal epithelial cells

The epithelium mediates interaction between the physical environment, the local microbiome and the body (53). It serves as a crucial barrier system in the body, covering surfaces and lining cavities in organs such as the respiratory, gastrointestinal, and urogenital tracts. It protects underlying structures from pathogens, allergens and pollutants utilizing various cell junctions like tight junctions, adherens junctions, and desmosomes to maintain structural integrity and regulate permeability. Tight junctions, made up of proteins such as claudins and occludins, restrict paracellular transport, playing a key role in immune defence and epithelial stability. The basement membrane beneath the epithelium provides additional support and facilitates important cell signalling for tissue repair and immune responses (54,55). Disruption of the epithelial barrier, often caused

by pollutants or inflammatory cytokines, can lead to increased permeability and immune activation, seen in conditions like allergic rhinitis and chronic rhinosinusitis, where degradation of tight junction proteins results in barrier dysfunction. Maintaining an effective epithelial barrier is essential to protect deeper tissues from external threats and prevent immune reactions (56).

1.2.1 The nasal epithelium as a physical barrier

In the nasal epithelium, the barrier is crucial due to constant exposure to airborne pathogens (57). This barrier is essential for maintaining homeostasis and preventing the entry of pathogens and allergens. Its structure is composed of tightly packed several specialised cell types, each of which contributes to the overall function of the nasal mucosa (55,58). Understanding the complexity of the nasal epithelium is essential for evaluating the early immune responses within nasal epithelial cells, which is the primary focus of this study.

An epithelium of the nasal passage is composed of three main types of cells: ciliated cells, goblet cells and basal cells that form a pseudostratified columnar epithelium (Figure 1.2)(55).

- Ciliated columnar cells are one of the defining cell types of nasal epithelium, (characterised by their FOXJ1 marker), featuring cilia that aid in moving mucus and trapped pathogens toward the throat (59). This process, called mucociliary clearance, is a critical defence mechanism that helps in restricting the spread of pathogens to the lower respiratory tract (60). These cells are also among the primary targets of respiratory viruses, such as SARS-CoV-2, during the early stages of infection (61)

- Goblet cells (expressing the MUC5AC and MUC5B markers), are interspersed among ciliated cells. They produce mucus that traps inhaled particles and pathogens, playing a crucial role in mucosal health, especially in conditions like chronic rhinosinusitis. Secreted mucus also contains various antimicrobial peptides, such as defensins and lysozyme, to neutralise invaders (62,63).
- Several basal cells (expressing KRT5 and KRT14) are present at the base of the nasal epithelium. These cells serve as progenitor cells and can differentiate into ciliated or goblet cells (59,64,65).

As a result of advances in single-cell RNA sequencing, additional types of airway epithelial cells have been identified, suggesting a more heterogeneous epithelium than traditionally believed. These include club cells, ionocytes, and suprabasal cells, among others. Club cells (marked by SCGB1A1) contribute to epithelial repair and immune regulation, and ionocytes, which express FOXI1 and are involved in maintaining ion balance and regulating mucus hydration (60,66).

There is evidence showing that the nasal epithelium also houses immune cells such as dendritic cells (DCs), macrophages, mast cells, natural killer (NK) cells, T cells, and innate lymphoid cells (ILCs), which enhance local immune responses(67).

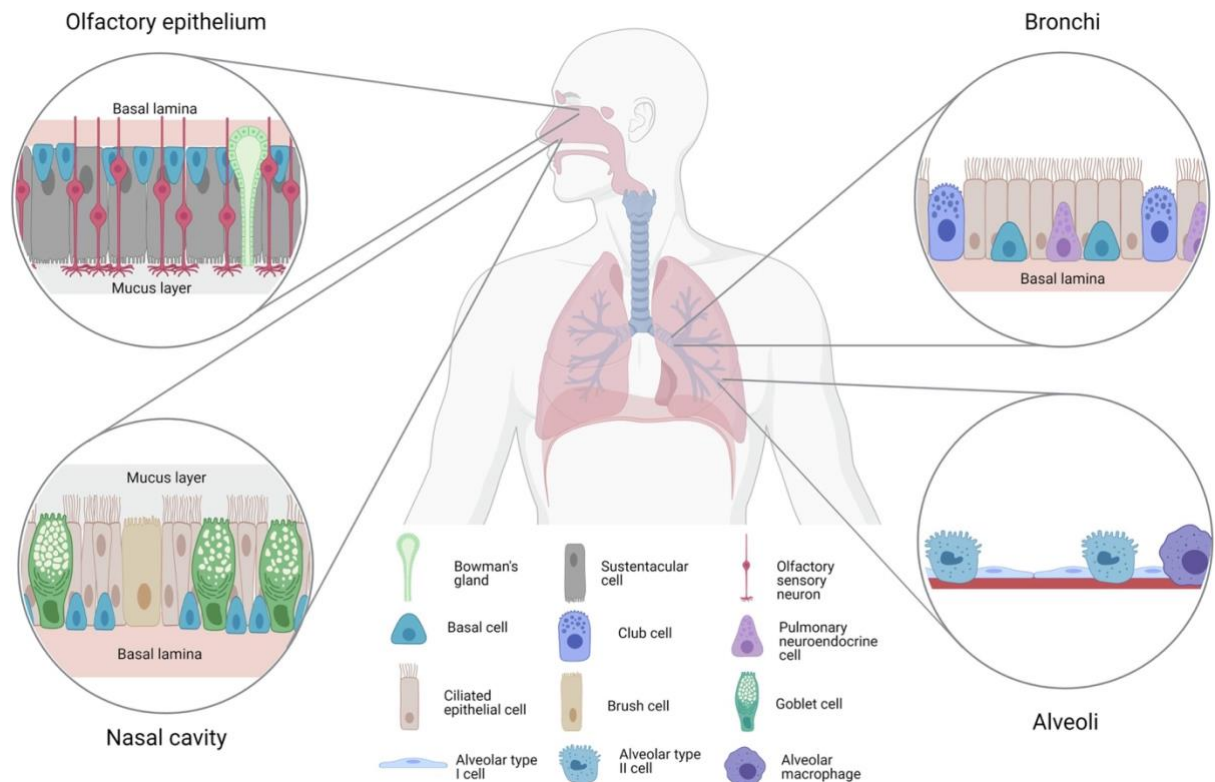


Figure 1.1 Cell composition of respiratory epithelium. The nasal epithelium consists mainly of pseudostratified columnar ciliated cells, goblet cells and basal cells, responsible for mucociliary clearance and filtering inhaled air. The bronchial epithelium is similar but has fewer goblet cells and more club cells, which help with surfactant production and repair. Alveoli have type I and II alveolar cells and alveolar macrophages. Figure from (68)

1.2.2 Nasal epithelial cells in antiviral immunity

Nasal epithelial cells in addition to their role in physical barrier are also active participants in antiviral immunity by expressing pattern recognition receptors (PRRs), including TLRs, RLRs, and NLRs, which recognize viral components such as dsRNA(67,69,70).

Type I and III IFNs are the major cytokines produced by epithelial cells during viral infection. They play crucial roles in establishing cellular resistance to viruses and activating adaptive immune responses(71). Recent studies have

highlighted the differences between the two classes of IFNs, especially in the respiratory tract (72–74). While type I and III IFNs induce a comparable interferon-stimulated gene (ISG) signature, type I IFN signalling results in a more rapid onset and decline of ISG expression(8).

Type III IFNs are especially prevalent at barrier surfaces such as the respiratory tract (48,75,76). They are less potent in inducing an inflammatory response compared to type I IFNs, thus minimizing tissue damage. Notably, clinical studies on pegylated interferon lambda (pegIFN- λ) show reduced hospitalisation rates in COVID-19 patients, demonstrating the therapeutic potential of type III IFNs in respiratory infections(77).

IFN- ϵ belongs to the type I IFN class and has been shown to be expressed in nasal epithelium (78). IFN- ϵ plays a distinct role in viral infection and disease manifestation and it has shown to exert antiviral activity. Even though IFN- ϵ induces a weaker pro-inflammatory activation, compared to IFN- α/β , it plays crucial but often overlooked role during viral infections (78,79)

A recent study revealed a pre-activated innate immunity in children, leading to a more efficient anti-SARS-CoV-2 response upon infection compared to adults (80–82) Strikingly, separate research showed that both IFN- ϵ and IFN- λ responses were found to be upregulated in children upon SARS-CoV-2 infection (83). Whether the two IFNs, and especially the less characterised IFN- ϵ , are fundamental for antiviral protection is still not clear. Understanding IFN- ϵ and IFN- λ roles in the innate antiviral response of nasal epithelial cells is necessary to develop novel therapeutic approaches. We aim to investigate early IFN responses in nasal epithelial cells, examining how

various cell types contribute to immune defence; thus informing for the development of therapeutic strategies for viral respiratory diseases.

1.2.3 Model of primary nasal epithelial cells

Working with primary nasal epithelial cells presents several challenges, including variability between donors, rapid cell death in culture, and difficulties in experimental reproducibility. Primary cells, often derived from nasal brushings or biopsies, exhibit considerable heterogeneity depending on the individual's age, health status, and environmental exposures, making standardisation difficult (84). Additionally, the limited lifespan of these cells in culture exacerbates the problem, often leading to incomplete or inconsistent data (85).

One of the most promising models to address these challenges is the air-liquid interface (ALI) culture, where cells are grown on a porous membrane with an air interface, mimicking the *in vivo* environment of the airway, which allows epithelial cells to differentiate into a structure that closely mimics the *in vivo* pseudostratified epithelium of the nasal cavity (85,86). These ALI cultures retain many key characteristics of the nasal epithelium, including ciliated cells, goblet cells, and basal cells, and exhibit functional mucociliary activity, making them an invaluable tool for studying airway diseases and host-pathogen interactions. Despite these advantages, establishing and maintaining ALI cultures is time-consuming and expensive, requiring specialized equipment and expertise. While ALI cultures better recapitulate the airway epithelium compared to traditional submerged cultures, they may still not fully represent the complex *in vivo* environment (68,87).

An approach that we use in this study is the direct stimulation of nasal epithelial cells from nasal swabs, circumventing the need for long-term culture. This method preserves the cells' physiological state at the time of collection and provides a more accurate representation of the natural immune environment. Swab samples are readily accessible and commonly collected in clinical settings, making this approach highly translatable to clinical research. By utilising cells directly from nasal swabs, this approach could provide us with a better understanding of the very early stages of the innate immune response in the upper respiratory tract.

1.3 Hypothesis and Aims

Hypothesis: Direct stimulation of human nasal epithelial cells in swabs provides a useful model to study early IFN kinetics in response to viral ligands.

Aims

This study aims to investigate the innate antiviral response in nasal epithelial cells, with a focus on the role interferon production, using a novel approach of directly stimulating swabs.

1. Optimisation of:
 - nasal swab cell isolation
 - the flow cytometry of nasal swab cells
 - detection of IFN expression
 - stimulation of nasal swabs cells
2. Recruitment of healthy individuals, characterising their nasal epithelium and response to viral ligands

2. Chapter 2. Materials and methods

2.1 Ethics

An Ethical Approval for this proposed study was given by Trinity College Dublin Research Ethics Committee (REC). Ethical approval#: 2297_FSTEMREC_FG_MAY2024

This approval covered all aspects of the study, including the recruitment of participants, the collection of nasal swab samples, the use of viral mimic for stimulation, and the experimental methodologies described in this thesis. Detailed documents regarding the ethical application can be found in Appendix 1 and official permissions are included in Appendix 2.

There was a slight delay in receiving ethical clearance due to administrative processes; however, that allowed us to concentrate on optimisation of protocols. Once approval was obtained, the recruitment and study procedures proceeded as outlined.

2.2 Study design and participants

Participants were recruited through an institutional recruitment campaign targeting healthy adult volunteers. This recruitment strategy included a flier campaign (see Appendix 3). Participants provided written consent after reviewing the Participant Information Leaflet (PIL), which was explained to them in detail prior to sample collection (included in Appendix 4).

For the final study primary human nasal epithelial cells were obtained from nasal swabs of 14 healthy adult volunteers (8 males and 6 females; ages

(mean $\pm$ Standard Deviation) $29.1 \pm 3,6$ and $30 \pm 12,2$, respectively). Four swabs were collected in total:

1. One swab was taken for flow cytometry and processed as described below.
2. One swab was processed immediately for RNA extraction to assess baseline gene expression.
3. Two swabs were for Poly IC stimulation: one was as a control and one was stimulated with $10 \mu\text{g/mL}$ Poly IC for 4 hours at 37°C in ALI media.

However, this final cohort study represents only the culmination of a broader research effort that involved extensive optimisation. During this period, numerous samples were collected to refine the methods for nasal epithelial cell isolation, RNA extraction and immune response measurements. These optimisation steps included:

Cell yield and viability: over 60 samples were used during the initial phase to optimise cell yield and ensure maximum viability from nasal swabs. This process involved testing two different dissociation protocols on the same individuals three times using one method and three times the other one.

Microscopy: microscopy was done regularly to assess the quality and viability of the isolated epithelial cells during optimisation. This helped to determine the most effective isolation techniques.

RNA yields: multiple extraction protocols were tested to optimise RNA quality and concentration. The RNA extraction process was refined using a variety of RNA isolation kits, and adjustments were made based on preliminary results from over 30 trial samples.

Flow Cytometry: Initial flow cytometry experiments on over 40 trial samples were used to optimize staining protocols and ensure consistent identification of epithelial and immune cell populations in the nasal samples. While these samples are not part of the final cohort of 14 participants, the insights gained from this optimisation process were crucial for refining the experimental design and ensuring the reliability of the results presented in this thesis. The main details and results of the optimisation experiments included in chapter 3.

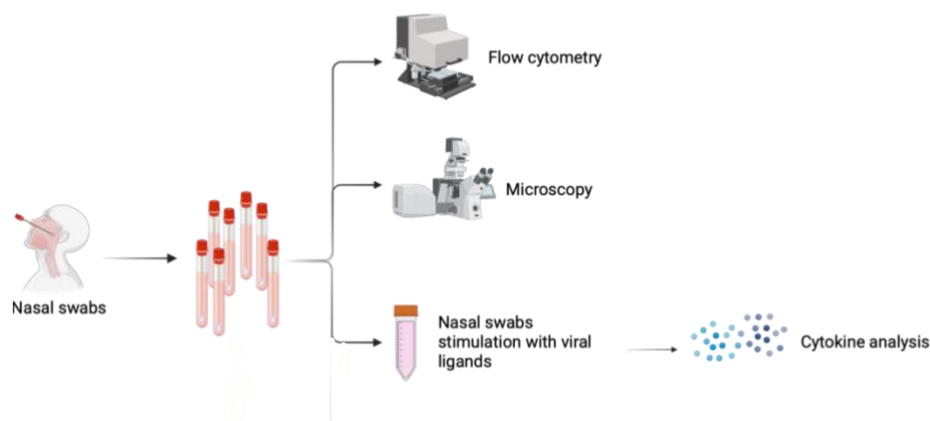


Figure 2.1 Study design. Nasal swabs were collected from healthy participants, followed by cell isolation. Isolated cells were characterized by flow cytometry (EpCAM+, CD45+) and microscopy. Nasal epithelial cells were stimulated directly on the swab with Poly I:C, RNA was extracted for gene expression analysis and qPCR was performed to assess baseline and induced interferon and ISG expression. Created in biorender.com

Tables with all the materials used can be found in Appendix 5.

2.3 NP swab collection

Donors were asked to blow their noses to remove any excess mucous from the nasal cavity. NP swabs were collected using a sterile polyester flocked swabs (Copan, Italy) from both nostrils of each participant twice and collected into sterile tubes containing ALI medium (PneumaCult™-ALI-S Medium, STEMCELL Technologies inc, Vancouver, Canada).

The patients were asked to tilt their heads slightly and the swab was inserted horizontally into one nostril, parallel to the hard palate, for approximately 2-3 cm until resistance was met. The swab was rotated 10 times each way, the swab was removed and placed into a sterile 15 ml Falcon tube containing 1 mL of ALI medium.

2.4 Cell Isolation

Two different cell isolation methods were evaluated and compared for efficiency:

1. Vortex dissociation: cells were dissociated from the swabs by vortexing.

The tube containing the swab tip was vortexed at speed 6 (Electro Scientific Industries SI-0286 Vortex Mixer (GENIE2)) for 15 seconds to elute the sample. The P1000 micropipette tip was trimmed by 2–3 mm, the swab was inserted into the trimmed tip, and the cellular material and mucous were carefully collected by pressing and rotating the swab tip against the sides of the Falcon tube. After pipetting gently up and down, the sample was passed through a 70 µm cell filter. The

tube was centrifuged at 500 x g for 10 minutes, supernatant was aliquoted into the separate Eppendorf tube for protein and the cell pellet was resuspended in 1 ml PneumaCult-ALI medium (STEMCELL Technologies) medium for counting and further processing.

2. ACF solution dissociation: cells were dissociated using the Animal Component-Free Cell Dissociation Kit (STEMCELL Technologies).

Nasal epithelial cells were isolated using a modified swab collection and dissociation protocol. Sterile nasal swabs were used to collect epithelial cells from each donor's nostril. The swab was immediately placed into a 1.5 mL Eppendorf tube containing 1 mL of Animal Component-Free (ACF) dissociation solution (STEMCELL Technologies) at 22°C (warmed up at the room temperature). To release cellular material from the swab (similar to vortex method), a P1000 pipette tip was trimmed by 2-3 mm from the end. The swab's handle was inserted into the pipette tip, and gentle pressure was applied to dislodge cells and mucus from the swab, collecting the swab in the pipette tip. The pipette tip was then pressed and rotated against the side of the Eppendorf tube to ensure that the material was fully detached from the swab in the ACF solution. Afterward, the swab and pipette tip were discarded. The Eppendorf tube was incubated at 37°C for 15 minutes, with gentle flicking of the tube halfway through the incubation and at the end. After incubation, 1 mL of ACF inhibition solution was added, and the tube was gently inverted several times to mix the contents. The cell suspension was centrifuged at 500 × g for 5 minutes at 22°C. Following centrifugation, the supernatant was

carefully removed and discarded. The cell pellet was resuspended in 1 mL of PneumaCult-ALI medium (STEMCELL Technologies) using a P1000 pipette, cells were counted and processed for further analysis.

2.5 Cell count and viability

To count and assess cell viability, trypan blue exclusion dye was used. Cells were diluted in Trypan blue at a ratio of 1:3. The cells were visualized with a Neubauer haemocytometer using microscopy. For a 0.1 mm x 1 mm x 1 mm grid section, four grid sections were counted and the average cell count was calculated. Cells touching the top and right borders of each grid section were not included in the calculations to avoid counting them twice. By multiplying the average number of cells in one section by 10^4 mL (converting each corner square's volume to one mL) and multiplying by the dilution factor, we calculated the number of cells per mL. The number of viable cells was counted. Viable cells excluded the dye, while non-viable cells took up the dye and appeared blue.

2.6 Cellular characterization, PI-staining

To visualize cell nuclei and assess cell viability, a combination of Hoechst 33342 and Propidium Iodide (PI) was used. 5000 of cells were resuspended in 200 μ L of growth media and transferred to a 96-well plate.

For staining, Hoechst 33342 and PI were added simultaneously to the samples at final concentrations of 1:200 and 1:1000, respectively. Cells were incubated with the dyes for 15 minutes at 37°C protected from light, without any subsequent wash steps.

Hoechst binds to the DNA of both live and dead cells, staining the nuclei and emitting blue fluorescence. The Hoechst signal was detected using the DAPI filter (excitation at 350 nm) on the fluorescent microscope. PI, which only penetrates the membranes of dead cells, stains the nuclei of non-viable cells, emitting red fluorescence. PI fluorescence was detected using the appropriate filter (excitation at 535 nm).

The stained cells were visualized directly using a fluorescent microscope, allowing differentiation between live cells (Hoechst-positive, PI-negative) and dead cells (Hoechst-positive, PI-positive). Images were captured to assess both nuclear morphology and cell viability.

2.7 Flow Cytometry Characterization:

To characterize the nasal epithelial cells, flow cytometry was performed using the following markers:

- To identify epithelial cells: EpCAM – BV605
- To distinguish immune cells: CD45 (Leukocyte Common Antigen) – BV510.

To identify specific immune cell subsets:

- CD3 – Spark Blue 550

- CD4 (Helper T-Cell Marker) – PE Fire640
- CD8 (Cytotoxic T-Cell Marker) – AF647
- CD56 (Natural Killer Cell Marker) – BV650
- CD16 (Fc Gamma Receptor III) – PECy7

Isolated cells were washed in PBS, centrifuged at 450 x g for 4 minutes and incubated with Live/Dead staining (Zombie-NIR), dilution 1:500, for 15 minutes and washed in 1 mL of PBS with 2% FBS and spun down at 450 x g for 4 minutes. Then, cells were incubated with fluorophore-conjugated antibodies (EpCAM, CD45, CD4, CD8, CD56, CD16), dilution 1:100, in the dark for 30 minutes at room temperature. At the same time, beads for single stained controls were incubated with the same antibodies for compensation. Unstained was used for compensation and gating control.

After washing at 450 x g for 4 minutes, cells were resuspended in PBS with 2% FBS and analysed on the flow cytometer (BD Fortessa or Aurora flowcytometer).

Data were analysed using FlowJo software and epithelial cells were identified by EpCAM positivity, while immune cells were gated on CD45+.

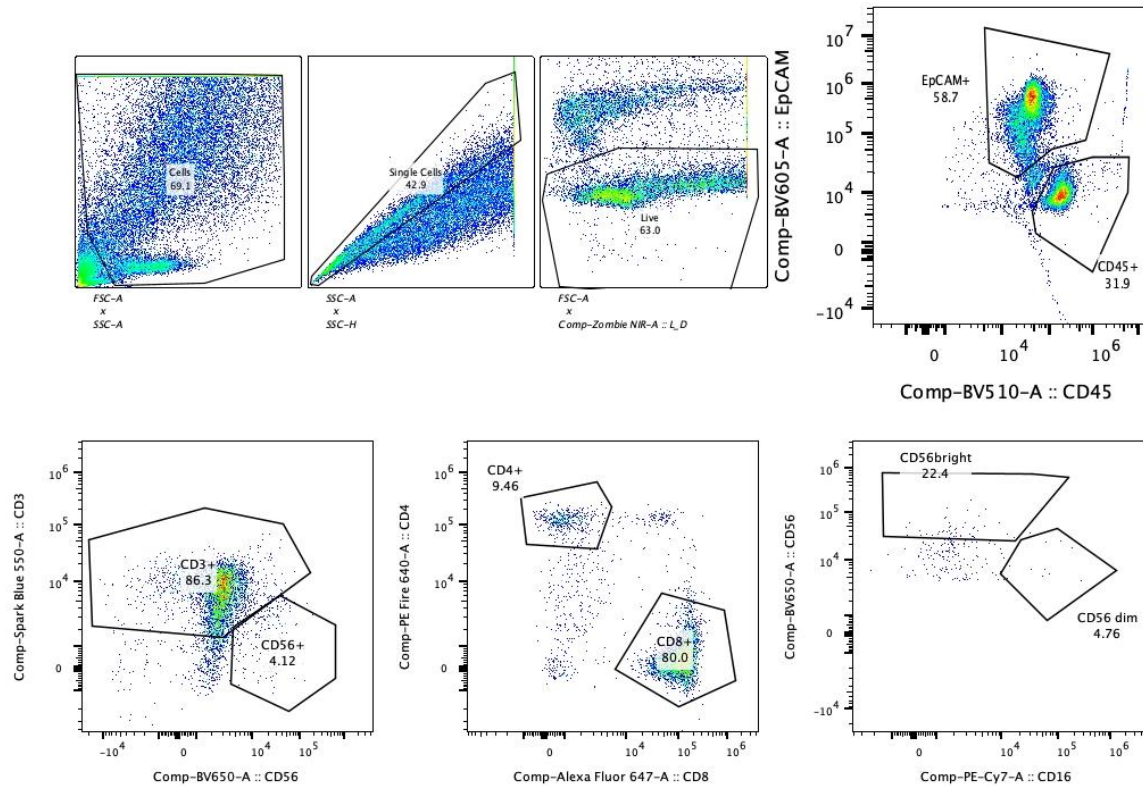


Figure 2.2 Flow cytometry gating strategy for nasal epithelial cells. *FSC vs. SSC*: Initial gating to exclude debris and select the main population of cells. *Single Cell Gate*: Doublets were excluded by gating on FSC-A vs. FSC-H. *Viability Gate*: Live cells (Zombie NIR-) were selected. *EpCAM vs CD45*: Epithelial cells (EpCAM+) and immune cells (CD45+) were identified. *Immune Subsets*: CD4+ T cells (CD45+CD4+), CD8+ T cells (CD45+CD8+), and NK cells (CD45+CD56+) were further gated from the CD45+ population.

2.8 RNA Extraction

Several RNA extraction methods were tested, including TRIzol, Invitrogen, and Qiagen kits. We compared extraction from both cells and swabs directly.

2.8.1 TRIzol (Invitrogen)

RNA was extracted from nasal swabs using a phenol-chloroform extraction method with TRIzol reagent. Following swab collection and cells extraction, the medium was carefully removed, and 1 mL of TRIzol was used per sample. The TRIzol-sample mixture was transferred into 1.5 mL Eppendorf tubes for further processing.

For phase separation, 200 μ L of chloroform was added for every 1 mL of TRIzol used. The samples were mixed vigorously for 15 seconds and incubated at room temperature for 2-3 minutes. After incubation, the tubes were centrifuged at 12,000 x g for 15 minutes at 4°C. This centrifugation resulted in three distinct phases: an upper aqueous phase containing RNA, an interphase with DNA, and a lower organic phase containing proteins and lipids. The upper aqueous phase (approximately 400 μ L per mL of TRIzol used) was carefully transferred to a new tube.

To precipitate the RNA, 500 μ L of isopropanol was added to the aqueous phase, and the mixture was vortexed for 30 seconds before being incubated at -20°C for 30 minutes. After incubation, the samples were centrifuged at 12,000 x g for 10 minutes at 4°C. The supernatant was gently removed, ensuring the RNA pellet, which appeared as a white gel-like substance, was not disturbed.

The RNA pellet was then washed by resuspending it in 500 μ L of 75% ethanol (prepared using nuclease-free water) and centrifuging at 8,000 x g for 10 minutes at 4°C. The supernatant was discarded, and a second ethanol wash was performed. After the second wash, the RNA pellet was allowed to air dry for approximately 7 minutes to remove any residual ethanol.

Finally, the pellet was resuspended in 30 μ L of nuclease-free water. The RNA was dissolved by incubating the samples at 56°C for 5 minutes, and the extracted RNA was stored at -80°C until further use.

2.8.2 PureLink RNA Mini Kit (Thermo Fisher Scientific)

Total RNA was extracted from nasal epithelial cells using the PureLink RNA Mini Kit according to the manufacturer's instructions.

Cells were lysed by adding 350 μ L of Lysis Buffer (provided in the kit) directly to each sample. The lysate was homogenized by vortexing, and an equal volume of 70% ethanol was added and vortexed again to dissolve any precipitate. The mixture was transferred to a spin cartridge and centrifuged at 12,000 x g for 15 seconds to allow RNA to bind to the silica membrane.

The column was then washed with Wash Buffer I and centrifuged again at 12,000 x g for 15 seconds. Two additional washes were performed using Wash Buffer II, each followed by centrifugation at 12,000 x g for 15 seconds. To remove residual ethanol, a final centrifugation was performed at 12,000 x g for 1 minute.

RNA was eluted by adding 30 μ L of RNase-free water to the membrane and centrifuging at 12,000 x g for 1 minute, twice to improve the yield. The RNA concentration and purity were assessed using a NanoDrop spectrophotometer, and samples were stored at -80°C for further analysis.

2.8.3 RNeasy Mini Kit (Qiagen)

RNA was extracted from nasal epithelial cells using the RNeasy Mini Kit (Qiagen) alone or a combination of TRIzol (Invitrogen) and the RNeasy Mini Kit (Qiagen). Swabs were immersed in 700 μ L of lysis buffer RLT buffer supplemented with 1% β -mercaptoethanol to ensure complete cell lysis and RNA stabilization (for kit alone processing) or directly in 1000 μ L of TRIzol reagent (for combined method), vortexed briefly, and incubated at room temperature for 5 minutes. Following TRIzol lysis, chloroform (200 μ L) was added, and the mixture was centrifuged at 12,000 x g for 15 minutes at 4°C. The aqueous phase was transferred to new Eppendorf tube. Equal amount of 70% ethanol was added to the lysate or the aqueous phase and mixed thoroughly by pipetting. Both mixtures were then transferred to an RNeasy spin column placed in a 2 mL collection tube, and centrifuged at 8,000 x g for 15 seconds at 4°C. The column was washed with 700 μ L of RW1 buffer, followed by centrifugation at 8,000 x g for 15 seconds. Two additional washes with 500 μ L of RPE buffer were performed, first followed by centrifugation at 8,000 x g for 15 seconds and second at 8,000 x g for 1 minute. A centrifugation for 3 minutes was done to ensure complete removal of buffers.

RNA was diluted in 30 μ L of DNase/RNase free water and centrifuged at 8,000 x g for 1 minute twice to improve the yield. RNA concentration and quality were assessed using a Nanodrop spectrophotometer (ThermoFisher Scientific), and samples with high purity (A260/A280 ratio between 1.6-2.0) were used for downstream analysis.

The combination of Trizol and the Qiagen kit extracted straight from the swab provided the highest RNA yield and quality, which was selected as

the final method.

2.9 cDNA Synthesis

Variable amounts of RNA, ranging from 100 ng to 1000 ng, depending on sample availability, were used for reverse transcription. To remove any residual genomic DNA, RNA was treated with DNase I (Invitrogen). This step was critical for accurate detection of IFN- ϵ due to the absence of introns in the gene (41). The lack of introns makes it impossible to design intron-spanning primers for distinguishing cDNA from genomic DNA during RT-PCR. DNase treatment was done to ensure that subsequent amplification and analysis captured only RNA signals, preventing genomic DNA interference. Specifically, the RNA was incubated with 1.8 μ L of DNase I (1 U/ μ L) and 1.8 μ L of 10x DNase I buffer and nuclease-free water to a final reaction volume of 15 μ L for 15 minutes at room temperature. The reaction was terminated by adding 1.8 μ L of 50 mM EDTA and heating the mixture at 65°C for 10 minutes to inactivate the DNase.

For cDNA synthesis, the DNase-treated RNA (100-1000 ng) was mixed with components of the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). The reaction mixture consisted of 2.7 μ L of 10x RT Buffer, 1.08 μ L of 25x dNTP Mix (100 mM), 2.7 μ L of 10x RT Random Primers, 1 μ L of MultiScribe™ Reverse Transcriptase, total volume was 27.88 μ L.

The reverse transcription reaction was carried out under the following thermal cycling conditions:

- 25°C for 10 minutes (primer annealing),
- 37°C for 120 minutes (reverse transcription),

- 85°C for 5 minutes (reaction termination).

The resulting cDNA was diluted to a concentration of 100 ng and either used immediately for qPCR or stored at -20°C for later use.

2.10 Real-time quantitative PCR

Quantitative PCR experiments were performed using PowerUp SYBR Green Master Mix with the StepOnePlus Real-Time PCR System, utilizing StepOne Software v2.3 for data analysis. Each reaction was set up in a total volume of 10 µL, which consisted of 8 µL of pre-prepared qPCR master mix, specific primers, nuclease-free water, and 2 µL of cDNA template. A non-template control was included for each gene assay to check for possible molecular contamination. All reactions were run in triplicate to ensure accuracy and reproducibility.

The thermocycling conditions were as follows: an initial denaturation step at 95°C for 20 seconds, followed by 40 cycles of 95°C for 30 seconds and annealing at 60°C for 30 seconds. After the amplification phase, the melt curve analysis was performed with a final step at 95°C for 15 seconds, 60°C for 1 minute, and 95°C for 15 seconds to confirm the specificity of the products.

Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method. The cycle threshold (CT) values were obtained for each sample and normalised to the reference gene β -actin, which served as the housekeeping gene. The ΔCT for each target gene was calculated as the difference between the CT of the gene of interest and the CT of β -actin. $\Delta\Delta CT$ was then determined by comparing the ΔCT values of treated samples with the control condition. Fold

changes in gene expression relative to the control were determined using the formula $2^{\Delta\Delta CT}$.

2.11 Stimulation of nasal epithelial cells in the swab

Nasal epithelial cells were stimulated using the viral ligand Poly IC to mimic a viral infection.

Two swabs were taken for Poly IC stimulation: one was as a control and one was stimulated with 10 µg/mL Poly IC for 4 hours at 37°C in ALI medium in a 15 mL Falcon tube. Then swab was removed from the tube using trimmed P1000 and the tube was spun down at 500 x g for 10 minutes at RT. Supernatant was removed and saved for subsequent protein analysis and the cell pellet was lysed in 1 mL of TRIzol and transferred to 1.5 mL Eppendorf tube.

RNA was then extracted from both the baseline and stimulated swabs to measure interferon (IFN) and interferon-stimulated gene (ISG) expression.

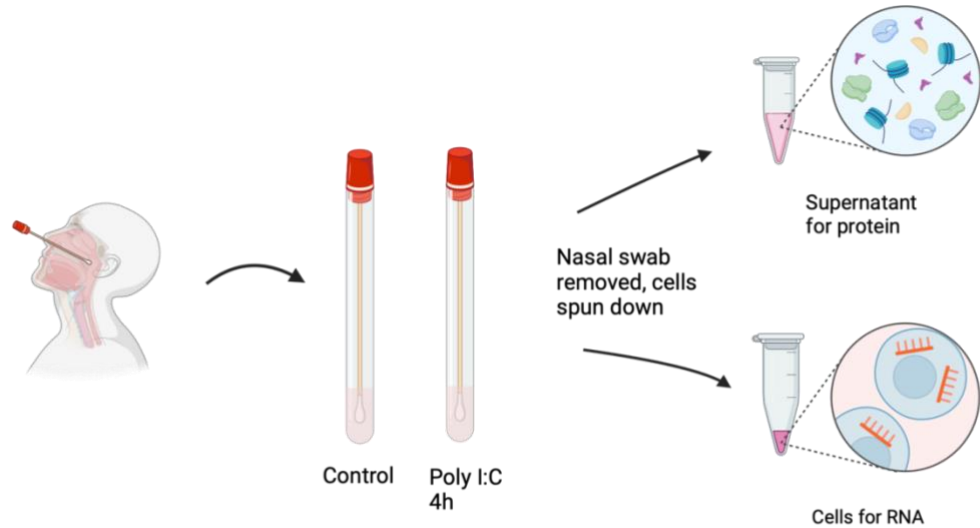


Figure 2.3. Schematic illustration of stimulation of nasal epithelial cells in the swab.

2.12 Lactate Dehydrogenase (LDH) cytotoxicity assay

To assess cell death following stimulation, the CyQUANT™ LDH Cytotoxicity Assay Kit (Invitrogen, Thermo Fisher Scientific) was used. This assay measures the release of lactate dehydrogenase (LDH) from damaged cells into the culture medium, reflecting the level of cytotoxicity.

After stimulating nasal epithelial cells in the swab under various conditions, 50 μ L of supernatant was transferred to a fresh 96-well plate. An equal volume (50 μ L) of the LDH reaction mixture was added to each well and mixed thoroughly. The plate was incubated at room temperature for 30 minutes in the dark to allow the enzymatic reaction to occur. After incubation, 50 μ L of stop solution was added to each well to halt the reaction.

Absorbance readings were taken at 490 nm using a microplate reader, with a reference reading at 680 nm to correct for background noise. The

spontaneous LDH release (from unstimulated cells) and maximum LDH release (from LDH control) served as controls to determine the baseline and maximal cytotoxicity levels, respectively.

The percentage of cytotoxicity was calculated using the following formula:

$$\% \text{ Cytotoxicity} = \left[\frac{\text{Compound-treated LDH activity} - \text{Spontaneous LDH activity}}{\text{Maximum LDH activity} - \text{Spontaneous LDH activity}} \right] \times 100$$

This allowed us to evaluate cell death across the different stimulation conditions.

2.13 Statistical analysis

Data was analysed using the GraphPad Prism 9 software. Unpaired t test was used to compare normally distributed data between two groups. Statistical analysis between two groups of not normally distributed data was performed by Wilcoxon test. One-way ANOVA test was used for comparisons between more than two groups with normally distributed data. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 were considered significant. The test used can be found in each respective figure legend.

3. Chapter 3. Optimisation of isolation and PAMP stimulation of nasal epithelial cells

3.1 Introduction:

Nasal epithelial cells (NECs) are the first point of contact with airborne pathogens, making them a crucial field of research in respiratory immunology. These cells are an excellent model for researching the body's defences against viruses and bacteria (69)

One of the major challenges in studying nasal epithelial cells is ensuring that their extraction and processing do not impair their viability or function. Optimising cell extraction is the first critical step in ensuring high cell viability and yield. Nasal swabs provide a great non-invasive method to collect epithelial cells, but obtaining a consistent and efficient dissociation of cells from the swab is essential for downstream applications (84,88).

Freshly isolated NECs contain a mix of differentiated epithelial and immune cells, representing the native nasal mucosa. This baseline state is heterogeneous and reflects the natural variability of human immune responses. Monolayer cultures, grown for 3–7 days, consist of undifferentiated epithelial cells. These cells lose their specialised functions (e.g., mucus production, cilia formation), resulting in altered immune functionality compared to the *in vivo* state. ALI cultures (21–29 days) allow NECs to re-differentiate, mimicking the mature nasal epithelium. These cultures restore key features like cilia and mucus, providing a more accurate model for studying immune responses. Although many publications use the methods of culturing primary human nasal epithelial cells to perform stimulations, this approach can still lead to changes in their *in vivo*

characteristics due to culturing for several days, including their responsiveness to pathogen and differentiation level which may not reflect the original cell population in the nasal mucosa (68,85,89–91). Performing the stimulation directly on the nasal swab can allow to get the most physiological model and obtain a more precise timing of antiviral response induction.

3.2 Optimisation of epithelial cells isolation from nasal swabs

We used primary human nasal epithelial cells obtained from nasal swabs as models. First, we optimised nasal swab cells isolation and characterise cells in nasal mucosa, using previously described protocols (84,88,92,93). Our optimisation focused on key criteria such as achieving high cell viability and yield, consistent dissociation efficiency, minimal cell debris and mucous.

Cell viability was assessed using both trypan blue exclusion and flow cytometry. Given the inherent variability of nasal epithelial cells during optimisation stage, each donor underwent three separate nasal swabs with each method with an interval of 2-3 weeks between swabbing to assess the variability and identify potential factors contributing to it.

3.2.1. Comparison of cell counts following NEC isolation using vortexing versus ACF method

Firstly, we decided to compare cell counts isolated by cells dissociated from the swabs by vortexing and cells dissociated using the Animal Component-Free (ACF) Cell Dissociation Kit.

While both methods yielded comparable numbers of cells, we observed a trend toward higher cell counts with the ACF method (Figure 3.1A). However, this increase was not statistically significant ($p > 0.05$), suggesting that while ACF may provide a slight advantage in cell recovery, the variability in cell yield between the two methods was not substantial.

3.2.2. Intra- and inter-donor variability of primary nasal epithelial cells

A significant finding during this optimisation was the considerable variability in cell yield between different donors and even within repeated swabs from the same donor. For some individuals, cell counts varied greatly across samples, regardless of the isolation method used (Figure 3.1B). This variability highlights the inherent differences in nasal epithelial cell populations among individuals, as well as potential technical factors related to swab collection or sample processing. This variability underscores the need for careful standardisation of the swabbing technique and cell dissociation processes in future experiments.

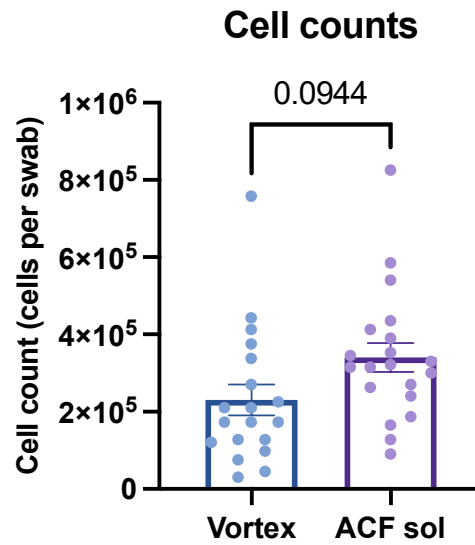
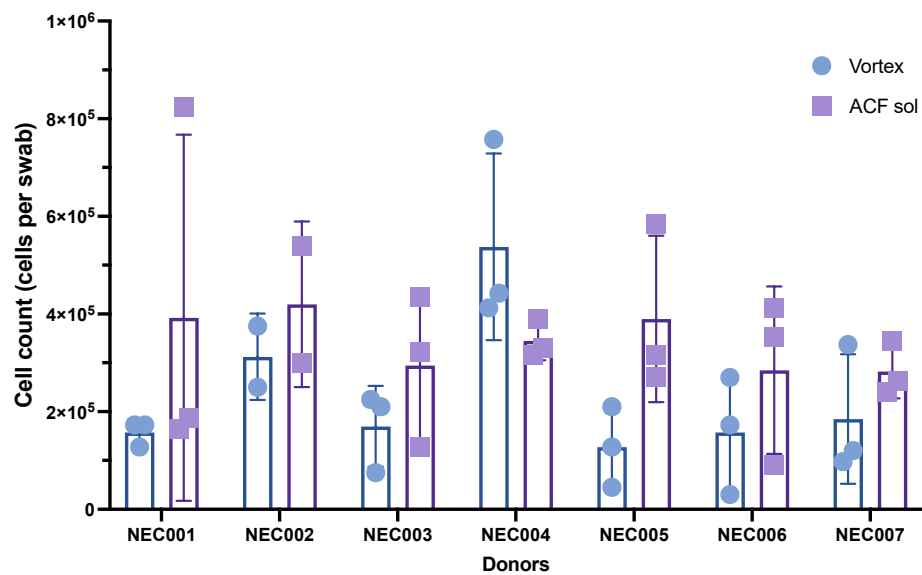
A**B**

Figure 3.1. Cell count of cells isolated from nasal swabs.

A. Comparison of cell yield obtained using Vortex and ACF solution dissociating methods. **B.** Inter- and intra-donor cells count variability. Each data point represents a separate swab. Statistical analysis was performed by unpaired T-test; results are statistically non-significant. ($p > 0.05$).

3.2.3 Comparison of cell death following NEC isolation using vortexing vs. ACF method

Next, we compared cell death using trypan exclusion and flow cytometry. Both methods had similar level of cell death with ACF method resulting in apparently higher cells death, however this was not significant (Figure 3.2 A,B).

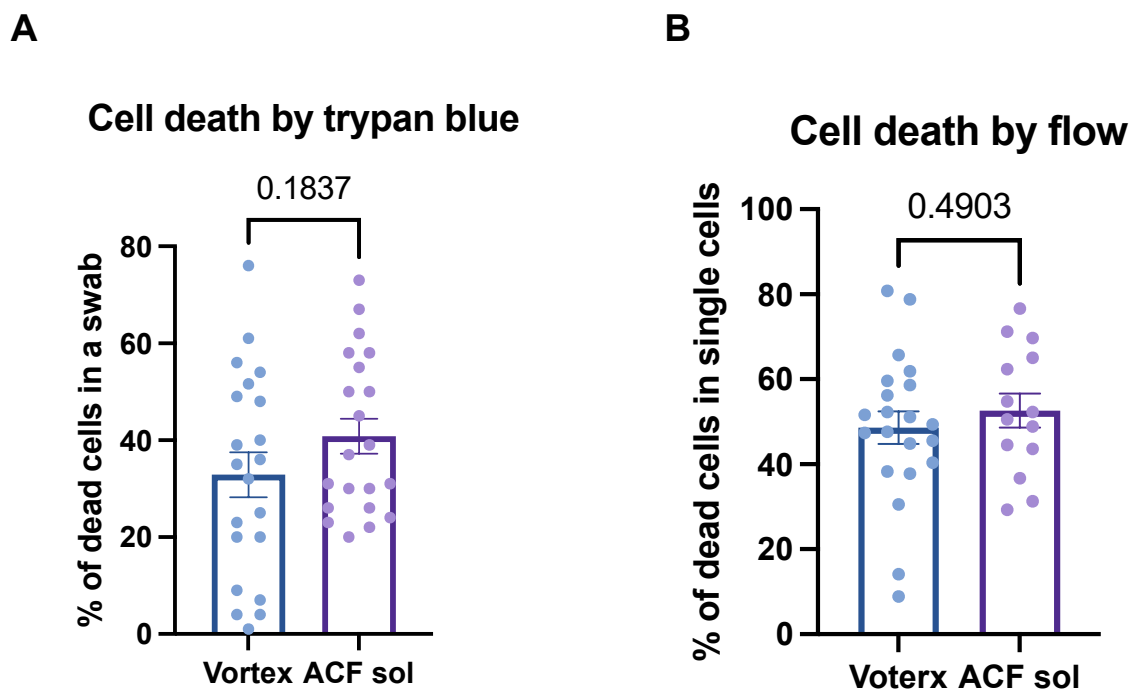


Figure 3.2. Cell death of cells isolated from nasal swabs. A. Comparison of cell death using Vortex and ACF solution dissociating methods by trypan blue and **B.** Cells were stained by Zombie NIR dye and analysed by flow cytometry. Each data point represents a separate swab. Statistical analysis was performed by unpaired T-test; results are statistically non-significant. ($p>0.05$).

3.2.4 Comparing dissociation efficiency and amount of debris between methods

We used PI and Hoechst staining to further visualise cells under the fluorescent microscope and assess dissociation efficiency and amount of debris between methods. We were able to visualise ciliated epithelial cells (Figure 3.3). Processing time appeared to have an impact on the number of ciliated cells seen under a microscope; quick sample processing appears to minimise cilia loss in NECs. Consistent with previous results on cell yield and cell death, we observed substantial cell death along with considerable inter- and intra-donor variability. The vortex approach outperformed the ACF solution in terms of both dissociation efficiency and cell debris count. Compared to cells separated by vortex, those separated by ACF solution were more aggregated and full of debris.

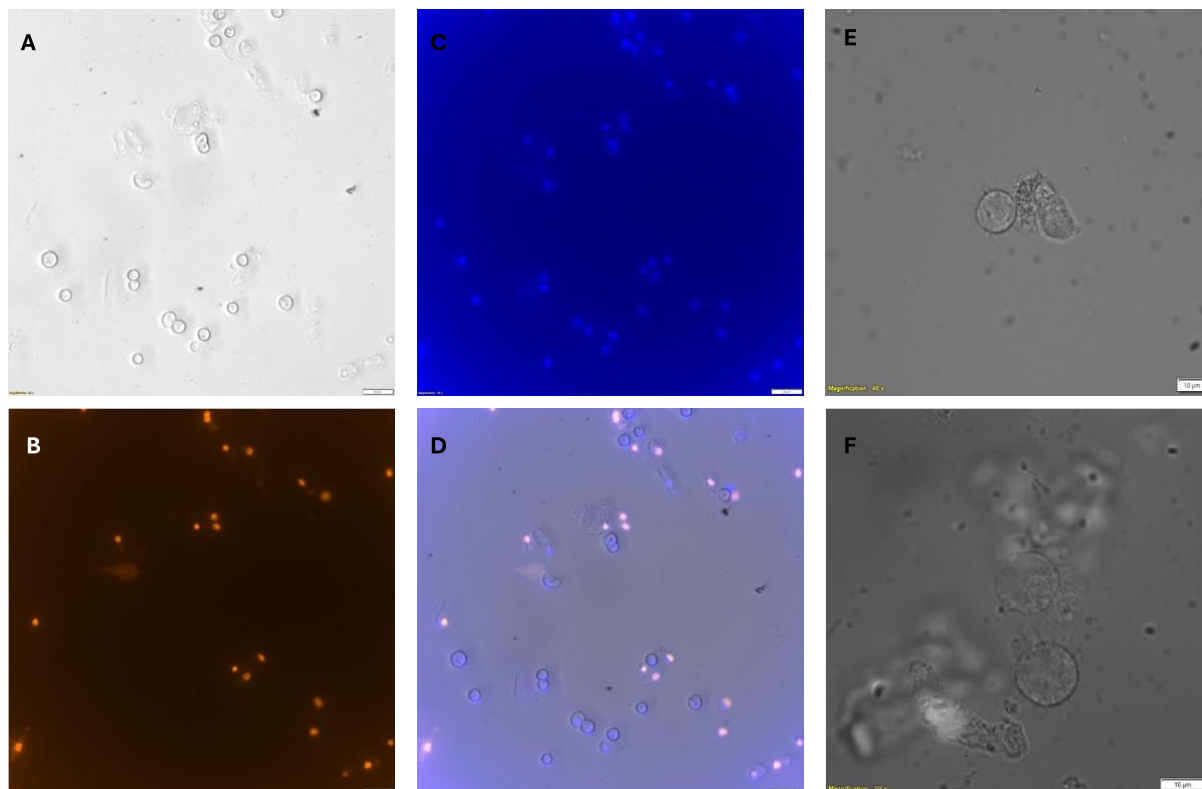


Figure 3.3 Cellular characterization with Olympus BX61WI Upright Microscope. **A.** Bright field-epithelial cells are round because they are not attached, magnification 20x (scale bar = 50 μm). **B.** DNA staining with the not permeable stain PI, red dots represent cells whose cell membrane is permeable, magnification 20x (scale bar = 50 μm). **C.** Hoechst stain the DNA of both living and dead cells blue magnification 20x (scale bar = 50 μm). **D.** Merge of bright field, PI and Hoechst, magnification 20x (scale bar = 50 μm). **E.** Cilia could be observed on freshly isolated cells, magnification 40x (scale bar = 10 μm) and **F** 60x, (scale bar = 10 μm).

We subsequently decided to use the vortex method for our future experiments because even though the yield and viability of the cells did not

significantly differ; still, the cells were easier to visualise and expanded more readily when cultured using this method.

3.3 Optimisation of RNA isolation from nasal swabs

Obtaining both high quality and quantity of RNA from primary epithelial cells is essential, thus we experimented with several techniques to find the most effective one.

Concentrations varied significantly among the different RNA extraction methods. First, the TRIzol extraction method was used, which yielded low concentrations of RNA with mean of 18,3 ng/ μ L (Figure 3.4). A second method was based on the PureLink RNA Mini Kit (Thermo Fisher Scientific), which presented a comparable yield to the Trizol method, with a RNA concentration mean of 23,5 ng/ μ L. A third method was based on the use of Qiagen kit. Although the mean was 17, 6 ng/ μ L, by dividing the results into two groups by the dissociation method, we found that cells that were isolated using the vortex method produced higher RNA concentrations, which further supported our decision to use this method. Furthermore, this observation strengthened our decision to use the Qiagen kit for whole swab RNA extraction as well.

Overall, the methods of extraction directly from the swab yielded higher RNA concentrations compared to those from the cells, possibly due to material loss during cells extraction and cell stress. Combination of TRIzol lysis and column purification has shown the best results, therefore this method was chosen as default in all the further experiments.

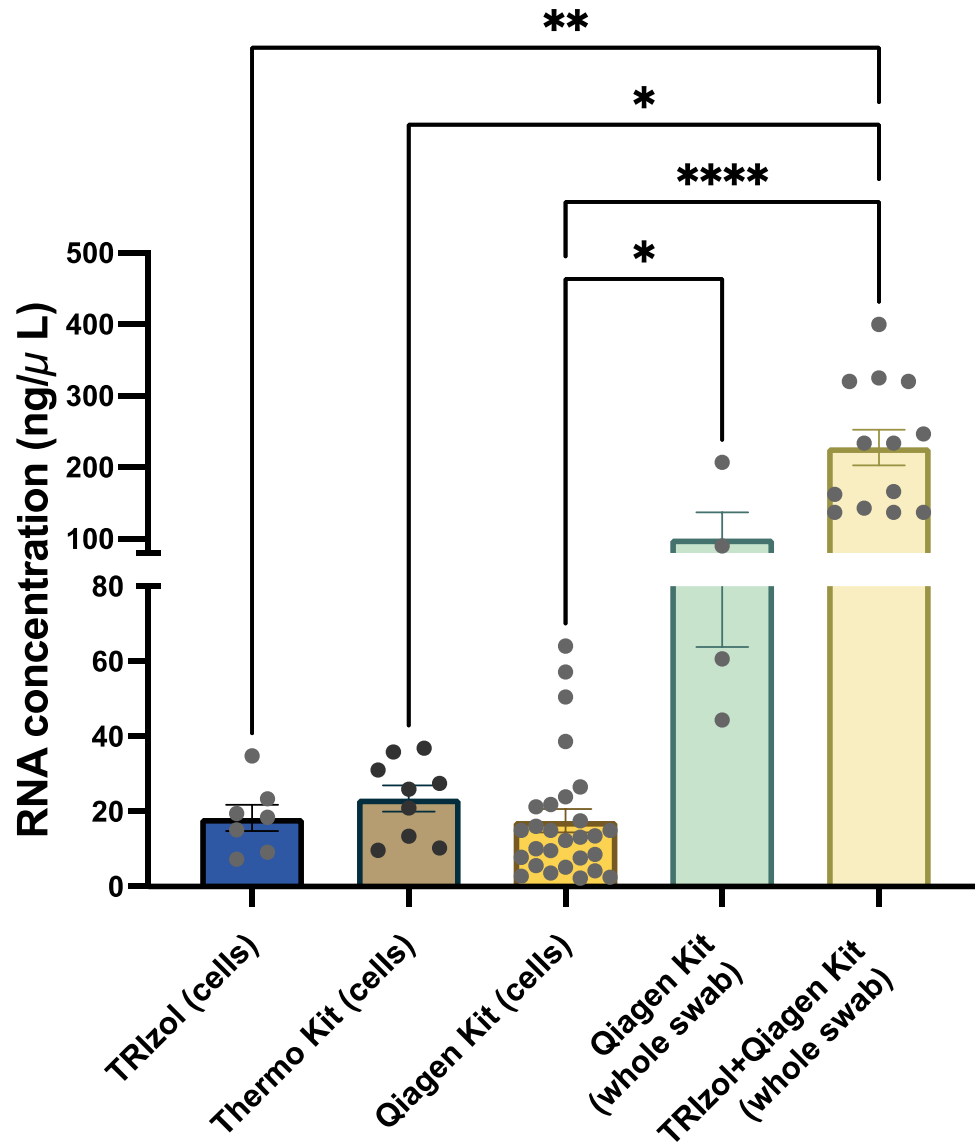


Figure 3.4. Comparison of RNA concentration obtained using different extraction methods. Each dot represents individual RNA concentration measurements from separate samples, with the bars indicating the mean with SEM RNA concentration for each extraction method. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared between each other using Kruskal-Wallis test.

RNA extracted from the nasal swab was used to assess the gene expression of PRRs, interferons and cytokines by quantitative PCR analysis (qPCR). Multiple housekeeper genes have been tested for optimal stability

using the BestKeeper software. This ultimately revealed that β -actin was the most stable housekeeping gene among those tested making it the optimal choice for normalization in qPCR experiments focused on nasal epithelial cells.

The analysis using BestKeeper revealed that β -actin is the most stable housekeeping gene among those tested, making it the optimal choice for normalization in qPCR experiments focused on nasal epithelial cells (Figure 3.5).

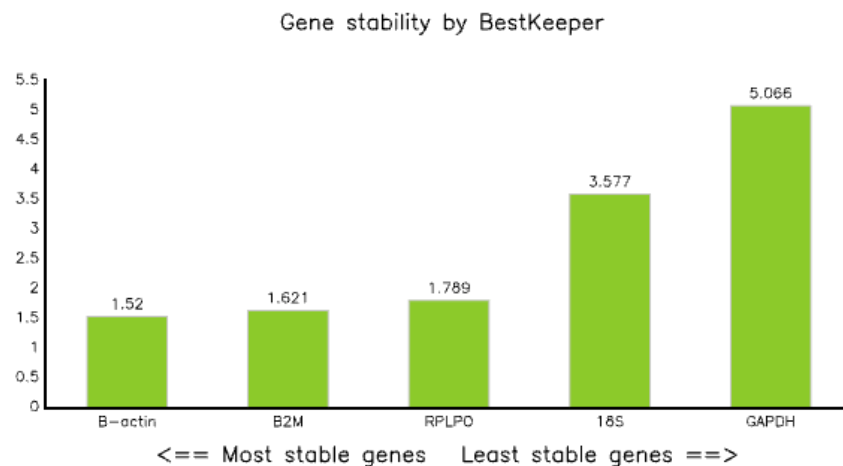


Figure 3.5. Assessment of housekeeping genes of NECs for qPCR normalization

The stability of various housekeeping genes assessed for their suitability as reference genes in qPCR experiments. The data shows the expression stability of multiple housekeeping genes, including β -actin, GAPDH, B2M, 18S and RPLPO, evaluated using the BestKeeper software. Among the tested genes, β -actin exhibited the highest stability, indicating its reliability for normalizing target gene expression in subsequent qPCR analyses.

3.4 Flow cytometry optimisation

To further characterise the cell populations present in the nasal swabs, flow cytometry was conducted, focusing on CD45 and EpCAM markers to identify immune and epithelial cells, respectively (Figure 3.6 A). Each donor was swabbed at 3 different time points to assess variability (Figure 3.6 B). Both CD45+ and EpCAM+ cells were detected, confirming the presence of immune and epithelial cells in the swabs. We also identified CD4+, CD8+ and CD56+ cells among immune cell population (Figure 3.6 C). However, the proportions of these cell types varied between donors, suggesting individual differences in the cellular composition of nasal samples. These findings support the utility of nasal swabs for sampling both immune and epithelial cells from the nasal mucosa, though cell yields and compositions may vary across individuals.

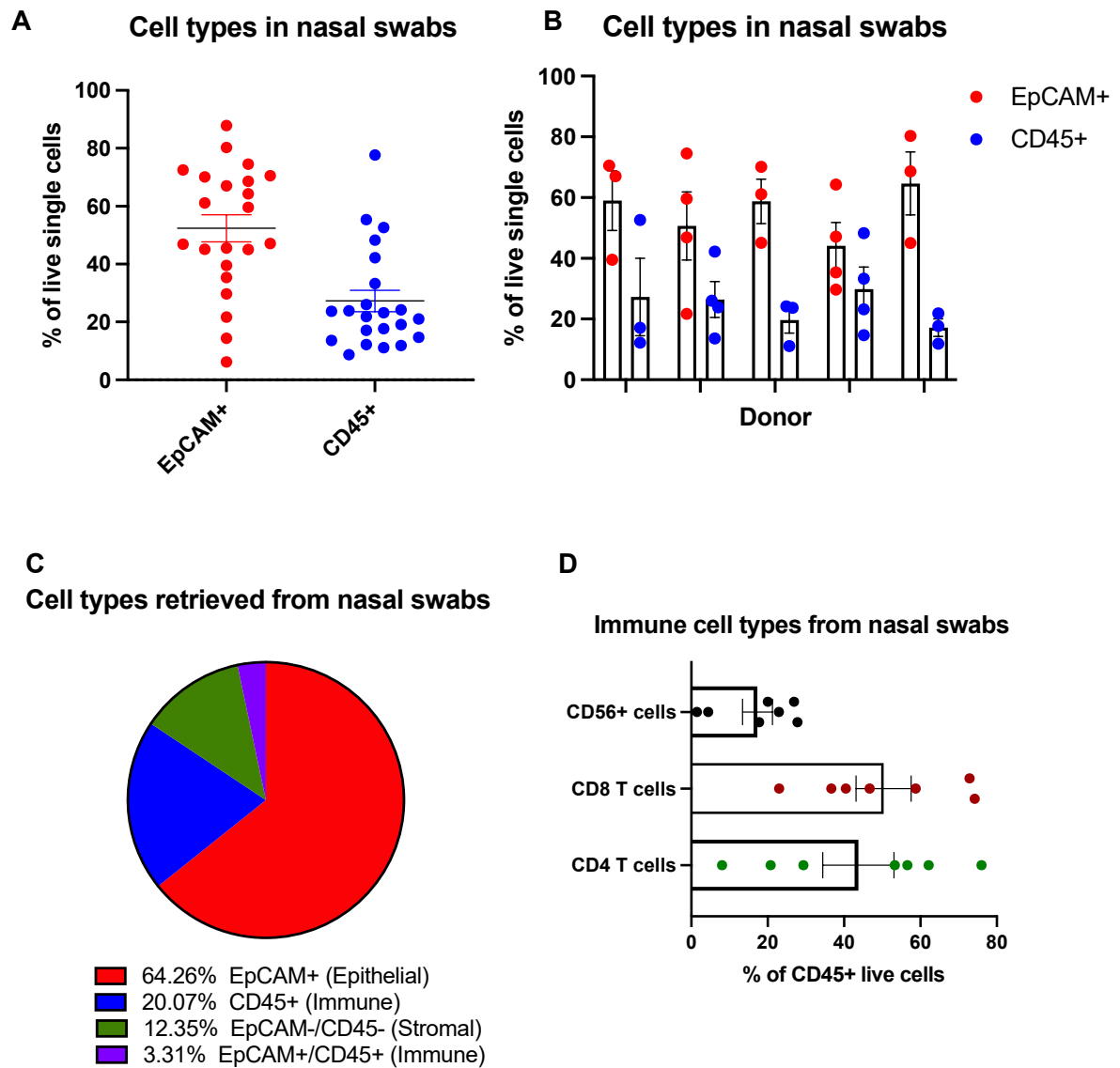


Figure 3.6. Flow cytometry analysis of cells from nasal swabs. A. Epithelial and immune cells were identified in nasal swabs based on EpCAM and CD45 expression respectively. Data is presented as individual data points for each swab, with cell types determined based on specific gating for CD45 and EpCAM markers. **B.** Cell types grouped by donor, each pair of columns represents a donor and each dot represents a different time point. Data is mean $\pm$ SEM. **C.** Cell types retrieved from nasal swabs. **D.** Immune cell types from nasal swabs. Data is mean $\pm$ SEM. Analysed by flow cytometry using FlowJo software.

3.5 Optimisation of PAMP stimulation on nasal swabs

3.5.1 Cell type ratio remains stable across multiple swabs despite variable cell yield

To ensure consistency in our sampling and to validate the reproducibility of our method, we first compared the cell counts, viability, and cell type ratios across four swabs collected from the same donor (Figure 3.7). Although the total cell counts and viability varied between swabs (Figure 3.7 A,B), we observed that the ratio of cell types—specifically epithelial cells (EpCAM+) and immune cells (CD45+)—remained consistent across all four swabs (Figure 3.6 C, D). This confirmed that despite the variability in cell recovery and viability, the overall cellular composition between swabs was relatively stable, ensuring that subsequent experiments would not be influenced by differences in cell type distribution.

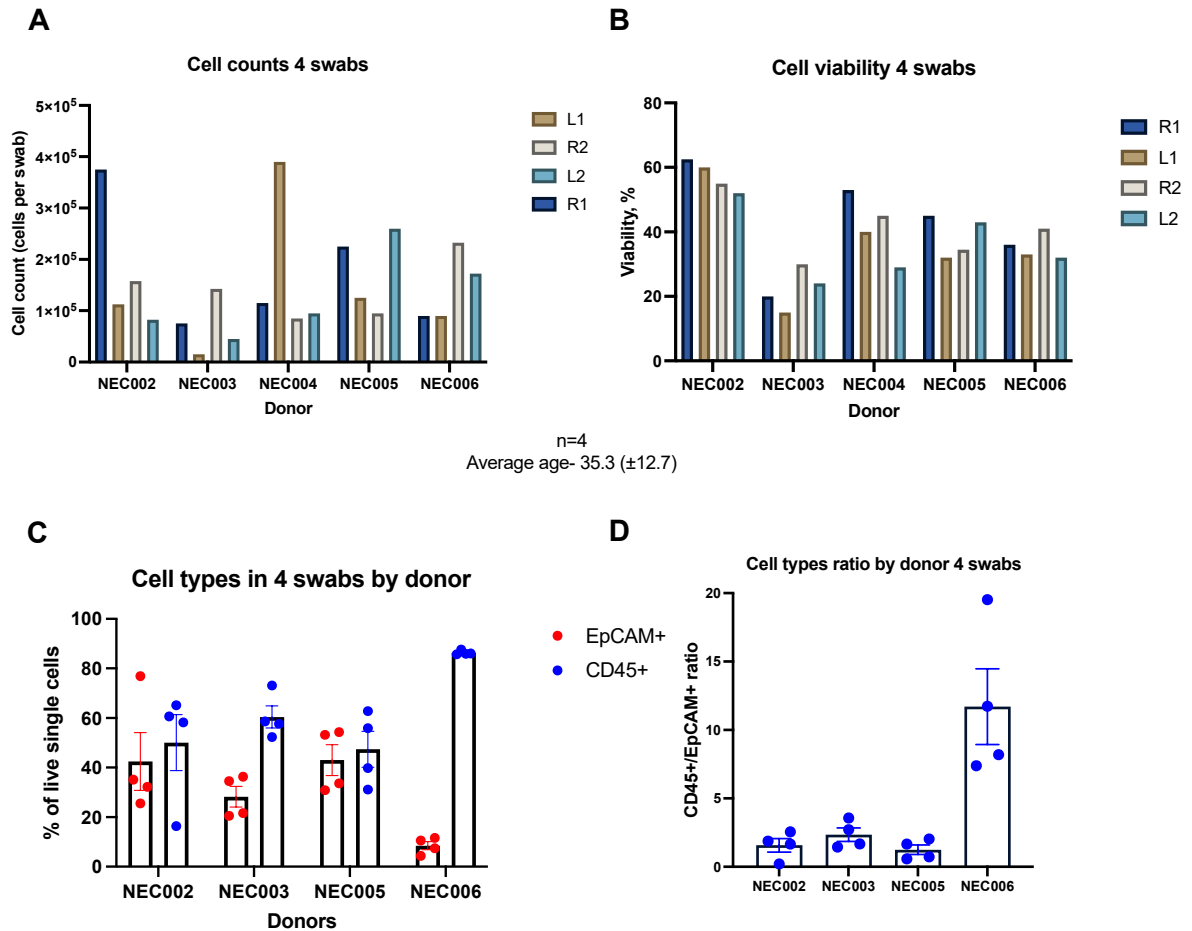


Figure 3.7. Cell counts (A), viability (B), cell types (C) and cell type ratios (D) across four nasal swabs. Panels (A) and (B) display cell counts and viability assessed using trypan blue exclusion. Each column represents a separate swab from one donor: L1 and R1 (left and right nostril, swabbed first time), and L2 and R2 (left and right nostril, swabbed a second time). Panels (C) and (D) show cell types and cell type ratios, analysed by flow cytometry using FlowJo software. Data is presented as mean $\pm$ SEM.

Since the quantity of cells retrieved from each nasal swab is variable, we needed to make sure to test for reproducibility of this approach by sampling the same individuals several times to see whether cell number would affect the observed responses.

3.5.2 Response of NECs to IFN- β stimulation

Next, we aimed to confirm the responsiveness of the cell in nasal swab to stimulation. We stimulated cells in the swab with IFN- β for 24 hours, a treatment expected to induce antiviral signalling without severely compromising cell viability(94,95). Following stimulation, we observed a significant upregulation of PKR and trend to upregulation in other PRRs and ISGs, indicating that the cells responded appropriately to IFN- β stimulation (Figure 3.8).

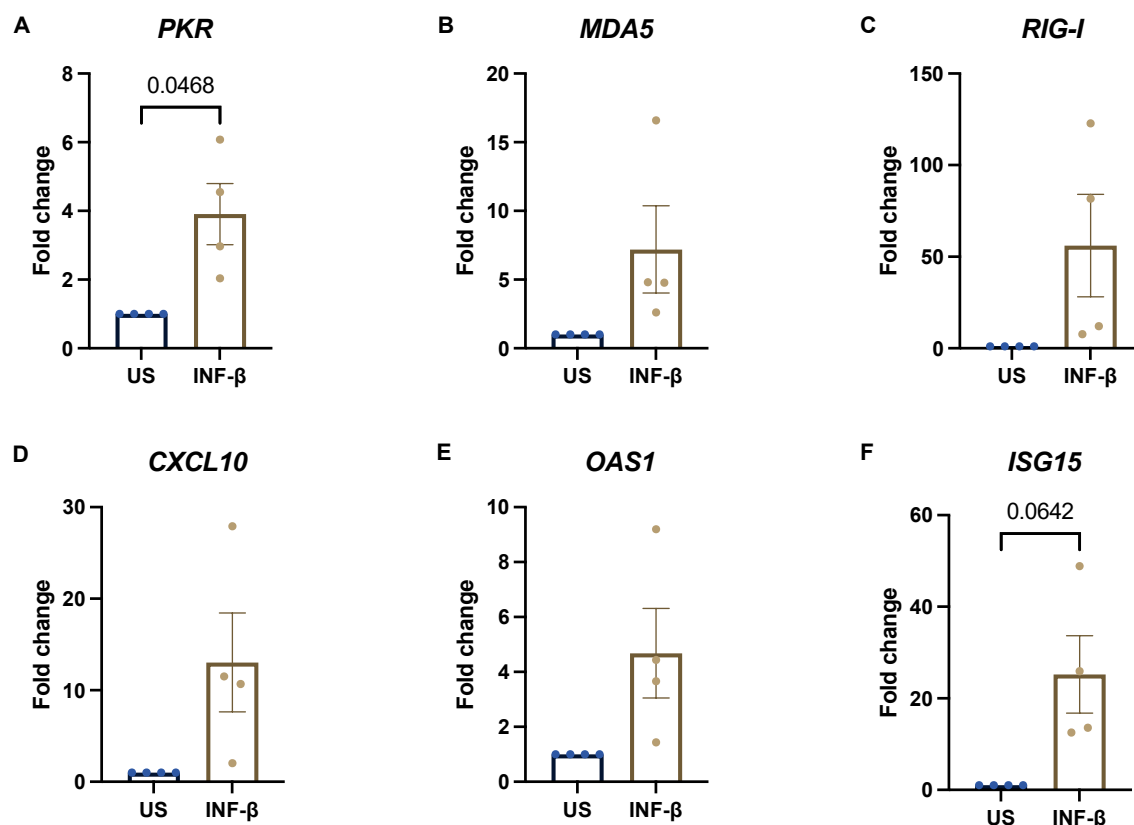


Figure 3.8. PRR and ISG expression in nasal epithelial cells following IFN- β stimulation. Nasal epithelial cells were assessed for antiviral PRRs and ISGs either at baseline or following stimulation with 100 IU/mL IFN- β for 24h. PRRs and ISGs expression was determined by RT-qPCR, and β -actin was used

for normalisation. Data are mean $\pm$ SEM. Statistical analysis was performed by paired t-test.

3.5.3 Stimulation of half-swabs

To maximise utilisation of nasal swabs, we tried cutting them in two and stimulating them with different concentrations of Poly I:C on two time points. We tested stimulation with 5 $\mu\text{g/mL}$, 10 $\mu\text{g/mL}$, and 20 $\mu\text{g/mL}$ concentrations of Poly I:C over 4 and 24 hours (Figure 3.9). Panels A-D show the mRNA expression levels of IFNs, while panels E-H represent ISGs.

While some donors exhibited responses to Poly I:C stimulation, the overall response patterns were inconsistent across both time points and concentrations, resulting in variable expression levels across donors. These results highlight the lack of reproducibility and indicate that using half swabs for stimulation may not yield reliable data. Therefore, subsequent experiments were conducted using whole swabs to improve consistency.

The additional rationale for this transition to whole swabs rests on the assumption that collecting a full swab from the nostril may capture a broader and more consistent cellular representation. By using the entire swab, we hypothesised that we could reduce sampling inconsistencies and improve cell recovery, which would, in turn, enhance the reproducibility of the data.

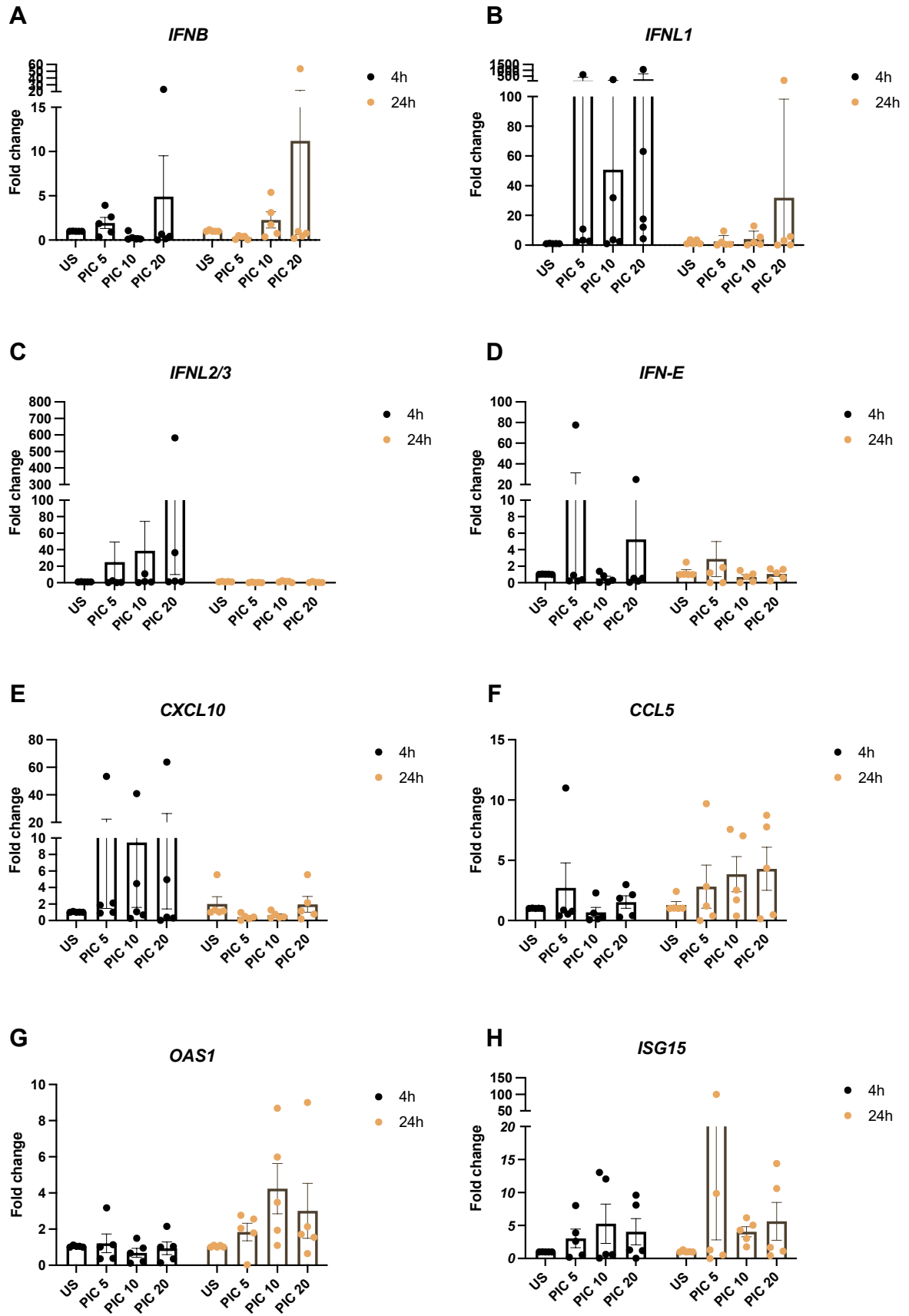


Figure 3.9. Effect of Poly I:C stimulation on IFNs and ISGs expression in cells from half of nasal swabs over 4 and 24 hours. The first four panels (A-D) display mRNA expression levels of various IFNs, while the last four panels (E-H) show mRNA expression levels of ISGs. Nasal swabs were cut in two and stimulated with varying concentrations of Poly I:C (5, 10, and 20 $\mu\text{g/mL}$) at two time points: 4 hours (first four columns) and 24 hours (last four columns). The four columns within each time point represent the following: control (unstimulated), Poly I:C 5 $\mu\text{g/mL}$, Poly I:C 10 $\mu\text{g/mL}$, and Poly I:C 20 $\mu\text{g/mL}$. IFNs and ISGs expression was determined by RT-qPCR, and β -actin was used as housekeeping gene. Data is presented as individual data points per donor, with mean $\pm$ SEM. Statistical analysis was performed by 2-way ANOVA, results are statistically non-significant ($p>0.05$).

3.5.4 Viability of cells upon stimulation, using LDH assay

To check cells condition and the impact of different medium and Poly I:C on cell viability during nasal swab stimulation, we performed an LDH assay after a 4-hour stimulation period. As shown in Figure 3.10, all conditions using DMEM medium exhibited high levels of cell death across donors. In contrast, switching to ALI medium resulted in a significant reduction in LDH levels, indicating improved cell viability. This finding suggests that ALI medium provides a more suitable environment for nasal swab cell stimulation, reducing cytotoxic effects associated with DMEM. Based on these

observations, ALI medium was chosen for subsequent experiments to ensure better cell viability and more reliable data outcomes.

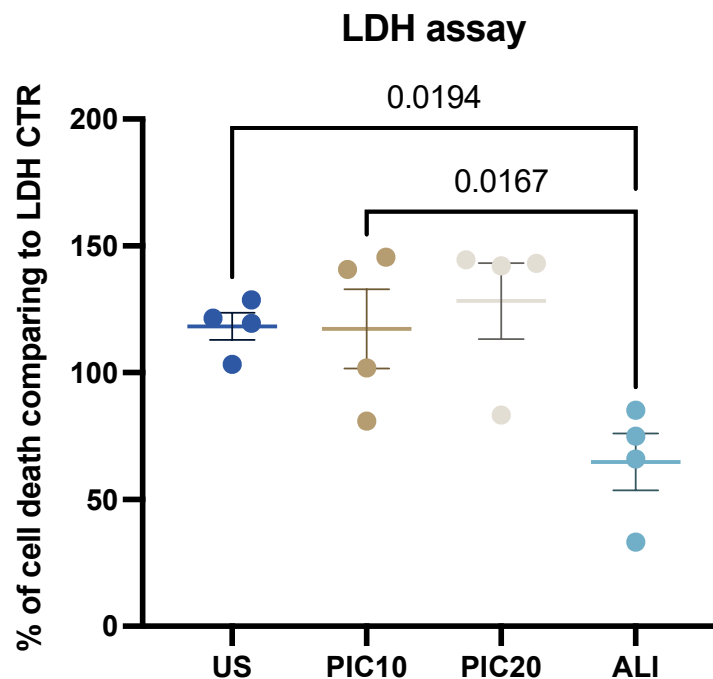


Figure 3.10. LDH assay shows high cells death upon 4 hour stimulation of nasal swabs in DMEM medium, with ALI medium improving reducing cell death

The graph presents the levels of LDH release comparing to LDH control, an indicator of cell death, following a 4-hour stimulation of nasal swab cells in DMEM across various conditions (unstimulated, Poly I:C 10 $\mu\text{g}/\text{mL}$, and Poly I:C 20 $\mu\text{g}/\text{mL}$) and ALI medium (unstimulated). Each point represents an individual donor sample, with data shown as mean $\pm$ SEM. High levels of cell death were observed in all conditions using DMEM even without stimulation, while switching to ALI medium significantly reduced cell death. This reduction indicates improved cell viability in ALI medium. Based on these results, ALI medium was selected for future nasal swab stimulations. Statistical analysis was performed using one-way ANOVA.

3.6 Discussion

In this chapter, we focused on optimising methods for isolating and stimulating nasal epithelial cells, aiming to establish a model that reflects in vivo conditions while maintaining cell viability and functional integrity.

Initial comparisons between vortex and ACF dissociation methods showed that, although ACF yielded slightly higher cell counts, the difference was not significant. Additionally, RNA extraction from cells isolated with ACF was less efficient, suggesting greater cell stress. The vortex method produced fewer cell clumps and less debris, while also yielding higher-quality RNA, making it the preferred dissociation approach for subsequent experiments.

We observed great variability in cell counts across and within donors, highlighting the biological heterogeneity of nasal samples. These differences emphasize the need for standardised swabbing and dissociation techniques to minimise technical variability. Cell recovery across samples appeared to be influenced by donor-specific factors, such as mucus composition and epithelial cell turnover.

In Fig. 3.6A, the EpCAM⁺ cell population is predominant, suggesting that the majority of isolated cells from the nasal swabs are epithelial cells. This finding is consistent with the expected profile of nasal epithelial cell enrichment. However, in Fig. 3.7C and D, CD45⁺ cells, which represent immune cells, are more frequent than EpCAM⁺ cells. This is an intriguing observation, as it suggests that while the majority of cells isolated from swabs are epithelial in nature (as seen in Fig. 3.6A), there is also a significant population of immune cells present in the nasal epithelium in some donors, which further highlights donor variability.

The culture medium impacted cell viability. DMEM led to high cytotoxicity, whereas ALI medium significantly reduced cell death, indicating that it maintained cell integrity better during stimulation. Therefore, ALI medium was chosen for all subsequent experiments to minimize cell stress.

In terms of stimulation, IFN- β effectively induced an antiviral response, confirming the cells' ability to respond. While both 10 $\mu\text{g/mL}$ and 20 $\mu\text{g/mL}$ concentrations of Poly I:C were initially tested, preliminary data variability and high cell death with 20 $\mu\text{g/mL}$ led us to standardise on 10 $\mu\text{g/mL}$ for future use. This concentration appeared to balance effective stimulation with reduced cytotoxicity, enhancing the reliability of our model.

In conclusion, the methods optimised in this chapter—vortex-based dissociation, use of ALI medium, and whole-swab stimulations—despite inherent variability, can be used for studying nasal epithelial cell responses.

4. Chapter 4: Variation of the interferon response in nasal epithelial cells in healthy individuals

4.1 Introduction

Nasal epithelial cells (NECs) are the first line of defence against respiratory viral infections (96). These cells have a robust antiviral defence system, capable of triggering a rapid and potent response upon viral recognition. Production of IFNs is a key aspect of this response that play a crucial role in clearance of respiratory pathogens (48). IFNs set off a cascade that activates ISGs to inhibit viral replication. By initiating this antiviral state, IFNs not only restrict viral spread but also enhance other immune responses to clear pathogens effectively(7,34).

However, recent studies suggest significant variability in IFN responses, which may influence individual susceptibility to respiratory infections. Various researches have highlighted how mutations in genes associated with IFN signalling, like TLR3 and IRF7, can increase vulnerability to severe viral infections, including COVID-19, by impairing IFN production(97–99). These studies show also how genetic differences in baseline IFN responses may explain why some individuals experience more severe infections despite similar exposure levels (100).

Other studies focused on immune variability in healthy individuals, showed that the interplay of genetic, environmental, and epigenetic factors influence immune responses to infections (101). This findings indicate that variability in IFN signalling and cytokine production can affect susceptibility to pathogens, even among individuals without identifiable immune defects(101–103). Despite advancements in understanding immune variability, the

knowledge about variability in the early IFN response and particularly in nasal epithelial cells is still very limited.

The rapid innate immune response, particularly within the first few hours of infection, is critical in determining the outcome of viral infection (5). The first hours can be decisive in determining whether the immune system can control the infection before it spreads. However, much of the current literature focuses on immune responses occurring beyond 24 hours post-infection, overlooking these early stages (104,105). In particular, while nasal epithelial cells are the first point of contact with airborne pathogens, there is limited research on how these cells respond during the initial hours of infection (80). Therefore, studies of IFN responses within nasal epithelial cells can provide a framework to help understand individual susceptibilities to viral infections (106).

Additionally, the methods commonly used to study these innate antiviral pathways in nasal epithelial cells, such as culturing primary cells in vitro for number of days, can introduce changes that may not accurately reflect the physiological response (68,86). This raises concerns about whether traditional in vitro models fully capture the dynamic early immune responses of these cells in their natural environment.

To address these limitations and knowledge gaps, in this chapter, we investigate the baseline interferon expression and response to Poly I:C in nasal epithelial cells from a cohort of healthy individuals. Poly I:C is commonly used to simulate viral infection in vitro, as it mimics the presence of viral RNA, triggering pattern recognition receptors such as TLR3 (4). By stimulating nasal epithelial cells with Poly IC directly in the swab, we aimed to characterise the

natural variability in IFN responses across individuals and to identify factors that may influence this response, particularly the immune cell composition.

4.2 Results

4.2.1 Variation of Immune cell population in nasal swabs from healthy donors

There is noticeable variability in the proportions of CD45+ immune cells and EpCAM+ epithelial cells in nasal swabs across donors, with most of them showing a higher prevalence of epithelial cells (EpCAM+) and some showing a higher proportion of immune cells (CD45+) (Figure 4.1 A). This variability suggests individual differences in the cellular composition of the nasal mucosa, which may influence immune responses to stimuli.

Figure 4.1B further breaks down the CD45+ immune cell population into subtypes, including CD4+ T cells, CD8+ T cells, and CD56+ cells. The distribution of these subtypes varies between donors, indicating diversity in immune cell composition within the nasal compartment. CD8+ T cells tend to be the most abundant, followed by CD4+ T cells and CD56+ cells.

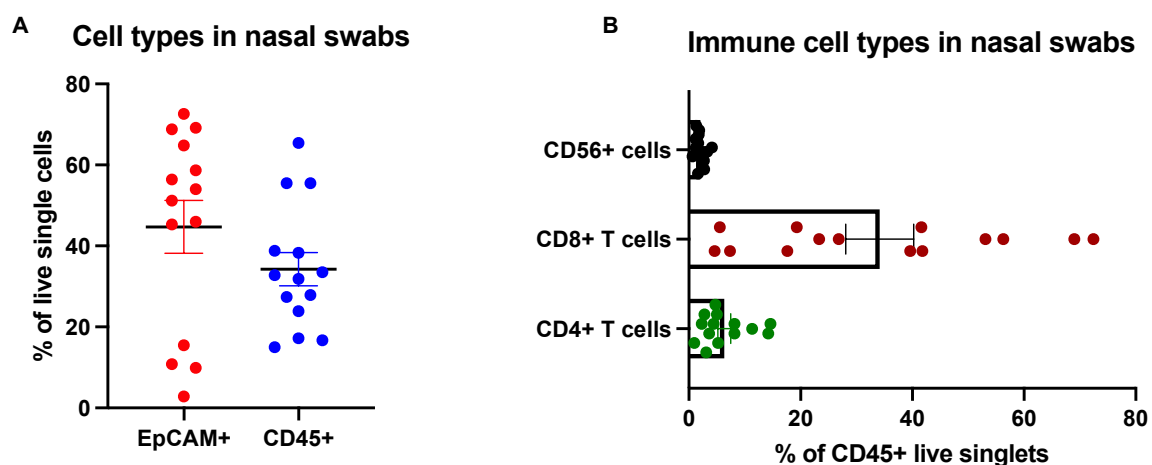


Figure 4.1. Variation in epithelial (EpCAM+) and immune (CD45+) cell populations, and distribution of CD45+ cell subtypes in nasal swabs from healthy donors.

(A) Scatter plot representing the distribution of total CD45+ immune cells and EpCAM+ epithelial cells in nasal swab samples from healthy donors. Each point represents a donor. (B) Bar graph showing the relative proportions of CD45+ subtypes—CD4+ T cells, CD8+ T cells, and CD56+ cells—across the donor cohort. Data are presented as mean $\pm$ SEM, with individual data points for each subtype. Analysed by flow cytometry using FlowJo software.

Interestingly, after performing correlation analysis between EpCAM+ epithelial cells, total CD45+ immune cells and immune cell subtypes, a moderate positive correlation was observed ($r = 0.61$) between CD8+ T cells and EpCAM+ epithelial cells. Within the immune cell subtypes, there is a strong positive correlation between CD4+ T cells and CD56+ cells ($r = 0.83$) (Figure 4.2).

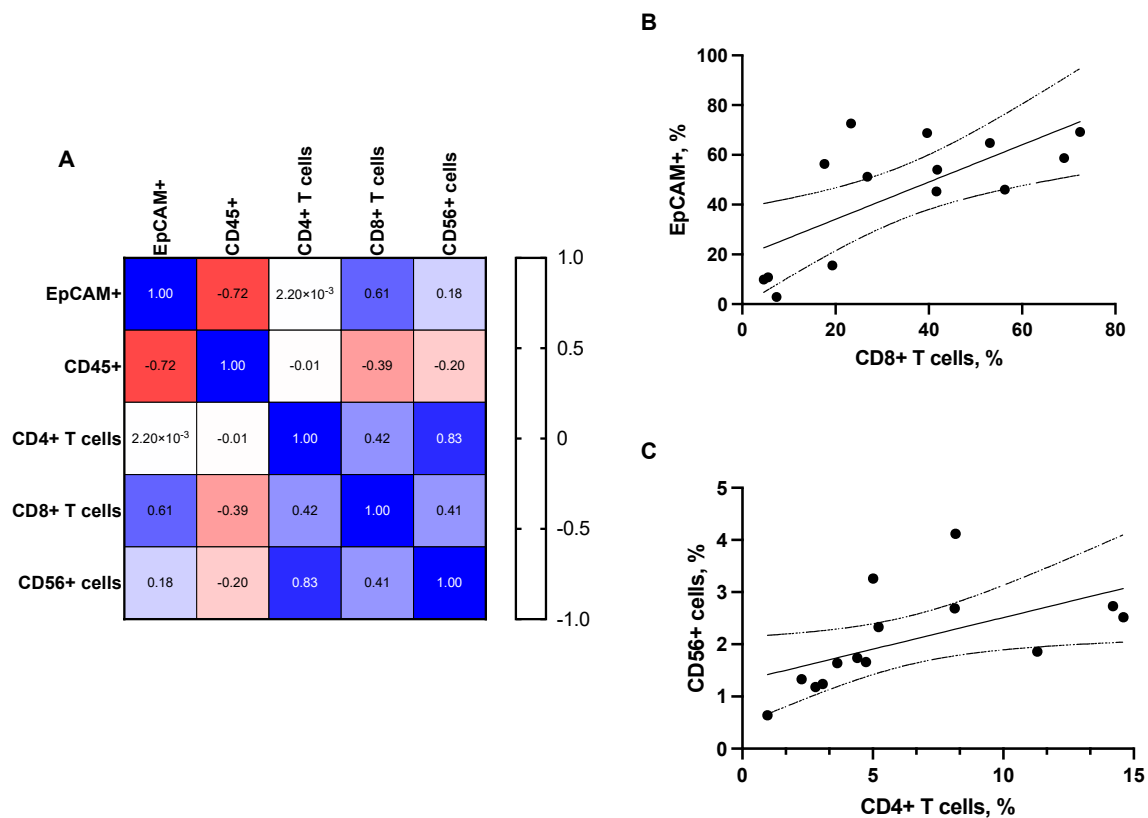


Figure 4.2. Positive correlations between EpCAM+ and CD8+ cells (A) and between CD4+ T cells and CD56+ cells (B). A. The heatmap shows Spearman correlation coefficients (r) between EpCAM+ epithelial cells and CD45+ immune cells (including subtypes) in nasal swabs from healthy donors. Positive correlations are shown in shades of blue, and negative correlations are shown in shades of red. The intensity of colour corresponds to the strength of the correlation, with values closer to 1.00 indicating a strong positive correlation, and values closer to -1.00 indicating a strong negative correlation.

4.2.2 Variation in basal expression of T1 and T3 IFNs in the nasal epithelium

The expression levels of *IFNB1*, *IFNL1*, and *IFNL2/3* were consistently low at baseline across all donors, with minimal variability between individuals. In contrast, *IFNE* exhibited the most significant variability, with some donors

showing relatively high levels of expression compared to the other interferons. This suggests that *IFNE* may have a more dynamic or donor-specific expression pattern compared to other type I and type III interferons.

When comparing interferon expression in monolayer and air-liquid interface (ALI) cultures, a similar pattern was observed, with *IFNE* showing higher expression than at baseline compared to the other IFNs. This suggests that *IFNE* might be predominantly expressed by epithelial cells. Given that other cell types tend not to survive as well in monolayer or ALI cultures, the higher expression of *IFNE* in these culture conditions implies that it may be preferentially produced by epithelial cells (Figure 4.3).

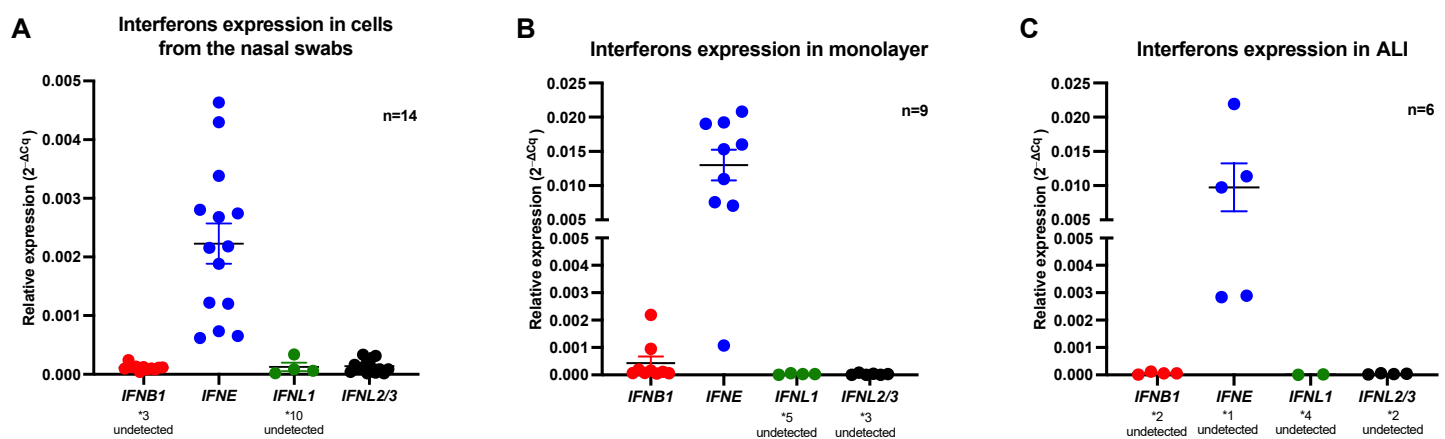


Figure 4.3. Interferons expression in the cells from nasal swabs (A), in monolayer (B) and ALI (C). A. *IFNB1*, *IFNE*, *IFNL1*, and *IFNL2/3* were measured in swabs at baseline. *IFN-ε* was the prevalently expressed *IFN* in nasal epithelium. Each point represents an individual donor sample, β -actin was used as housekeeping gene, data shown as mean $\pm$ SEM. B. *IFNB1*, *IFNE*, *IFNL1*, and *IFNL2/3* were measured in cells after growing them in monolayer for ~7 days. C. *IFNs* were measured in cells in ALI (21-29 day of growing), data shown as mean $\pm$ SEM. Data in B and C were generated by Dr Federica Giangrazi.

4.2.3 Variation in PRRs expression in nasal epithelium from healthy donors

At baseline, different PRRs expression showed high variability across donors, which decreased after culturing in monolayer. This greater variability at baseline could be due to the presence of a more heterogeneous population of cells in freshly collected samples. When cells are cultured in a monolayer, the reduced variability reflects the loss of differentiation and homogeneity of cell types. As the cells re-differentiate during ALI culture, variability in PRR expression returns, suggesting the presence of multiple cell types (Figure 4.4).

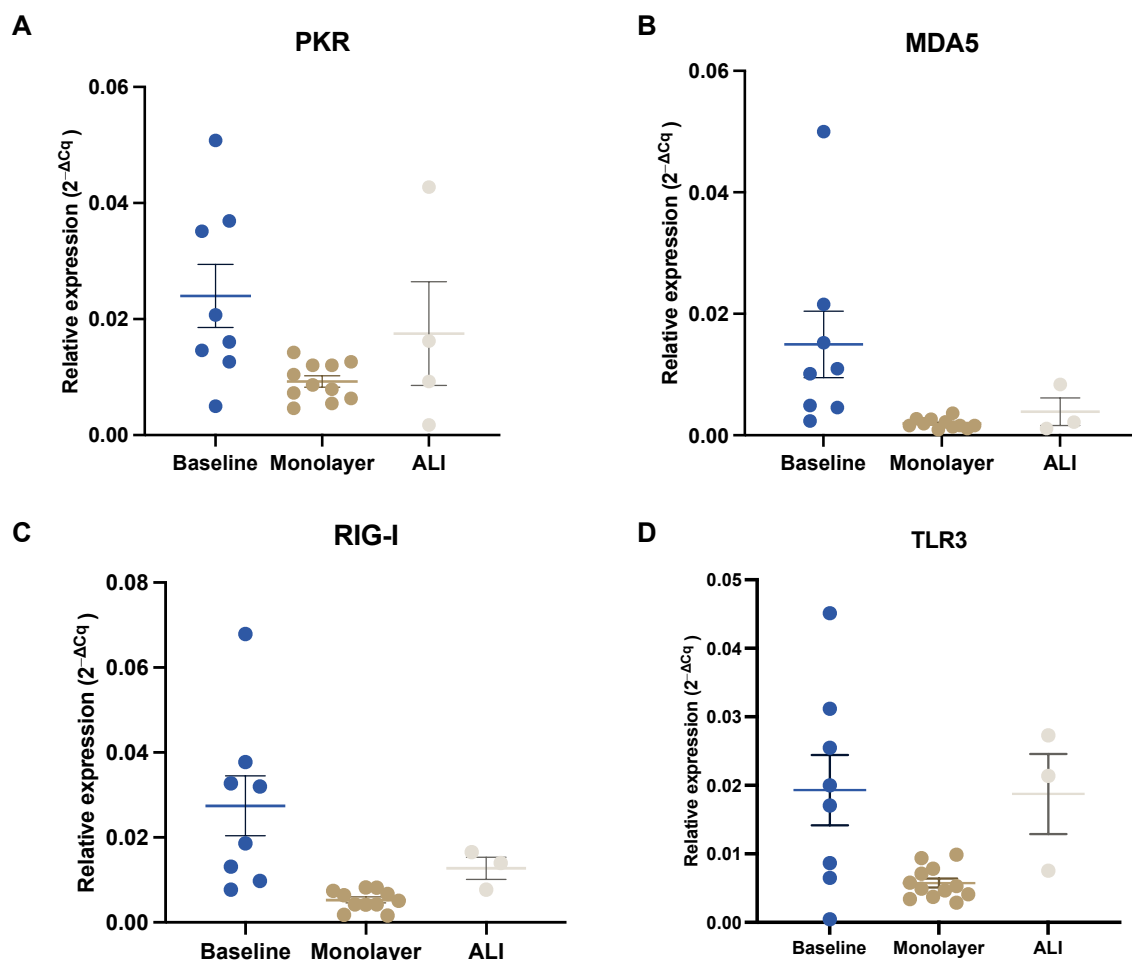


Figure 4.4. Variation in pattern recognition receptor (PRR) expression in the nasal epithelium at baseline, in monolayer and ALI. The graphs show the expression levels of four different PRRs in nasal epithelial cells, including

PKR (A), MDA5 (B) RIG-I (C) and TLR3 (D) at three different stages: baseline (immediately after collection, in blue), monolayer culture (3–7 days of culturing, in yellow), and air-liquid interface (ALI) culture (21–29 days of culturing, in grey). Each data point represents an individual donor, with data presented as mean $\pm$ SEM. RNA from monolayer and ALI were generated by Dr Federica Giangrazi.

4.2.4 Variation in type I and Type III interferon and ISGs to soluble Poly I:C from healthy donors

The response to Poly I:C in nasal epithelial cells from healthy donors demonstrates substantial variability, particularly in the upregulation of Type III interferons (*IFNL1* and *IFNL2/3*), which show the most significant fold changes (Figure 4.5 C, D). However, type III IFNs *IFNL1* and *IFNL2/3* showed a significant upregulation after 4h stimulation with Poly I:C. Some genes, such as *IFNB1* and *CXCL10* show moderate upregulation, while other ISGs (*CCL5*, *OAS1* and *ISG15*) display limited induction in response to Poly I:C stimulation, probably due to a short time point of 4 hours. Interestingly, we observed a bimodal distribution of expression for *IFNE*, with a group of individuals downregulating the gene in response to Poly I:C.

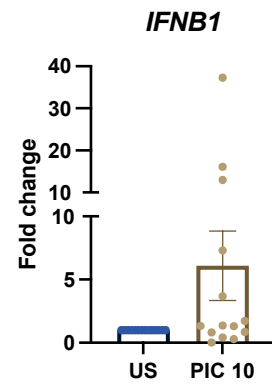
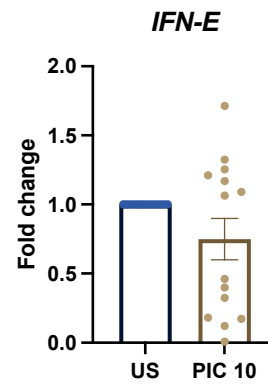
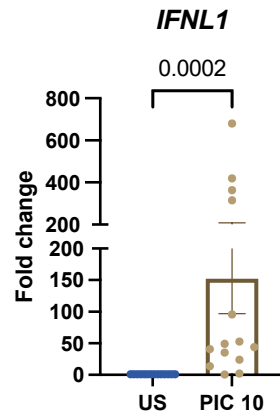
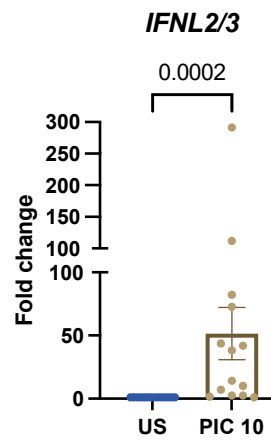
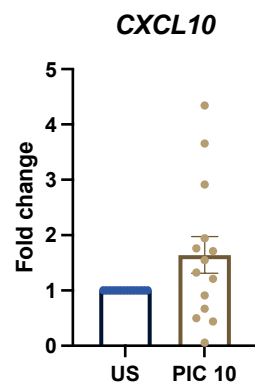
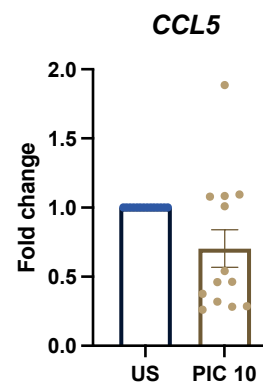
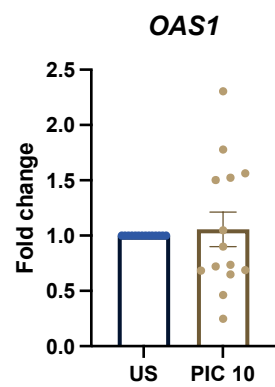
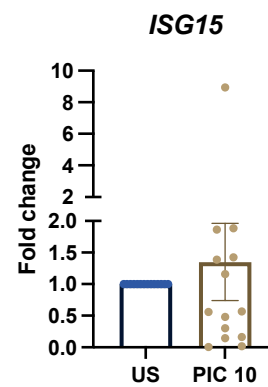
A**B****C****D****E****F****G****H**

Figure 4.5. Response of cells in nasal swabs upon stimulation with Poly I:C. Nasal swabs were stimulated for 4 hours stimulation with 10 μ L /mL of Poly I:C, PRRs and ISGs expression was determined by RT-qPCR, and β -actin was used as housekeeping gene. Data are mean $\pm$ SEM. Statistical analysis was performed by Wilcoxon test.

4.2.5 EpCAM expression is positively correlated with IFNL1 responses

Correlation between cells composition and IFNs expression in nasal epithelium upon stimulation with Poly I:C revealed that EpCAM+ epithelial cells exhibit a moderate positive correlation with *IFNL1* ($r = 0.47$), suggesting that higher epithelial cell populations might be associated with increased expression of *IFNL1*. This relationship indicates that epithelial cells could contribute to *IFNL1* producing in the nasal mucosa (Figure 4.6, 4.7 A).

Additionally, CD56+ cells show a stronger positive correlation with *IFNL1* ($r = 0.58$), indicating that that immune cells, such as Natural Killer cells may play a significant role in enhancing *IFNL1* expression in the nasal epithelium (Figure 4.6, 4.7 B).

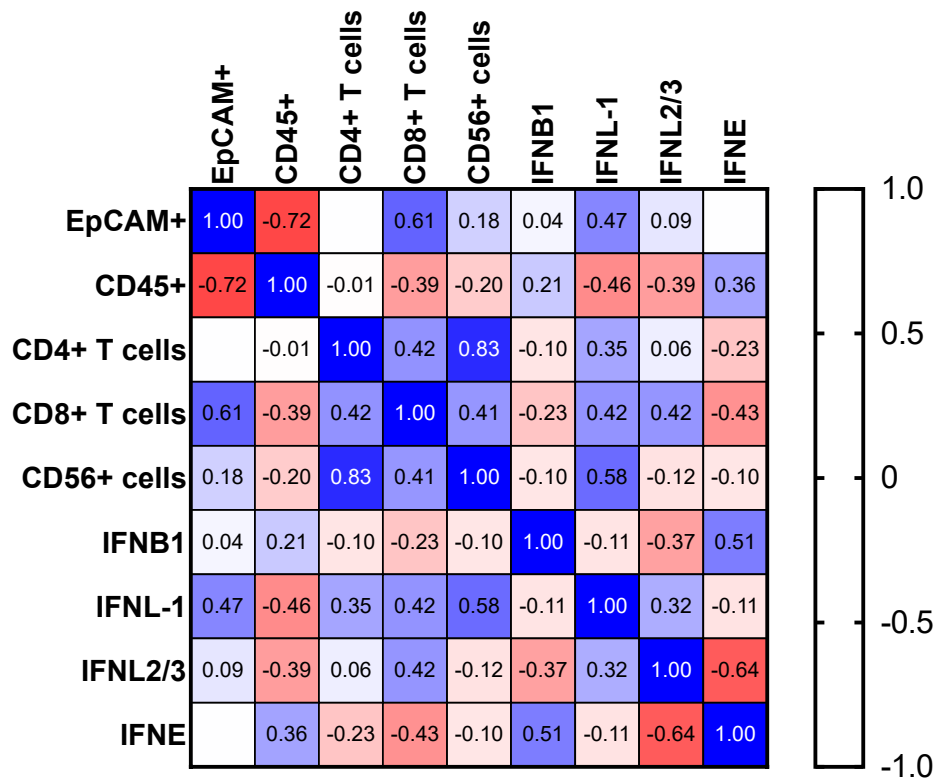


Figure 4.6. High EpCAM expression is associated with increased IFNL1 responses. The heatmap displays the Spearman correlation coefficients between immune cell populations (CD45+, CD4+, CD8+, CD56+, and EpCAM+) and interferons expression (IFNB1, IFNE, IFNL1, IFNL2/3) in the nasal epithelium. Positive correlations are shown in blue, and negative correlations in red, with the intensity representing the strength of the correlation. Positive correlations are shown in shades of blue, and negative correlations are shown in shades of red. The intensity of colour corresponds to the strength of the correlation, with values closer to 1.00 indicating a strong positive correlation, and values closer to -1.00 indicating a strong negative correlation.

4.2.6 INFE responses are negatively correlated with IFNL1

The inverse relationship between IFNE and IFNL2/3 expression (Figure 3.6 C) suggests potential regulatory interactions or distinct roles within the

nasal epithelial immune response. IFN- ϵ is known for its role in mucosal immunity and is often constitutively expressed in some tissues, unlike the inducible IFN- λ 2/3. This negative correlation may imply that higher levels of one type of IFN may suppress or balance the production of the other, potentially to regulate inflammation and avoid excessive immune activation in the respiratory tract.

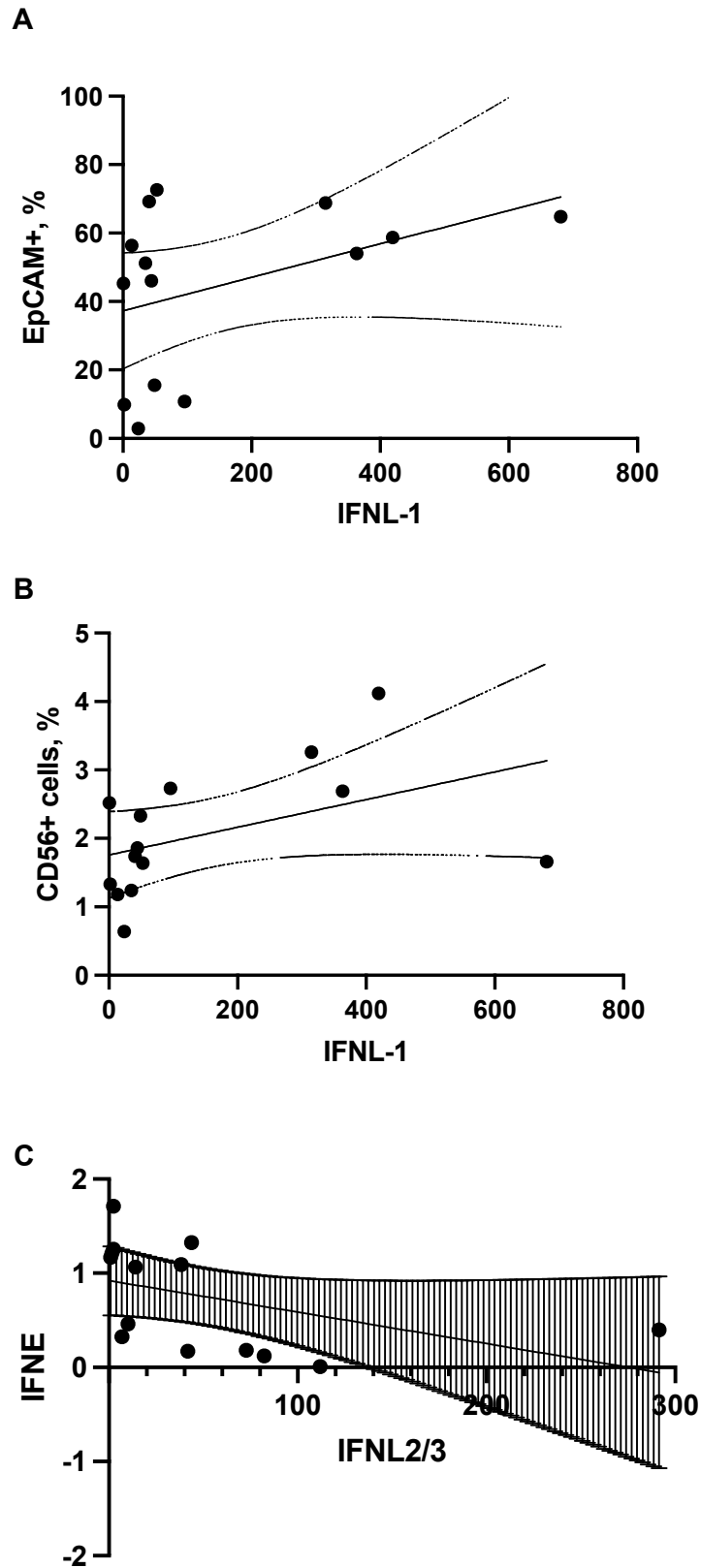


Figure 4.7. Positive correlation between IFNL1 production and percent of epithelial (A) and CD56+ (B) cells in nasal swab and a negative correlation between IFNE and IFNL2/3. Simple linear regression.

4.3 Discussion

In Chapter 4, we investigated the variability in cellular composition and interferon responses within the nasal epithelium among healthy individuals, using Poly I:C (TLR3 agonist) stimulation to mimic viral infection. Our findings demonstrate significant interindividual differences in baseline and stimulated levels of Type I (IFN- β , IFN- ϵ) and Type III (IFN- λ 1, IFN- λ 2/3) interferons, as well as variability in epithelial and immune cell populations within the nasal mucosa. These results underscore the inherent heterogeneity in nasal immune defences, which may contribute to differing levels of susceptibility to respiratory infections across people.

One critical observation is the variability in the cellular composition of nasal swabs, specifically the proportion of EpCAM+ epithelial cells and CD45+ immune cells, including subpopulations like CD8+ T cells and NK cells (CD45+ CD56+ cells). This heterogeneity could have important implications for immune readiness, as epithelial cells are the primary producers of interferons in mucosal barriers. Previous research has emphasised the importance of epithelial and immune cell interactions in barrier tissues, with evidence suggesting that the overall health and functionality of these cells can impact immune responses, including interferon production(107,108). Our findings suggests that the density of epithelial and immune cells in barrier tissues can influence interferon production and overall immune responsiveness. The positive correlation between EpCAM+ cells and IFN- λ levels upon stimulation observed here suggests that higher epithelial cell counts might enhance the tissue's capacity to maintain a low-level antiviral state, potentially reducing susceptibility to infections by facilitating quicker responses to viral pathogens.

The significant variability in IFN- β and IFN- λ baseline expression across individuals reflects innate differences in immune readiness, as baseline IFN levels can prime the immune system for rapid responses to pathogens. The findings of Yoshida et al. (2021), showing that higher baseline interferon levels in children correlate with better outcomes in SARS-CoV-2 infections, support the idea that pre-existing interferon activity plays a protective role (81). Surprisingly, in our study, we did not see the positive correlation between baseline production of IFNs and the response to stimulation. This may be due to several factors, including the type of stimulation used, as we relied on a viral mimic (Poly I:C) rather than an actual infection. Additionally, the small number of donors may have limited our ability to detect a significant correlation.

Upon stimulation with Poly I:C, we observed a variable induction of interferon and interferon-stimulated gene (ISG) expression, indicating that individuals mount differing levels of immune response to viral mimics. This variability may stem from differences in PRR expression levels or downstream signalling efficiencies among individuals, which would need further investigation.

In conclusion, Chapter 4 demonstrates that baseline and upon stimulation (Poly I:C), IFN responses vary significantly across individuals, with these differences likely contributing to individual susceptibilities to respiratory viral infections.

5. General discussion

This study explored the role of early innate immune responses within the nasal epithelium, particularly the production and variability of interferons, using the direct stimulation of nasal swabs. The early response (within the first hours) could be decisive in determining whether a viral infection is contained at the site of entry or allowed to spread further into the respiratory tract, where it could cause more severe disease (48). Some researches have shown that the early immune responses, particularly in the nasal epithelium, tend to be stronger and more rapid in children compared to adults. These studies suggest that children have a heightened innate immune readiness of nasal epithelium, which may explain their relatively better outcomes in some respiratory infections, such as COVID-19(67,81,109–111). Despite the well-established antiviral function of interferons, a gap exists in understanding the timing and individual differences in these responses, especially during the crucial initial hours following viral exposure. The objective of this investigation was to examine the early innate immune responses of nasal epithelial cells, with a focus on interferon production and the potential variations across individuals. The underlying hypothesis was that the early induction of interferons, especially within the first few hours of exposure to a viral mimic, could reveal key differences in immune readiness that could influence susceptibility to respiratory infections and that the whole swab stimulation could be a useful model for studying these responses.

Optimising cell isolation techniques from nasal swabs, including comparing mechanical dissociation and animal component-free kits, improved cell viability and data reliability. We faced challenges with RNA concentration, likely due to variability in cell yield of primary cells and

extraction efficiency, but through optimisation of the extraction protocols and cell isolation techniques, we improved RNA quantity. Additionally, flow cytometry was optimised to better characterize cell populations from nasal swabs, allowing for more accurate downstream analyses of interferon responses. These optimisations contributed to more robust and reliable results throughout the study.

A key finding of this study was the rapid induction of IFN- λ within 4 hours of Poly I:C stimulation, that highlights the ability of nasal epithelial cells to mount a quick antiviral response. This early production of IFN- λ , which plays a crucial role in limiting viral replication without excessive inflammation, could be a decisive factor in containing viral infections locally before they spread further into the respiratory tract and potentially lead to more severe outcomes.

We found notable variability in baseline and induced interferon responses among donors. While early induction of IFN- λ at 4 hours was observed across samples, the magnitude of the response varied, suggesting some individuals have a stronger capacity to mount a robust antiviral defence at the mucosal surface. This variability in interferon production could come from differences in pattern recognition receptor expression, immune cell composition or genetic factors that influence immune readiness(71). These findings highlight the importance of understanding how early immune responses differ between individuals and the potential impact on the severity of respiratory infections.

The positive correlation between high EpCAM expression and increased IFNL1 responses supports the existing literature showing that epithelial cells

are the main producers of IFN- λ (112,113). The inverse relationship observed between IFN- ϵ and IFN- λ 2/3 expression suggests a potential regulatory interaction or distinct functional roles for these different interferon subtypes within the nasal epithelial immune response. These findings indicate that the nasal epithelial cells in the nasal mucosa play a crucial role in orchestrating the early antiviral response through the production of type III interferons, while the balance between type I and III interferons may be important for regulating inflammation and immune activation in the respiratory tract. It is also possible that the balance between type I and III interferons is not solely driven by epithelial cells, but may rely on a complex interplay between multiple immune cell populations and signalling pathways. Simply concluding that epithelial cells play a "crucial role" in the early antiviral response may oversimplify the nuanced dynamics at play within the nasal mucosa. Further investigations are needed to fully understand the relative contributions of different cell types and interferon subtypes in orchestrating the respiratory immune response.

5.1 Limitations of the study

While this study provides valuable insights into the variability of nasal epithelial immune responses, several limitations should be addressed. The study's sample size, although sufficient to detect variability, could be expanded to strengthen statistical power and enable deeper stratification of immune cell subtypes. Additionally, we did not take into account the potential role of the nasal microbiome in influencing immune responses. The composition of the local microbiome can have a profound impact on epithelial and immune cell activation, including the production of interferons and other

cytokines. Recent studies suggest that commensal microorganisms in the respiratory tract can modulate host immune responses, potentially enhancing or dampening the antiviral defences of nasal epithelial cells (53,114,115).

It is also important to acknowledge potential limitations of direct swab stimulation. Swab samples, while easily accessible and highly translatable to clinical research, contain a mix of different cell types, including immune cells. This cellular heterogeneity can complicate the interpretation of results when the focus is specifically on nasal epithelial cell responses. The high cell death that we observed in our study might as well impact results. Changing medium for stimulation from complete DMEM to ALI improved the viability, suggesting the latter for future experiments. Variable number of cells obtained from nasal swabs affects the standardisation of the method across individuals. Factors influencing cell yield include swabbing technique, anatomical differences and variability in epithelial and immune cell populations. This inconsistency complicates the accuracy and comparability of immune response measurements, as it impacts cytokine concentrations detected after stimulation. Lower cell yields may underrepresent the innate immune response, while higher yields could exaggerate it. Future research should consider normalising data based on cell counts or using alternative methods to standardise cell numbers in assays.

Another limitation of this study is the restricted number of nasal swabs that can be reasonably collected from each participant, finite number of swabs that can be obtained restricts the number of different stimulations or time points that can be studied. In this research, we obtained four swabs per individual, which allowed us to assess baseline and stimulated responses; however, this is not ideal for repeated or more extensive stimulation studies.

Collecting multiple swabs can be uncomfortable for participants, and not everyone may be willing to undergo several rounds of sampling.

While the use of Poly I:C in this study as a surrogate for viral infection has provided valuable insights into the innate immune responses of nasal epithelial cells, it is important to acknowledge the inherent limitations of this approach. Poly I:C is a synthetic analogue of double-stranded RNA, which mimics the molecular pattern associated with viral replication. However, it does not fully replicate the dynamic interaction between actively replicating viruses and host cells, particularly the modulation of host innate immune responses during an actual viral infection.

Despite these limitations, the direct swab stimulation method remains a viable and efficient approach for rapidly assessing immune responses in nasal epithelial cells, especially in clinical or time-sensitive settings. This method is relatively non-invasive, allowing for frequent sampling, and provides a quick capture of the natural immune state and response without the need for extensive cell culture. Traditional cell culture model in monolayer doesn't accurately represent the complexity of what happens in vivo, as it lacks the multi-layered structure and cells interactions present in the nasal epithelium. Similarly, while air-liquid interface (ALI) cultures can mimic in vivo conditions more closely, lacking immune component, they still require extensive preparation and maintenance, and prolonged culturing can introduce changes in cellular behaviour that differ from the natural state of the nasal epithelium. In contrast, swab samples allow us to assess immune responses as they occur in the body, providing more accurate reflections of how nasal epithelial cells respond to viral mimics or pathogens in real-time. This approach circumvents the cost and time associated with culturing cells,

making it advantageous for large-scale studies and adaptable for various downstream analyses.

5.2 Future research directions

This study was conducted as preliminary work for a larger SARS-CoV-2 resistance project aimed at exploring the mechanisms of innate immune responses in nasal epithelial cells and identifying potential markers of resistance to respiratory viruses. The global impact of the COVID-19 pandemic has underscored the importance of understanding individual variability in immune responses, particularly in mucosal tissues like the nasal epithelium, which serves as the first point of entry for many respiratory pathogens, including SARS-CoV-2 (96). A more detailed exploration of the molecular mechanisms driving differences in baseline interferon expression, including genetic factors such as single-nucleotide polymorphisms (SNPs), could help identify biomarkers for immune readiness. Furthermore, the impact of external factors such as air pollution, smoking, or previous infections on epithelial health and interferon production warrants further investigation.

This preliminary work shows that direct swab stimulation is a practical and accessible approach for quickly assessing innate immune responses in the nasal epithelium. The observed variability in interferon responses across individuals suggests that this method could be used in the larger resistance study to identify individuals with more robust antiviral responses at the mucosal surface. This variability in immune readiness could help pinpoint key factors contributing to natural resistance to SARS-CoV-2 and other respiratory viruses.

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Appendices

Appendix 1: Ethics application

Applicant & Collaborators

Applicant Details

- Applicant Name : Federica Giangrazi
- Is applicant applying as a member of staff or a student? : Staff
- School / Department : School of Biochemistry & Immunology - Biochemistry
- Role on the Project : Non-Principal Investigator
- Primary Employer (if not TCD) :
- Other affiliations (if applicable) :
- Course (for student applicant only) :
- Part time / full time :
- I have read and understood Trinity policy on Good Research Practice : Yes
- I have read and completed the Integrity module : Yes
- I have read and completed the Data Protection Training module within the last 24 months : Yes

Trinity Collaborators

User	Role
CLIONA O'Farrelly	Trinity Principal Investigator
Marie Lambe-Costello	Stakeholder :Academic / Clinical / Professional / Industrial Collaborator
Valeriia Korovanenko	Stakeholder :Academic / Clinical / Professional / Industrial Collaborator
Cian Reid	Stakeholder :Academic / Clinical / Professional / Industrial Collaborator
Vasile Mihai Sularea	Stakeholder :Academic / Clinical / Professional / Industrial Collaborator
Andreea Atanasescu	Stakeholder :Academic / Clinical / Professional / Industrial Collaborator

Other Collaborators

Name	Email Address	User
Darragh Duffy	darragh.duffy@pasteur.fr	Translational Immunology unit lead
András Spaan	A.N.Spaan@umcutrecht.nl	Physician scientist
Evangelos Andreakos	vandreakos@bioacademy.gr	Assoc. Professor at the Centre for Clinical, Experimental Surgery and Translational Research

Project Details

Main Project Details

- Title of research :Defining the immune potential of nasal epithelium
- Data Collection Start Date : 01/06/2024
- Data Collection End Date : 31/12/2026
- Project End Date : 31/12/2026 12:00:00 AM

- Does the research involve :Humans (or their data)

- Does this Animal research involve :

- Is this AREC research a first application or an amendment :

- Is the research WHOLLY (i) an analysis of statutes/legal provisions AND/OR (ii) legal judgments/cases which have been made public by a judicial process? : No

- Trinity Researchers will have access ONLY to aggregate data or data that otherwise fall outside of the remit of the GDPR. : No

- Could the research have detrimental legal, economic or social consequences for either the participant or their establishments : No

- Intentions of the research: Does the research : None of the above

- State research aim(s) and objective(s), research question or hypothesis : We hypothesize that epithelial cells located at mucosal sites are able to respond and fight infections similarly to immune cells. For this reason, the immune potential of nasal epithelial cells will be assessed using several approaches to determine immune modulator expression and secretion and compare whether there are differences in between male and female or between young adults and elderly people.

- Lay Summary: including background/ rationale/ justification, research approach, study design (exclude detail of measurement instruments and intervention and analysis (if applicable) (Word limit: 250 words) : Many studies have highlighted the protective role of the epithelium in the sites in contact with the outer world. The nasal epithelium is the first site where bacteria and viruses interact and some researchers have found differences in the nasal epithelium of people who resisted infections. Thus, we aim to understand the protective potential of nasal epithelial cells and if these protection is different between male or female or between young adults and elderly people.

- Identify all countries where data is collected or processed : IE,FR,GR
 - Does the project involve :Human biological samples taken in a non-invasive manner
 - Is the project funded : Yes
-

Details on Human Participants and their Data

- Does your research involve Mixed Methods : No
- Does the project use data from :Primary sources only
- Will you obtain Consent from participants (or in the case of children or adults at risk of vulnerability from a parent / legal guardian) for their participation and/ or the use of their data? : Yes
- Provide further information :
- Will payment be made to research participants? :No
- Provide further information :
- Is the research Health Research as defined in the Health Research Regulations? : Yes
- Does the research require a Consent Declaration form as defined by the Health Research Regulations 2018 and amendments 2021? : NO
- Which of the following best describes the research Secondary Data Source(s)? :
- Could any of the research data directly identify any participant? : No
- Could any of the research data indirectly identify any participant? :No

- Do you process Personal Data for study administration purposes? e.g. contacting individuals : No

- If yes please outline how you will store the information, keep it confidential, and destroy it in line with the data retention policy. :

- Which of the following best describes the general characteristics of the target population?
:Staff of Trinity, Adults currently not at risk of vulnerability

- Do any of the following further describe the characteristics of the target population? :
health controls/participants, e.g. those used in the control arm of a health trial study

- Please describe :

- List the inclusion/ exclusion criteria for selection of research participants : An email will be circulated in the department of Biochemistry and Immunology to recruit 100 participants (50 males and 50 females of broad range ages) who wish to donate nasal swabs (3 swabs in total) and a matched blood sample from a subgroup. Inclusion criteria: healthy individuals above 18 years old. Exclusion criteria will be: people who have acute symptoms of infection, allergic rhinitis or asthma, recent usage of NSAIDs and immunomodulatory therapies (aspirin, ibuprofen ...) or steroids.

Research Sources and Sites

Name	Req
Department of Biochemistry and Immunology, Comparative Immunology lab	NOT-APPLICABLE

Outline of research methods

Method / Measurement

Nasal swabs and anonymised data on age and sex will be obtained from the study participants (n=100, 50 males and 50 females, broad age range) by a certified research staff in the department of Biochemistry and Immunology. Three nasal swabs will be taken from each donor, one to obtain protein and RNA, one for immunophenotyping through flow cytometry and one for establishing primary human nasal epithelial cell culture, which will be used to monitor cell growth and immune activation following in vitro stimulations. RNA and protein extracted from the nasal swab will be used to assess expression/presence of cytokines and chemokines. Matched PBMCs will be obtained from a whole blood sample to be used as a control for immunophenotyping for flow cytometry.

-
- Does your research use any of the following methods exclusively? : No

 - Does the research include an intervention? : No

 - Please select which of the following best describes the intervention :

 - Other :

 - Outline the intervention :

 - Outline the method of analysis (Word limit :100 words) : All analysis will be carried in the Comparative immunology lab (4.11, TBSI). Three nasal swabs will be obtained from participants to obtain: RNA and protein to assess expression and concentration of immune molecules; immunophenotyping of local immune cell composition will be carried using matched blood sample by flow cytometry; culturing nasal epithelial cells to assess responses by in vitro stimulations. A database with study ID, age and sex will be created in TCD-owned Microsoft Office 365 Excel and stored in a password protected and encrypted laptop. The results of analysis will be correlated with age and sex of the individuals.

 - What is the approximate size of the target population? : 100

 - What is the proposed sample size -how many participants are involved in the study? : 100

- What is the justification for the sample size? : This is a first study to characterize the model to obtain epithelial cell readouts from nasal swabs and see if any difference can be inferred between certain populations based on age and sex, therefore having a total of 100 individual would give us a good idea of the robustness of the method. We expect that having 100 participants will guarantee enough power for statistical analysis after dividing our groups by sex or by age.

Risk

Risk , benefit, harm to researcher

- What setting/s will be used for data collection : Participants' place of work

- Other elaborate : Data and samples will be collected in the Phlebotomy room in the department of Biochemistry and Immunology, room B1.18

Type	Other Type	Impact	Probabilty	Mitigation
NONE				

Risk , benefit, harm to site environment or Society

Type	Impact	Probabilty	Mitigation
NONE			

Risk , benefit, harm to participants

- Do you or any of the research team have any dependant relationship to the participants?
: : No

- Detail the role of the researcher/s, the relationship of the researcher/s to the participants normally, and how you will mitigate against coercion of participants to participate in the research or in influencing their responses :

Type	Other Type	Impact	Probability	Mitigation
PHYSICAL		LOW	LOW	Discomfort derived from nasal swabbing and needle injury will be limited as much as it could be possible
PHYSICAL		LOW	LOW	Bruising derived from needle injury will be limited by allowing only certified phlebotomist performing the blood drawn.
PHYSICAL		LOW	LOW	Risk of fainting derived from blood drawn will be limited by inclining the phlebotomy chair until participants feels better.

Participant Benefits

- Is it foreseeable that participants could potentially reveal information that you have a legal obligation to disclose (e.g. child protection policy, malpractice, etc.) :No
- What information may be disclosed, why, and to whom. :
- Outline any direct benefits to research participants of participation : There will be no direct benefits to the participants

Conflict of Interest

- Are you aware of any Conflict of Interest from the PI or any collaborator, processor or other person involved in the conduct of the project, that could arise in the course of the project? : No
- Give details of the Conflict of Interest and what mitigation measures are in place :

Health Research
Health Related

- Please indicate which of the following apply to your Health Research project : The data are irreversibly anonymized.

- Is the PI a medical doctor / dentist covered by the state claims agency (SCA) Clinical Indemnity Scheme (CIS) for research conducted within a designated state authority (HSE hospital or Service Provider) : No

- Will the project involve the administration of any substances or require participants to refrain from taking any substance : No

- Please detail substance, amount, desired effect, possible side effects and measures for minimising risk. :

- Will there be ongoing clinical supervision of the participants by a duly insured clinical practitioner during the project : No

- Will the research participant's GP be informed that they are taking part in the project : No

- Will permission be sought from the research participants to disclose information (for example, information about adverse outcomes) to their GP : No

- Please enter contact details of GP. :

Consent

Consent

- Do your participants require support to give Consent : No

- How will Assent be obtained and by whom :

- Provide Further Information :

- Do you require Assent or Proxy Consent from participants e.g. because of their vulnerability : No

- Provide Further Information :

- Are you required to have Garda Clearance : No

- What is the time interval between giving information and securing Consent : Less Than 7 Days

- Provide Further Information : Consent and PIL will be sent via email to the participants with the recruitment email sent by the school secretary (biochem@tcd.ie). This email will be sent 2 days before sample collection. The day of sample collection, the phlebotomist in charge of collecting samples will make sure participants have understood the study and answer any question that may arise. If participants are happy to sign the consent form, then the sample collection will occur. If participants do not wish to continue with the study, no data on age and sex nor samples will be collected from them. The consent forms signed by the study participants are going to be stored within the departmental secretary office (biochem@tcd.ie) as an electronic copy on the BIDS server.

- Describe how you will inform participants about the use of their Personal Data : No personal data will be recorded as the samples and data will be fully anonymised at source. Only age and sex information will be collected and stored in an anonymised TCD Microsoft Office 365 database. All information regarding storage and data processing will be given in detail in the participant information leaflet and any questions arising can be answered by the phlebotomist or by emailing the research team.

- Describe how participants can withdraw their Consent and/or their data : After anonymisation, withdrawal would not be possible since would be impossible to locate the samples/data linked to each individual. Therefore, if participants want to withdraw from the study it would be impossible for us since the data and samples are anonymized already at collection.

Funding

Funding

- Insert RPAMS number if applicable and available :

- Outline sources of funding, list names of all confirmed sources of funding or support (including in-kind benefit). For all, state if they are industry/commercial, state/public, philanthropic/charitable, other : This study is being funded by Science for Ireland and by the European Union (HORIZON Research and Innovation Actions (RIA)).

- Please specify any funder specific requirements or obligations which should be brought to the attention of the ethics committee and or Trinity Innovation & Enterprise : The funding agencies will ask for report of progresses of the project. Therefore, the results obtained from the study will be compiled in presentations or reports in a combined and anonymized manner.

- Will the results of the project be used or disclosed for commercial purposes : No

- Please clarify which party shall have the commercialisation and/or intellectual property rights : None

Conflict of Interest

- Are you aware of any possible Conflict of Interest arising from the funding or commercialisation of this project : : No

- Give details of the Conflict of Interest and what mitigation measures are in place :

- It there likely to be possible Conflict of Interest between the funders of the project and the aims and results of the project : No

- Give details of the Conflict of Interest and what mitigation measures are in place :

Sampling & Recruitment

Sampling and Recruitment

- Outline the sampling method : An email will be circulated by the secretary (biochem@tcd.ie) in the department of Biochemistry and Immunology seeking for volunteers belonging to the inclusion criteria. The email will contain as attachment the consent form and patient information leaflet. The study participants are asked to liaise with the phlebotomist to decide the date and time suitable for sample collection. On the day, the phlebotomist will collect the informed consent form which will be stored with the secretary office in the department of Biochemistry and Immunology (biochem@tcd.ie). If the consent form has been signed, the phlebotomist will be collecting samples (swabs and blood) from participants. The name of the participant will only be recorded on the consent form, while on the sample tube will be only present a study ID and age and gender will be recorded on the tubes by the phlebotomist. The samples will be then delivered to the Comparative Immunology lab for further processing.

- Describe the time commitment of the participant : The consenting and sampling procedure would take approximately 30 minutes. Where possible the collection of samples will occur as part of normal working day hours, and it will only happen once.

- Will the research require/use a gatekeeper :Yes

- Outline the position/role of the gatekeeper within the organisation : The gatekeeper is the secretary office of Biochemistry and Immunology (biochem@tcd.ie).

- Detail the role of the gatekeeper in the research : The secretary of Biochemistry and Immunology (biochem@tcd.ie) will be responsible to circulate email to staff members to seek study participants. They will also held the consent in order to store them in a separate location (school BIDS server) and make sure full anonymisation is maintained.

- Is there a dependant relationship between the gatekeeper and the participants : No

- Outline how this is going to be managed to mitigate against the dependencies :

- Please give a detailed step by step description of how participants will be recruited and append the recruitment material (Word limit: 100 words). : An email will be circulated two days before sample collection by the secretary office in the department of Biochemistry and Immunology (biochem@tcd.ie) seeking for volunteers fulfilling inclusion criteria. The email will provide details of the certified phlebotomist responsible for collecting the

samples whom participants can contact to arrange sample collection. The phlebotomist will not be involved directly in the project to maintain anonymity of the individuals.

Human Biological Samples

Human Biological Samples

- Will the samples in any form be stored for any period after the research completes : No
- State what type of samples will be stored :
- Where they will be stored (name specific location, ownership etc.) :
- Does the research involve the use of genetic data? : No

Appendix 2: Ethics approval letter



Coláiste na Tríonóide, Baile Átha Cliath
Trinity College Dublin
Ollscoil Átha Cliath | The University of Dublin

Dáimh na hInnealtóiríocht, na Matamaitice agus na hEolaíochta
Faculty of Science, Technology, Engineering and Mathematics (STEM)

7th May 2024

RE: Approval letter for REC application re: Defining the immune potential of nasal epithelium

Reference No: 2297

Dear Federica Giangrazi,

Thank you for your recent REC submission to the Trinity College Faculty of Science, Technology, Engineering and Mathematic (STEM) research ethics committee (REC). This has been reviewed. Following the review, we are pleased to grant you ethical approval from the Faculty of Science, Technology, Engineering and Mathematic (STEM) research ethics committee (REC) pending consideration of the below suggestion and conditions arising from the review on the following points:

- We would suggest that in Part 4 - Future Work section in the PIL, the following is repeated. The current wording suggests that the samples may be kept for future research indefinitely.
 - o *Your consent form will be retained for a period of 10 years to allow extended research on the field of mucosal immunology to take place. Once this time has passed or if the study finishes sooner, your consent and your samples will lawfully disposed of.*
- The end date for your ethical approval is 31/03/2027. This includes an automatic additional 3 months in case your project runs overtime slightly. After date 31/03/2027 you will no longer have ethical approval.
- Extensions can be applied for under exceptional circumstances and if appropriate must be made 2 months prior to your original ethics approved end date.
- It is only under extenuating circumstances (e.g. an unforeseen event) that extensions are granted (applying for an extension assumes there has been no scientific changes in your original REC submission) and evidence must be provided by the applicant via the online form [here](#)
- If you have major changes you must submit a new REAMS application.
- Please keep us updated on any changes to your REC application over the course of the study. You are required to submit a progress report every 6 months to the REC (for level 2 and 3) or every 12 months if your ethics is a level 1.
- The progress report is to be submitted via the online form at [here](#)
- The first progress report is due in December 2024.

Please don't hesitate to contact me if you require further information. Congratulations, your ethical approval reference code is 2297_FSTEMREC_FG_MAY2024. We hope your research project goes well.

Yours sincerely,

Dr Katie O'Connor (Convenor)

on behalf of the Chair and the members of the

Faculty of STEM Research Ethics Committee and subject experts

Dáimh na hEolaíochta, na
Teicneolaíochta, na hInnealtóiríocht
agus na Matamaitice
Coláiste na Tríonóide, Baile Átha Cliath,
Ollscoil Átha Cliath,
Baile Átha Cliath 2,
Éire,

Faculty of Science,
Technology, Engineering
and Mathematics (STEM)
Trinity College Dublin,
The University of Dublin,
Dublin 2,
Ireland.

T: +353 1 896 2291
STEM@tcd.ie
www.tcd.ie/stem
@TCDFacultySTEM

Why are some people resistant to SARS-Cov-2?



Why is this study being done?

We think some people's nasal epithelial cells are particularly good at keeping SARS-Cov-2 away. But very little is known about the anti-viral activity of nasal epithelial cells.



How can you help?

Volunteer for this study!

Contact vkorovan@tcd.ie



What will be collected?

- Nasal swabs
- Blood
- Data on age, sex, COVID infection exposure, vaccination history

*All collected data will be anonymised



Funder: Science Foundation Ireland
Study Leader: Cliona O'Farrelly



Researchers: Valeriia Korovanenko
Federica Giangrazi



Room 4.12, Trinity Biomedical Sciences Institute

Ethical approval#: 2297_FSTEMREC_FG_MAY2024



Appendix 4:Participant Information Leaflet

Study Title	Defining the immune potential of nasal epithelium
Research Site(s)	Comparative Immunology lab, room 4.11, Dept. of Biochemistry and Immunology, Trinity College Dublin
Principal Investigator(s) and Co-Investigator(s) (Study Team) (insert names, titles and contact details including Trinity School/Department/Unit)	Principal Investigator: Prof. Cliona O'Farrelly (email: ofarrecl@tcd.ie) Research team: Dr. Federica Giangrazi (postdoctoral researcher, email: fgiangra@tcd.ie);Valeriia Korovanenko (research assistant, email: korovanv@tcd.ie) Marie Lambe-Costello (Lab. Admin, email: lambecom@tcd.ie) Dr. Cian Reid (postdoctoral researcher) Dr. Vasile Mihai Sularea (postdoctoral researcher) Andreea Valeria Atanasescu (research assistant)
Study Organiser/ Sponsor (If applicable - delete if not required)	Science for Ireland, European Union (HORIZON Research and Innovation Actions (RIA))
Data Controller	Trinity College Dublin (research data)
Data Protection Officer (Research Data)	Data Protection Officer Secretary's Office Trinity College Dublin Dublin 2

Invitation

We would like to invite you to take part in a research study that is being carried out by the Comparative Immunology laboratory led by Prof. Cliona O'Farrelly at the Dept. of Biochemistry and Immunology in Trinity College Dublin. Before you decide whether or not you wish to take part, please take time to read this information leaflet carefully and discuss it with your family, friends or GP if you wish. Please feel free to ask the phlebotomist if there is anything which is not clear. If you would like more information, please ask the researchers whose names and contact details are provided on page 1 above. Don't feel rushed or under pressure to participate or to make a quick decision. You should understand the risks and benefits of taking part in this study so that you can make a decision that is right for you.

This leaflet has six parts:

Part 1 - The Study

Part 2 - Data Protection

Part 3 - Approval, Organising and Funding

Part 4 - Future Research (if personal data will be used in future research)

Part 5 - Further Information

Part 6 - Next Steps

Part 1 - The Study

Why is this study being done?

The cells located at the interface with the extern (mouth, skin), named epithelial cells, possess powerful activities to prevent bacterial and viral infections. In this sense, these cells can act like cells belonging to the immune system, even if they are not listed as part of it. Very little is known about the protective activity of nasal epithelial cells, and we aim to better understand their role in protecting against viral infection.

Why have I been invited to take part in this study?

We are interested in understanding the immune potential of nasal epithelial cells and see if there are any differences between male and female participants and between young and older individuals. You are being invited to participate in this study because you have voluntarily responded to the email stating your willingness to be enrolled in the study.

*We are hoping to recruit **100 participants** in the study, **50 male and 50 females (broad age range)**. We ask you not to participate if you **are feeling unwell, tested positive for COVID-19 or if you have allergic rhinitis or asthma, recent usage of NSAIDs and immunomodulatory therapies (aspirin, ibuprofen...) or steroids.***

Do I have to take part? What happens if I change my mind and wish to withdraw from the study?

No, you do not have to take part. Your participation in this study is voluntary and you can change your mind at any stage. However, since your information is anonymised when samples are collected, we will no longer be able to identify you.

If you change your mind and wish to withdraw this will not affect your education/employment in any way. If you would like to withdraw from the study, please contact Marie Lambe-Costello (lambecom@tcd.ie) who can take you through the process and organise this for you.

Please note that we will not be able to remove personal data which has been shared or pooled for use in publication before your request for deletion. Any samples already shared with collaborators or used up cannot be destroyed.

What does taking part in the study involve?

*This study will be carried out in TBSI from **June 2024** to **December 2026**.*

*If you decide to take part, you will receive an electronic copy of this document in the recruitment email and you will arrange sample collection day and time in **TBSI phlebotomy room (B1.18)**. On the scheduled day, this information leaflet and consent form will be discussed with you in **TBSI phlebotomy room (B1.18)**. If you agree to participate in the study and sign the consent form, the biological samples (blood and nasal swabs) will be collected by the phlebotomist. It should take about half an hour to take your consent and collect the samples. You will be given a copy of your signed consent form and this leaflet to keep for your records.*

If you are happy to take part, the phlebotomist will collect the following samples from you:

Nasal swabs: we will need to collect **three nasal swabs** (one on the left, one on the right and a third swab on the nostrils which is less uncomfortable to you) using standard Minitip nitrile swabs (same as used for SARS-CoV-2 testing) from up to **100 individuals**;

Blood samples: we will need to collect a **blood sample**, of up to **20ml** (equivalent of two tablespoons), on **100 participants** (50 males and 50 females) .

Appropriate PPE will be provided.

The phlebotomist will also record your age and gender on the pre-labelled ID-coded tubes, therefore all details will be fully anonymised and your sample will be unidentifiable throughout the research.

Where possible the collection of samples will occur as part of your normal working day hours, and it will only happen once. However, you may be asked to attend for sampling outside of your working hours if this will help facilitate sample collection and if you agree to this.

What will happen to my data and samples?

The **three nasal swabs** obtained from each donor will be used as follows: one to obtain **protein and RNA** will be obtained and the type of cells present in the nose (epithelial cells or immune cells) will be assessed through flow cytometry. We will also grow in the laboratory **nasal epithelial cells** which we will then stimulated to mimick a viral infection and record their reaction. RNA and protein obtained from the nasal swab will be used to assess presence of immune molecules. No genotyping will be performed on the samples, except for analysis on the expression of immune-related genes at the transcriptional level.

For the matched **blood samples** (20mL), peripheral blood mononuclear immune cells will be obtained to be used as controls for understanding the cellular composition of the sample by flow cytometry.

Microsoft office-based programs (Excel) provided by TCD will be used to populate a database with study ID, age and gender of the individuals and will be stored in a password protected and encrypted laptop belonging to TCD.

Results obtained from the analysis will be correlated with age and gender data to determine differences in nasal mucosal immunity between male and female or between younger or older individuals.

What are the possible benefits of taking part?

Taking part in this study **may not directly benefit you**. However, we hope that this research may help us to better understand the immune potential of mucosal sites and may result in new policies/guidelines/tests, drugs or treatment approaches. By participating, you are helping to advance science and medicine/education for future generations.

By taking part in this study, you are contributing to an extremely valuable information resource that will help the research team in Trinity to continue to examine and further understand the innate mucosal immunity in the years to come.

Are there any possible disadvantages or risks from taking part?

Nasal swab and blood sampling can sometimes cause minor **discomfort** in individuals; however, be assured that the phlebotomist collecting the samples will have full training on the procedure and any possible risks will be minimised.

Blood sampling can sometimes cause **bruising or fainting**. The phlebotomist who will collect the specimen will ask you about your previous reactions to blood sampling. The phlebotomy chair can be reclined until you fully recover if you may feel fainting.

*Health Information (Data): Only data related to age and sex will be recorded manually by the phlebotomist on the sample collection tubes. Your samples will be collected on pre-labelled tube with the study ID which will be used thereafter for the entire study. The only place where your name will appear is the consent form, which will not be accessible to the research team nor linked to your study ID, therefore the **risk you of a breach of confidentiality** is considered almost **null**.*

We will not be able to report back any findings to you directly, as this is a research study, and the tests are not clinically validated and all the data will be fully anonymised, so the researchers would not know your identity.

What will happen to the results of the study?

The results of the study will be reported in medical/scientific/educational journals and disclosed at medical/scientific conferences. No information which reveals your identity will be disclosed.

Part 2 - Confidentiality

What information about me will be used for this study?

Your name will only be recorded in the consent form.

*All data collected will be **anonymised**. Your sample is given a study ID instead of your name and the study ID will **never** be linked to your name on the consent form. Your age and sex will be recorded manually on the tubes by the phlebotomist.*

This is to protect your anonymity.

All research data is held securely on TCD Microsoft Office 365 provided platforms and TCD licensed software, with restricted access to the research team listed above, standard encryption and two step security verification for those who have access to it.

Your consent form will be retained for a period of 10 years to allow extended research on the field of mucosal immunology to take place.

Once this time has passed or if the study finishes sooner, your consent and your samples will lawfully disposed of up to a period of 10 years.

Part 3 - Approval, Organising and Funding

Has this study been approved by a research ethics committee?

Yes, this study has been approved by Trinity College Dublin Research Ethics Committee (REC). Approval was granted on [INSERT DATE]. An annual report will be provided to the REC and on completion of the study.

Who is organising and funding this study?

This study is being funded by Science for Ireland and by the European Union (HORIZON Research and Innovation Actions (RIA)). They will be provided with an anonymous report. They will not have access to your personal data.

Is there any payment for taking part?

No, we are not paying participants to take part in the study.

Part 4 - Future Research

Will my biological samples be used in future studies?

*Yes, with your permission, we would like **to keep samples for use in further research in the field of mucosal immunity** by the research team (or by collaborators in Europe).*

We ask your consent to keep your samples and basic information about your age and gender to be used for future research .

By permitting use of this information for future research, you are contributing to a valuable information resource that will help researchers (including our collaborators (Dr. Darragh Duffy, Institute Pasteur Paris; Dr. Andr s Spaan, UMC Utrecht; Dr. Evangelos Andreakos, Academy of Athens) to further understand mucosal immunity in the years to come.

No-one will know who you are as samples are anonymised at source. We keep no link between your sample and your name.

Will I be contacted again?

No, we will not contact you again since the samples, and information we collect is fully anonymised and it would be impossible for us to find your details.

Part 5 - Further Information

Who should I contact for information or concerns?

If you have any concerns or questions, you can contact:

Principal Investigator: Cliona O'Farrelly, ofarrecl@tcd.ie

Thank you for taking the time to read this Participant Information Leaflet.

You will be given a copy of this Leaflet and the signed Consent Form to keep. Please retain these in case they are needed for future reference.

CONSENT FORM

Study Name: Defining the immune potential of nasal epithelium
Investigator/PI: Prof. Cliona O'Farrelly
Department/School: School of Biochemistry and Immunology, TBSI
Ethical approval number for the study:
_2297_FSTEMREC_FG_MAY2024_____

Thank you for participating. There are **2 sections** in this form.

Section 1 Each section has a statement and asks you to tick if you agree.

Section 2 asks for your consent, Please initial if you agree

The end of this form is for the researchers to complete.

Please Initial the box if you agree with the statement. Please feel free to ask any questions if there is something you do not understand.

General	Tick Box
I confirm I have read and understood the Information Leaflet for the above study. The information has been fully explained to me and I have been able to ask questions, all of which have been answered to my satisfaction.	
I understand that this study is entirely voluntary , and if I decide that I do not want to take part, I can stop taking part in this study at any time without giving a reason, until samples have been anonymized. I understand that deciding not to take part will not affect my current or future education/employment.	
I understand that all information will be kept private and confidential and that my name/identity will not be disclosed to the researcher or to collaborators.	
I understand that I will not be paid for the use of my samples	
I understand that there are no direct benefits to me from participating in this study. I understand that results from analysis of my samples will not be given to me.	
I understand that my age and sex will be recorded on each tube.	

Consent	Initial
I agree to take part in this research study having been fully informed of the risks, benefits and alternatives which are set out in full in the information leaflet which I have been provided with.	
I agree to allow my anonymized data to be reported in medical/scientific/educational journals and disclosed at medical/scientific conferences. No information which reveals my identity will be disclosed.	
I agree to provide a blood sample up to 20mL as outlined in the information leaflet. The risks have been explained to me.	
I agree to provide four nasal swab samples as outlined in the information leaflet. The risks have been explained to me.	
I agree to allow my samples to be shared with other Academic or not for profit collaborators within the EEA for the purpose of nasal mucosal immune response characterization research, as described in the Information leaflet.	

Donor Name (Block Capitals)

Donor Signature

Date

To be completed by the phlebotomist (nominated by the investigator)

I, the undersigned, have taken the time to fully explain to the above patient the nature and purpose of this study in a way that they could understand. I have explained the risks and possible benefits involved. I have invited them to ask questions on any aspect of the study that concerned them. I have given a copy of the information leaflet and consent form to the participant with contacts of the study team

Phlebotomist Name (Block Capitals)

Phlebotomist Signature

Date

2 copies to be made: 1 for donor, 1 for gatekeeper records

Appendix 5

Table 1. List of general reagents

Reagent	Supplier	Ref number
TRIzol® Reagent	Invitrogen	15596018
Chloroform	Honeywell	33539-2.5L-M
2-Propanol purist. p.a., ACS reagent, reag. ISO, reag. Ph. Eur., ≥99.8% (GC)	Sigma-Aldrich	33539-2.5L-M
Ethanol, Absolute (200 Proof), Molecular Biology Grade	Fisher Bioreagents	BP2818-500
UltraPure™ Distilled Water DNase/RNase Free	Invitrogen	10977-035
High-Capacity cDNA Reverse Transcription Kit	Thermo Fisher Scientific	4368814
10x Random primers	Thermo Fisher Scientific	4319979
10x RT Buffer	Thermo Fisher Scientific	4319981
dNTP Mix 100 mM	Thermo Fisher Scientific	4367381
MultiScribe Reverse Transcriptase	Thermo Fisher Scientific	4308228
PowerUp™ SYBR™ Green Master Mix	Applied Biosystems	A25742
Gibco™ Trypan Blue Solution, 0.4%	Thermo Fisher Scientific	15250061
Gibco™ Fetal Bovine Serum, heat inactivated	Thermo Fisher Scientific	A3840001

1x DMEM (Dulbecco's Modified Eagle Medium)	Thermo Fisher Scientific	11965084
Gibco™ Penicillin-Streptomycin (5,000 U/mL)	Thermo Fisher Scientific	15070063
Dulbecco's Phosphate Buffered Saline (DPBS 1X)	Thermo Fisher Scientific	14190-094
Poly(I:C)	Invivogen	tlrl-pic
Nylon® Flocked Swab	COPAN Diagnostics	503CS01
PneumaCult™-ALI Medium	STEMCELL Technologies	05001
PureLink™ RNA Mini Kit	Invitrogen	12183018A
RNeasy Mini Kit	Qiagen	74106
DNase I, Amplification Grade	Invitrogen	18068015

Table 2. Forward and reverse primer sequences of target genes.

Target gene	Forward primer (5' - 3')	Reverse primer (5' - 3')	Conc. per reaction
Beta actin	ATTGGCAATGAGCGGTTC	TGAAGGTAGTTTCGTGGATGC	500 nM
IFNB1	AAACTCATGAGCAGTCTGCA	AGGAGATCTTCAGTTTCGGAGG	300 nM
Il29 (IFNL1)	TCCAAGCCCACCACAACCTG	TGAGTGACTCTTCCAAGGCGT	300 nM
IFNL2/3	CAGCTGCAGGTGAGGGA	GCGGTGGCCTCCAGAACCTT	300 nM
IFNE	TCAGCCTCTTCAGGGCAAATA	GAGGAATTTCTCCGTGTGGTTT	500 nM

CXCL10	CCTGCAAGCCAATTTTGTCCA	TGTGGTCCATCCTTGGAAGC	300 nm
CCL5	CCCATATTCCTCGGACACCA	GGTGACAAAGACGACTGCTG	300 nm
OAS1	TGTCCAAGGTGGTAAAGGGTG	CCGGCGATTTAAGTATCCTG	300 nm
ISG15	TTTGCCAGTACAGGAGCTTGTG	GGGTGATCTGCGCCTTCA	300 nm
PRK	GGTACAGGTTCTACTAAACAGGAA GC	CAGGTAGTCAGATTTCACTGAGGT TTC	300 nm
MDA5	GCCCGCTACATGAACCCTG	CAGCAATCCGGTTTCTGTCTT	300 nm
RIGI	CTGGACCCTACCTACATCCTG	GGCATCCAAAAAGCCACGG	300 nm

Table 3. Reagents used for flow cytometry

Antibody	Conjugate	Ref number	Supplier
EpCAM	BV605	563182	BD Biosciences
CD45	BV510	563204	BD Biosciences
CD4	PE/Fire 640	344664	BioLegend
CD8	AF647	344726	BioLegend
CD56	BV650	564057	BD Biosciences
CD16	PECy7	557744	BD Biosciences
CD3	Spark Blue 550	344853	BioLegend
Live/Dead	Live/dead NIR	L10119	Thermo Fisher Scientific
Compensation beads	-	01-1111-42	Thermo Fisher Scientific