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Characterisation and culture of primary human nasal epithelial cells

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List of abbreviations

AIC	Air-interfaced culture
ALI	Air liquid interface
APC	Antigen presenting cell
BPE	Bovine pituitary extract
CCL	Chemokine ligand
CRS	Chronic rhinosinusitis
DC	Dendritic cell
DNase	Desoxyribonuclease
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
FBS	Fetal bovine serum
GM-CSF	Granulocyte/macrophage colony-stimulating factors
HC	Hydrocortisone
H&E	Hematoxylin and Eosin
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
IP	Interferon-induced protein
LC	Langerhans cell
LCC	Liquid covered culture
MCP	Monocyte chemotactic protein
MHC	Major histocompatibility complex
NP	Nasal polyposis
RA	Retinoic acid
RANTES	regulated on activation normal T cell expressed and secreted
rpm	Rounds per minute
TER	Transepithelial electrophysiological resistance

T_H1 or T_H2	T helper cell 1 or 2
TJ	Tight junction
TNF	Tumor necrosis factor

1 Introduction

The nasal epithelium with its tight junctions (TJs) represents an important barrier against the penetration of exogenous substances (e.g. allergens, pollutants and pathogens). Damage of the epithelium allows higher amounts of inhaled allergens and pollutants to penetrate the mucosa. Our laboratory has previously used a bronchial epithelial cell line to investigate epithelial damage by various factors [cigarette smoke (Gangl et al., 2009) and cytokines (Reisinger et al., 2005)]. Cultures of primary epithelial cells obtained from the nasal epithelium may be superior in resembling the natural situation in the nose and should allow a comparison of epithelia from non-allergic and allergic patients. We compared different epithelial cell culture systems to investigate damage and repair in primary human nasal epithelial cells.

1.1 Aim of the study

The aim of this master thesis project was to establish primary human nasal epithelial cell cultures using a transwell system [air liquid interface culture (ALI)] to investigate the epithelial barrier function and the properties of the nasal epithelium. Therefore we assessed the transepithelial electrophysiological resistance (TER) of the epithelium, investigated the epithelial permeability using the cytokine interferon gamma (IFN-γ) as an impairing test substance and established the scratch test method as a measurement of physical damage and the proliferation and growth rate of primary human nasal epithelial cells. To characterise the cell types present in the cultured epithelium and the morphological features of the cultured cells, flow cytometry and immunohistological assessments were employed.

1.2 The nasal airway

Besides functioning as the principal olfactory organ, the nose filters, warms and humidifies the inspired air. The air has to pass the nose before entering the more sensitive tracheobronchial airways and alveolar parenchyma of the lung (Cole, 1993). The important nasal passages in the upper respiratory tract defend the lower respiratory tract by absorbing gases, capturing particles and metabolising airborne xenobiotics (Brain, 1970). In fact the nasal epithelium constitutes the first barrier of entry for inhaled air in the respiratory

system of humans and other mammals and has therefore various essential functions. Due to the exposure to high concentrations of allergens, pathogens, medications, and infectious toxic and carcinogenic agents the nose is vulnerable to acute and chronic diseases (Harkema et al., 2006; Polosa et al., 2000). Therefore many diseases of the nasal airways and associated paranasal sinuses can be due to allergic reactions, microbial infections or natural aging (Harkema et al., 2006).

1.3 Anatomy of the nasal cavity

The median nasal septum divides the human nasal airway in two symmetrical passages, which extends anterior through the nostrils to the face and posterior to the nasopharynx (see Figure 1) (Chien et al., 1989). Each half of the nasal cavity is restricted by the septal wall and the lateral wall (Dahl and Mygind, 1998). The nasal vestibule, the respiratory region, and the olfactory region represent the nasal cavity (Fig. 2) and have a total volume of 15-20 ml. The total nasal area covers 150 cm^2 (Kim, 2008).

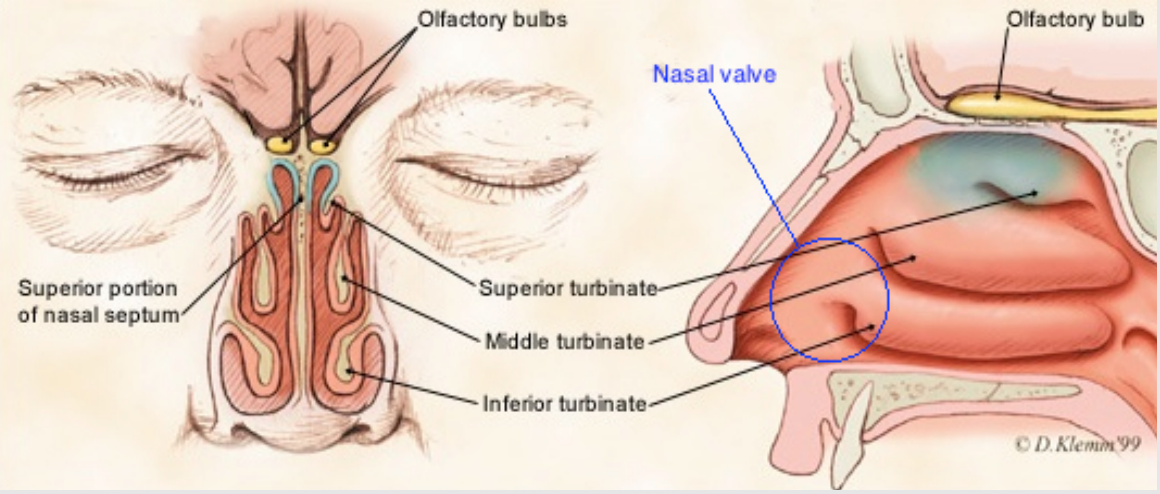


Figure 1: Anatomy of the nasal cavity

Nasal septum and symmetrical turbinates (superior, middle and inferior) are displayed and nasal valve region is marked in blue.

[Adapted from (Bromley, 2000)]

1.3.1 The nasal vestibule

The nasal vestibule (Fig. 2A) serves as the most anterior section just inside the nostrils with a surface of about 0.6 cm^2 and borders to the intermediate part, the atrium (Morrison and Costanzo, 1990). It filtrates large particulate matter with its thick hairs without piloerector muscles, called vibrissae. In contrast to the other regions of the nasal cavity, the vestibule is constituted with lightly keratinised, stratified squamous epithelium without microvilli. The basal cells along the basal lamina and some layers of squamous cells get flatter near the luminal surface. The squamous epithelium changes in its more posterior region into nonciliated transitional epithelium (Fig. 2B) with short microvilli (Cauna, 1982; Harkema et al., 2006; Sahin-Yilmaz and Naclerio, 2011). By comprising thermoreceptors the nasal vestibule has the most important role in sensing the nasal airflow (Clarke and Jones, 1992; Jones et al., 1989).

1.3.2 The respiratory region of the nose

The superior, middle, and inferior turbinates or conchae represent the respiratory region and cover about 85% of the total nasal area. The scroll-like and bony turbinates protrude into the airway lumen from the lateral walls of each symmetrical half into the main chamber of the nasal cavity (Fig. 1). By substantially increasing the nasal surface to around 100 to 200 cm and preserving a slit-like cavity the turbinates ease the crucial humidification and temperature regulation of the inhaled air. The conchae are important for generating a turbulent airflow through the nasal passages and therefore providing a better contact between the inspired air and the mucosal surface (Dahl and Mygind, 1998; Harkema et al., 2006; Morrison and Costanzo, 1990; Sahin-Yilmaz and Naclerio, 2011; Swift and Proctor, 1977).

The inferior and middle turbinates have the most important functions in this context, as they ease the humidification and fast heat transfer from the nasal mucosa (Doorly et al., 2008; Pless et al., 2004). Because of slowing down the rapid laminar flow of the inhaled air through the nasal valve (Fig. 1), the air is exposed to tight contact with the warmer nasal mucosa. The nasal mucosa warms the air to around 34°C and moisturises it to 100% when it arrives at the nasopharynx. During the expiration of the respired air, the nasal mucosa gains the lost humidification and heat again. The nasal mucosa shows the highest temperature at the end of expiration and the lowest at the end of inspiration (Lindemann et al., 2004; Lindemann et al., 2002).

Additionally the inferior concha is crucial in defending the lungs against inhaled factors, e.g. allergens, pollutants and pathogens, and is important for the physiology of the nose. A trimming turbinoplasty reduces the anterior part of the inferior turbinate and expands the nasal valve. This surgery can decrease the total nasal resistance to airflow while maintaining the turbinate function (Berger et al., 2003; Polosa et al., 2000; Polosa et al., 2004; Sahin-Yilmaz and Naclerio, 2011; Wight et al., 1988).

Highly vascularised mucosal tissue overlays the bony turbinates, i.e. the respiratory epithelium, which presents ciliated cells, nonciliated cells, columnar cells, goblet cells, and basal cells. The respiratory epithelium is of the pseudostratified ciliated columnar type (Fig. 2) (Baroody, 2007; Harkema et al., 2006; Kim, 2008). Around 20% of all cells in the lower turbinate part are covered with about 100 cilia per cell on the apical cell surface. The 4-6 µm long fine extensions are mobile, beat with a frequency of 1000 strokes per minute and therefore transport the mucus to the nasopharynx. Approximately 300 microvilli per cell are located on ciliated and nonciliated columnar cells and benefit in enlarging the surface area. The nonciliated cells are considered to be important for high metabolic activity and transportation of liquid across mucosal cells (Baroody, 2007; Kim, 2008).

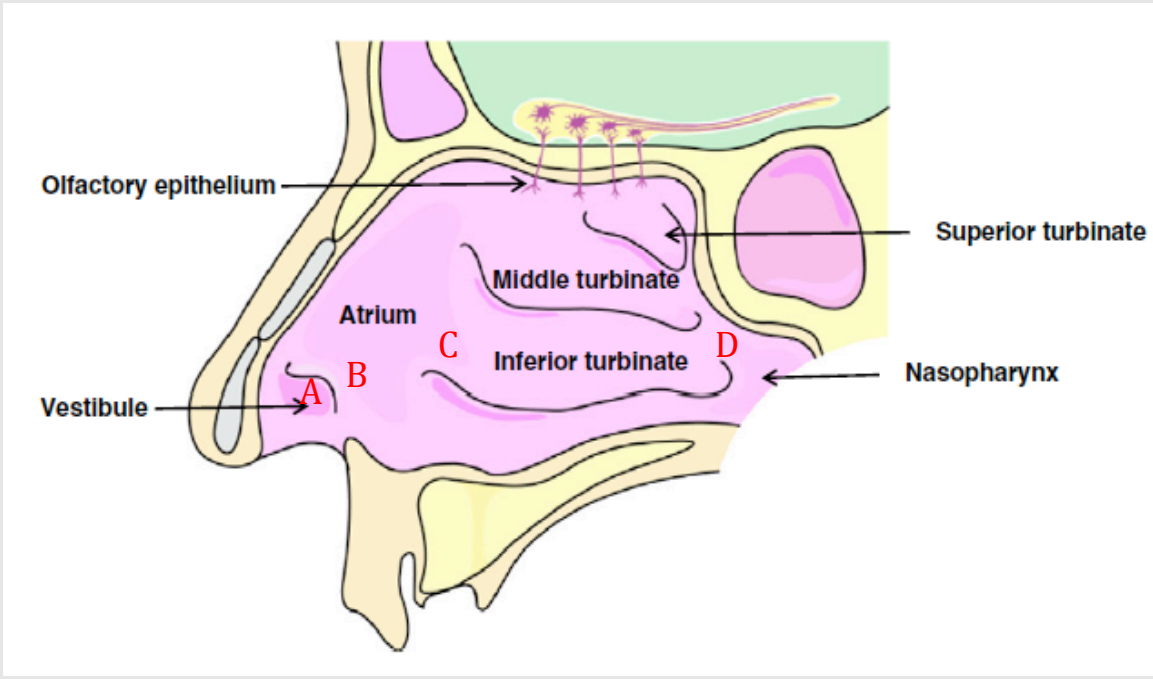


Figure 2: Schematic picture of a sagittal section representing the different areas of the human nasal cavity: Nasal vestibule, atrium, inferior, middle and superior turbinates, nasopharynx and olfactory region.

Letters indicate epithelial type at specific locations: (A) vestibule with squamous epithelium without microvilli, (B) atrium with transitional epithelium with short microvilli, (C) respiratory pseudostratified columnar epithelium with few ciliated cells at inferior turbinate, (D) respiratory pseudostratified columnar epithelium with many ciliated cells at inferior turbinate

[Adapted from (Grassin-Delyle et al., 2012) and modified according to (Proctor and Anderson, 1982)]

1.3.3 The olfactory region

The top of the nasal cavity is the olfactory area, which represents only 10% of the nasal area (Kim, 2008). Only 10% of the inhaled air gets to this region. Bipolar neurons from the first cranial nerve are situated in the olfactory epithelium. This epithelium is lined by mucus containing immunoglobulin A and M (IgA, IgM), lactoferrin, and lysozyme, which can help to defend the cranial cavity against pathogens (Mellert et al., 1992; Sahin-Yilmaz and Naclerio, 2011). Inflammatory diseases, e.g. allergic rhinitis, rhinosinusitis, or viral infections may cause olfactory disturbances such as dysfunctional differentiation and recognition (Hornung, 2006; Sahin-Yilmaz and Naclerio, 2011).

1.4 The nasal respiratory epithelium

This epithelium is composed of various specialised cells, which feature important functions for maintaining the normal homeostasis of the human nasal mucosa. The tasks of the cells are regulating the balance of fluid, metabolising and/or clearing inspired substances, responding to damage by attracting and activating inflammatory cells, and secreting mediators for the airway smooth muscle activity. As already mentioned, the respiratory epithelium represents the first barrier of entry for inhaled air containing environmental and inflammatory agents (Knight and Holgate, 2003). Around 500 ml air and a higher amount than one million particles are inspired with every breath. Therefore 10 000 liter of air with around 300 million particles per day are exchanged (Gehr et al., 2010).

Respiratory diseases, e.g. inflammatory disorders and oedema, can therefore be related to injuries of the nasal epithelium (Knight and Holgate, 2003). In this case the nasal area is negatively affected in defending the lower respiratory tract against exogenous agents (Proctor, 1977). However the nasal epithelium has the ability to repair itself after injuries to provide the integrity (Polosa et al., 2000).

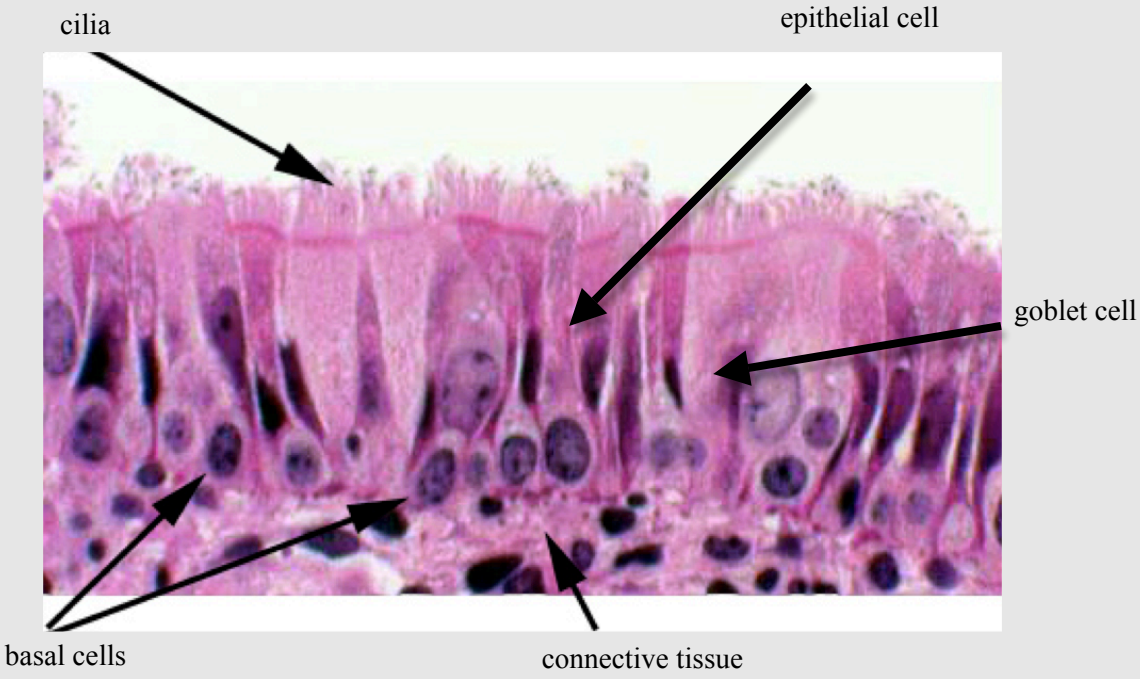


Figure 3: Hematoxylin and eosin (H&E) stain of respiratory ciliated epithelium
Epithelial cells, goblet cells, cilia, basal cells and an underlying connective tissue can be distinguished. [Adapted from (Caceci, 2008)]

1.5 Respiratory epithelial cells

Respiratory epithelial cells are functionally, ultrastructurally and biochemically divided into three groups, i.e. basal, ciliated and secretory cells (Fig. 2 and 3) (Spina, 1998). Supplementary immune cells, inflammatory cells and phagocytic cells get to the epithelium and persist there or pass through to the lumen (Knight and Holgate, 2003).

1.5.1 Basal cells

This cell type is located on the basement membrane and is not in contact with the airway lumen. It is thought that the basal cells are the precursors of the other cells. The basal cells fill 51% of the proliferating compartment in large (diameter of ≥ 4 mm) and 30% in small airways (diameter of < 0.5 mm). Due to this fact there is a direct correlation between the diameter size of the epithelium and the amount of present basal cells. The number of the basal cells decreases with the airway size (Boers et al., 1998).

The basal cells feature electron-dense cytoplasm and tonofilament-clusters. This cell type is believed to be responsible for anchoring the higher located cells of the respiratory epithelium to the basement membrane as they are the only cells, which are constituted of hemidesmosomes with the indispensable integrins (α6β4). Above all it is thought that the basal cells aid columnar cells to attach to the basement membrane, because columnar cells do not show/have hemidesmosomes and connect with the basement membrane only with the cell-adhesion molecules. Additionally, the basal cells have interepithelial cell adhesion mediating desmosomes. Furthermore it is suggested that the basal cells release bioactive molecules, such as cytokines, neutral endopeptidase and 15-lipooxygenase products (Baroody, 1997; Dahl and Mygind, 1998; Evans and Plopper, 1988; Knight and Holgate, 2003).

1.5.2 Columnar ciliated epithelial cells

Basal or secretory cells are the progenitors of the columnar cells (Ayers and Jeffery, 1988). Fifty % of all epithelial cells in the nasal area are ciliated epithelial cells and are therefore the prevalent cells (Spina, 1998). A high number of mitochondria is present in the upper area of the columnar cells, which indicates a high metabolism and transport of mucus (Dahl and Mygind, 1998; Harkema et al., 1991). Tight junctions bind the columnar cells

intercellularly on the apical side and the cell membrane interdigitations bind the cells on the top. Around 300 short, thin and cytoplasmic microvilli enlarge the epithelial surface area of each cell and improve the fluid transport into and out of the epithelium. The moisturisation by microvilli is crucial for maintaining the epithelial function and preventing the epithelium from drying out. Hundred cilia (0.3 µm width x 5 µm length) per cell begin to appear with the second-third of the nasal cavity, i.e. with the inferior concha. At the back part of the nasal area the cilia build a tight blanket over the epithelial cells (Dahl and Mygind, 1998). Cilia do not occur in the anterior area because of the high nasal airflow. It is thought that the cilia prefer the low tempo of the airflow, the higher temperature and high humidity in the posterior part of the nasal area (Cole, 1982; Dahl and Mygind, 1998).

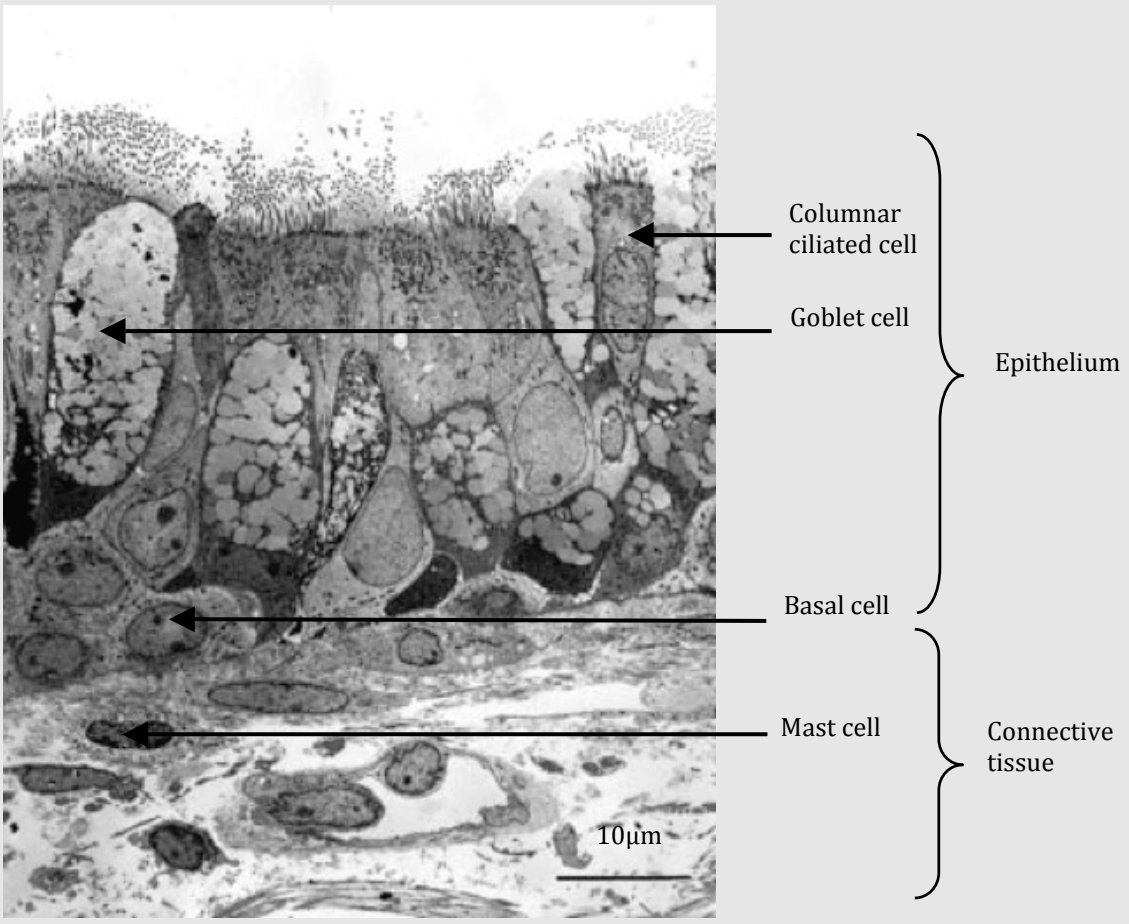


Figure 4: The respiratory epithelium in a transmission electron micrograph

Imaging the epithelium, which is constituted of goblet, ciliated and basal cells. A mast cell is characterised in the underlying tissue.

[Adapted from (Jeffery and Li, 1997)]

1.5.3 Goblet cells

This cell type covers about 10% of the turbinate mucosa and is assumed to be self-renewing and differentiating into ciliated epithelial cells (Baroody, 2007; Evans and Plopper, 1988). The goblet cells are responsible for producing the appropriate quantity of vicsoelastic mucus for the crucial mucociliary clearance. The released mucin granules are membrane-bound and acidic, because of the sialic acid containing glycoproteins. It is believed that the acidic and viscoelastic properties of the mucus enable the goblet cells to capture particles in the airway lumen and to transport across the cilia (Harkema et al., 1991; Jeffery, 1983, 1991). Contact with exogenous substances, biochemical mediators and cytokines may promote the mucin secretion of the supposed 4000-7000 mucous goblet cells/mm^2 of the surface epithelium (Ellefsen and Tos, 1972; Lumsden et al., 1984).

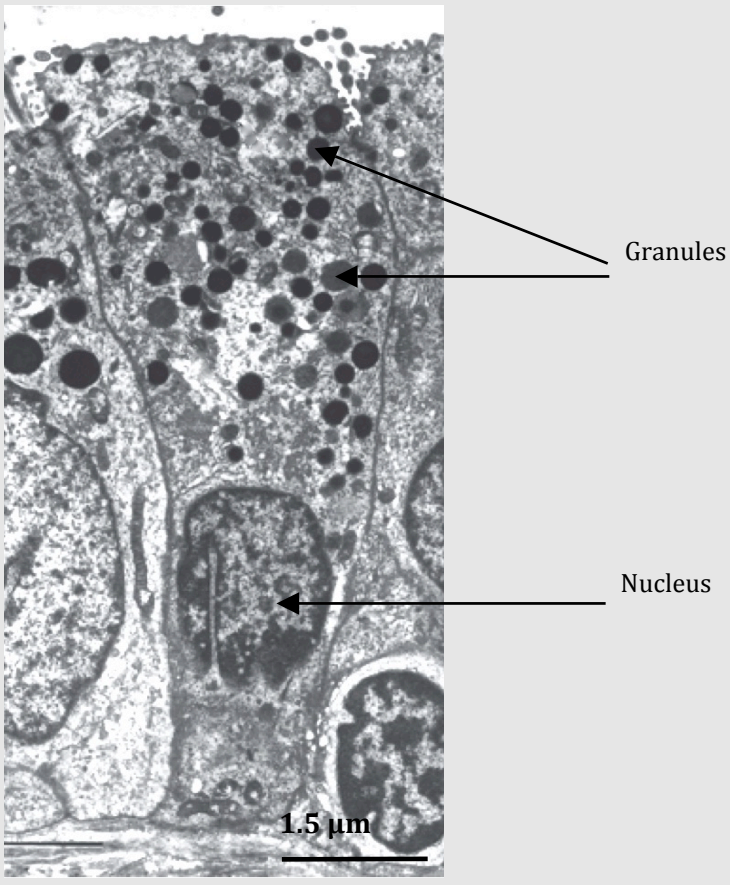


Figure 5: Goblet cell with secretory granules in a transmission electron micrograph
[Adapted from (Jeffery and Li, 1997)]

1.6 Basement membrane of respiratory epithelium

This collagen fibril layer is the anchor for the respiratory epithelial cells as it promotes the migration and adhesion of the cells. Additionally the membrane is responsible for providing the appropriate polarity and adjusts the phenotype of the epithelial cells. The basement membrane represents a barrier between the epithelium and the mesenchymal compartments underneath (Boudreau et al., 1996; Terranova et al., 1980). Epithelial cells are normally not or barely adjacent to other resident structural cells. However, they are in contact with the infiltrating and freely moving inflammatory and immune cells. The upper layer (the lamina densa) of the membrane is constituted of collagen type IV and laminin type V, which are released by the epithelial cells. The broader and lower located lamina reticularis is mostly produced by subepithelial fibroblasts and compounds collagen type III and V and fibronectin (Paulsson, 1992). The basement membrane comprises around 800 pores per mm^2, which feature about 1.8 μm diameter (Howat et al., 2002). Underneath an elastic lamina propria, also called subepithelium, supports the surface epithelium, where blood vessels, nerve bundles and free cells are located (Jeffery and Li, 1997).

1.7 Respiratory epithelial function

The respiratory epithelium is important for regulating various airway functions. Diverse mediators are produced constantly or by stimuli and contribute significantly in changing the epithelial performance (Holgate, 1998). The epithelial cells synthesise various cytokines, chemokines, growth factors, lipid mediators, and peptides (Barnes et al., 1988). The barrier is physically constituted of the mucociliary layer with an underlying submucosal layer and intercellular adhesion structures, such as tight junctions between the neighboring cells at the apical sides, thus limiting paracellular routes for molecules and electrolytes. Additionally desmosomes, gap and intermediate junctions occur in the epithelium supporting the epithelial structure and integrity (Churg, 1996; Knight and Holgate, 2003; Sparrow et al., 1995)

1.7.1 Mucociliary clearance

Mucociliary clearance is an important function of the nasal area for clearing itself of released and captured substances. The mucus transport system is mainly composed of the mucous layer and ciliated epithelial cells (Sahin-Yilmaz and Naclerio, 2011).

The nasal area is lined by a 10 – 15 µm mucous blanket, which is produced by goblet cell and submucosal gland secretions, and transepithelial transferred liquid (Wilson and Allansmith, 1976). The periciliary and watery sol phase and the uppermost gel phase compose the mucous blanket (Fig. 6) (Ali and Pearson, 2007). The covering mucus gel phase of the nasal epithelium is in contact with the external environment (Kim, 2008).

The nose resembles a filter as it filtrates particles in the nasal valve region (Fig. 1), which are bigger than 3 µm. The nasal mucosa filtrates particles of 3 to 0.5 µm and the filtrated substances are transported to the nasopharynx by ciliary flow. For smaller than 0.5 µm particles and gases it is easier to get to the lower respiratory tract (Sahin-Yilmaz and Naclerio, 2011; Wiesmiller et al., 2003).

Besides the function as a filter, the mucous layer provides fluid for humidification and also has excellent defense abilities. Because it serves as the primary site of contact with exogenous agents, the mucous blanket contains immunoglobulin A (IgA). This immunoglobulin is mainly contained in nasal secretion and is able to protect against pathogens (Bellanti et al., 1965; Sahin-Yilmaz and Naclerio, 2011).

Goblet cells and submucosal glands produce around 0.1 – 0.3 mg/kg mucus per day. Mucins are high molecular weight glycoproteins and their amount in mucus determines the gel-like properties (Sleigh, 1977). A lubricating and mucosal surface protecting matrix is established by binding water to oligosaccharide side chains of the macromolecules mucin (Rubin, 2002; Sahin-Yilmaz and Naclerio, 2011). Besides mucin the mucus consists of IgA, IgG, IgE, histamine, bacteria, albumin, lysozyme, lactoferrin, ions and cellular debris (Bascom et al., 1988; Baumgarten et al., 1985; Butler et al., 1967; Naclerio et al., 1983; Watson et al., 1962).

The mucus is transported towards the nasopharynx while the mucous layer is moving. Cilia are able to transport inhaled particles and therefore clear the nasal area and renew the mucus layer in 10 – 20 minutes (Andersen et al., 1974; Kim, 2008). This transport system is negatively impacted by nasal septal deviations and chronic sinusitis (Cohen, 2006; Keojampa et al., 2004; Ulusoy et al., 2007). However, blowing the nose, sneezing and sniffing promote the mucociliary clearance of substances (Hounam, 1975).

The ciliary beat system is divided in two stroke patterns. The cilia straighten, touch the gel

phase and set the mucus in motion while effector stroking. While bending of the cilia and moving in the sol phase, the cilia are in the recovery stroke (Sahin-Yilmaz and Naclerio, 2011).

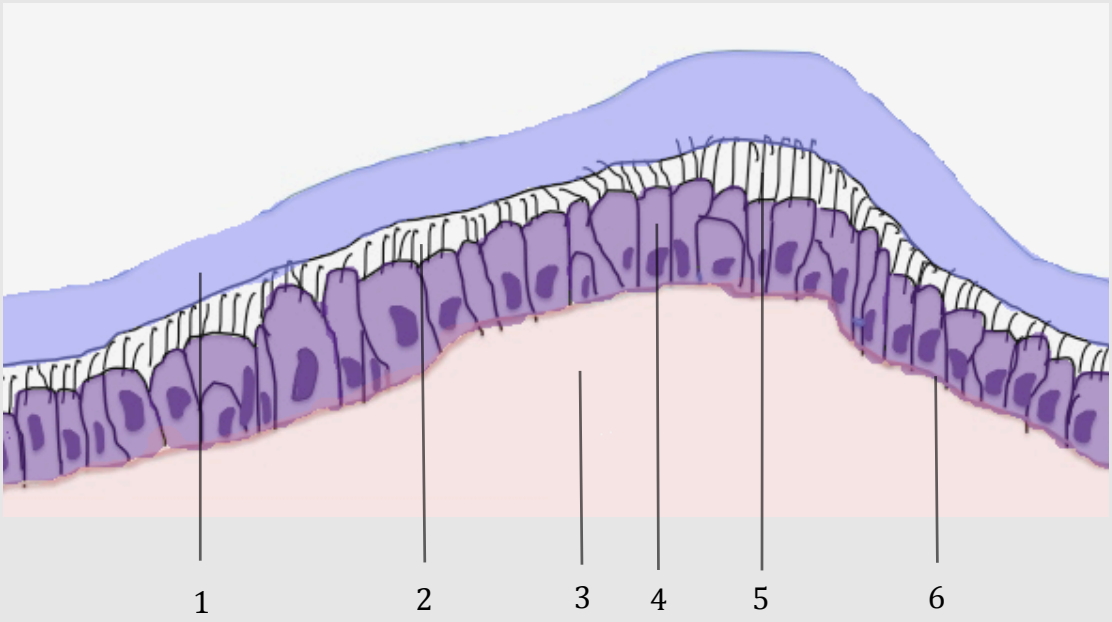


Figure 6: Mucus layer of nasal airway

1. Gel Phase, 2. Sol Phase, 3. Submucosal layer, 4. Epithelial cell, 5. Cilia, 6. Basement membrane

[Adapted from (Ali and Pearson, 2007)]

1.7.2 Nasal epithelial barrier function

As the most apical intercellular junctions between epithelial cells, tight junctions prevent exogenous particles (e.g. allergens) from penetrating the epithelium. These junctions are constituted of transmembrane and scaffold adaptor proteins and play an important role in the paracellular flux regulation and epithelial impermeability. Draining of inflammatory cells toward the lumen and resolution of inflammatory reactions can be consequences of tight junction openings. Tight junction proteins, e.g. claudins, occludin, tricellulin and junctional adhesion molecules, create homo- and heterodimers between adjacent cells (Chiba et al., 2008; Soyka et al., 2012). Tight junctions attach to the actin cytoskeleton with related proteins, e.g. zonula occludens and cingulin. When tight junctions are disrupted, antigens (e.g. allergens) or pathogens can get through the epithelium and therefore many inflammatory diseases could develop due to altered or defective tight junctions (Holgate, 2007; Soyka et al., 2012). Not many studies are focusing on the nasal epithelial tight junctions, but decreasing transepithelial resistance (TER) and disturbed zonula occludens-1 (ZO-1) proteins were shown in individuals suffering from acute rhinitis (Sajjan et al., 2008).

1.8 Allergy

Approximately 25% of the population of industrialised countries are affected by IgE-mediated allergic diseases (Wüthrich et al., 1996).

According to Coombs and Gell (1963) four types of hypersensitivities can be distinguished by their type of immune response and cell and tissue damage effector mechanisms. Besides IgE-associated type I hypersensitivity, i.e. the immediate hypersensitivity response, type II is IgG-mediated against surface antigens, type III is immune complex-mediated and type IV is the delayed hypersensitivity and is T cell-mediated (Averbeck et al., 2007; Coombs and Gell, 1963).

Different IgE-associated allergic reactions, e.g. acute rhinoconjunctivitis, asthma, skin reactions, food allergy and fatal systemic reactions (anaphylaxis) occur when allergic individuals get in contact with allergens against which they are sensitised. Young children with a genetic atopic predisposition become sensitised with encountered allergens. The allergic immune response depends on the amount, route and frequency of the allergen contact (Wills-Karp et al., 2001).

During sensitisation allergens are taken up by antigen presenting cells (APC), processed and presented by major histocompatibility complex (MHC) class II molecules to T cells. In this way the T_H2 cell differentiation is induced and as a result they produce B cell activating cytokines (e.g. IL-4, IL-13). Subsequently the B cells undergo immunoglobulin class-switch recombination and therefore IgE antibodies are produced. IgE binds to the IgE high affinity receptor FcεRI on mast cells and sensitises them for a reexposure at a later point of time. Memory T cells and IgE memory B cells are developed. Allergens cross-link FcεRI-bound IgE and therefore mast cells and basophils are activated. They secrete vasoactive amines (e.g. histamine), inflammatory mediators, cytokines and chemokines, which lead to immediate allergic reactions (Bischoff, 2007; Galli et al., 2008).
An allergic inflammation starts with the sensitisation by allergen exposure, an immediate phase reaction follows and in many individuals a late phase reaction succeeds. Chronic allergies can be acquired with repeated or persistent allergen contact (Galli et al., 2008).
Allergen contact mainly occurs in the respiratory tract (by inhalation), the skin or the gastrointestinal tract of the body (Kay, 2008).

1.8.1 Nasal allergy

Nasal epithelial cells play an important role in the regulation of airway inflammation, host defense and function in normal and diseased airways as they are the first line of entry for inhaled environmental factors (Proud and Leigh, 2011). In the nasal epithelium of a healthy individual there are primarily T cells, some other lymphocytes and very few mast cells and eosinophils. A lot of mast cells and many lymphocytes [T to B cell ratio of 3:1 and T helper cells ($CD4^+$) and T effector cells ($CD8^+$) in a ratio of 2-3:1] are present in the lamina propria (Dahl and Mygind, 1998; Winther et al., 1987). Additionally antigen presenting cells (APC) such as macrophages, dendritic cells (DCs) and B cells occur in the epithelium and lamina propria and are involved in innate and/or adaptive immune responses (Fokkens et al., 1990; Godthelp et al., 1996; Goldsby et al., 2003; Juliusson et al., 1991). The epithelium of the nasal mucosa of allergic individuals has many dendritic cells with granules, significantly more Langerhans cells (LCs) and numerous IgE+/CD1+ cells in contrast to nonallergic individual controls (Fokkens, 1999; Fokkens et al., 1989).
It has been suggested that antigen-presenting Langerhans cells (LCs, which are DCs) are also present in the healthy nasal mucosa and lamina propria. Therefore the mucosa is

supposed to be highly antigen recognising and initiating immune reactions towards foreign or non-self macromolecules, that are inhaled into the nose (Fokkens et al., 1989).

Epithelial cells secrete soluble cytokines and chemokines [interleukin (IL)-6, IL-8, tumor necrosis factor (TNF)-α, eotaxin (eosinophil chemotactic protein) also known as chemokine ligand (CCL)-11, regulated on activation normal T cell expressed and secreted (RANTES) also known as CCL-5, monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1, interferon gamma induced protein (IP)-10, lipid mediators (e.g. prostaglandins) or granulocyte/macrophage colony-stimulating factors (GM-CSFs) for attracting, activating and regulating immune cells and neighboring epithelial cells (Adachi et al., 1997; Brydon et al., 2003; Goldie et al., 1990; Robertson, 2002; Rollins, 1997; Rossi and Zlotnik, 2000; Sanders et al., 2011; Sim et al., 1995; Walzer and Vivier, 2011).

Furthermore, it has been shown that proallergic cytokines were detected in nasal secretions after antigen challenges (Sim et al., 1995).

The direct interaction of environmental substances and pathogens with nasal epithelial cells is important considering activating epithelial innate immune responses and the support of epithelial cells in immune regulation through their receptor and ligand expression (Fig. 7). It is thought that epithelial cells contribute to structural changes, i.e. airway remodelling. Additionally the epithelium gains more attention for being the main target of many inhaled drugs that treat inflammatory airway diseases. Certain medications (e.g. steroids) are supposed to modify the epithelial function (Müller and Jaspers, 2012; Proud and Leigh, 2011).

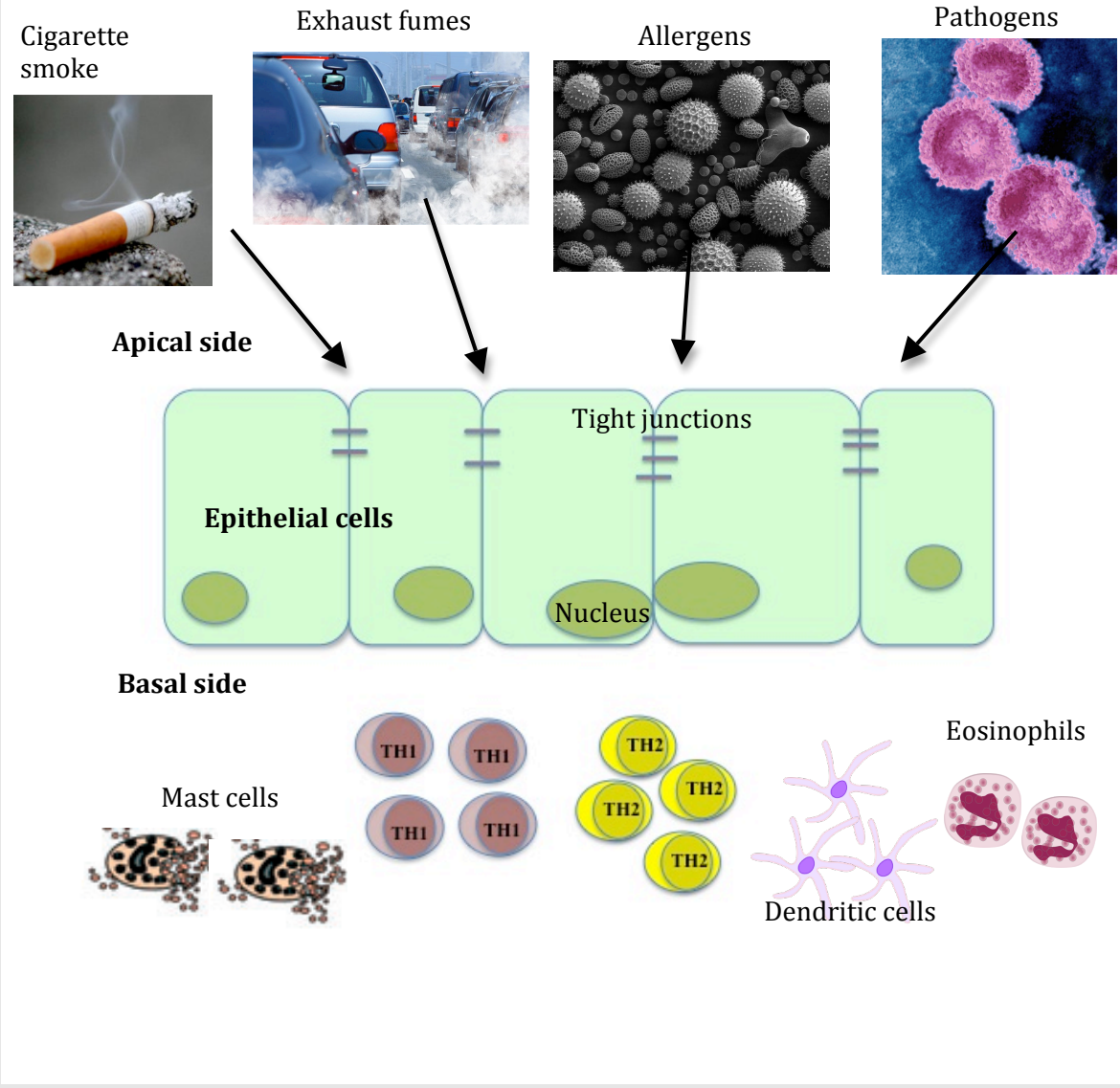


Figure 7: Interplay of nasal epithelial cells with environmental factors (allergens, pathogens and pollutants) and immune cells

[Adapted from (Holgate, 2011; Müller and Jaspers, 2012)]

1.8.2 T_H1 cells and the T_H1-derived cytokine IFN-γ

Allergic inflammation primarily develops at mucosal surfaces (Durham et al., 1997). The contact of allergic patients with allergens leads to an activation of mast cells and basophils in the submucosa within minutes. Therefore a release of inflammatory mediators, such as histamine, leukotrienes and tryptase follows (Kawakami and Galli, 2002). A late-phase inflammatory response subsequently occurs after an immediate allergic reaction. Cytokines like IL-3, IL-4, IL-5, IL-8 and TNF-α are secreted by mast cells and start the late-phase allergic response. Thus T_H2 lymphocytes, eosinophils and basophils are recruited to the site of inflammation (Durham et al., 1992; Kay et al., 1991; Robinson et al., 1993).

It is thought that mainly T_H2 cells occur in IgE-mediated allergies, but it also has been shown, that in chronic atopic diseases also allergen-specific T_H1 cells appear at the sites of repeated allergen exposure and chronic reactions (Grewe et al., 1998; Hansen et al., 1999; Yssel and Groux, 2000).

The T_H1-derived cytokine IFN-γ is secreted during chronic allergic inflammations and influences the epithelial integrity and barrier function against inhaled respiratory allergens. It has been shown, that IFN-γ, derived from allergen-specific T_H1 cell clones, severely decreases the respiratory epithelial barrier function and leads to increased transepithelial allergen penetration (Fig. 8). This discovery indicated also an increased basophil activation in the presence of IFN-γ through increased allergen contact. Therefore it is affirmed, that T_H1-mediated IFN-γ induces tissue damage and increases allergic inflammations (Reisinger et al., 2005).

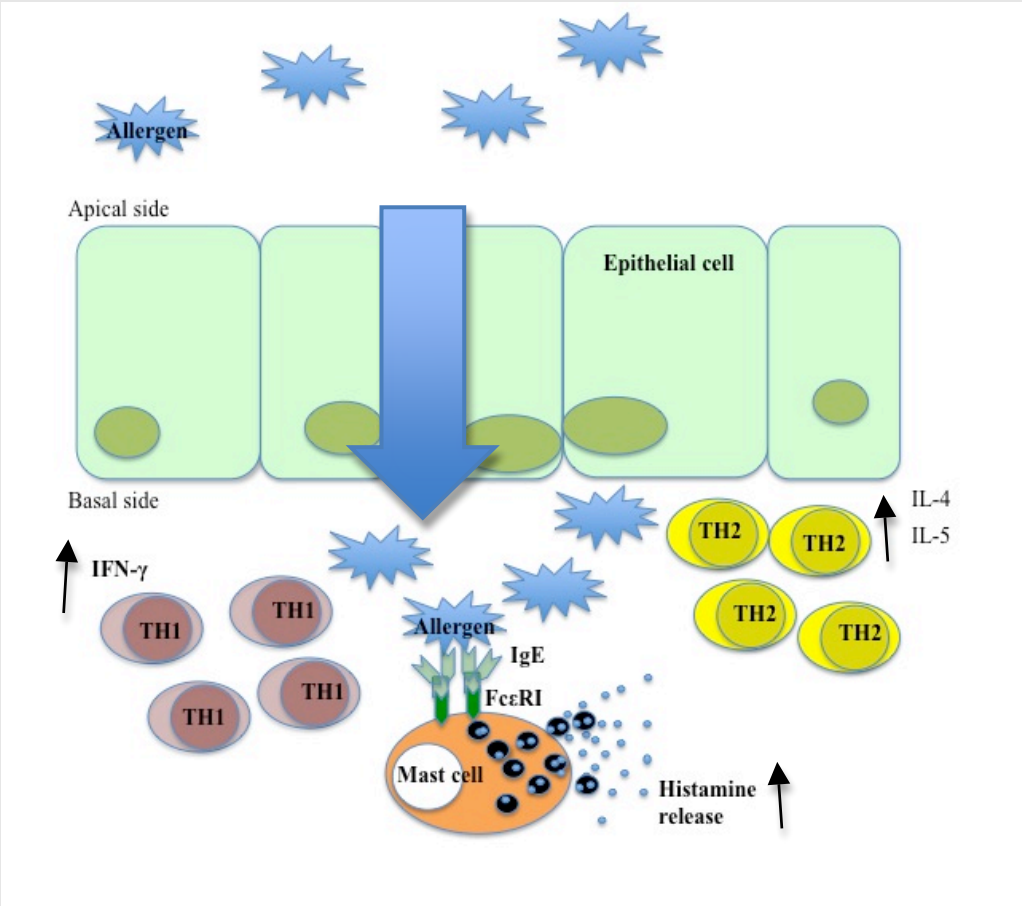


Figure 8: Impaired epithelial barrier function due to TH1-derived cytokine IFN-γ
Allergens can therefore penetrate the epithelium more easily and can increase IgE-mediated allergic reactions.
[Adapted from (Reisinger et al., 2005)]

1.9 Primary human nasal epithelial cells

The laboratory of Prof. Dr. Niederberger-Leppin has been studying the barrier function of the respiratory epithelium for several years. To that aim, a human bronchial epithelial cell line (16HBE14o-) as a surrogate for the respiratory epithelium has been employed to test the influence of various substances (e.g. cigarette smoke and IFN-γ) on the epithelial barrier function in a two-chamber model (Gangl et al., 2009; Reisinger et al., 2005). This model bears the advantage of high reproducibility and relatively easy handling, but several interesting questions, such as potential differences in epithelial properties between allergic and non-allergic subjects on the barrier function, can not sufficiently be assessed in this

system. These questions may only be answered by directly culturing cells derived from human nasal epithelium. However the culture of primary cells, in contrast to cell lines, bears several challenges. So far, few research groups worked with the primary nasal epithelium, thus information on culture conditions are rare and variable.

The nasal epithelium is a highly-organised ciliated epithelium and culture conditions need to be optimised to ensure that it does not lose these properties during weeks of culturing *in vitro*. Furthermore, the nose is one of the first places of encounter with antigens, thus potential infections of primary cultures with bacteria or fungi derived from the human samples need to be prevented. The work with primary cells is more time consuming than with cell lines as these cells need to be cultured for several weeks before they can be used for experiments. The interindividual differences between patients of different age, gender or suffering from different diseases need to be borne in mind and thus a larger number of samples and experiments are required to get consistent results. However, these challenges are outweighed by the great advantage of being able to investigate and compare the properties of the nasal epithelium in allergic and non-allergic patients and to discover important differences.

2 Materials and Methods

2.1 Description of patients

With consent from the Ethics Committee of the Medical University of Vienna (EK Nr. 1476/2013) human nasal tissue samples from patients undergoing routine nasal surgery at the ENT Department of the General Hospital of Vienna were obtained. All patients gave written informed consent to donate their tissue if it was not needed for diagnostic histological investigations. Male or female individuals aged between 18– 65 years were included in the study and the tissue samples were anonymised and were consecutively numbered.

Only tissue, which was obtained routinely during surgery and would otherwise have been discarded, was used for this study. Human nasal mucosa was only removed if indicated for medical reasons and not specifically for this study. The samples were stored in physiologic saline solution (0.9 % sodium chloride) until the operation was completed and were then immediately transferred to the laboratory.

2.2 Cell culture method of primary human nasal epithelial cells

Nasal mucosa samples were transferred on ice to the laboratory and were immediately processed for tissue culture. The following protocol was adapted and modified from (Coste et al., 2000; Fulcher et al., 2005).

To prevent infections of primary cultures, nasal tissue samples were first washed with 30 ml MEM medium (Gibco, USA) including dithiothreitol (DTT) (0.5 mg/ml, Sigma, USA), desoxyribonuclease (DNase) (10 µg/ml, Sigma) and antibiotics [100 U/ml penicillin, 100mg/ml streptomycin and 0,1g/ml, 2.5 µg/ml amphotericin B (Sigma), 50µg/ml gentamicin (Gibco)]. The nasal tissue sample was soaked in medium for 5 min in a Petri dish and this step was repeated. Afterwards the sample was washed three times in MEM medium containing the named antibiotics. All steps were carried out under a laminar flow hood. Next the nasal mucosa was transferred in to 15 ml tubes containing 8 ml MEM medium with antibiotics plus 1 ml protease (1%, Sigma)/ desoxyribonuclease (0.01 %) mix.

Single cells were obtained by dissociating the tissue sample 24 hours placed on a platform rocker at 4°C with 50-60 cycles per min. The following day the tissue sample was poured in a Petri dish and 10% v/v fetal bovine serum (FBS) (Gibco) was added. The epithelial surface was scraped with a convex surgical scalpel to harvest primary epithelial cells. The nasal mucosa sample was rinsed with cell culture PBS. The solution containing the scraped cells was transferred into a 50 ml Falcon tube. The tissue sample was discarded and the cell solution was centrifuged with 1000 rounds per minute (rpm) for 5 minutes. Cells were resuspended and were treated with MEM medium including a 0.25 % trypsin/EDTA mixture (10 ml) for 3 minutes. The mixture was diluted 1:1 with bronchial epithithelial growth medium (BEGM) BulletKit medium (Lonza, USA). Cell solution was centrifuged again with 1000 rpm for 5 min. Cells were resuspended in bronchial BEGM medium (4°C).

T25 cell culture flasks were coated for 2 hours at 37°C with 1:25 dilution of collagen type I from calf skin (Sigma) in distilled autoclaved water (dH_2O). Aliquots of 1.5 ml of the collagen solution were used per T25 flask. After 2 hours of incubation the remaining coating solution in the flasks were aspirated and the flasks were exposed to Ultraviolet (UV) light in a laminar flow hood for 30 minutes. T25 flasks can be stored afterwards for up to 6 weeks at 4°C. 6-well plates were coated the same way for the scratch test.

Cells (4×10^6) were cultured in the type I collagen coated flasks with 10 ml BEGM medium per T25 flask at 37°C and 5% CO_2. BEGM contained specific hormones [bovine pituitary extract (BPE), epinephrine, hydrocortisone (HC), insulin, triiodothyronine)] and growth factors [epidermal growth factor (EGF), retinoic acid (RA), transferrin]. Twenty-four hours afterwards the medium was changed to eliminate dead and not culture flask-attached cells. Medium was subsequently changed every 2-3 days. Cells reached confluence after approximately 7 days of incubation. Primary human nasal epithelial cells were then once washed with prewarmed cell-culture PBS and were then incubated with 1.5 ml Accutase (Gibco) per T25 flask for 10-15 min. at 37°C to detach the cell culture flask attached epithelial cells. Cells were transferred in 4°C BEGM medium (10 ml) and were spun down with 1000 rpm for 5 min. Afterwards cells were passaged again in type I collagen coated T25 flasks. Two to three days later the cells were confluent again.

Permeable transwell supports (Costar, Corning, USA) were coated with collagen type IV from human placenta (Sigma) 1:10 diluted with dH_2O. Inserts in the 12-well plates were coated with 150 µl collagen solution per well and were dried open overnight at 21°C in a

laminar flow hood. The next day the transwell supports were exposed to UV light for 30 min. in a laminar flow hood.

As a second passage cells were transferred in collagen type IV coated porous supports in a transwell system. This system was chosen to establish an air liquid interface (ALI) mimicking the physiological environment of the nasal epithelium. The second passage was also transferred in collagen type I coated 6-well-plates for the scratch test.

For the comparison of different cultering media DMEM/F12 medium (Lonza) with 10 % FBS and LHC-9 medium (Gibco) were used.

2.3 Transwell system

The second passage of the human nasal epithelial cells were transferred in collagen type IV coated porous supports of a transwell system. Cells (2 x 10^5) per 12-well insert were cultured with BEGM medium with all supplements (1.5 ml in the lower well and 0.5 ml in the upper well). The following 2-3 days after passaging the cells were confluent and the BEGM medium was removed from the upper well to establish an air liquid interface mimicking the physiological environment of the nasal epithelium. The BEGM medium in the lower well was removed and was replaced by BEGM and DMEM (Gibco) (1:1) medium with all supplements including 100 mM retinoic acid (Sigma) according to (Soyka et al., 2012). Medium was changed every 2-3 days.

The transwell system allowed transepithelial resistance (TER) measurement and penetration experiments. Resistance measurement using an Ohm-Volt meter (Merck Millipore – Millicell ERS-2, Germany) was carried out at periodical intervals. For this purpose, 0.5 ml BEGM and DMEM (1:1) medium without retinoic acid was added to the upper well to measure TER with the Ohm-Volt meter. Two days after reaching the maximum TER human IFN-γ (50 ng/ml) (eBioscience, USA) was added and hydrocortisone was removed from the supplemented BEGM and DMEM (1:1) medium.

To determine the influence of IFN-γ on epithelial resistance, the substance was added to the lower well of the transwell system. As the medium was changed every 2-3 days, IFN-γ was added also in this intervals. TER was measured in 24-hour intervals.

2.4 Scratch test

To measure cell growth, a scratch test was established. Cells (3 x 10^5) per well in 3 ml BEGM were transferred in type I collagen coated 6-well plates. Two to three days afterwards the cells were confluent. Twenty-four hours before scratching the cells hydrocortisone was removed from the BEGM medium with supplements and IFN-γ (50 ng/ml) was added to the wells. A scratch (cross) was performed using a 1 ml pipette tip after cells were grown as a monolayer (about 2-3 days of incubation). By scratching the cell monolayer some cells were detached and were removed by changing the medium.
The scratch was marked at three positions and thus it was possible to take photographs at the exact same positions at different points of time using a light microscope (4x objective) with phase contrast after performing the scratch. Medium was changed every 2-3 days.

Scratch tests of primary human nasal epithelial cells of samples NM-6, NM-8 and NM-9 were performed and the same marked positions were photographed every 24 hours. Untreated and IFN-γ (50 ng/ml) -treated cells were compared and the scratch surface areas were calculated with the Nikon Imaging Software (NIS) and after calibration of the scale. IFN-γ was supplemented 24 hours before performing the scratch in the according wells. Additionally hydrocortisone was excluded from the supplemented BEGM Lonza medium 24 hours before scratching the epithelial cell monolayer (Soyka et al., 2012).
BEGM Lonza medium with supplements was changed every 24 hours for eliminating dead cells and feeding the cells. Therefore IFN-γ (50 ng/ml) was added as well every 24 hours in the according wells. Optimal points of time for measurement of the scratch test were established by comparing the photographs taken in 24 hour intervals.

2.5 Flow cytometry

For flow cytometry, the second passage of the primary human nasal epithelial cells or the human bronchial epithelial cell line (16HBE14o-) cultured in type I collagen coated T25 flasks were used. Cells were prepared as described in section 2.2 and were used for staining with fluorescently labelled monoclonal antibodies. Primary human nasal epithelial cells were stained for pan-Cytokeratin (e488, eBioscience, USA) and E-cadherin (e660, eBioscience) to identify epithelial cells (van Meegen et al., 2011) or the respective isotype

controls mouse IgG_1 488 (eBioscience) and rat IgG_1 660 (eBioscience). Additionally cells were stained for CD45 PE (eBioscience) or the respective isotype control mouse IgG_1 PE (eBioscience) to determine possible contamination of the cultured cells with haematopoietic cells.

In detail, cells (4×10^5) per U-bottom well were blocked with 50µl/well PBS with 2 % w/v BSA (flow cytometry buffer) containing 10 % v/v mouse serum at 4°C for 20 minutes. Next 50 µl/well flow cytometry buffer were added and cells were spun down with 1000 rpm for 5 minutes. Cells were resuspended in 50 µl/well flow cytometry buffer and were stained with anti-pan-Cytokeratin and anti-CD45 or the istopye controls IgG_1 488 and IgG_1 PE were added. Viability dye e780 (eBioscience) was added to every well. Cells were stained in the dark at 4°C for 40 minutes. Afterwards 50 µl/well flow cytometry buffer was added again and cells were spun down with 1000 rpm for 5 minutes.

For the intracellular staining with anti-E-Cadherin cells were resuspended with 100 µl/well fixation permeabilisation solution (BD) and were permeabilised for 20 minutes at 4°C. Cells were washed with 100 µl/well flow cytometry buffer containing 0.1 % w/v Saponin. Cells were centrifuged with 1000 rpm for 5 minutes and were resuspended in 50 µl/well flow cytometry buffer containing 0.1 % Saponin and 10 % v/v rat serum. Cells were blocked for 20 min. at 4°C. Fifty µl/well flow cytometry buffer with 0.1 % Saponin was added and cells were centrifuged again with 1000 rpm for 5 minutes. Anti-E-Cadherin or IgG_1 660 was added to the cells and cells were stained at 4°C for 40 minutes. Fifty µl/well flow cytometry buffer was added and cells were spun down with 1000 rpm for 5 minutes.

For flow cytometry cells were washed with 100 µl/well flow cytometry buffer. Cells were resuspended in 300 µl/well flow cytometry buffer and were measured with Beckman coulter flow cytometer. A minimum of 10 000 cells was counted. For compensations universal CompBeads (eBioscience) were used. CompBeads were vortexed and 1 drop was added in 100 µl flow cytometry buffer. One µl of the respective antibodies was added and beads were stained for 20 minutes at room temperature. For washing 900 µl flow cytometry buffer was added and spun down with 2500 rpm for 5 minutes. Beads were taken up in 500 µl flow cytometry buffer and were also measured with the flow cytometer (BD FACS Canto II). Stainings were performed in triplicates. Samples were analysed

using FlowJo Software (Version 7.2.5, Tree Star, USA) and dead cells were excluded using the viability dye e780 (eBioscience).

2.6 Immunohistology

2.6.1 Frozen samples

Primary human nasal epithelial cells grown on membranes were embedded in TissueTek, snap frozen in liquid nitrogen and kept at -80°C. Then membranes were cryo-sectioned (4 µM sections), transferred onto slides and fixed with 4% paraformaldehyde for 15 minutes at room temperature. Cells were incubated with anti-JAM-A antibody (eBioscience) and were diluted in PBS (Lonza) containing 0,1% TritonX-100 (Bio-Rad) for permeabilisation. At first the antibody was incubated for one hour at room temperature and afterwards cells were washed twice with PBS containing 0.05% v/v Tween 20 (Bio-Rad). As a second step the antibody was Alexa Fluor 488-labelled with goat anti-mouse IgG (Invitrogen) and was diluted in PBS. The fluorescence-labelled antibody was incubated for 1 hour at room temperature followed by two washing steps with PBST. The actin cytoskeleton was visualised with direct labelled phalloidin-TRITC (Sigma). Nuclei were stained with DAPI (4',6-diamidino-2-phenylindole, Sigma) in a concentration of 1µg/ml.
Membranes were analysed with a confocal laser scanning microscope LSM780 (Carl Zeiss) using the 63x lens with oil immersion (numeric aperture 1.4).

2.6.2 Paraffin samples

Sections of 4 µm were obtained from paraffin-embedded membranes, were transferred on slides and were deparaffinised in xylene for 15 minutes. After clearing in ethanol, sections were rehydrated in distilled water and an H&E staining was performed, which included one minute of staining with hematoxylin (Merck) and a washing step. Next membranes were stained for one minute with eosin (Merck), which was followed by intensive washing in distilled water. Samples were mounted in Aquatex (aqueous mounting agent, Merck Millipore, Germany) and imaging was performed using an Axio Imager (Carl Zeiss).

3 Results and Discussion

3.1 Cell culture of primary human nasal epithelial cells

3.1.1 Harvesting of cells

After scraping a nasal mucosa tissue sample (average sample size around 1 cm^2 and an average weight of about 0.6 g) with a scalpel, an average cell number of 3 x 10^7 cells was obtained per sample (Table 1). Nasal mucosa samples NM-1 to -4 were not weighed.

Table 1: Obtained cell number after scraping human nasal mucosa tissue samples

Sample	Weight (g)	Cell number after scraping
NM-1	n.d.	8 x 10^5
NM-2	n.d.	4,8 x 10^7
NM-3	n.d.	1,5 x 10^7
NM-4	n.d.	6 x 10^7
NM-5	0,51	1,6 x 10^7
NM-6	0,74	3,6 x 10^7
NM-7	0,12	2 x 10^6
NM-8	0,57	3,8 x 10^7
NM-9	1,07	6,8 x 10^7

n.d.: not determined

The size of nasal mucosa samples were in direct correlation with the obtained cell number. The weight corresponded with the cell number. However, the nasal mucosa samples accumulated from routine surgeries get to the laboratory one time with a piece of turbinate bone and the other time without, the weight depended on the amount of bone as well.

3.1.2 Comparison of different culture media
– Identification of BEGM as optimal culture medium

In the first experiments the primary human nasal epithelial cells were cultured in DMEM/Ham's F12 medium containing antibiotics (penicillin, streptomycin and gentamicin) as well as fungicide (amphotericin B) to prevent infections and either supplemented with or without 10% v/v fetal bovine serum (FBS). The cells did not gain confluence when they were cultured without FBS as a growth supplement. However, with FBS cells gained confluence. Optimal growth was achieved when 4 x 10^6 cells were seeded in type I collagen-coated T25

flasks. For resistance measurements the primary human nasal epithelial cells were transferred directly after scraping in transwell inserts as described by (Coste et al., 2000), but experiments using cells that were first grown in T25 flasks to confluence before transferring them onto transwell supports (Fulcher et al., 2005), displayed a more consistent and tighter cell growth. After about 2 weeks of incubation, the cells were confluent and were passaged from type I collagen-coated T25 flasks again in type I collagen-coated flasks. Following this step the cells were confluent again 2-3 days after passaging.
However, primary human nasal epithelial cells did not look well-defined and gained low and rapidly decreasing transepithelial resistance values (up to 600 Ohm). Furthermore we aimed to work with a serum-free cell culture system for the primary cells as serum contains many unknown und undefined substances that may influence the result. Therefore, different media with growth factors and hormones were evaluated.

It has been reported that bronchial epithelial cell growth medium (BEGM) containing specific hormones [bovine pituitary extract (BPE), epinephrine, hydrocortisone (HC), insulin, triiodothyronine)] and growth factors [epidermal growth factor (EGF), retinoic acid (RA), transferrin] show optimal conditions for growing, viability and morphology of primary human nasal epithelial cells and their passages (Mattinger et al., 2002).
LHC-9 medium of Gibco and BEGM medium with single quot kit supplements of Lonza are specific human bronchial epithelial cell media including the above mentioned hormones and growth factors. For this reason these media were compared with the previously used DMEM/F12 10% FBS medium conducting the experiments with 4 x 10^6 seeded cells per type I collagen-coated T25 flasks. Figure 9 shows passage 0 cells of sample NM-6 cultured for 7 days with different media. DMEM/F12 10 % FBS, LHC-9 and BEGM media were compared with each other.
In comparison to the previously used culturing medium DMEM/F12 with 10 % FBS (Fig. 9A) the primary human nasal epithelial cells had a more defined appearance when cultured with LHC-9 medium (Fig. 9B). However, cells cultured with BEGM medium led to higher cell growth and well-defined cells (Fig. 9C).
The primary human nasal epithelial cells reached confluence already after 7 days of incubation with the BEGM medium, around 50% of confluence grown with LHC-9 medium and around 40% of confluence cultured with DMEM/F12 10% FBS medium.
Because of this result BEGM medium was judged as the most suitable medium and is the only growth medium used for further experiments. BEGM medium delivered by far the best results

regarding confluence, definition of the cells and also for time-saving reasons as cells have to be cultured for several weeks.

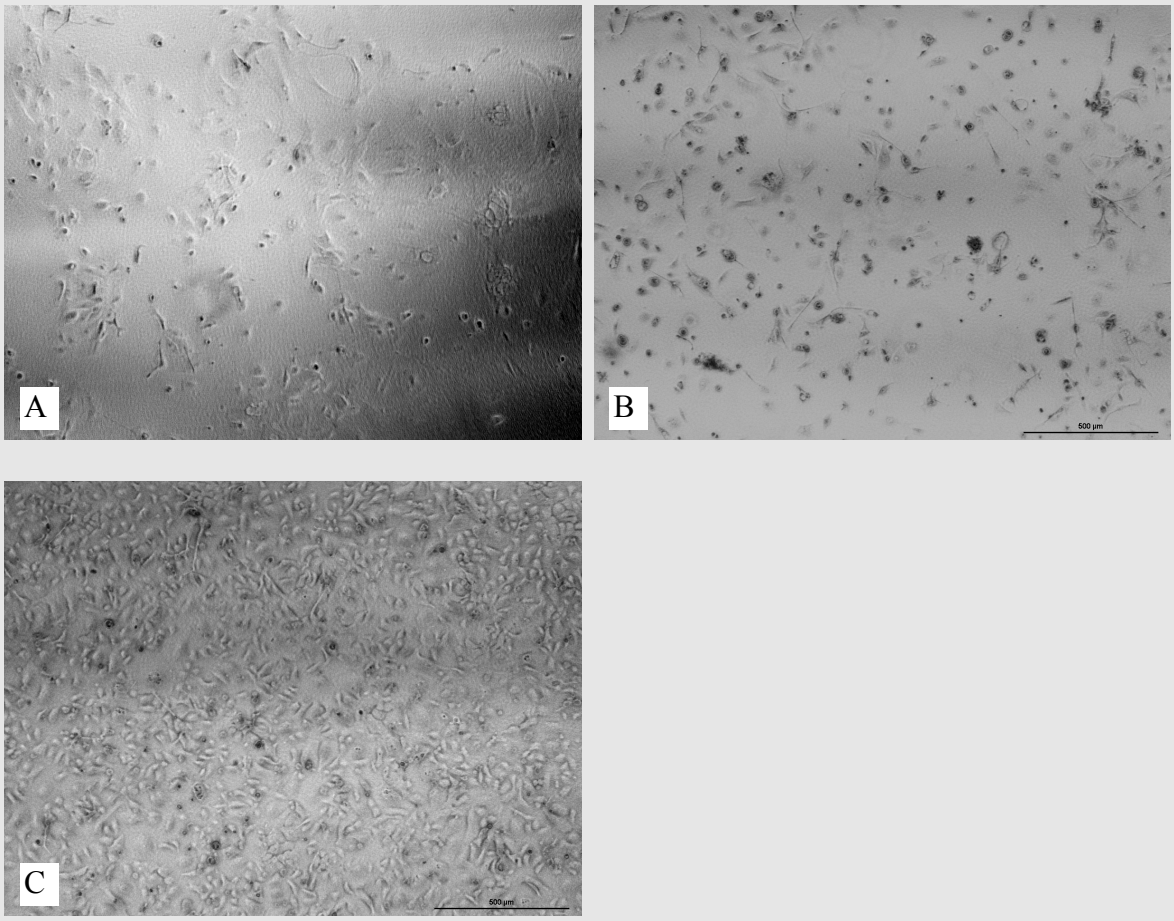


Figure 9: Cells of sample NM-6 and identification of BEGM as optimal culture medium
Cells of passage 0 were grown with DMEM/F12 10 % FBS (A), LHC-9 (B) and BEGM (C) medium for 7 days.

Cells of passage 0 grown with BEGM medium (Fig. 9C) were transferred in collagen-coated flasks and continued to be cultured with BEGM as passage 1 (Fig. 10). These cells were confluent 2 days after passaging and appeared well defined.

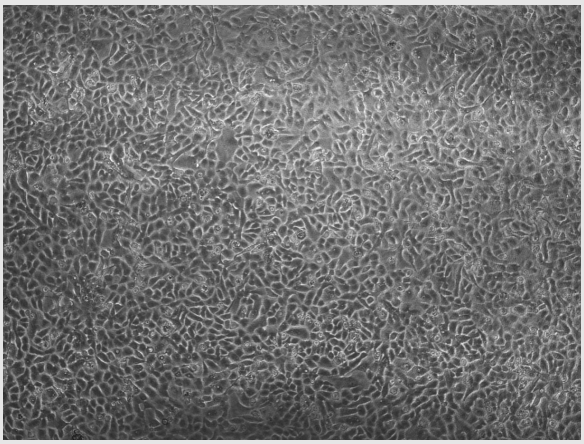


Figure 10: Passage 1 cells of sample NM-6
Photograph shows passage 1 of primary human nasal epithelial cells grown with BEGM medium for 2 days.

3.1.3 Patient samples NM-6, NM-8 and NM-9

In the following chapter experiments with three nasal mucosa samples (NM-6, NM-8 and NM-9) are demonstrated. Sample NM-6 was obtained from a 45-year-old male patient, which underwent a septoplasty and turbinoplasty surgery. A cell number of 3,6 x 10^7 was obtained from the 0.74 g weighing sample NM-6 (Table 1). Patient of sample NM-8 was male, 40 years old and was also operated for septoplasty and turbinoplasty. The sample weighed 0.57 g and 3.8 x 10^7 cells were obtained (Table 1). 35-year-old female patient underwent a turbinoplasty and 6,8 x 10^7 cells were obtained from her nasal mucosa sample NM-9 of 1.07 g (Table 1). All three patients were non-allergic.

Samples NM-1 to NM-5 were only cultured in DMEM/F12 10% FBS medium. Therefore these samples were not included in further experiments. As the comparison of the different media was done for the first time with sample NM-6, only NM-8 and NM-9 were also cultured in BEGM medium. The primary human nasal epithelial cells of these three samples were included in further experiments.

3.1.4 Discussion of cell culture establishment

After 7 days of incubation, cells of sample NM-6 showed different growing effects with DMEM/F12 10 % FBS, LHC-9 and BEGM media. Cells cultured with BEGM reached confluence already after 7 days of incubation while cells grown with DMEM/F12 10 % FBS or LHC-9 were not confluent yet (Fig. 9). In Table 2 the contents and growing effects of the tested media are listed.

Table 2: Contents of different cell growth media

Medium	**DMEM/F12**	**LHC-9**	**BEGM**
Producer	Lonza	Gibco	Lonza
Supplements	10 % FBS Penicillin Streptomycin Gentamicin Amphotericin B	 Penicillin Streptomycin Gentamicin Amphotericin B	 Penicillin Streptomycin Gentamicin Amphotericin B
Hormones contained in medium		BPE Epinephrine HC Insulin Triiodothyronine EGF RA Transferrin	BPE Epinephrine HC Insulin Triiodothyronine EGF RA Transferrin
Effects	- Cells not defined - Not confluent after 7 days of culturing	- Defined cell appearance - Not fluently confluent after 7 days of culturing	- Well-defined cells - Confluent after 7 days of culturing - time-saving

The hormones (BPE, epinephrine, HC, insulin and triiodothyronine) and growth factors (EGF, RA, transferrin) were added freshly to the BEGM medium as they were delivered as separate single quots of the producer Lonza. As opposed to this the LHC-9 medium contains already

the same hormones and growth factors. This might be the reason that BEGM worked better and cultured cells were more defined and were reaching confluence earlier than LHC-9 cultured cells. Therefore, BEGM medium was used for further experiments although it is more expensive than the LHC-9 medium. The concentrations of the supplements in the media are not known as the producers do not quote these information and did not provide these information when requested. DMEM/F12 10 % FBS medium contains neither hormones nor growth factors, which appear to be important for the growth of primary human nasal epithelial cells.

3.2 Two-chamber transwell system

3.2.1 Optimisation of culture conditions in the transwell system

At first we cultured cells with DMEM/F12 medium containing 2% v/v Ultroser G (PALL Life Science, USA) as a serum substitute in the outer well and DMEM/F12 10% FBS medium in the insert as described by (Coste et al., 2000). However, as mentioned in chapter 3.1.2, when these culture conditions were used, primary nasal epithelial cells did not look well-defined and did only gain low transepithelial resistance. To overcome these problems, different culture conditions and passage times were compared to establish the best culture conditions for our primary cells.

After comparing cells from different passages we found that the best results were achieved when culturing passage 2 (transferred from type I collagen-coated T25 flasks) in type IV collagen-coated porous 12 mm-inserts (2 x10^5 cells/well) of the transwell system with BEGM and DMEM (1:1) medium with supplements. Cells were again confluent 2-3 days after passaging. These culture conditions were therefore used for the experiments described below.

3.2.2 Transepithelial resistance and influence of IFN-γ in the transwell system

3.2.2.1 TER values of samples NM-6, NM-8 and NM-9

Cells of NM-6 built up resistance up to 2150 Ω/cm^2 after 8 days of incubation in transwell inserts with air liquid interface culturing method. Cells of sample NM-8 showed highest TER

values after 6 days (1133 Ω/cm^2) and sample NM-9 after 10 days (4100 Ω/cm^2) culturing them the same way as sample NM-6.
Thereafter TER values were slowly decreasing when left untreated. After two days of TER decrease IFN-γ was added to part of the wells while other cells were left unexposed as control. In Figure 11 and in Table 3 TER measurements of samples NM-6, NM-8 and NM-9 are displayed.

It has previously been shown that the T_H1-derived cytokine IFN-γ is able to disturb the protective function of epithelial cells by opening of tight junctions (Reisinger et al., 2005). It is important for us to establish reproducible ways to disturb the barrier function of the epithelium as it is planned for a later stage of this project to search for factors which are able to protect the respiratory mucosa from damage or to support repair mechanisms. Therefore, the effect of IFN-γ on our cell layers was investigated.
For this purpose 50 ng/ml IFN-γ was added to the basolateral side of epithelial cell layers in the transwell system as it was previously described by (Reisinger et al., 2005). Treatment with IFN-γ led to a reduction in TER already after 24 hours. This result was reproducible in all three patient samples and was observed in duplicate wells. IFN-γ-treated cells were exposed to IFN-γ for 120 hours. Medium control cultures and wells with medium without cells were used as a control and were also measured in duplicates.

TER values of NM-6 did not show substantial changes in untreated cells and ranged between TER values of 1917 Ω/cm^2 to 1567 Ω/cm^2 during the whole observation period. Untreated cells of samples NM-8 and NM-9 delivered similar constant results during the same period of measurement.
By contrast the IFN-γ-treated cells of sample NM-6 showed a TER decrease of almost 50 % after 24 hours of incubation (1850 Ω/cm^2 to 1017 Ω/cm^2) and were almost as low as medium control after 48 hours. Sample NM-8 showed almost the same result as NM-6. Sample NM-9 demonstrated a decrease of almost 50 % after 48 hours and TER values were similar to medium control after 120 hours.
Numbers of the TER values of each of the three patient samples are shown in Table 3 and are demonstrated graphically in Figure 11.

Table 3: TER average values of samples NM-6 (A), NM-8 (B) and NM-9 (C)

Hours	Untreated cells (Ω/cm^2)	IFN- γ-treated cells (Ω/cm^2)	Medium control (Ω/cm^2)
0	1667	1850	500
24	1917	1017	417
48	1617	583	450
72	1700	717	467
96	1567	533	467
120	1617	467	400

A

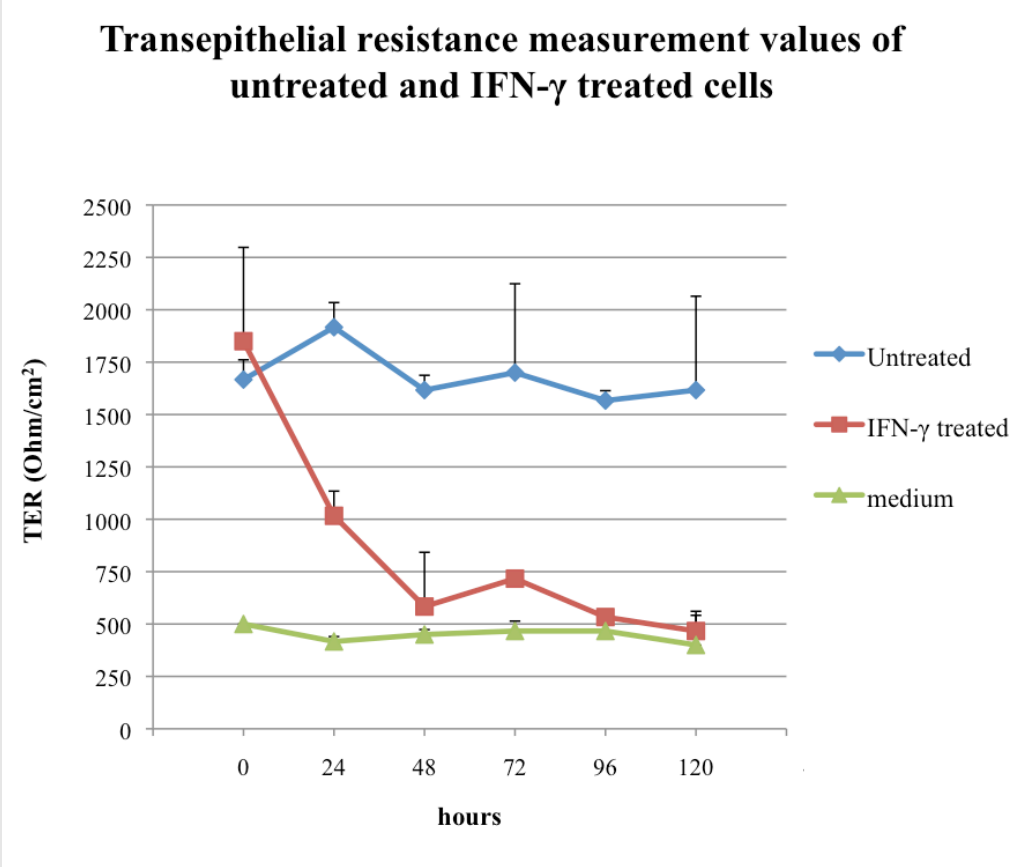
Hours	Untreated cells (Ω/cm^2)	IFN-γ-treated cells (Ω/cm^2)	Medium control (Ω/cm^2)
0	917	950	517
24	833	567	517
48	967	600	500
72	917	583	517
96	1050	533	517
120	1000	475	533

B

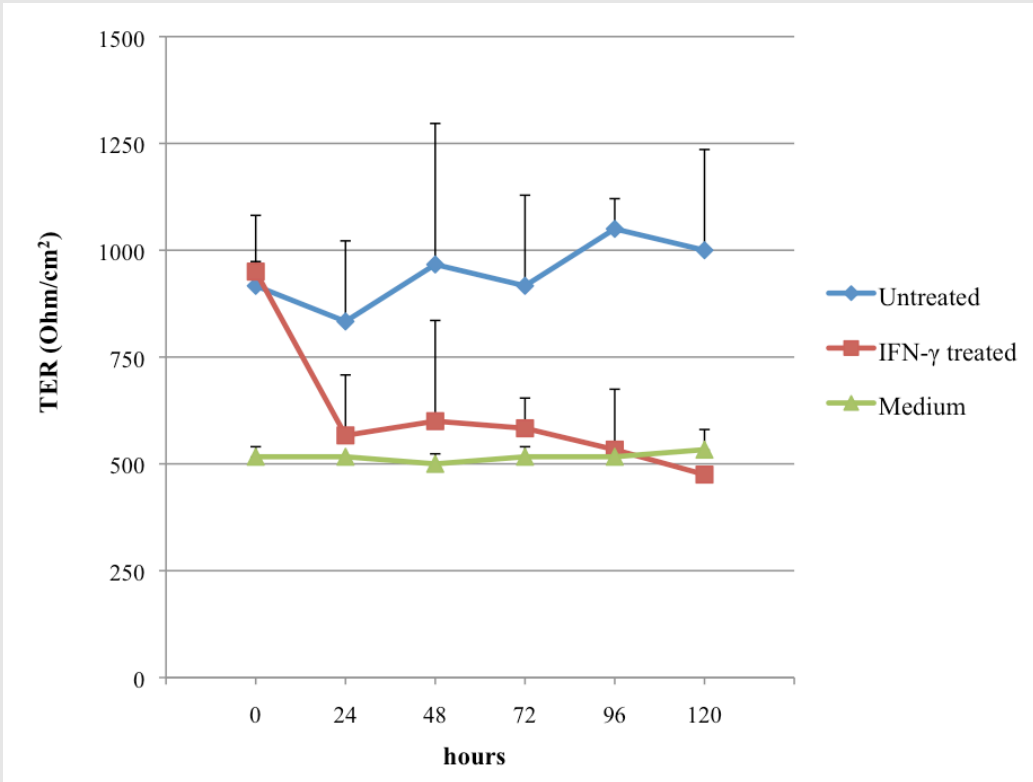
Hours	Untreated cells (Ω/cm^2)	IFN-γ-treated cells (Ω/cm^2)	Medium control (Ω/cm^2)
0	3033	2833	417
24	3417	2650	433
48	2883	1983	433
72	3233	1083	367
96	2683	833	400
120	2300	617	400

C

A


Transepithelial resistance measurement values of untreated and IFN-γ treated cells
TER (Ohm/cm²)
2500
2250
2000
1750
1500
1250
1000
750
500
250
0
0
24
48
72
96
120
hours
Untreated
IFN-γ treated
medium

B


TER (Ohm/cm²)
1500
1250
1000
750
500
250
0
0
24
48
72
96
120
hours
Untreated
IFN-γ treated
Medium

C

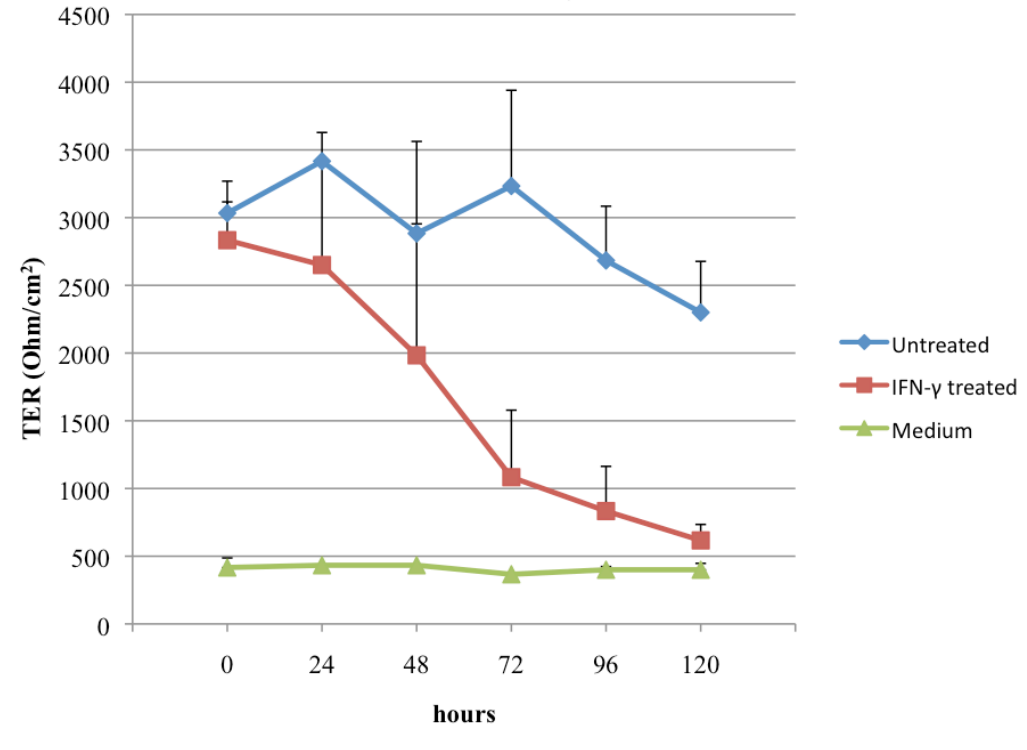


Figure 11: TER average values of samples NM-6 (A), NM-8 (B) and NM-9 (C)

IFN-γ induced a time-dependent decrease of TER in primary human nasal epithelial cells. IFN-γ was added to monolayers of primary human nasal epithelial cells. TER (y-axe) was measured 24, 48, 72, 96 and 120 hours (x-axe) after incubation of primary epithelial cell monolayers with medium with or without 50 ng/ml IFN-γ. TER of untreated cells are shown in the blue lines, of cells treated with 50 ng/ml IFN-γ in the red lines and of wells with medium control without cells in the green line. The change of average TER values is shown for triplicate determinations and standard deviations are displayed.

3.2.2.2 Images of samples NM-6, NM-8 and NM-9

After 96h of IFN-γ treatment primary human nasal epithelial cells of all three patients looked very disrupted, which corresponded to the results of the TER measurements (see red lines in Fig. 11) and photographs B, D and F (Fig. 12). Untreated cells were well-defined and confluent (Fig. 12 A, C and E) and as appropriate their TER values were still high after 96 hours of incubation (see blue lines in Fig. 11).

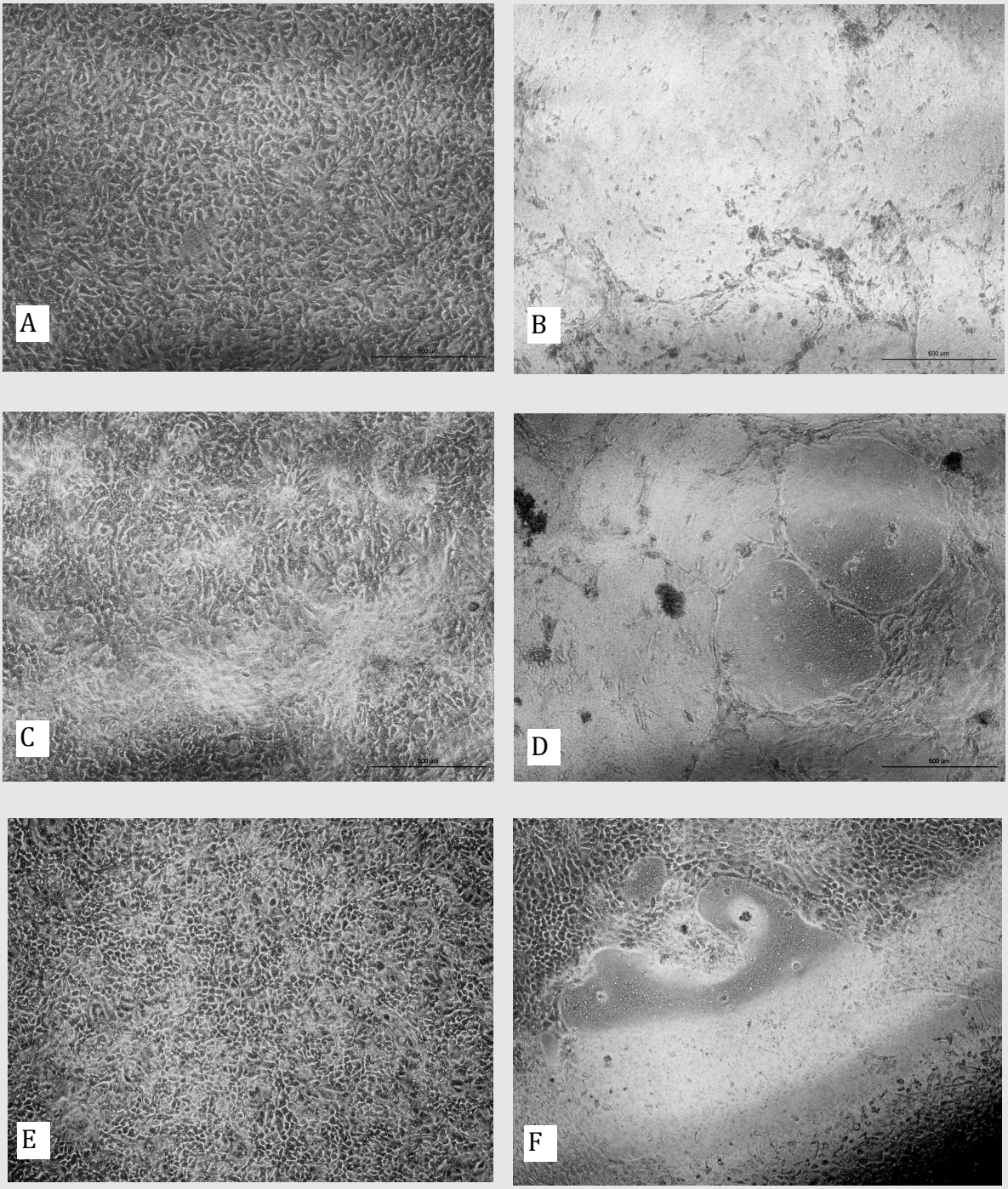


Figure 12: Untreated and IFN-γ-treated cells of samples NM-6, NM-8 and NM-9

Primary human nasal epithelial cell cultures of three patient samples were photographed after 96 hours of incubation with air-liquid interface cultured in the transwell system. Photographs A and B demonstrate sample NM-6, C and D sample NM-8 and D and F sample NM-9. Images A, C and E show untreated cells and images B, D and F display IFN-γ-treated cells.

3.2.3 Discussion of the transwell system and transepithelial resistance

Our results demonstrate that cultured primary human nasal epithelial cells showed a significant decrease of transepithelial resistance when treated with IFN-γ (Table 3 and Fig. 11 and 12). This finding is along the same line as results obtained with cell lines (Reisinger et al., 2005) and a recent publication (Soyka et al., 2012), which investigated the effect of IFN-γ in primary human nasal epithelial cells.

In cultures of all three individuals a decrease of TER upon IFN-γ treatment was observed. Lower initial TER values showed a quicker response than higher starting TER values. It is likely that cells are more vulnerable when they show lower TER and are not as intact as cells having higher TER values.

Therefore the initial TER values were associated with the decreasing effect of IFN-γ treatment. The most substantial effect of IFN-γ on TER was seen in sample NM-8 as its TER values after 24 hours exposure to IFN-γ (567 Ω/cm^2) were almost at the same level as the medium control value (517 Ω/cm^2). Although its starting TER value was only 950 Ω/cm^2. This result corresponds to a complete breakdown of the barrier function already after 24 hours of incubation with IFN-γ.

The slowest effect of IFN-γ treatment was seen in sample NM-9 as its starting value was 2833 Ω/cm^2 and its TER value after 24 hours of IFN-γ exposure was 2650 Ω/cm^2. In all results the direct correlation of the TER initial values with the decreasing effect of IFN-γ was affirmed. The TER values of all three patient samples tended towards the medium control values after IFN-γ treatment after 120 hours of IFN-γ exposure.

Untreated cells of sample NM-9 looked even more defined (Fig. 12E) than the untreated cells of sample NM-6 (Fig. 12A). This result was also matching with higher TER values of sample NM-9 than the TER values of sample NM-6 after 96 hours of incubation.

Untreated cells of sample NM-8 did not look as well-defined (Fig. 12C) as the untreated cells of sample NM-6 (Fig. 12A), but were also confluent. Accordingly the TER values of NM-8 are not as high as the TER values of NM-6 after 96 hours of incubation.

In our experiments we did not evaluate the minimum dose of IFN-γ to open the tight junctions and to lead to a decrease of TER. However, we noticed that the susceptibility of cells of different individuals varied. Further experiments are planned in which we will compare different doses of IFN-γ.

The results of these experiments showed that high TER values and reproducible TER measurements can be observed from primary epithelial cell cultures of three persons. IFN-γ reduced the barrier function of primary human nasal epithelial cells by decreasing TER values of all tested cell samples. Cell disruptions might be due to apoptosis in cells and weakening of intercellular tight junctions. Because of the disruption of the cells, the penetration of allergens may be facilitated and therefore can lead to immediate allergic inflammation (Fig. 8). As future projects it would be interesting to evaluate differences between allergic and nonallergic individuals regarding TER values and IFN-γ damage distinctions. Additionally it may be identified and used for drug development. Furthermore factors required for building up intact cell layers can be analysed by eliminating certain components from the culture medium.

3.3 Scratch test of primary human nasal epithelial cells

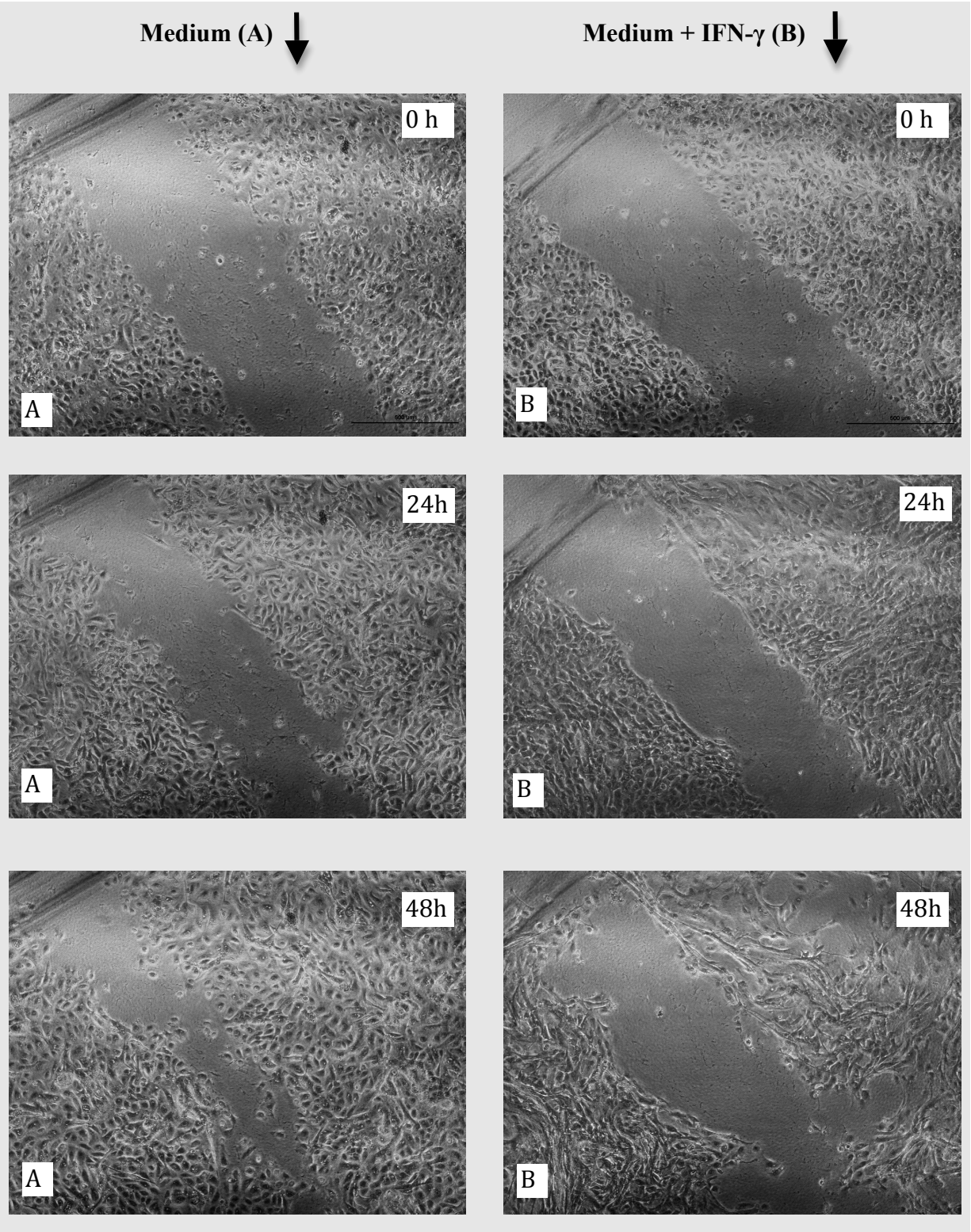
The nasal epithelium can be damaged by various factors. When the epithelium is wounded the barrier function or structural integrity can be lost and therefore the normal epithelial repair processes are important. The remaining epithelial cells migrate and repopulate the damaged area as a quick process by proliferating and differentiating until epithelial integrity has been reconstituted (Rennard, 1998). It is suggested that cells near the injury autonomously respond by repairing the wound faster than cells further away from the wound (Erjefalt et al., 1995). Repair processes are difficult to study *in vivo* and therefore it is worthwhile to work with the primary human nasal epithelial cells applying the scratch test method *in vitro*.

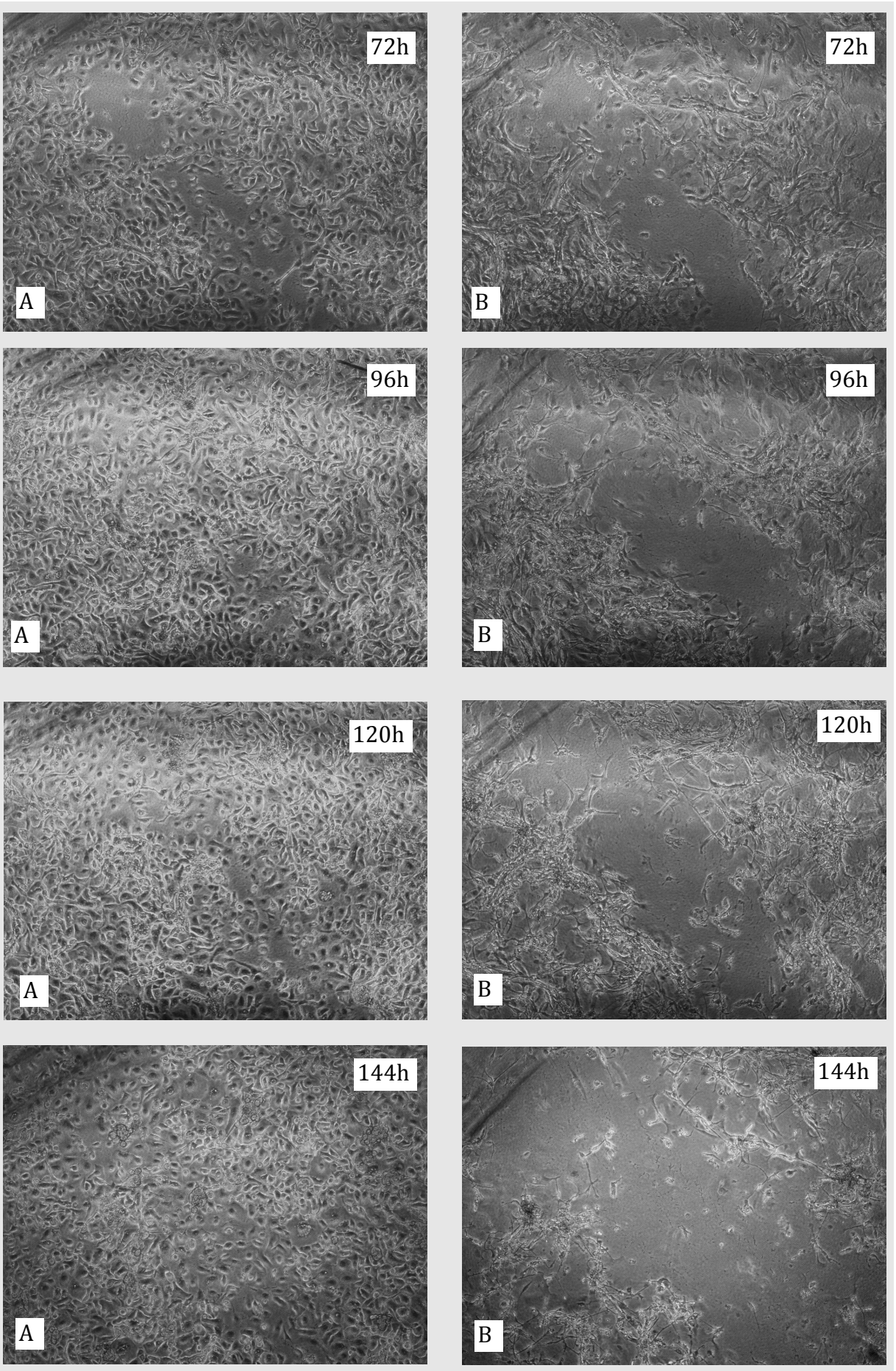
We found that in our experiments primary human nasal epithelial cells formed a confluent monolayer, when 3 x 10^5 cells were transferred as a second passage in type I collagen coated 6-wells and cultured with BEGM medium.

The *in vitro* scratch test method involves performing damage by "scratching" the cell monolayer, marking three positions per well and taking photographs of these positions in 24 hour intervals during cell migration closing of the scratch. By this method images can be compared to quantify the migration rate of the cells.

We were able to show reproducible and similar results of the scratch tests in samples NM-6, NM-8 and NM-9 regarding the damage repair of untreated cell monolayers and the increase of damage of IFN-γ-treated cell monolayers. Therefore photographs taken in 24 hour intervals of sample NM-6 are all displayed in the following chapter and only photographs of three significant points of times were shown of samples NM-8 and NM-9.

3.3.1 Images of scratch test of samples NM-6, NM-8 and NM-9




72h
A
72h
B
96h
A
96h
B
120h
A
120h
B
144h
A
144h
B

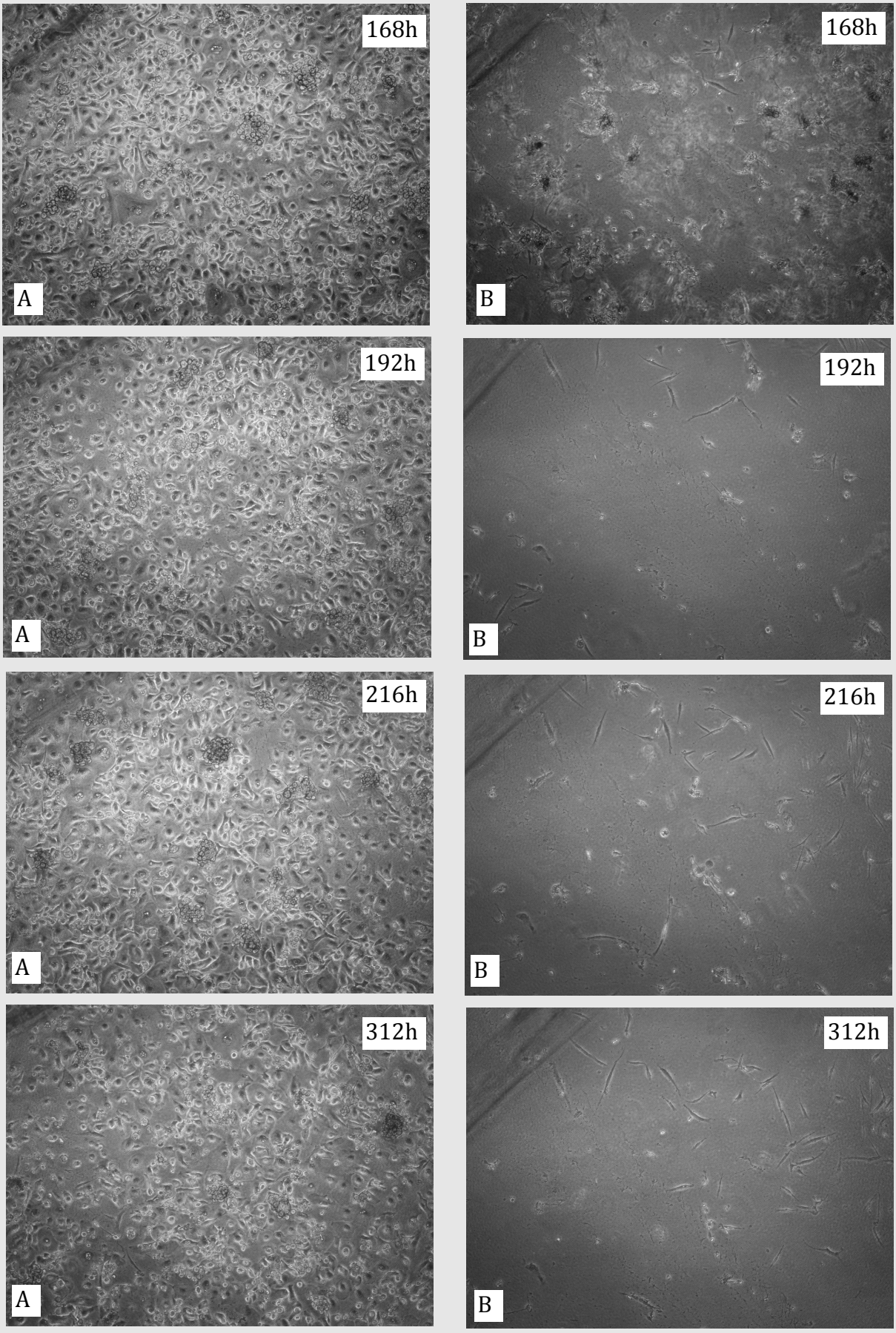


Figure 13: Untreated (A) and IFN-γ-treated (B) primary human nasal epithelial cells of sample NM-6 photographed in 24 hour intervals after scratch damage

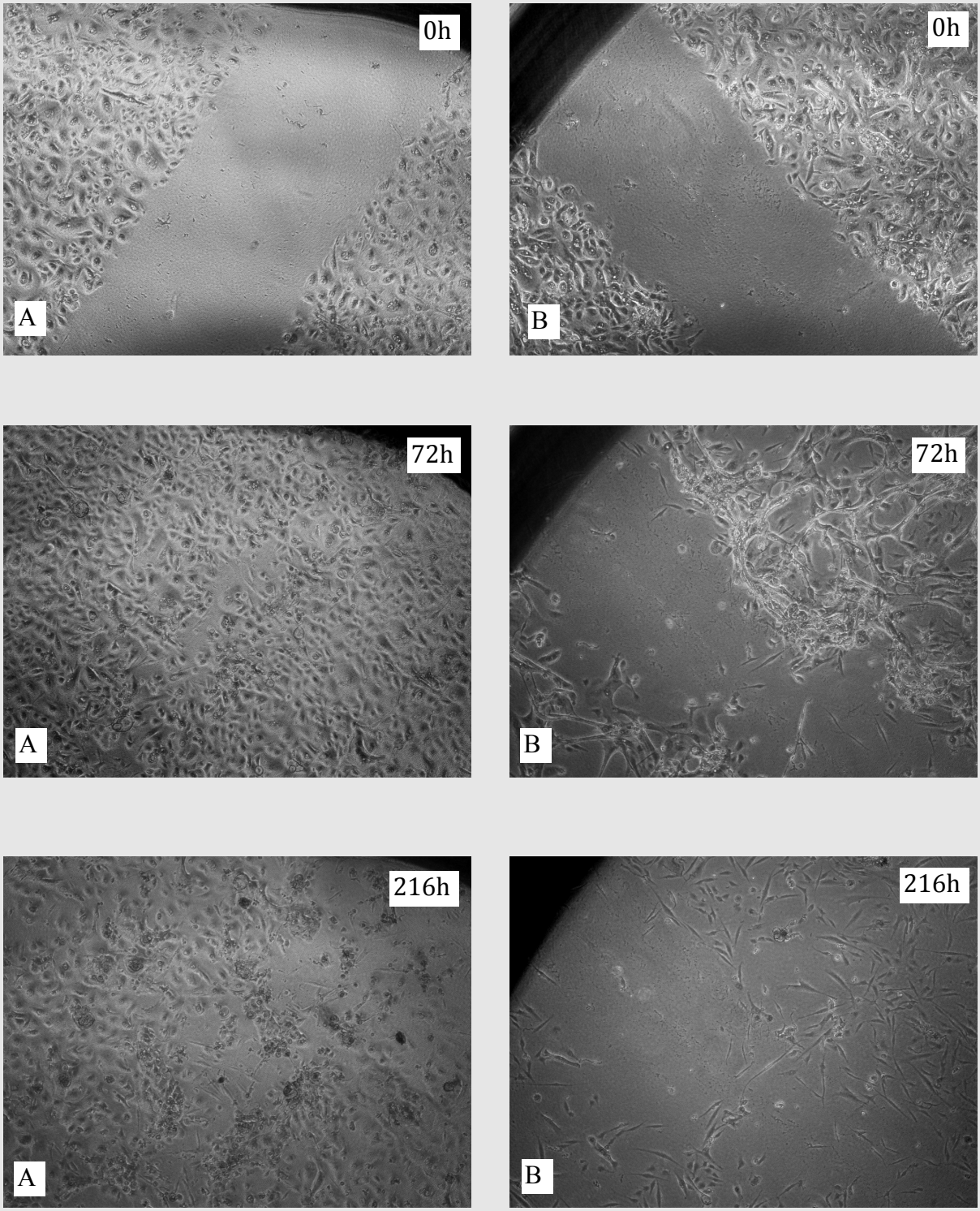


Figure 14: Scratch of untreated cells (A) and IFN-γ-treated cells (B) of sample NM-8 immediately, 72 and 216 hours (9 days) after performing damage

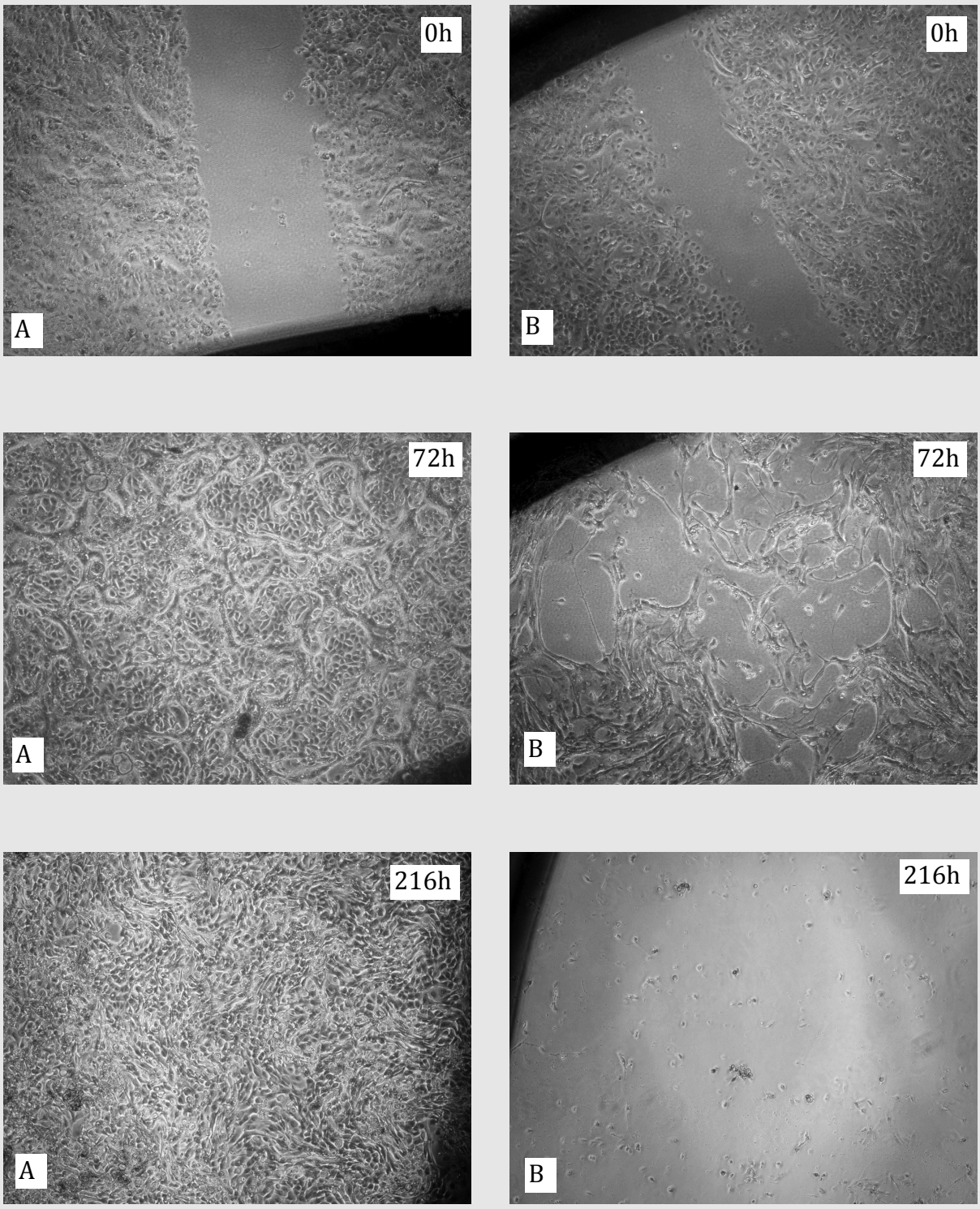


Figure 15: Untreated cells (A) and IFN-γ-treated cells (B) of sample NM-9 immediately, 72 and 216 hours after performing the scratch

3.3.2 Surface area of scratch of samples NM-6, NM-8 and NM-9

Hours of incubation	Untreated cells (mm^2)	IFN-γ-treated cells (mm^2)
0	1206	1179
24	897	946
48	652	680
72	351	633
96	177	606
120	129	718
144	143	1491
168	132	2414
192	143	3445
216	116	3447
240	127	3447
312	188	3447

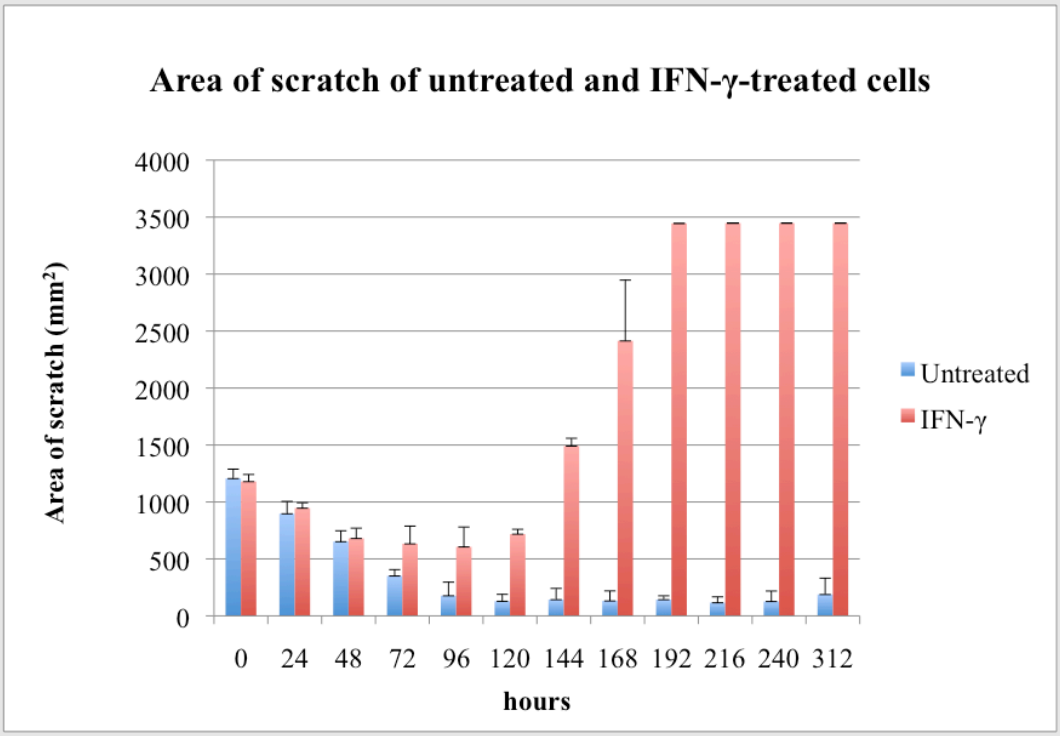


Table 4 and Figure 16: Scratch area values of sample NM-6

IFN-γ induced a time-dependent increase of the scratch surface areas. IFN-γ was added to monolayers of primary human nasal epithelial cells in 6-well plates. Photographs were taken 0, 24, 48, 72, 96, 120, 144 and 216 hours (x-axis) after incubation of primary epithelial cell monolayers with BEGM and DMEM (1:1) medium or 50 ng/ml IFN-γ supplemented medium. The change of average scratch surface area values is shown in duplicate determinations and standard deviations are displayed.

Hours	Untreated cells (mm^2)	IFN-γ-treated cells (mm^2)
0	1455	1385
24	652	1123
48	392	1161
72	567	1256
96	376	1352
120	550	1256
144	487	1990
216	703	1994

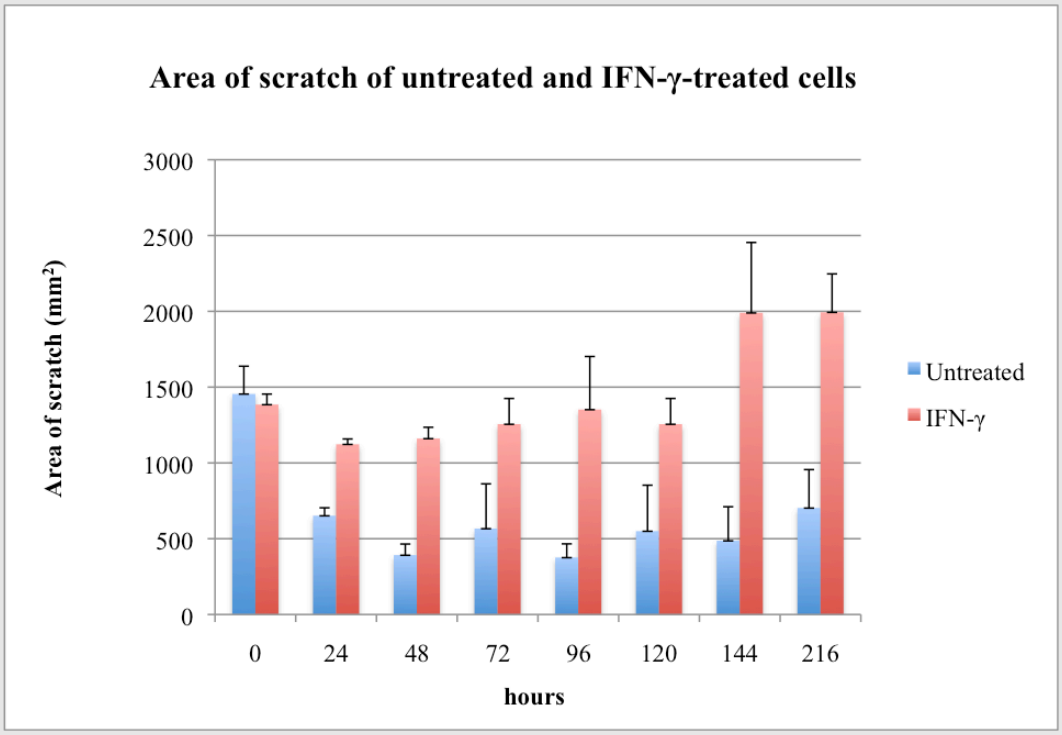


Table 5 and Figure 17: Scratch area values of sample NM-8

Hours	Untreated cells (mm^2)	IFN-γ-treated cells (mm^2)
0	767	756
24	108	499
48	13	536
72	0	785
96	0	1527
120	0	1280
144	0	3375
216	0	3375

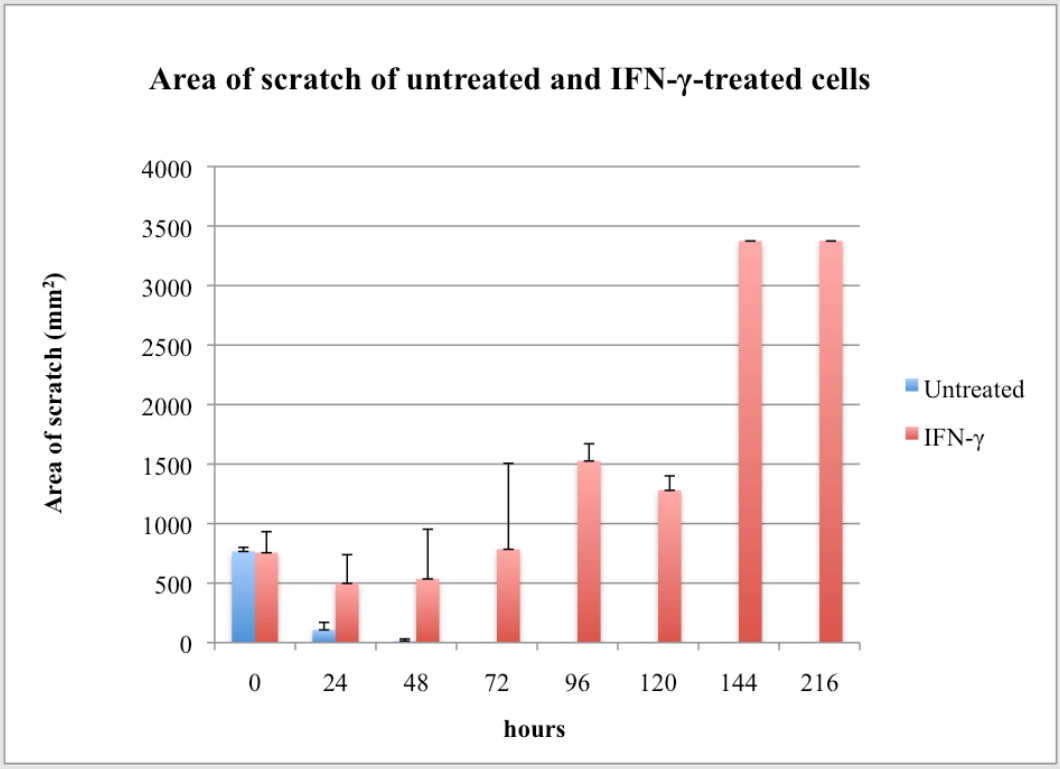


Table 6 and Figure 18: Average scratch area values of sample NM-9

3.3.3 Discussion of scratch test results

Scratch surfaces of untreated cells were growing in all samples almost together and therefore a satisfying *in vitro* repair of the epithelial cell monolayers occured.
In all three samples IFN-γ led to a severe increase of the damage areas. According to results obtained for cell lines, this result may be due to weakening of tight junctions by the soluble cytokine IFN-γ.
The results of the scratch tests corresponded well with the results of transepithelial resistance measurements with IFN-γ treatment. TER decreased and scratch areas increased by the influence of IFN-γ. Samples with high TER resulted in higher cell growth rates. For example cells of sample NM-9 showed the highest TER values (Fig. 11C and Table 3C) of all three nasal mucosa cell samples and had also the fastest repair of damage areas (Fig. 15 and 18) of all three tested individuals.

The area of damage can be clearly seen in all photographs of untreated (A) and IFN-γ-treated (B) cells immediately after performing the scratch (0h images of Fig. 13, 14 and 15). In Tables 4, 5 and 6 it is shown, that the area of scratch had approximately the same size immediately after scratching the cell monolayers. The same areas of damage of the cultures are displayed in Fig. 16, 17 and 18 and showed that the untreated cells already minimised the scratch to around 50 % of the scratch surface after 24 hours of incubation. Compared to samples NM-6 and NM-8 the scratch of the untreated cells of sample NM-9 grew already almost together after 24 hours of incubation.
As opposed to this the IFN-γ-treated cells did not reduce the scratch surfaces as well as the untreated cells did, but they minimised the scratch slightly after 24 hours. It is noticeable that the IFN-γ-treated cells did already look disrupted after 24 hours of incubation, which might be due to weakening of tight junctions.

After 48 hours of incubation open scars of the scratch surface areas were still seen. After 72 to 96 hours of incubation the damage area of the untreated cells had almost recovered from damage in all cell samples and confluent cell monolayers were almost regained. The damage areas of the IFN-γ-treated cells were still wide open and the cells did not look well defined anymore as the untreated cells did after 72 hours of incubation (Fig. 13, 14 and 15).
After 144 hours of treatment with IFN-γ the damage areas increased up to 50 % and subsequently the scratches opened continuously as the treated cells detached and were eliminated by changing the medium every 24 hours.

Interestingly untreated cells overlaid each other after approximately 120 hours of incubation and started to detach slightly from the 6-well plate. This was especially visible in sample NM-8 in Fig. 14A after 216 hours of incubation and was also visible in sample NM-6 in Fig. 13A after 216 and 312 hours of incubation. The scratches started to open again. This result might also be due to the liquid-liquid culturing method in the 6-well plates. Primary human nasal epithelial cells could be cultured up to 6 weeks in the transwell system with air-liquid interface and were detaching from 6-well plates and T25 culture flasks already after 2 or 3 weeks of incubation.

3.4 Characterisation of cultured cells by flow cytometry

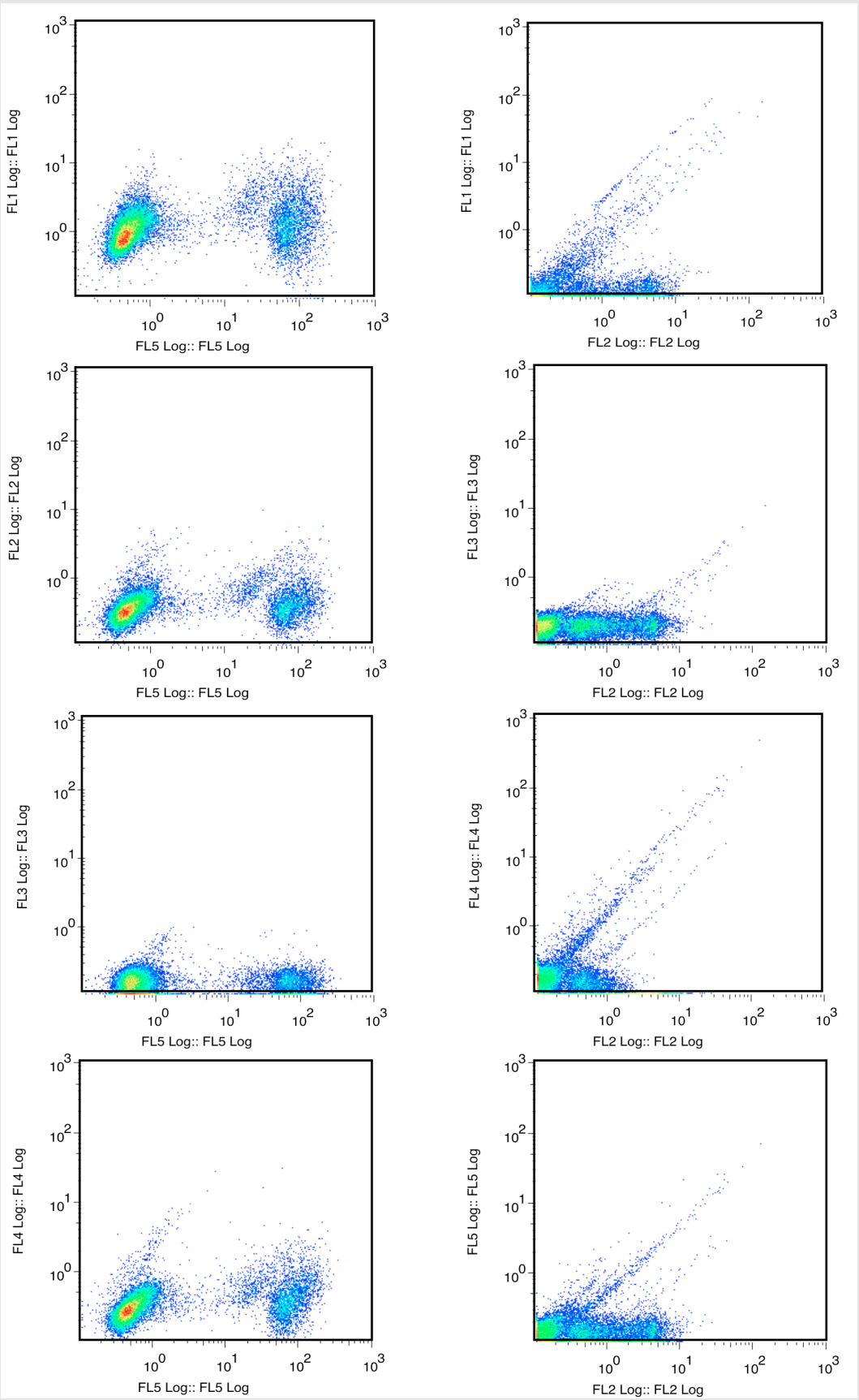
Fluorescently labelled monoclonal antibodies were employed to determine which cell types are present in the cells cultured with the specific bronchial epithelial cell growth medium (BEGM) with single quot supplements of Lonza. To this aim cells were detached from type I collagen coated T25 flasks as a second passage as used for the TER and scratch test experiments and were stained with antibodies for flow cytometry.

Primary human nasal epithelial cells were stained for pan-Cytokeratin (e488) and E-cadherin (e660) to identify epithelial cells (van Meegen et al., 2011). Pan-Cytokeratin recognises many of the cytokeratin family members, which are intermediate filament proteins of the cytoskeleton expressed in epithelial cells. E-cadherin is a transmembrane glycoprotein, which is important for intercellular adhesion and interacts with catenins and actinins. Catenins connect E-cadherin with the cytoskeleton. E-cadherin is situated at epithelial intercellular boundaries of many tissues and are considered to be important for maintaining tissue integrity. Cells were additionally stained for CD45 (PE) to determine if any haematopoietic cells were present in the cultured cells.

As a control a human bronchial epithelial cell line (16HBE14o-) was stained with the same three antibodies.

3.4.1 Results of flow cytometry

In Figure 19 it is shown that all channels [FL-1 (e488; Pan-Cytokeratin), FL-2 (PE; CD45), FL-4 (e660, E-cadherin), FL-5 (life/death; E780)] were compensated properly before starting to analyse the cells by flow cytometry.


FL1 Log:: FL1 Log
FL5 Log:: FL5 Log
FL1 Log:: FL1 Log
FL2 Log:: FL2 Log
FL2 Log:: FL2 Log
FL5 Log:: FL5 Log
FL3 Log:: FL3 Log
FL2 Log:: FL2 Log
FL3 Log:: FL3 Log
FL5 Log:: FL5 Log
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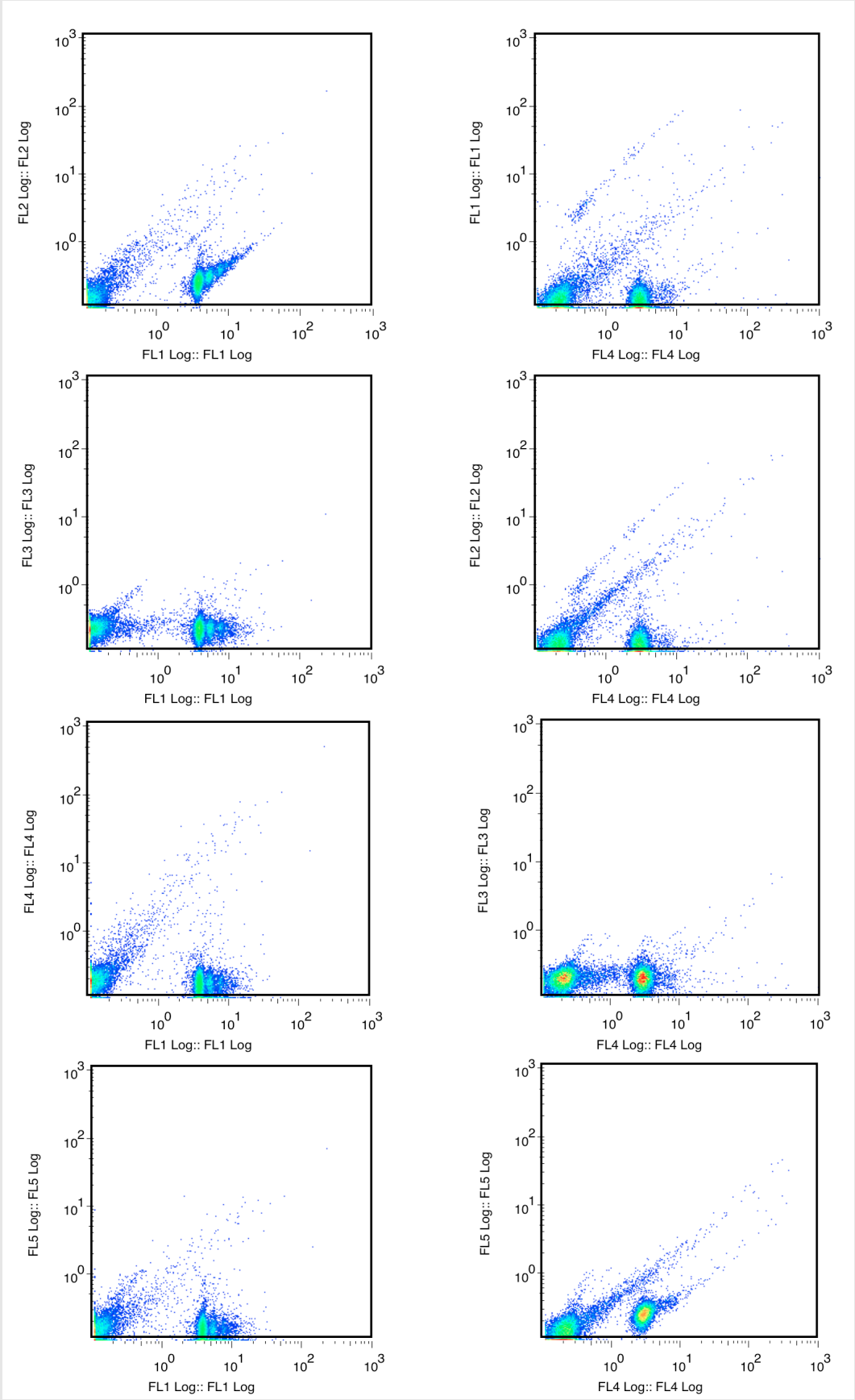

Figure 19: Compensations of all channels

All channels were compensated

Primary human nasal epithelial cells of sample NM-9 were stained in triplicates. Compared to isotype control primary epithelial cells were highly pan-Cytokeratin positive (Fig. 20).

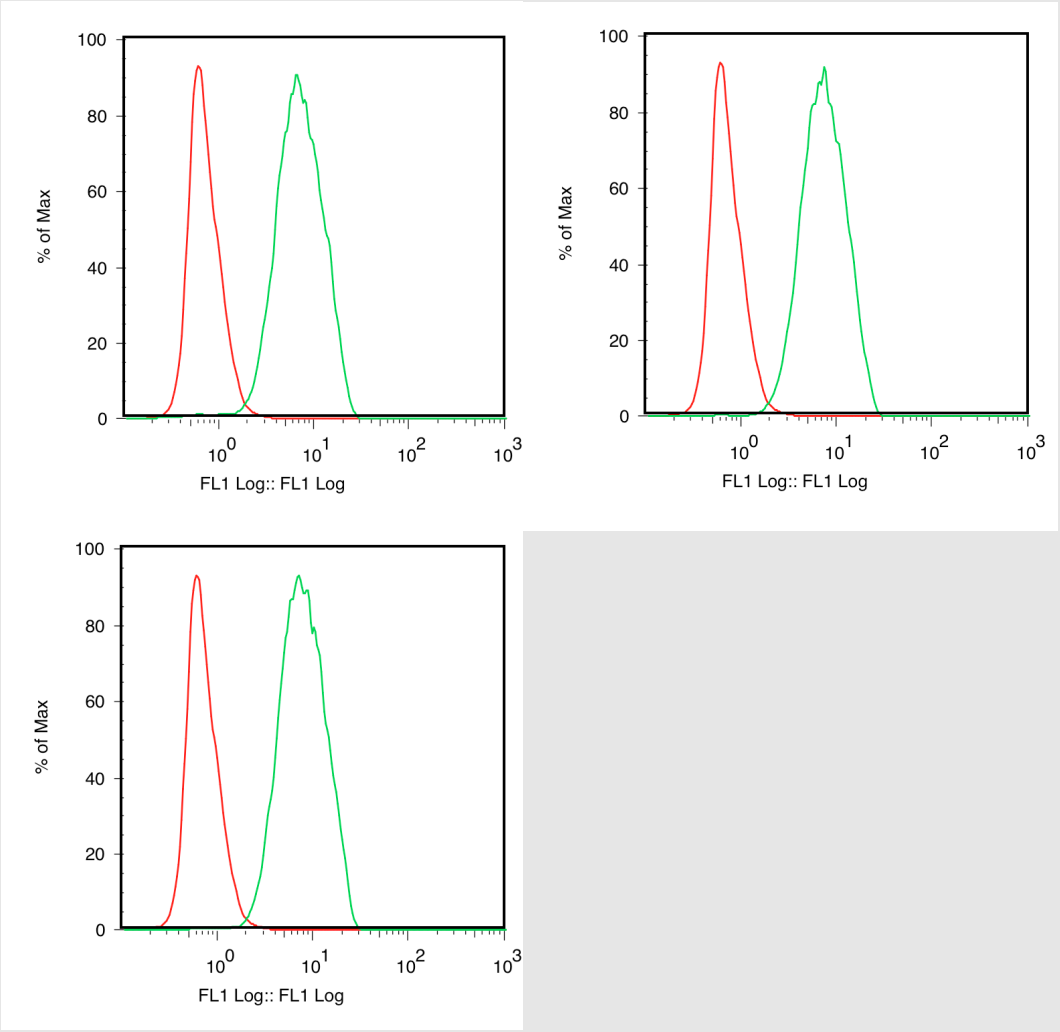

Figure 20: Triplicate histograms showing staining for pan-Cytokeratin (e488, in green) of primary human nasal epithelial cells compared to the isotype control (IgG_1 488, in red) by flow cytometry

Primary cells of NM-9 were only slightly positive stained with anti-E-Cadherin in comparison to the isotype control (Fig. 21). Cells were again stained in triplicates.

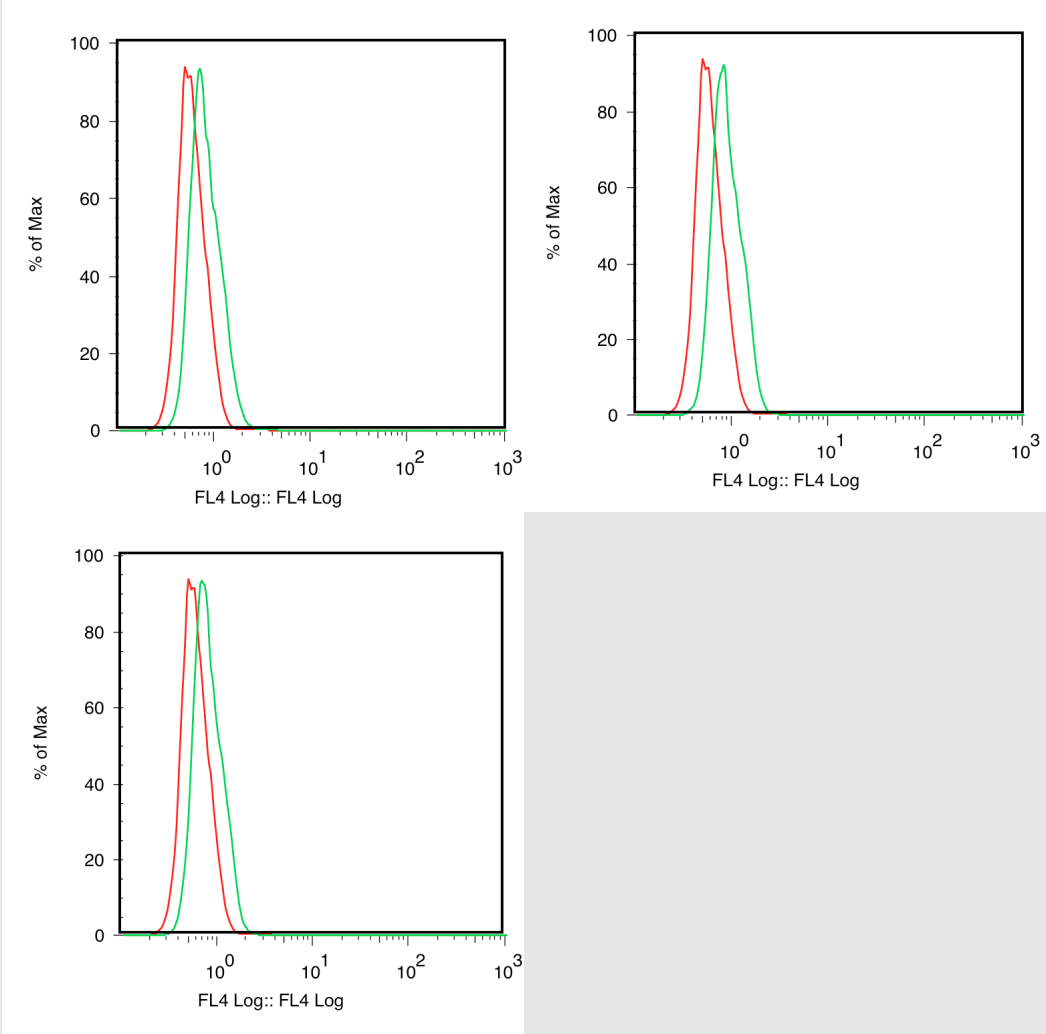

Figure 21: Triplicate histograms showing E-cadherin staining intensity of primary human nasal epithelial cells (e660, in green) compared to isotype control (IgG_1 660, in red)

Human bronchial epithelial cell line (16HBE14o-) was also stained in triplicates. Compared to isotype control the cells of the epithelial cell line were Pan-Cytokeratin positive (Fig. 22).

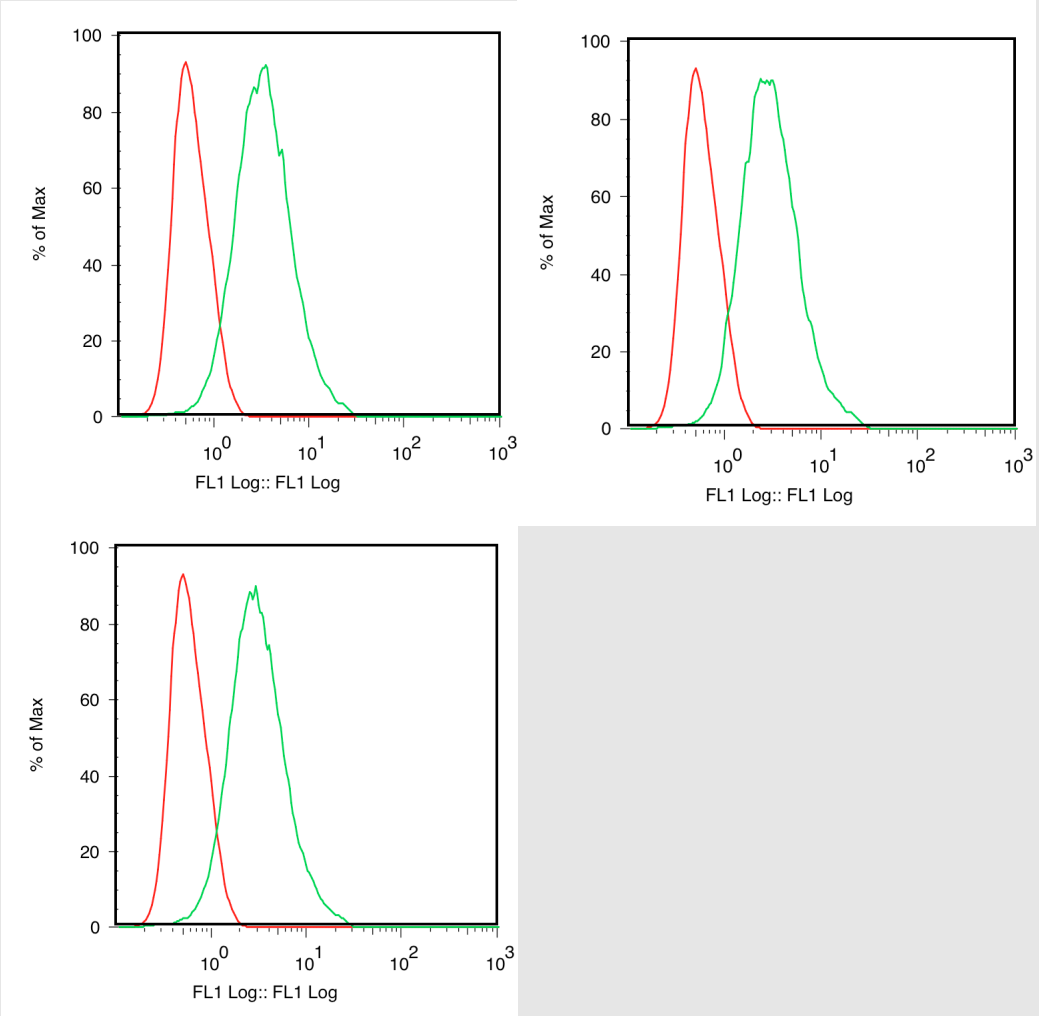

Figure 22: Triplicate histograms of bronchial epithelial cell line (in green) in comparison to isotype control (IgG_1 488, in red) stained with anti-Pan-Cytokeratin (e488)

Cells of bronchial epithelial cell line (16HBE14o-) were only slightly positive stained with anti-E-Cadherin in comparison to the isotype control (Fig. 23). Cells were again stained in triplicates.

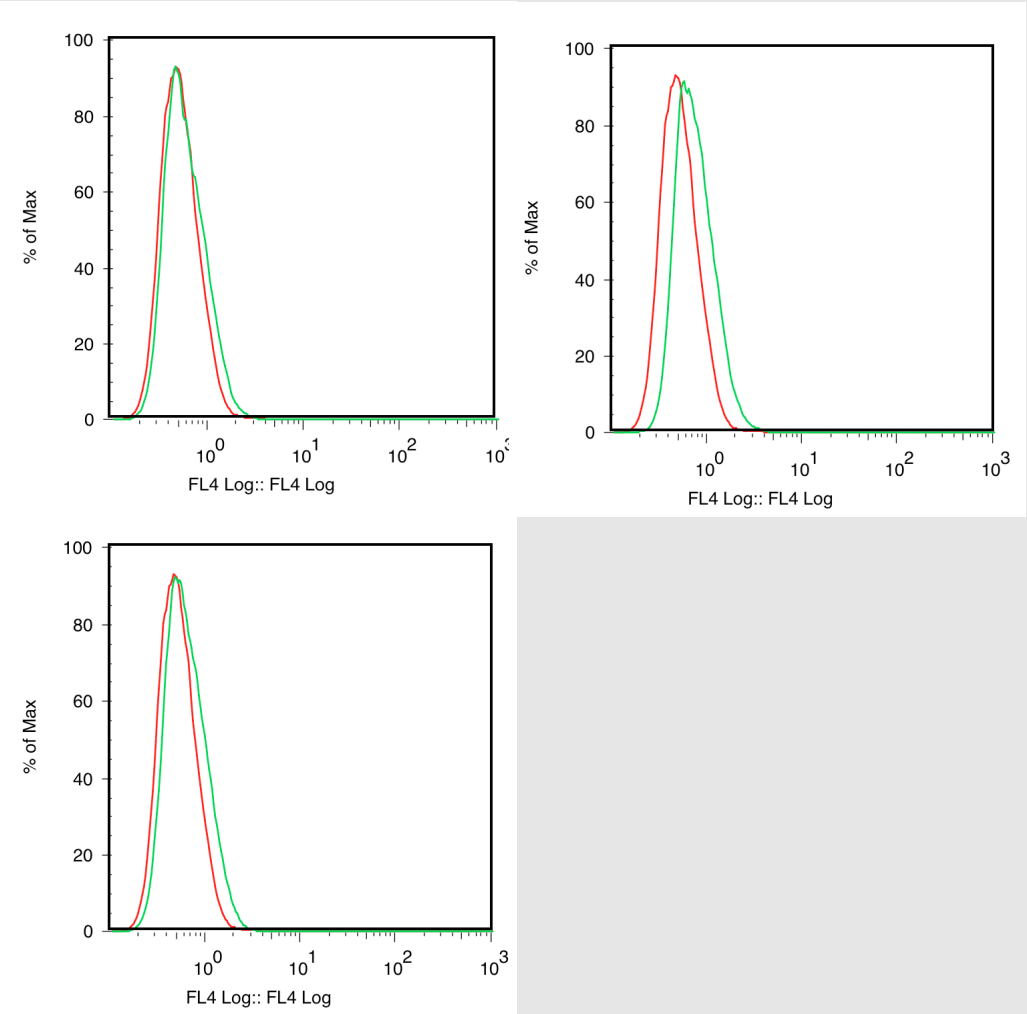

Figure 23: Triplicate histograms of bronchial epithelial cell line (in green) in comparison to isotype control (IgG_1 660, in red) stained with anti-E-cadherin (e660)

To examine if any haematopoietic cells were present in the cultured primary human nasal epithelial cells, cells were stained with anti-CD45 (Fig. 24) and the epithelial cell line as a control (Fig. 25).

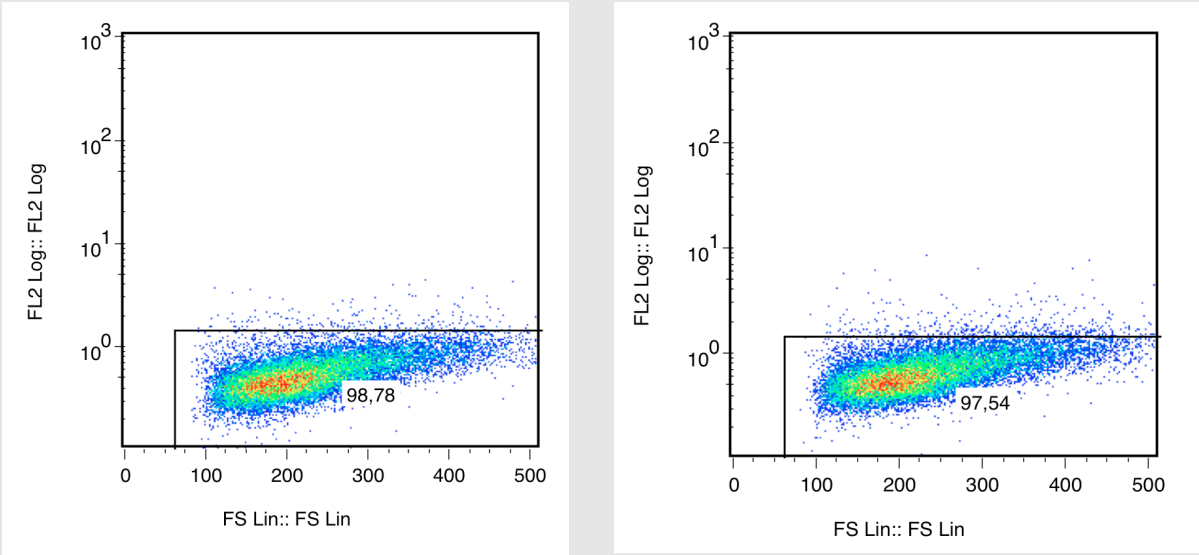

Figure 24: Dot blots demonstrate CD45 (PE) negative cells in cultured primary human nasal epithelial cells

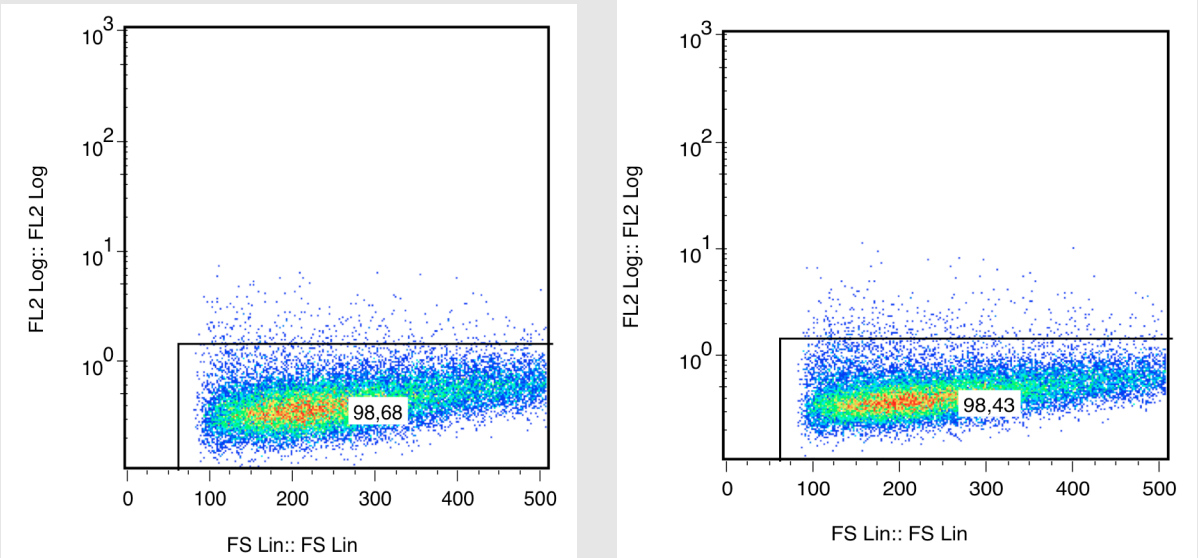

Figure 25: Dot blots show CD45 (PE) negative cells also in cultured human bronchial epithelial cell line

Blood cells were stained with anti-CD45 as a control, which can be seen in Fig. 26.

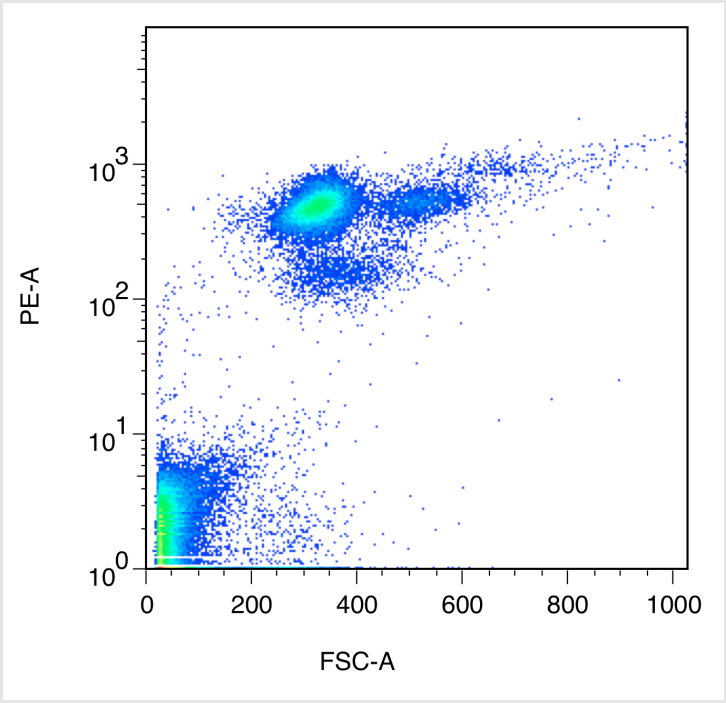

Figure 26: Anti-CD45 stained blood cells with high intensity

3.4.2 Discussion of flow cytometry

Primary human nasal epithelial cells were highly pan-Cytokeratin positive (Fig. 20) and as a control a human bronchial epithelial cell line (16HBE14o-) showed almost the same result (Fig. 22).

It is possible that the anti-E-cadherin antibody used for the experiment did not stain the cells as the primary human nasal epithelial cells as well as the epithelial cell line both showed few E-cadherin positive cells. It is equally possible that cells did not express tight junction proteins after resuspension. A weak shift can be seen in primary epithelial cells (Fig. 21) in comparison to the epithelial cell line (Fig. 23). Therefore anti-E-cadherin antibody from other companies will be tested and also cultured cells will be stained directly.

Primary epithelial cultured cells were not CD45 positive, which indicates that there are no haematopoietic cells present in the cultures. The specific epithelial culturing methods, including specific bronchial epithelial cell growth medium (BEGM) with specific growth factors and hormones and passaging of the cells seem to be responsible for the elimination of

haematopoietic cells. The epithelial cell line was also CD45 negative. To prove if the anti-CD45 antibody works, blood cells were stained with anti-CD45 PE antibody and were highly positive for CD45.

In conclusion we demonstrated that the cultured primary human nasal epithelial cells highly expressed Pan-Cytokeratin and may therefore be indeed epithelial cells. E-cadherin could not be well detected in both primary epithelial cells and the cell line by the antibody used at present. Due to the fact that no CD45 positive cells were stained it can be assumed that no contamination of blood cells were in the cultured primary human nasal epithelial cells.

3.5 Immunohistology

For morphological evaluation of the cultured primary human nasal epithelial cells, immunohistological assessment was employed. Frozen or paraffin sections of cells grown on membranes (Fulcher et al., 2005) were fixed with 4% paraformaldehyde and were morphologically assessed for the presence of ciliated epithelium as a sign of an intact epithelium using H&E staining. Furthermore morphological features such as tight junctions between epithelial cells were stained using various fluorescent antibodies and were assessed using confocal microscopy (collaboration with S. Rauscher and M. Gröger of the Core Facility, Anna Spiegel Research Institute, Medical University of Vienna).

Cells were cultured for 28 days on semipermeable membranes of 12- well inserts of the transwell system with BEGM and DMEM (1:1) medium with all supplements and air-liquid interface culturing (AIC) method. According to (Kim, 2008) ciliated cells should become detectable after 3 weeks of incubation with AIC. Liquid covered culture (LCC) showed undifferentiated epithelial cells and few flattened ciliated epithelial cells. Furthermore AIC method mimics the physiological environment of the nasal epithelium *in vivo* and greater and prolonged TER values were observed (Gray et al., 1996; Kim, 2008).

3.5.1 Results of Immunohistology

The membranes with the cultured primary human nasal epithelial cells were embedded in paraffin vertically. Four µm sections were stained with H&E and were transferred on a microscope slide to take photographs with the confocal microscope (Fig. 27).

Figure 27: H&E staining of primary human nasal epithelial cells grown on a semipermeable membrane of the transwell system

Hemalum colours nuclei blue and eosin stains cytoplasm light blue.

Some frozen slices of the primary human nasal epithelial cells grown on membranes were also stained with fluorescent antibodies. Fluorescent colour DAPI stains the nuclei (in blue), Anti-Actin stains the cytoskeleton (in red) and transmembrane marker Anti-JAM-A was used to colour epithelial tight junctions, including ZO-1 and occludin (in green). In Fig. 28 single stains of these three fluorescent antibodies and on the bottom on the right a merged photograph are shown.

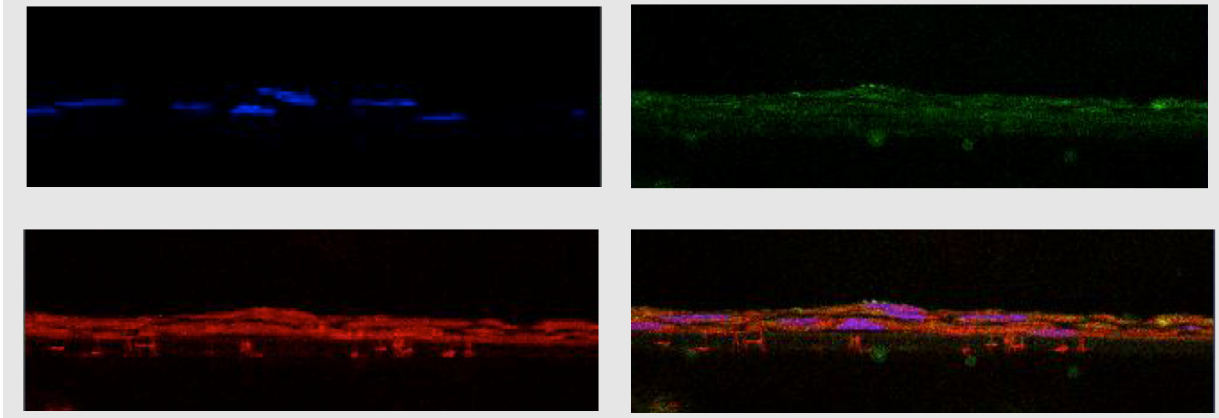

Figure 28: Fluorescently stained primary human nasal epithelial cells

Nuclei were stained blue by DAPI, tight junctions were stained green by anti-JAM-A, cytoskeleton was stained red by anti-Actin and merge photograph shows staining of all named antibodies.

3.5.2 Discussion of Immunohistology

The immunohistology needs technical improvement. It was difficult to embed the semipermeable membranes of the transwell inserts as they are thin, filigree and highly flexible. Additionally many cells were detached from the filter by holding them with a forceps and embedding them in paraffin. Furthermore it was challenging to take clear and sharp pictures as the membranes are difficult to get in a straight and even level. Moreover the enlargement of the confocal microscope did not fulfil the requirements to observe a differentiated ciliated epithelium.

For these reasons a series of technical improvements is planned in the future. Nevertheless we were able to show a tight epithelium and cells with numerous tight junctions between the cells (Fig. 28).

3.6 Future projects

Within the current master thesis it has been possible to culture primary human nasal epithelial cells as a surrogate of the human nasal mucosa. So far, culture conditions were found which allowed to build up intact epithelial cell layers exhibiting barrier function and to disrupt the barrier using substances which affect epithelial barrier (e.g., IFN-γ). For this purpose two types of assays were used, one in which barrier was measured in transwell culture systems by recording TER, and another simple but versatile, i.e., by using a scratch test method. The latter method provided interesting results in as much as it suggested that epithelial damage by certain factors not only temporarily damage epithelial cell barrier and disturbs the barrier function but leads to sustained and large injuries which do not heal. Furthermore, a preliminary characterization of the cultured cells was performed by immune-phenotyping of the cells showing their primary epithelial origin.

Next goals will be to compare the artificial cell layers with primary nasal epithelium by morphology and detailed immunological characterization. For this purpose, cultured cell layers will be compared with biopsies using immune histochemistry and electron microcopy. Next steps will be to study in detail nasal mucosa from allergic and non-allergic individuals and to compare cultured nasal epithelium from both groups of individuals. In this context, the presence of epithelial surface markers (e.g. CD23) involved in allergic inflammation and the ability of the epithelium to secret pro- and anti-inflammatory cytokines depending on the atopic background of the donor will be explored.

Results obtained in this thesis highlighted that certain factors are critical for the building up of an intact epithelial barrier and it is therefore planned to identify the importance of individual factors for barrier formation with the goal to identify substances that may be used for the development of drugs which strengthen the epithelial barrier. This may be important for the treatment and prevention of allergic diseases because it has been shown that disruption of the epithelial barrier is a critical factor for increased penetration of allergens, a process involved in allergic sensitization, in the elicitation of allergic symptoms and in the increase of IgE responses in allergic patients. According to the obtained results, insulin, retinoic acid, EGF, hydrocortisone, epinephrine and transferrin contributed to cell growth and barrier formation. Results obtained in the laboratory also suggested livostin as a possible factor enhancing barrier. Furthermore, it will be attempted to identify environmental factors which disturb the epithelial barrier function (e.g., rhinovirus infections) and develop therapeutic strategies which interfere with these processes.

4 Summary

The nasal epithelium consists of basal cells, ciliated epithelial cells and goblet cells, which are associated by tight junctions. It represents an important barrier against the penetration of environmental allergens, pollutants and pathogens. Inhaled exogenous factors can more easily penetrate the nasal mucosa if the epithelium is damaged. Our laboratory has previously worked with a bronchial epithelial cell line to investigate epithelial damage by various factors (e.g. cytokines, cigarette smoke). With primary epithelial cells, obtained from the nasal epithelium, additional interesting investigations can be done. For example epithelia from non-allergic and allergic patients can be compared. Primary human nasal epithelial cells may be superior in resembling the natural situation in the nose and it was therefore the aim of the work presented in this Master thesis to establish a culture system for these cells in our laboratory.

Different epithelial cell culture systems were compared to establish optimal conditions for the study of damage and repair in primary epithelial cells. A transwell system with air liquid interface culture was used to study the properties of the nasal epithelium as well as epithelial barrier function. Epithelial permeability was examined by measuring transepithelial electrophysiological resistance (TER). Damage to the epithelium was determined using the cytokine interferon gamma (IFN-γ), which is known to disturb the barrier function of nasal epithelial cells. Furthermore, the scratch test method was performed for analysing physical damage and the proliferation and growth rate of the cells. Additionally, cell types and morphological features of the cultured epithelium were investigated by flow cytometry and immunohistological assessments.

In this Master thesis, experiments with cells from 9 different nasal mucosa samples from human donors were performed, which were obtained during routine surgery. After the basic techniques had been established, further experiments were performed with three different samples, which are shown in detail and demonstrate the reproducibility of the used techniques.

We showed a significant decrease of TER in epithelial cells and a substantial decrease of epithelial repair after treatment with IFN-γ. Cells with lower TER were more vulnerable than cells with higher TER values. Therefore the initial TER values corresponded with the decreasing effect of IFN-γ-treatment.

Scratch surfaces of untreated cells closed in all samples at the latest after 96 hours after performing damage and therefore a satisfying *in vitro* repair of the epithelial cell monolayers was observed. In all three samples IFN-γ led to a severe increase of the damage areas.

In conclusion, we were able to establish the cell culture system of primary human nasal

epithelial cells in our laboratory. Reproducible techniques to investigate the epithelial barrier function, to analyse physical damage and proliferation and growth rate of cells were set up.

5 Zusammenfassung

Das nasale Epithel stellt eine wichtige Barriere gegen das Eindringen von Allergenen, Schadstoffen und Pathogenen dar. Es besteht aus Basalzellen, Flimmerepithelzellen und Becherzellen, die durch Tight Junctions verbunden sind. Die Aufnahme von eingeatmeten exogenen Faktoren wird durch ein beschädigtes Epithel der Nasenschleimhaut erleichtert. Um epitheliale Schäden durch verschiedene Faktoren (z.B. Zytokine, Zigarettenrauch) zu untersuchen, arbeitete unser Labor bis vor kurzem mit einer bronchialen Epithelzelllinie. Da primäre humane nasale Epithelzellen die natürliche Situation in der Nase besser widerspiegeln können, haben wir im Laufe dieser Masterarbeit die Etablierung dieser Zellkultur in unserem Labor durchgeführt. Primäre nasale Epithelzellen können zum Beispiel verwendet werden um Epithelien von nicht-allergischen und allergischen Patienten miteinander zu vergleichen.

Verschiedene epitheliale Zellkultur-Systeme wurden verglichen um optimale Bedingungen für die Untersuchung der Schädigung und Reparatur der primären Epithelzellen zu erreichen. Ein Transwell System wurde zur Untersuchung der Eigenschaften und der epithelialen Barrierefunktion des nasalen Epithels verwendet. Durch Messung des transepithelialen elektrophysiologischen Widerstands (engl. TER) wurde die epitheliale Permeabilität ermittelt. Epithelschäden wurden durch Verwendung des Zytokins Interferon gamma (IFN-γ) analysiert, da IFN-γ die Barrierefunktion der Nasenepithelzellen bekannterweise stört. Zusätzlich wurde die Scratch-Test-Methode für die Analyse von physischen Schäden, der Proliferation und des Wachstum der Zellen durchgeführt. Zelltypen und morphologische Eigenschaften des kultivierten Epithels wurden mittels Durchflusszytometrie und immunhistologische Untersuchungen bestimmt.

Die Experimente wurden mit Zellen von 9 verschiedenen humanen Nasenschleimhaut-Proben durchgeführt, welche bei Routine-Operationen entnommen wurden. Nachdem wir die grundlegenden Techniken etabliert hatten, wurden weitere Experimente mit drei Proben durchgeführt, welche in dieser Masterarbeit veranschaulicht sind und die Reproduzierbarkeit der verwendeten Techniken belegen.

Die Ergebnisse zeigen eine signifikante Abnahme des transepithelialen elektrophysiologischen Widerstands und eine deutliche Abnahme der epithelialen Reparatur in Epithelzellen nach der Behandlung mit IFN-γ. Zellen mit niedrigem TER waren anfälliger für die IFN-γ Behandlung als Zellen mit höheren TER Werten.

Die Scratch-Flächen der unbehandelten Zellen sind in allen Proben spätestens 96 Stunden nach der Durchführung des Scratches zusammen gewachsen und zeigten eine überzeugende *in vitro* Reparatur der epithelialen Zell-Monolayer. IFN-γ hat in allen drei Proben zu einer starken Zunahme der geschädigten Flächen geführt.

Im Laufe dieser Masterarbeit ist es uns gelungen das Zellkultursystem von primären humanen Nasenepithelzellen zu etablieren. Außerdem konnte die Reproduzierbarkeit der Techniken, zur Untersuchung der epithelialen Barrierefunktion, zur Analyse von physisch zugefügten Schäden und zur Beobachtung der Proliferation und des Zellwachstums, gezeigt werden.

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Curriculum Vitae

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2011 - 2013	Master studies of Immunobiology and Molecular Microbiology at the University of Vienna, Austria
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1999 - 2007	Grammar School in Sankt Johann in Tirol, Austria, A-levels
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