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Direktor: Prof. Dr. Christian Sina

ROLE OF IL-5 IN AIRWAY REMODELING IN SEVERE ASTHMA

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Rola AY Abujabal

aus Ländliches Gazza, Palestainene

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1. Berichterstatterin*in: PD Dr. rer. nat. Yves Laumonnier Ko-Betreuer*in: Prof. Dr. med. vet. Jennifer Hundt

2. Berichterstatterin*in: Prof. Dr. rer. nat. Hauke Busch

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LIST OF ABBREVIATIONS

A

- **AEC** – Airway Epithelial Cell
- **AHR** – Airway Hyperresponsiveness
- **Ap-1** – Activator protein-1 (Ap-1)
- **ASMC** – Airway Smooth Muscle Cell

B

- **BALF** – Bronchoalveolar Lavage Fluid

C

- **CAM** – Cell Adhesion Molecules
- **CDC** – Centers for Disease Control and Prevention
- **cDNA** – Complementary DNA
- **COPD** – Chronic Obstructive Pulmonary Disorder
- **CRD** – Chronic Respiratory Disease

D

- **DC** – Dendritic Cell

E

- **ECM** – Extracellular Matrix
- **EGF** – Epidermal Growth Factor
- **EMT** – Epithelial Mesenchymal Transition

F

- **FEV** – Forced Expiratory Volume

G

- **GAG** – Glycosaminoglycan
- **GINA** – Global Initiative for Asthma
- **GRα** – Glucocorticoid Receptor Alpha

H

- **H&E** – Hematoxylin and Eosin
- **HDM** – House Dust Mite

I

- **Ig** – Immunoglobulin
- **IL** – Interleukin
- **ILC** – Innate Lymphoid Cell

J

- **JAK** – Janus Kinases

L

- **LAMA** – Long-acting Muscarinic Antagonists
- **LTRs** – Leukotrienes

M

- **MAIT** – Mucosal-Associated Invariant T (MAIT)
- **MBP** – Major Basic Protein

- **MENA** – Middle East and North Africa
- **MHC** – Major Histocompatibility Complex
- **MMP** – Matrix Metalloproteinases

N

- **NF-B** – Nuclear Factor-B
- **NK** – Natural Killer

P

- **PAF** – Platelet-Activating Factor

S

- **SNP** – Single Nucleotide Polymorphism
- **STAT** – Signal Transducer and Activator of Transcription

T

- **TGFβ** – Transforming Growth Factor β
- **TIMP** – Tissue Inhibitor of Metalloproteinases
- **TLR** – Toll-Like Receptor
- **TNFα** – Tumor Necrosis Factor α
- **TSLP** – Thymaic stromal ymphopoietin

V

- **VCAM-1** – Vascular Cell Adhesion Molecule-1

W

- **WHO** – World Health Organization

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Chapter I.

Introduction

Chronic Respiratory Diseases (CRD) encompass a range of pulmonary and airway disorders, including asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis, and lung cancer. Primary risk factors for CRD include tobacco smoke, along with exposure to air pollutants, chemicals, allergens, and dust. Globally, CRD rank as the third leading cause of death and continue to impose substantial socio-economic burdens on individuals, communities, and healthcare systems. Among the listed CRD, asthma is ranked as the second most prevalent CRD after COPD, with both conditions primarily affecting the lung airways. In the Middle East and North Africa (MENA) region, the United Arab Emirates (UAE) has reported the highest incidence and prevalence of asthma among the 21 countries in this region.

1.1.Asthma

1.1.1. Definition

Asthma is classified as non-communicable major chronic inflammatory disease of the lower respiratory tract, which is mainly characterized by cough, wheezing, and dyspnea. The word asthma is derived from the Greek word “aazein” which means the “noisy breath” (Marketos & Ballas, 1982).

Asthma is also defined as an allergic disease of the airways, due to the exaggerated immune response that takes place in response to common environmental allergens including air pollutants, dust mite, mold and pollens (Devereux et al., 2014).

In 2024, the Global Initiative for Asthma (GINA), defined asthma as “a history of respiratory symptoms including wheezing or repetitive coughing, dyspnea, and chest tightness plus variable expiratory airflow limitation all of which vary over time and in intensity” (GINA, 2024a)

1.1.2. Epidemiology

Asthma is a major respiratory disease with a major health challenge that causes socioeconomic burden at the level of both induvial and health care systems. Asthma ranks 16th worldwide as a leading cause of years lived with disability and 28th in terms of overall disease burden,

measured by disability-adjusted life years (Dharmage et al., 2019). In 2019, the World Health Organization (WHO) estimated that asthma affects over 262 million individuals, with the number of cases expected to rise by 100 million by 2025. Additionally, asthma was the leading cause of death for approximately 500,000 people (WHO, 2024). However, the mortality rate from asthma has decreased in children and young adults due to improved management. Despite this, mortality rate remains high in patients aged 65 and older, particularly among adult females. Asthma is more frequently reported in boys compared to girls during their childhood **(Figure 1.1)** (Arshad et al., 2014), while during adulthood, asthma is more prevalent in adult females compared to adult males. However, the geographical distribution of asthma varies in terms of prevalence, severity and mortality. In 2021, US reported 25 million cases as published by the Centers for Disease Control and Prevention (CDC) (Centers for Disease Control and Prevention, 2021). However, locally in UAE, around 7.4% percent of the population suffers from various severities of asthma while other reports show that 14% of the total UAE population suffers from asthma, and 14% of the cases were reported in children mostly leading to sleep disturbance and speech limitation (Ibrahim et al., 2021). It worth mentioning that UAE has the highest asthma cases prevalence across the gulf region with a relatively high economic burden that reached up to 29 million US dollars which equals 105 million dirhams (Alzaabi et al., 2014).

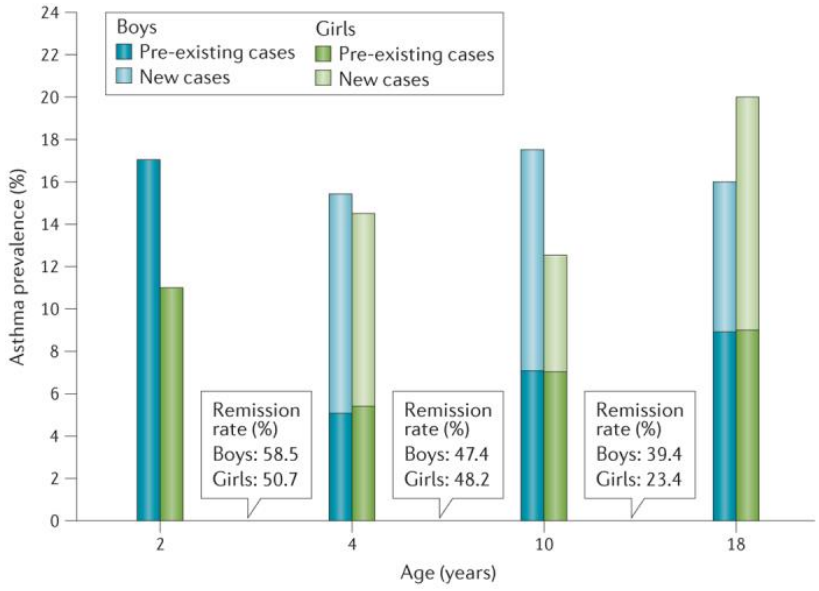


Figure 1.1 Prevalence of asthma in boys vs girls considering the new vs the pre-existing cases (Dharmage et al., 2019)

1.1.3. Asthma Phenotypes and Endotypes

Historically, asthma is an old disease called “a disease of the pipes of the lungs” as defined by the Belgian researcher Jean Baptiste in 1579–1644, and Bernardino Ramazzin was the first to observe the exercise induced asthma and he acknowledged the relation between asthma and dust as an allergic disease, considering it as a single disease of the airways (Kapri et al., 2023). However, in 2017, as declared by GINA, asthma was redefined as a heterogeneous and complex lung condition encompassing multiple distinct diseases under the umbrella term of asthma (“GINA,” 2017).

Asthma was initially classified into two broad categories: extrinsic (atopic) asthma, which typically manifests in childhood or early adulthood and is associated with a higher tendency to develop allergies to environmental factors, and intrinsic (non-atopic) asthma, which usually appears in adulthood and is primarily triggered by weather conditions, exercise, infections, and stress (Pham et al., 2023). However, this classification has become outdated as new phenotypes have been identified based on clinical symptoms, such as persistent airflow limitation or exacerbation-prone asthma, airway inflammation (eosinophilic, neutrophilic, mixed, or paucigranulocytic), and cluster analysis. This has led to the definition of a phenotype as "observable characteristics” resulting from a combination of genetic and environmental influences." With advancements in biological therapies and the shift towards personalized medicine, a newer classification system has emerged, focusing on the molecular characteristics of the disease, known as endotypes (Agache & Akdis, 2019). Two major endotypes have been well established, a T2-High and a T2-Low (**Figure 1.2)**.

1.1.3.1 T2-High:

The T2h-high endotype is the most common and well-understood form of asthma. Generally, asthma is characterized by an exaggerated immune response dominated by the type 2 immune response, which is mainly marked by airway eosinophilia and a surge of type 2 cytokines (Habib et al., 2022). T2-high asthma is mostly atopic and strongly associated with remodeling (Coverstone et al., 2020; Laprise et al., 1999). The pathogenesis of T2h-high asthma occurs in two phases: the sensitization and the challenge phases.

Sensitization Phase

This phase begins with the initial entry of an allergen into the lower airways. The allergen is captured by airway-residing dendritic cells and presented to naïve T cells, leading to their activation. Upon activation, these cells secrete Interleukin (IL)-4, IL-5, and IL-13. Another

cellular subset called innate lymphoid cells 2 (ILC-2) is contributing to the type 2 immune response through secreting IL-5 and IL-13. IL-5 is a potent activator and recruiter of eosinophils to the airways, while IL-4 and IL-13 prompt B-cells to switch to IgE production. The IgE produced is specific to the allergen and binds to the FcERI receptors on mast cells. During this phase, no signs or symptoms are typically observed (Habib et al., 2022; Harker & Lloyd, 2023).

Challenge or Reaction Phase

This phase occurs upon re-exposure to the same allergen. The allergen binds to IgE, bridging it with mast cells, which triggers mast cell activation (Habib et al., 2022). This activation leads to the release of mediators such as histamine, leukotrienes (LTRs), and other interleukins, resulting in airway smooth muscle contraction, leading to bronchoconstriction (Yeh & Schwartzstein, 2010)(Yeh & Schwartzstein, 2010). Eosinophils will get activated as well in response to IL-5, secreting mediators including major basic protein (MBP), and stimulating mast cells to release histamines and LTs (Hussain & Liu, 2024). Additionally, MBP inhibits M2 receptors, promotes acetylcholine release from cholinergic nerves, and induces bronchospasm (Habib et al., 2022). The T2-High itself is further divided into either allergic or non-allergic subtypes or phenotypes (Ricciardolo et al., 2021). In the allergic subtype, the secretion of these cytokines is mainly mediated by innate and adaptive immune ILC2 and Th2 cells (Porpodis et al., 2022). While the non-allergic phenotype is orchestrated solely by ILC-2 through an innate immune response with a non-specific mechanism (Nelson et al., 2020). this activation is explained due to the secretion of a set of cytokines called alarmins from the injured AEC, these cytokines are IL-25, IL-33 and Thymic stromal lymphopoietin (TSLP). these alarmins will activate ILC-2 leading to the secretion of IL-5 and IL-13 resulting in an augmented Type 2 immune response thus a T2h high asthmatic phenotype (Kuruvilla et al., 2019). The allergic phenotype is mainly recognized during childhood (early onset), while the non-allergic phenotype developed during adulthood (late-onset) (Novak & Bieber, 2003).

1.1.3.2 T2-Low:

On the other hand, there is another class of asthma that lack the classical characteristics of asthmatic patients including normal eosinophilic count, normal IgE levels and no signs of allergic immune response, yet with a persistent airflow limitation and poor response to steroids (Hinks et al., 2021). This subtype of the disease is still not yet well understood, but it has been proposed to be mediated mainly by IL-17 (Lambrecht et al., 2019). This IL-17 has been

reported to be secreted mostly by a subtype of CD4+ cells known as Th-17, and other cells like natural killer T cells, mucosal-associated invariant T (MAIT) cells, ILC3 cells, and B-cells (Huangfu et al., 2023). IL-17 reported to stimulate epithelial cells and fibroblasts recruiting neutrophiles to the airways through the release of chemoattractant including CXCL1, 5 and 8. IL-17 also directly acts on airway smooth muscles inducing their contraction, migration, and proliferation thus contributing to airway hyperresponsiveness as well as in airway remodelling (Ramakrishnan et al., 2019). Asthma patients with T2 Low known to be classified as moderate to severe asthma and usually manifests during late adolescence (late-onset), and mostly hard to control with a poor response to classical therapy (Peri et al., 2023).

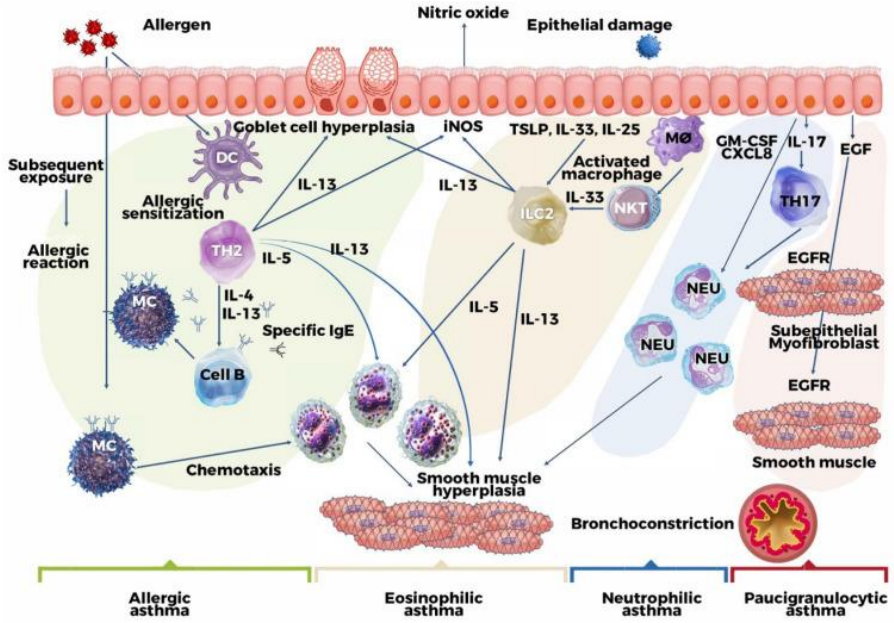


Figure 1.2 Complexity of the pathophysiology of the different asthma endotypes. T2-high encompasses allergic and eosinophilic asthma represents, while the T2-low is represented by the neutrophilic and paucigranulocytic asthma (Gonzalez-Uribe et al., 2023).

1.2. Asthma Severity

Based on severity, asthma could be classified as mild, moderate and severe, all of which is determined based on the frequency of exacerbations and the level of response to steroids (**Figure 1.3**). Mild and moderate cases are well known with their high response to steroids,

however, the major challenge in the clinic is mostly managing the severe cases (*2022 GINA Report, Global Strategy for Asthma Management and Prevention*, 2022).

There is no absolute definition of severe asthma (Lommatzsch & Virchow, 2014), however, they could be defined as "The uncontrolled asthma symptoms despite the adherence with the maximal optimized high dose of ICS-LABA treatment and management of contributory factors or that worsens when high-dose treatment is decreased". It's also referred to as refractory asthma, difficult-to-treat asthma, and uncontrolled asthma. (GINA, 2024b)

Table 1 Classification of asthma based on the severity of the case, PEFR = Peak Expiratory Flow Rate. FEV1 = Forced Expiratory Volume in 1 second (2022 GINA Report, Global Strategy for Asthma Management and Prevention, 2022).

	Frequency of symptoms		% predicted	Variability
	Day	Night	FEV_1/PEFR	PEFR%
Intermittent	<1/wk	≤2/m	≥80%	<20%
Mild persistent	≥1/wk <1/day	>2/m	≥80%	20-30%
Moderate persistent	Daily	≥1/wk	60-80%	>30%
Severe persistent	Daily	Frequent	≤60%	>30%

Classified according to the Global Initiative for Asthma [GINA] guidelines

1.3. Genetic and environmental factors linked to the risk of asthma.

Asthma is a complex, multifactorial condition in which genetic predisposition and environmental factors interact in the disease's pathology. Some individuals are hypersensitive to certain environmental substances—mainly house dust mite (HDM) as 85% of asthmatics are allergic to HDM (Gregory & Lloyd, 2011), air pollutants, respiratory viruses, tobacco smoke, endotoxins, airborne allergens, and diet—triggering an inappropriate immune response, whereas others are not ('Chabra & 'Gupta, 2023). These hypersensitive individuals are referred to as atopic. In addition, a group of genes have been showed to be associated with atopy including HLA-DR, HLA-DQ, HLA-DP, FcεRIβ, RANTES, IL-4 receptor α, β-adrenergic receptor, T cell receptor α, and mast cell chymase (Mahdi et al., 2018; Mukherjee & Zhang, 2011; Vercelli, 2008). Additionally, evidence indicates that genetic loci on human chromosomes 5, 6, and 11 are likely to contain genes associated with atopy (Mukherjee & Zhang, 2011). Furthermore, Single Nucleotide polymorphisms (SNP) in genes like

TGFB1(Sharma et al., 2009) and *IL10* (Hunninghake et al., 2008) alter the gene-environmental interaction thus increasing the susceptibility to allergic asthma.

1.4. Pathophysiology of Asthma

Asthma is an umbrella of inflammatory disease, and its pathophysiology encompasses three major features: airway inflammation, airway hyperresponsiveness and airway remodeling.

1.4.1 Airway inflammation

Airway inflammation is widely discussed in literature, as discussed earlier various immune cells and their mediators are shown to drive the pathogenesis of both T2-high and T2-low asthma,

1.4.2 Airway hyperresponsiveness (AHR)

Airway Hyperresponsiveness is defined as the excessive abnormal narrowing of the airways in response to a stimulus which the normal subject has little to no reaction against it (Chapman & Irvin, 2015). AHR is a cardinal feature of asthma and it's mainly associated with an exaggerated airway smooth muscle contraction thus narrowing the airway lumen leading to bronchoconstriction (Brannan & Lougheed, 2012). The reduction in the measurement of spirometric parameters, mainly the forced expiratory volume (FEV) is what reflects the degree of AHR and is usually associated with the severity of the case (Brannan & Lougheed, 2012). AHR is so far classified into either narrowing or the closure of the airways (Busse, 2010).

1.4.3 Airway Remodelling

Airway Remodelling is defined as the permanent/semi-permanent structural changes of both small and large airways.

1.5. Airway remodelling in asthma

Airway remodelling, as the name suggests, constitutes the structural changes that take place in both the large and small airway walls due to repeated cycles of injury and repair (**Figure 1.4**), because of chronic inflammation in patients with chronic lung diseases including asthma, chronic obstructive pulmonary disease (COPD) and cystic fibrosis (Bergeron et al., 2009).

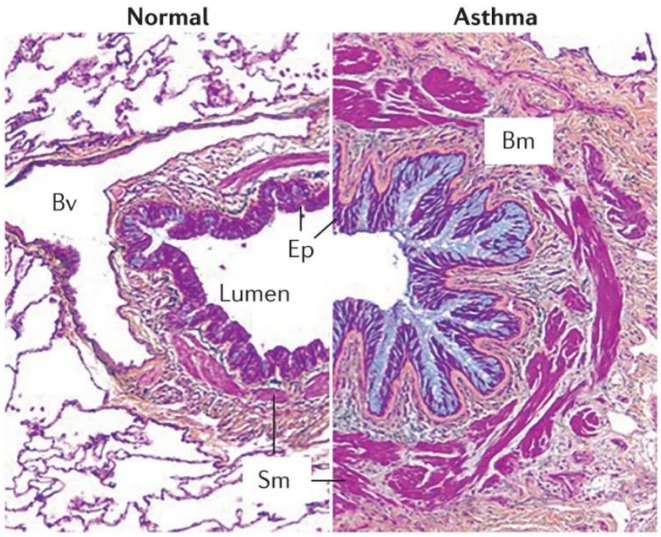


Figure 1.4 Histological differences in asthmatic vs normal airways. Ep: Epithelial cell layer, Bm: Basment membrane, Sm: Smooth muscle, Bv: Blood vessele (Holgate et al., 2015)

Airway remodelling was initially thought to start in advanced stages of asthma but then it was reported that airway remodelling may occur during early phases of the disease even before symptom manifestations or any airway hyperresponsiveness. Airway remodelling is a detrimental event in the pathogenesis of asthma (Barbato et al., 2003; Boulet, 2018; Laprise et al., 1999) which worsens with time due to the chronicity of the inflammation and the development of symptomatic phase of the disease. In addition, remodelling is not a feature of severe asthma exclusively, but it takes place in mild asthmatics as well with a tendency to worsen with the severity of the disease (Hough et al., 2020).

1.5.1 Epithelial layer alteration

Airway epithelium is composed mainly of ciliated columnar epithelial cells, along with goblet cells, the mucus secreting cells, and other cells such as pluripotent basal cells, which act as primary stem cells giving rise to the previously mentioned cells (Hiemstra et al., 2015; Knight & Holgate, 2003). The airway epithelial cells (AEC) are known to provide a physical and chemical immunological barrier against antigen/allergen invasion, thus playing a critical role in maintaining an anti-inflammatory environment in the airway in response to an innocuous antigen. On the other hand, they can sense a pathogen through their expressed pattern recognition receptors (PRRs), mainly the toll like receptors (TLRs), which result in recruitment of other pro-inflammatory cells such as dendritic cells (DCs), which will further stimulate and

engage paving way to an adaptive immune response orchestrated mainly by Th2 cells (Banno et al., 2020; Lambrecht & Hammad, 2012).

In severe asthmatic patients, alterations in this layer including epithelial cell shedding, loss of ciliated cells, goblet cell hyperplasia and upregulation of growth factor expression and their corresponding receptors, are commonly observed. These changes contribute to weakened attachment of the epithelial cells to the basement membrane because of dysfunctional tight junctions (Al-Muhsen et al., 2011; Bergeron et al., 2009). Bronchoconstriction and bronchospasm stimulate epithelial cells to secrete TGF-β and GM-CSF, that will recruit DCs further exaggerating the inflammation through both innate and adaptive immune responses (Ramsey & Becker, 2014). Mechanical stress will also increase the expression of some other growth factors such as Epidermal Growth Factor (EGF), and Early growth response (Egr-1), which will act on goblet cells inducing hyperplasia and thereby increasing mucus production (Ressler et al., 2000).

Upon injury or death to the AECs, these cells release first order cytokines such as IL-33, IL-1α, IL-1β, IL-25 and (TSLP), which will recruit pro-inflammatory effector cells including dendritic cells and Th2 cells, which exaggerate the inflammation (Iwasaki et al., 2017).

1.5.2 Goblet cell hyperplasia and mucus hypersecretion

Goblet cell hyperplasia is a major source of mucus production in the inflamed airways along with the dysregulated mucin gene expression mainly MUC5A and MUC5B which strongly correlates with severe type 2 high asthma phenotype (Bonser & Erle, 2017; Veerati et al., 2020). Previous reports showed that fatal asthma biopsies showed 30-fold increase of goblet cells (Bonser & Erle, 2017), in addition mild and moderate cases showed increased numbers of goblet cells as well (Ordoñez et al., 2001). Besides the well-known role of goblet cells in mucus secretion, it has been shown that they harbour the capacity of secreting certain cytokines and chemokines such as IL-4, IL-5, IL-13 and CXCL8, making them another potential source to further exaggerate the airway inflammation (Tanabe & Rubin, 2016).

1.5.3 Subepithelial fibrosis

Another distinct structural alteration that asthmatic patients suffer from is subepithelial fibrosis, which is strongly correlated with the severity of the disease (Al-Muhsen et al., 2011), where the layer beneath the epithelium becomes thicker compared to normal airways. Due to chronic

inflammation in the airway environment, the residing airway fibroblasts get activated in response to several secreted cytokines and growth factors, mainly TGF-β1, a potent profibrotic cytokine that is secreted mainly from eosinophils and airway epithelial cells (Saito et al., 2018). The activation of fibroblasts leads to their differentiation into myofibroblasts resulting in increased ECM protein deposition in the lamina propria, reticular basement membrane region and submucosa. The basic nature and phenotypic profile of asthmatic fibroblasts differ from their normal counterparts, whereby they display higher proliferative capacity, express higher levels of TGF-β receptor with higher secretion of inflammatory and fibrotic cytokines, including IL-6, IL-8, TNF-α and TGF-β respectively (Michalik et al., 2018a).

1.4.3.1 Fibrotic layer composition: Role in airway remodelling

The ECM itself is composed of wide range of proteins and glycoproteins including: **1)** structural proteins such as collagens and elastin, **2)** adhesion proteins such as fibronectin and tenascin, **3)** glycosaminoglycan (GAG) and proteoglycans including lumican and biglycan (Royce et al., 2012). Collagen, especially types I, III and V, constitutes a significant proportion of the ECM component in asthmatic airways when compared to the normal airways, particularly in the subepithelial region, where collagen increases stiffness and worsens fibrotic development (Burgstaller et al., 2017). Adding to the increased ECM protein deposition, the enzymatic imbalance between matrix metalloproteinases (MMPs) and tissue inhibitor of matrix metalloproteinases (TIMPs) further favors fibrosis (**figure 1.5).**

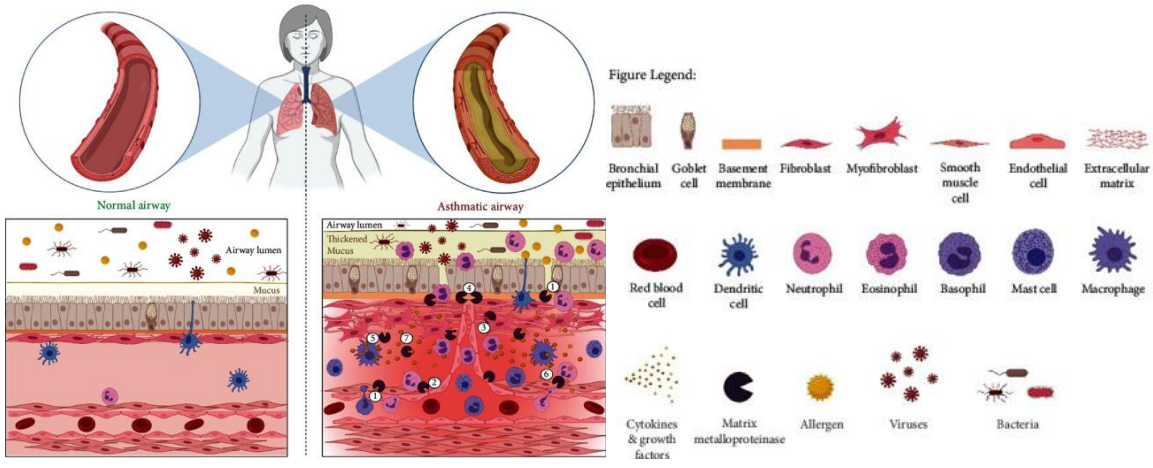


Figure 1.5 A schematic illustration of the role of MMPs in inflammation and remodeling (Bajbouj et al., 2021)

Among the 26 members of the MMP family of proteins, MMP-9 is the one mainly implicated in asthma as it is secreted by various cells including immune and non-immune cells in response to TGF-β and/or TNF signaling (Ohbayashi & Shimokata, 2005a) It has been reported to be at higher levels with increased activity in blood, sputum and BALF isolated from asthmatic patients experiencing exacerbations, while allergic individuals not diagnosed with asthma lack such an elevation in MMP-9 (Grzela et al., 2016) In addition, an elevation in MMP-2 facilitates the migration of fibroblasts/myofibroblasts along with trafficking of inflammatory cells (Kuwabara et al., 2018). The accompanying lower levels of TIMP-1, the inhibitor of MMP-9 (Gueders et al., 2006), together with the disturbance of the 1:1 ratio of MMP-9/TIMP1 and MMP-9/TIMP3 (Weitoft et al., 2014) will further favor fibrosis specially in severe asthmatic individuals (Chaudhuri et al., 2014; Grzela et al., 2016). Beside the role of MMPs in subepithelial fibrosis, they have a role in eosinophil trafficking and smooth muscle enlargement (Relevance et al., 2010). Finally, myofibroblasts are intermediate cells between fibroblasts and airway smooth muscles, which secrete ECM proteins such as collagen and fibronectin (Weitoft et al., 2014) similar to fibroblasts but also express alpha smooth muscle actin that provide contractility similar to airway smooth muscle cells (ASMs) (Michalik et al., 2018b), thus contributing to fibrosis and airway remodelling.

1.5.4 Increased airway smooth muscle mass

The most critical event that takes place in airway remodelling and bronchoconstriction in asthmatics is the increase in smooth muscle mass due to both an enlargement in the size (hypertrophy) and proliferation rate (hyperplasia) of the ASMs, the main cellular component of the airway wall. This is reported to be either due to the prolonged survival or increased proliferation of the existing ASM cells, through switching their phenotype from contractile into synthetic profile with potent secretion of proinflammatory cytokines, chemokines and extracellular matrix proteins (Khan, 2013). Nevertheless, the initial trigger for such a switch needs further investigation (Johnson et al., 2022; Wright et al., 2013). Furthermore, epithelial mesenchymal transition (EMT) is a process in which airway epithelial cells transdifferentiate into motile mesenchymal cells in a TGF-β rich environment (Berair et al., 2013).

Inflammatory eosinophils are hallmark players in asthma pathogenesis as they induce ASM proliferation via eosinophilic adhesion to ASM integrins which will induce the release of eosinophilic mediators such as Cysteinyl Leukotrienes and promoted ASM division (Halwani et al., 2013). In addition, infiltration of T-cells through the ASM bundles induce their

proliferation directly through intercellular contact, or indirectly through the release of IL-13 in the airways (Ramos-Barbón et al., 2010). Interestingly, structural cells such as airway epithelial cells (AEC) switch ASM towards a proliferative phenotype. ASMC and AEC when co-cultured result in the upregulation of the microRNA miR210 which is known to promote ASM proliferation rate. It is worth highlighting that this interaction increased the expression of proinflammatory cytokines such as IL-8, IL-6 and CXCL1, which further exaggerated the inflammatory environment in the airways (O'Sullivan et al., 2021). Mitogens including epidermal growth factor (EGF), fibroblast growth factor 2 (FGF2), platelet derived growth factor (PDGF) and insulin-like growth factor boost the proliferative capacity of ASM through subsequent activation of the tyrosine kinase (95). Proinflammatory cytokines such as IL-6, IL-1B (De et al., 1993) and TNF-α also modulate ASM function, proliferation rate and overall size (hypertrophy) through interactions with their cognate receptors (De et al., 1995; Knobloch et al., 2016).

1.5.5 Angiogenesis

Angiogenesis and vascular remodelling are other prominent features of asthmatic airways. Increased vascularity, vasodilation and microvascular leakage are observed in the airways of patients with asthma (Hough et al., 2020). New abnormal blood vessel formation takes place below the basal lamina in the space between the muscle layer and the surrounding parenchyma along with an enlargement of the pre-existing vessels (Al-Muhsen et al., 2011). It's mainly attributed to the imbalance between two major growth factors: vascular endothelial cell growth factor (VEGF) and angiopoietin-1 (ang-1) that will induce and promote endothelial cell proliferation (Makinde et al., 2006).

1.6.Asthma Management

There are several approaches that are used to manage and improve an asthmatic patient's quality of life and control their sporadic exacerbations as in **Figure 1.6** but unfortunately, these approaches do not cater to all subtypes of asthma.

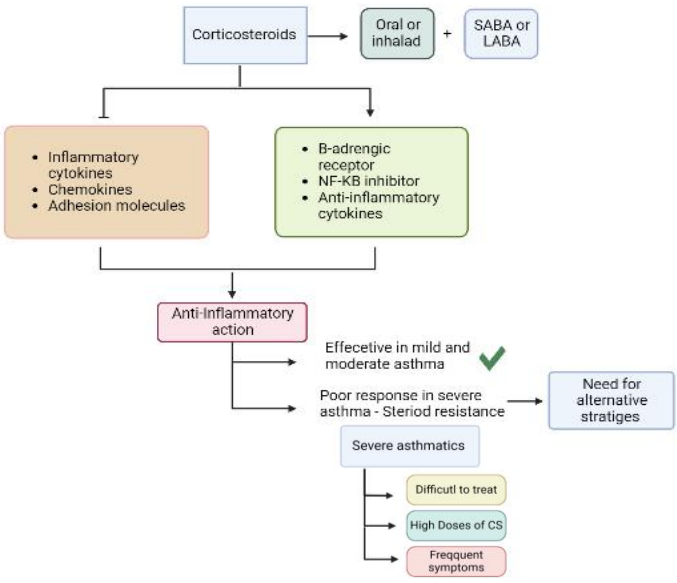


Figure 1.6 A summary of the classical therapeutic approach used to manage asthmatic cases.(GINA, 2024).

The treatment plan for each asthma patient is designed according to the severity of the case, the phenotypic and sub-phenotypic features of the disease, and following the guidelines of (GINA) (*2022 GINA Report, Global Strategy for Asthma Management and Prevention*, 2022). Classically, asthmatic patients are managed through evasion of their exacerbation trigger (Page, 2012) in addition to treatment with either inhaled or systemic corticosteroids that could be combined with long acting β2 agonists (LABA). Other medications such as leukotriene-receptor antagonists (LTRAs), slow-release theophylline, and long-acting muscarinic antagonists (LAMAs). The major issue with steroidal therapy is the development of hyporesponsiveness, and their limited effect on the progress of airway remodeling.

1.6.1. Biologics implicated in severe eosinophilic asthma

Recently, biological treatments that target IL-5 and its axis are gaining increasing interest in treating asthma cases of reduced response to classical therapy as they can overcome some of the limitations of current therapies (Manuyakorn et al., 2013). Table 2 summarizes the monoclonal antibodies that are currently available clinically to treat and manage asthma.

Table 2 Monoclonal antibodies that are used to block IL-5/IL-5Ra

Therapeutic Antibody	Target	Prescribed dosing	Main effects	Reference
Mepolizumab	IL-5	Up to 750mg	Low blood and sputum eosinophil, lower exacerbations, reduced doses of OCS, Improved lung function, maturation arrest of eosinophils	(Bel et al., 2014; Flood-Page et al., 2003; Menzies-Gow et al., 2003; Numata et al., 2019; Ortega et al., 2014; Pavord et al., 2012)
Reslizumab	IL-5	3mg/kg	Improved lung function, lower exacerbations in eosinophilic asthma, lower levels of blood and sputum eosinophils.	(Castro et al., 2011; Deeks & Brusselle, 2017; Lim & Nair, 2015; Reslizumab (Cinqair), n.d.)
Benralizumab	IL-5Ra	30mg	Low eosinophil infiltration, reduced exacerbation, improvement in FEV1, improved overall quality of life.	(Castro et al., 2014; Committee, 2018; FitzGerald et al., 2016; Kolbeck et al., 2010; Ricciardi et al., 2022)

1.6.2.1 Mepolizumab

Mepolizumab is a humanized monoclonal antibody that was raised against IL-5 preventing its binding to its IL-5Ra subunit, making it the first add-on targeted therapy to be approved in U.S in 2015 for antagonizing IL-5 to manage severe eosinophilic asthma cases aged ≥ 6 years old (Menzella et al., 2015; Poulakos et al., 2017).

1.6.2.2 Reslizumab

Reslizumab is another humanized monoclonal antibody that is directed against IL-5, it gained the FDA approval in 2016 for managing severe eosinophilic asthma patients aged ≥ 18 years old (Varricchi et al., 2017). It acts similarly to mepolizumab but Reslizumab is restricted only for adults.

1.6.2.3 Benralizumab

Benralizumab is another class of humanized monoclonal antibody that targets IL-5Rα subunit expressed mainly on eosinophils, unlike the above discussed antibodies which were tailored against IL-5 itself. Benralizumab was approved by the FDA in 2017 as an add-on therapy for patients with severe eosinophilic asthma aged 12 years old or above (Ricciardi et al., 2022).

1.6.2. Anti-IL-5/Anti-IL5R proposed mechanism of action

A study using bronchial biopsies from asthmatic patients before and after three doses of Mepolizumab, described a mechanism whereby anti-IL-5 therapy was characterized by

reduction in the ECM) thickness, including expression of tenascin, lumican, and procollagen III in the reticular basement membrane (RBM). Moreover, the reduction in the eosinophils correlated with a reduction in TGF-β in BALF which might explain the reduced rate of airway remodelling (Flood-Page et al., 2003; Mauad et al., 2007). In addition, another mechanistic study mentioned that subjects treated with mepolizumab reduced the expression of β2 integrins that mediate adhesion and migration of circulating eosinophils or the BALF cells to the lung airways in contrast to β1 integrins expression, in addition to blunting the upregulation of PSGL-1 receptor (Johansson et al., 2013). Interestingly, a recent report showed a secondary mechanism of using anti-IL-5 mAbs whereby mepolizumab and reslizumab upregulated miR-338-3p levels post administration (Rial et al., 2021). Benralizumab has been shown to reduce airway eosinophilia through the enhancement of antibody dependent cytotoxicity (ADCC) which induces eosinophilic apoptosis (Kolbeck et al., 2010) (**Figure 1.7**). Despite the available reports and clinical trials in this field, the mechanism of action of these biologics needs further elucidation to derive a comprehensive picture of its effect on the various cell types in the airways.

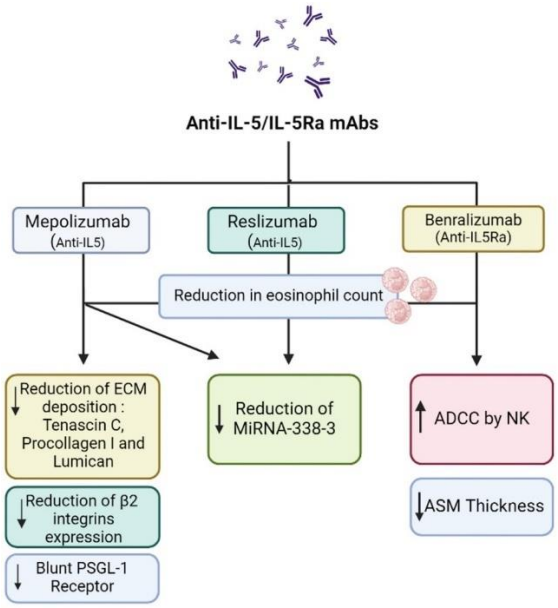


Figure 1.7 Proposed mechanisms of action that have been reported upon administration of IL-5/IL-5Ra biologics. (AbuJabal et al., 2024)

1.7. IL-5 and IL-5 Receptor

1.7.1 Interleukin-5

Interleukin 5 (IL-5), previously named as T cell-replacing factor (TRF), is a member of the group of hematopoietic cytokines (Tominaga et al., 1988). The IL-5 coding gene is located on chromosome 5 in a gene cluster near to the genes encoding IL-4, IL-3 and GM-CSF family of cytokines (A. T. C. Kotsimbos & Hamid, 1997; Takatsu & Tominaga, 1991). Structurally, IL-5 is a homodimer glycoprotein with a motif composed of four helix bundle. IL-5 is known to consist of 134 amino acids with a molecular weight of 40-50 kDa and is majorly produced by activated Th2 cells (Ying et al., 1993), mast cells, eosinophils, CD34+ progenitor cells, natural killer (NK) and ILC-2. As a result, IL-5 is a potent driver and promoter of type 2 immunity (Bagnasco et al., 2017). Initially it was thought that IL-5 acts on B-cell precursors to induce immunoglobulin switching from IgM to IgA in vitro, but a few studies showed no functional effect of IL-5 on the human B cells (Mahanty & Nutman, 1993; Tominaga et al., 1988). It was then found that IL-5 is strongly correlated with eosinophilia by acting mainly on cells of eosinophilic origin it stimulates their growth and differentiation, enhances their survival and promotes the release of their granular contents at the site of inflammation (Weltman & Karim, 2000). Moreover, IL-5 was shown to play a role in stimulating basophils by inducing the release of histamine and generation of leukotriene C4 from basophils in allergic conditions (Mahanty & Nutman, 1993).

1.7.2 IL-5 Receptor

IL-5 acts on their target cells through interaction with their IL-5 receptor, which is a complex heterodimer that consists of two subunits IL-5Rα (60 kDa) and IL-5Rβ (130 kDa). While the former is specific for IL-5 signalling, the latter subunit is shared among IL-3 and GM-CSF receptors since they share functional homology. (Dougan et al., 2019; Kotsimbos & Hamid, 1997a; Tominaga et al., 1988). Previously, it was shown that IL-5 binds to its specific IL-5Rα in a low affinity binding manner, and the Bc subunit mediates high affinity binding that promotes the intracellular signaling (Sakamaki et al., 1992). In contrast, *Scibek JJ* et al. showed that the alpha subunit mediates high binding affinity with low capacity to initiate a signal by their own and the Bc subunit is more involved toward intracellular signaling (Scibek et al., 2002) indicating that the dimerization of both subunits is a must for a signal to be initiated. In humans, IL-5Rα (CD125) is expressed in its membranous form exclusively on blood cells such as eosinophils and basophils. The expression of IL-5R on human B cells is still debatable (Adachi & Alam, 1998). However, it has been reported that functional IL-5Rs are expressed

on non-hematopoietic cells such as airway epithelial cells as well as on lung fibroblasts (Bajbouj et al., 2023) suggesting its potential role in lung disorders such as asthma (Barretto et al., 2020)

It has been reported that IL-5Rα is present in two isoforms, the transmembrane (TM-IL-5Rα) and soluble forms (sIL-5Rα), via the alternative splicing of the mRNA coding IL-5Rα (Byström et al., 2006; Kotsimbos AT, 1997). When it comes to the function of each form of the receptor, the transmembrane form mediates the signal transduction in response to ligand binding, while the soluble form is suggested to act as neutralizing receptor blocking the actual effect of IL-5 (Byström et al., 2006) However, what triggers the alternative splicing is still unknown.

1.7.3 IL-5/IL-5Ra signalling

Cognate binding of IL-5 to its specific α receptor subunit will enhance the recruitment of the common beta subunit, thus inducing the dimerization of both α and β subunits. This in turn will activate a series of downstream events to further transduce an intracellular signal and modulate gene expression (Adachi & Alam, 1998; A. T. Kotsimbos & Hamid, 1997). Since IL-5R doesn't harbour any intrinsic tyrosine kinase activity, the signal will be amplified through interaction with a juxta-membranous kinases, mainly the Janus kinases (JAKs) including JAK1, JAK2 and Lyn, that will phosphorylate tyrosine residues providing docking sites to recruit the signal transducer and activator of transcription (STAT) factors such as STAT1 and STAT5. Upon dimerization, the STATs translocate to the nucleus and activate genes related to other signaling pathways including MAP kinase, Btk and PI3K pathways as well as NF-kb (Ishino et al., 2005; Pelaia et al., 2019; Schwartz et al., 2015) as shown in **Figure 1.9**. The activation of these pathways further promotes the proliferation and survival of the corresponding cells, thus inhibiting any cellular apoptotic signaling (Takatsu, 2011).

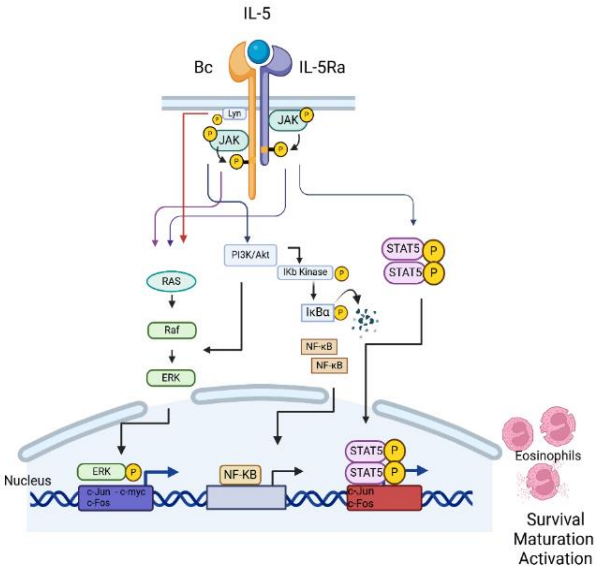


Figure 1.9 Major pathways activated by IL-5 signaling, all of which led to the recruitment, activation, maturation and survival of eosinophils to the airway.

1.8. Role of IL-5 and IL-5R in asthma pathophysiology

Considering that IL-5 is a potent pro-inflammatory cytokine, its elevation will mediate the recruitment of eosinophils to the airways and its subsequent maturation, activation, differentiation and survival, eosinophil will further be synergized with other chemokines such as eotaxin (CCL11) (Fulkerson & Rothenberg, 2013) along with upregulation of the integrin CD11b (Dougan et al., 2019).

Activated eosinophils will secrete IL-5 in an autocrine fashion acting as a positive feedback loop, thereby recruiting and activating more eosinophils to the airway wall resulting in a condition called eosinophilic asthma with increased severity of airway hyperresponsiveness. The link between IL-5 and eosinophils in the pathogenesis of asthma is well established (Danahay et al., 1999; Hogan et al., 1997; Sugita et al., 2003), and knocking out IL-5 or its successful blockade eliminated the elevated levels of eosinophils in the bronchi and reduced the asthmatic exacerbations in different tested mouse models of asthma (Leckie et al., 2000; Tanaka et al., 2004). IL-5 is mainly involved at the later-phase of the immune response where eosinophils play a more active role. Interestingly, higher levels of IL-5 in both the serum and bronchoalveolar lavage fluid (BALF) are confined to severe asthmatic patients whereas mild and moderate cases were shown to have lower levels of IL-5 (Greenfeder et al., 2001).

Accumulation of eosinophils from the circulation to the airway will exert their effect via secreting their mediators including Basic Granule Proteins such as BMP, fibrogenic/growth factors such as TGF-α/β and some other cytokines such as IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, and chemokines such as CCL5 and CCL11 (Hogan et al., 2008; Kay A Barry, 2005). This will contribute to damaging the airway structural cells, airway hyperresponsiveness and bronchial obstruction, airway remodelling and overall asthma exacerbations (Eng & Defelice, 2016; Gleich, 2000; Hogan et al., 2008).

1.9. Potential role of IL-5 in airway remodelling

Recent reports are shedding new light on the role of IL-5 in airway remodelling in asthma, below are some of the observations which were reported either In vivo or in human clinical trials. Cho and his colleague showed in their OVA-induced asthma mouse model that with IL-5 knocked out, two cardinal features of airway remodeling, namely peri-bronchial fibrosis and thickening of the peri-bronchial smooth muscle layer, were lacking when compared to their WT counterparts. The reduction in bronchial fibrosis was observed in terms of reduced collagen deposition in the lung (Cho et al., 2004). The same study showed that IL-5 deficient mice that were challenged with OVA repetitively have less eosinophils infiltrating the bronchial tissue along with low TGF-β expression. Another in-vivo study showed how targeting IL-5 using neutralizing antibodies would interfere with airway remodeling by suppressing the rate of subepithelial fibrosis and ameliorating goblet cell hyperplasia. This reduction in airway remodeling was attributed to the low infiltration of eosinophils into the airways and reduced release of their inflammatory mediators at the site of inflammation (Blyth et al., 1996).

Furthermore, a study conducted on asthmatic patients further signified the above in vivo findings, whereby patients with asthma intravenously injected with three doses of Resilizumab once monthly was sufficient in depleting sputum eosinophils (Castro et al., 2011). All patients involved in the study demonstrated reduced TGF-β1 expression and reduced ECM proteins in their bronchial tissue. There was also a strong correlation between reduced eosinophil count and tenascin deposition in particular and ECM proteins in general, and this effect may be mediated directly by neutralizing IL-5 albeit with an unknown mechanism (Flood-Page et al., 2003), Based on the above observations, the prolonged use of such targeted biological therapies could reverse the level of airway remodeling making it a reversible or semi reversible feature of asthma disease by which the patient could attain true remission.

Interestingly, a recent observation by Barretto et. al showed a functional IL-5Ra expression on AEC, which supports a direct role of IL-5 on airway structural cells as well as airway remodelling, this is mainly supported by to the down regulation of the cell adhesion molecules mainly *CDH1* (E-cadherin), Caveolin-1 and −2 (*CAV1*, *CAV2*), epidermal growth factor receptor (*EGFR*), which results in alteration of the epithelial layer integrity a major feature of airway remodelling (Barretto et al., 2020). Neutralizing IL-5 is suggested to improve the epithelial barrier integrity. At the same time, it should be warranted that using IL-5R neutralizing antibodies might induce cell mediated cytotoxicity similarly to what has been reported against other cells such as leukocytes (Barretto et al., 2020). In addition, human lung fibroblasts isolated from asthmatic and normal subjects express functional IL-5R, with higher expression levels in asthmatic tissues when compared to normal subjects. Stimulating these cells with recombinant IL-5 was shown to induce their proliferation (Bajbouj et al., 2023). Taking the observation of Flood-Page and his colleagues (Flood-Page et al., 2003) along with this finding, brings to the forefront the question of whether IL-5 has a direct potential role in fibrosis (**Figure 1.10**).

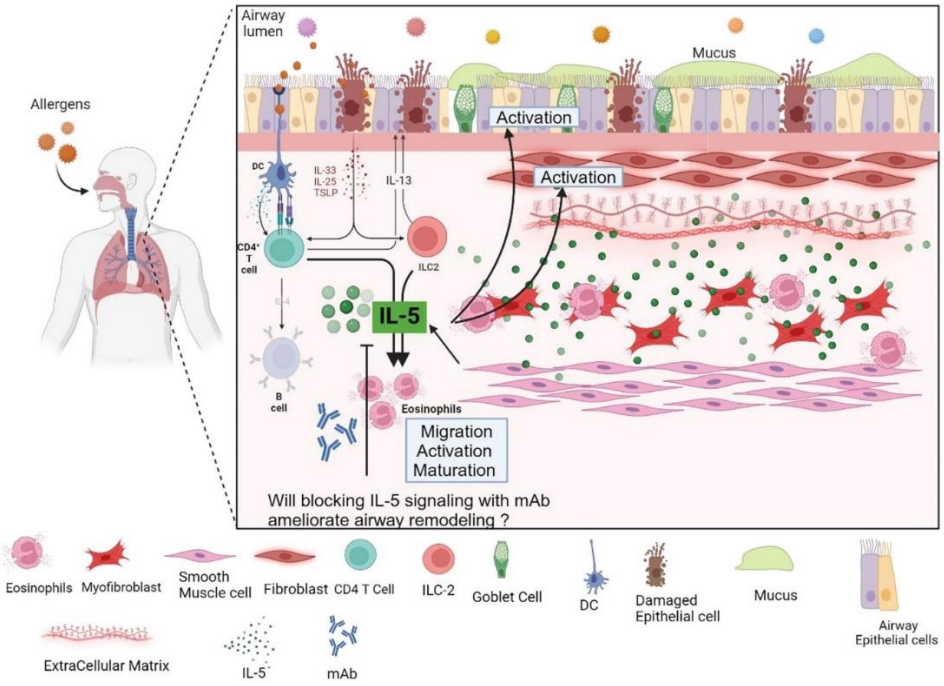


Figure 1.10 Complexity of the remodeling process and the new potential role of il-5 in its pathogenesis and how inhibiting the il-5/il-5ra could be involved in therapeutic purposes (AbuJabal et al., 2024)

1.10. Rational

Severe asthma, particularly in patients characterized with eosinophilic inflammation who respond poorly to corticosteroid therapy, presents a significant clinical challenge with few effective treatment options currently available. These patients often experience persistent airway inflammation, leading to progressive structural changes, or "airway remodelling". Airway remodelling is mainly characterized by fibrosis, increased smooth muscle mass, epithelial layer detachment and mucus gland hyperplasia.

The cytokine Interleukin-5 (IL-5) is known to play a critical role in the survival, activation, and recruitment of eosinophils, a key inflammatory cell type in eosinophilic asthma. IL-5 is widely secreted by immune cells, including CD4 Th2, ILC-2, and eosinophiles. Given IL-5's involvement in the pathogenesis of eosinophilic inflammation, there is growing interest in its potential to directly promote fibrosis within the airway.

Investigating IL-5's direct effects on lung fibroblasts and extracellular matrix production could shed light on how this cytokine contributes to airway remodelling independent of its effects on eosinophils. This highlights IL-5 as significant target to combat or mitigate airway remodelling progression through the use of the current biologics that tackle the IL-5/IL-5Ra signalling axis.

1.11. Hypothesis

We hypothesized that IL-5 directly contributes to tissue remodeling in severe asthma by directly affecting lung fibroblasts.

1.12. Aims and Objectives

Aim 1: **Assess the baseline expression of IL-5Ra on both severe asthmatic and normal derived fibroblasts**.

Objectives 1: Determine the baseline expression of IL-5Ra in fibroblasts obtained from asthmatic and normal subjects.

Objectives 2: Confirm the expression of IL-5Rα in tissues obtained from asthmatic and normal subjects.

Aim 2: Characterize the effect of IL-5 stimulation on primary human lung healthy and asthmatic fibroblasts

Objective 1: Investigate the effect of IL-5 stimulation on the IL-5Ra expression in lung derived fibroblasts

Objective 2: Identify the transcriptional landscape in human asthmatic fibroblasts and compare to healthy control upon exposure to IL-5 using next-generation sequencing (NGS).
Objective 3: Validate the involvement of the identified IL-5 regulated genes in asthmatic and healthy fibroblasts using functional assays for proliferation and survival.
Objective 4: Examine the effect of IL-5 on the expression levels of the remodeling markers in fibroblasts isolated from severe asthmatic patients compared to control.

Aim 3: Evaluate the effect of IL-5 inhibition on reversing IL-5-stimulated changes in an *in-vitro* model.

Objective 1: Investigate the inhibition of IL-5 on the proliferation and survival rate of fibroblasts obtained from asthmatic subjects.
Objective 2: Assess the effect of anti-IL-5 and anti-IL-5R to reduce the expression level of remodeling markers in the asthmatic lung fibroblasts.

Aim 4: Explore the role of IL-5 in airway remodelling in an ex vivo lung murine system.

Objective 1: Characterize the expression of IL-5Ra in isolated fibroblasts from murine lung.
Objective 2: Evaluate the effect of IL-5 on the proliferation rate of the isolated fibroblasts using the incucyte incubator.
Objective 3: Evaluate the expression of fibrotic related genes in response to murine IL-5 (mIL-5) in isolated fibroblasts from murine lung.
Objective 4: Evaluate the role of IL-5 in airway remodelling in an ex-vivo system using the Precision cut lung sections (PCLS).

Chapter II.

Methodology

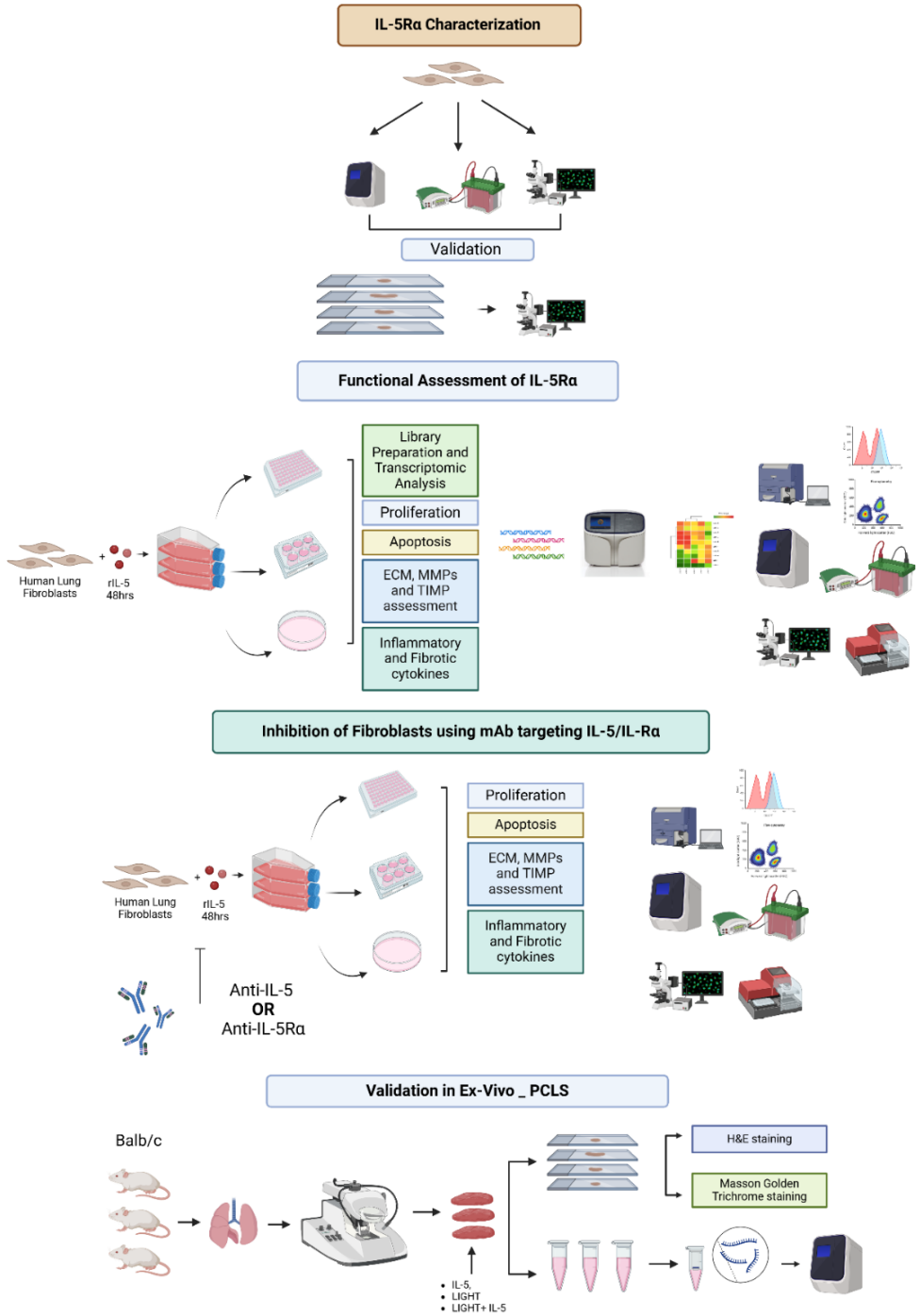


Figure 2.1 Experimental design summary (generated by Biorender)

2.1 Ethical Approvals

This study received multiple ethical approvals. Endobronchial biopsies were conducted following a study protocol approved by the Dubai Scientific Research Ethics Committee (Approval No. DSREC-11/2017_04). Mice used for in vitro and ex vivo experiments were kept in the specific-pathogen-free (SPF) animal facility of the University of Lübeck. Female and male mice were used ranging from 2-17 months of age. For the removal of the small intestine and/or colon, the life mice were handled by authorized personnel and sacrificed according to the appropriate institutional and national guidelines. All organs were harvested according to European (EU directive 2010/63) and German laws and approved by the Schleswig Holstein state authorities (Approval No. 3_2024-01-09_Laumonnier).

2.2 Human Cell Culture

Human primary lung fibroblasts isolated from both normal and asthmatic subjects used in this study were purchased from Lonza . Table 3 summarizes the subject characteristics used in this study. Cells were maintained in Gibco Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) (ThermoFisher Scientific) supplemented with 15% fetal bovine serum (FBS, Sigma Aldrich), 1% Penicillin/Streptomycin (ThermoFisher Scientific). Cells were grown in a 37° C humidified incubator (Thermo-Scientific HERA cell 150i Carbon Dioxide Incubator) containing 5% CO2 and 21% oxygen.

Table 3 Clinical characteristics of the subject involved

	Healthy	Asthmatic
	n=3	n=3
Mean Age (Years), (SD, Range)	58 (10.6, 25)	43 (1.6, 27)
Gender (M%:F%)	67:33	33:67
Ethinicity , Caucasian	100%	100%
BMI (Kg/m2) Mean (SD,range)	39.9 (1.6, 4)	39.9 (4.7, 10)

2.3 Cell Treatment/Stimulation:

Human Primary lung fibroblasts were seeded in different tissue culture plates, size of the plate being decided based on the experimental needs. After reaching 70 % confluency, cells were starved overnight using DMEM/F12 media supplemented with 0.1 % FBS and 1 % P/S. The next day, the starvation media was removed, and cells were stimulated with rh-IL5 (R&D), at an increasing concentration to determine the optimal concentration in complete DMEM/F12 medium. For anti-IL-5 treatment, 0.4 µg/ml of mepolizumab were used, referring to the IC50 used to target eosinophils (NA, 2008)

2.4 MTT Assay:

Human lung fibroblasts obtained from healthy and asthmatic subjects were seeded in 96 well plate at a density of 4 x $10^{3,}$ cells were left to reach 50% confluency and starved overnight using 0.01% DMEM-F12 media. Cells then were treated with increasing concentrations of hr-IL-5 for 24, 48 and 72 hrs. MTT (Sigma Aldrich-USA M5655) was prepared freshly by dissolving 50mg of MTT powder in 10ml PBS (Sigma Aldrich-USA D8537). To each well, 10 µl was added using multi-channel micropipette and plates were tapped to ensure mixing of the reagent. Cells were incubated with MTT for two hours in incubator supplemented with 5% CO_2. After that, the media was removed and the formazan was dissolved by adding 100 µl of DMSO (Sigma Aldrich, USA - D8418). The plate was incubated for 5 minutes on a plate shaker, protected from light. The plate was read using ELISA reader machine, at 570nm wavelength.

2.5 RT-PCR analysis from human fibroblasts

Asthmatic and healthy fibroblasts were seeded at 0.5-1 × 10^5 cells / mL in a 6 well cell culture plate and kept till ~70% confluency. Cells were then stimulated with rh-IL5 for 6 hours, collected and washed with ice cold PBS for RNA extraction. RNA extraction was carried on using Trizol (Sigma-Aldrich) following the manufacturer protocol. To determine the gene expression levels in fibroblasts, cDNA was synthesized from 1 µg of total RNA using the High-Capacity cDNA synthesis kit (Invitrogen) according to the manufacturer's instructions. RT-PCR was performed using the complementary DNA (cDNA), Verti thermal cycler machine was used as a thermal cycler, and the following specific primers for the used genes are provided

in the below table 4. HOT FIREPol® EvaGreen® qPCR Mix Plus (solisbiodyne Cat: 08-24-0000S) was used, Quantstudio 5 was used for RT-PCR,. Expression levels of the studied genes were normalized to 18S expression as DCt and fold changes were calculated as $2^{-(\Delta\Delta Ct)}$.

Table 4 Showing the Sequence of the primers used in the RT-PCR

Gene Name	Forward	Reverse
IL5RA	5′-CACACGCTGACTGTACTTGCAC-3′	5′-GGGCATTGAGAACGAACCTTA-3′
COL1A1	5'-GATTGACCCCAACCAAGGCTG-3'	5'-GCCGAACCAGACATGCCTC-3'
COL3A1	5'-GATCAGGCCAGTGGAAATG-3'	5'-GTGTGTTTCGTGCAACCATC-3'
COL5A1	5'-GTCGATCCTAACCAAGGATGC-3'	5'-GAACCAGGAGCCCGGGTTTTC-3'
FN1	5'-CTGGGAACACTTACCGAGTGGG-3'	5'-CCACCAGTCTCATGTGGTCTCC-3'
IL6	5'-GAAAGCAGCAAAGAGGCAC-3'	5'-GCACAGCTCTGGCTTGTTCC-3'
TGFb	5'-TACCTGAACCCGTGTTGCTCTC-3'	5'-GTTGCTGAGGTATCGCCAGGAA-3'
IL11	5’ GGACCACAACCTGGATTCCCTG-3’	5’ AGTAGGTCCGCTCGCAGCCTT-3’
TNF	5'-CTCCTACCCGAACAAGGTCA-3'	5'-CGGTCACCCTTCTCCAACT-3'
LUM	5'-AACATACCAACTGTCAATGAAAACC-3'	5'-TGCCATCCAAACGCAAATGCTTG-3'
TNC	5'-TCCCAGTGTTCGGTGGATCT-3'	5'-TTGATGCGATGTGTGAAGACA-3'
MMP7	5'-GCTGGCTCATGCCTTTGC-3'	5'-TCCTCATCGAAGTGAGCATCTC-3'
MMP2	5′-CTCTCCTGACATTGACCTTGGCAC-3′	5′-AAAAAGCTTACTCGCTGGACATCAG-3′
MMP3	5’AAACTCCCGCGTCATAGAAA-3’	5’- TGAGTCAATCCCTGGAAAGT-3’
MMP9	5′-GGCATCCGGCACCTCTATGGTCC-3′	5′-GCCACTTGTCGGCGATAAGGAAGG-3′
TIMP1	5'-AGTCAACCAGACCACCTTATACCA-3'	5'-TTTCATAGCCTTGGAGGAGCTGGTC-3'
TIMP2	5'-TGCAATGCAGATGTAGTGATCAGGG-3'	5' -GCTTATGGGTCCTCGATGTCGAGA-3'
FOXO1	5’ AAGAGCGTGCCCTACTTCAA 3’	5’ GCACACGAATGAACTTGCTG 3’
NR4A1	5’ ACTGCCAAACTGGACTACTC 3’	5’ AGAGCAGGTCGTAGAACTG 3’
18s	5'-TGACTCAACACGGGAAACC-3'	5'-TCGCTCCACCAACTAAGAAC-3'

2.6 Western Blotting

Cellular lysate of Human fibroblasts derived from normal and severe asthmatics subjects was used to determine and confirm protein levels of the genes of interest. Cells were lysed using ice-cold RIPA buffer (Cat: ab156034 Abcam) supplemented with protease cocktail inhibitor tablets (Sigma-Aldrich). Whole cell lysate protein concentrations were quantified using the Pierce BCA Protein Assay Kit. Lysate aliquots containing 30µg µg of protein was separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a PVDF (Bio-Rad) activated with Methanol. The membrane was blocked by 5% skimmed milk powder for 1 h at room temperature, washed with (1xTBST), and reacted with primary antibodies. overnight at 4°C as listed in table 5. The blot was then incubated with secondary (anti-mouse and anti-rabbit) antibodies (Cat: 7076S and 7074S Cell Signaling Technology) at 1:1000 dilutions for 1 h at room temperature. Chemiluminescence was detected using the ECL kit (Cat. 170-5060 BioRad). Protein band quantification was carried out using the Bio-Rad Image Lab software (ChemiDoc™ Touch Gel and Western Blot Imaging System; Bio-Rad). Tubulin was used as a normalization control.

Table 5 Summary of the primary Antibodies used for protein detection

Protein	Description	Dilution
IL-5Ra	Mouse Molyclonal, R&D, Cat: AF-253-NA	1:500
Collagen I/COL1A1	Rabbit Monoclonal, CST, Cat No. 72026S	1:1000
Collagen III/COL3A1	Rabbit Monoclonal, CST, Cat No.66887	1:1000
Collagen V/COL5A1	Rabbit Monoclonal, CST, Cat No.86903	1:1000
Fibronectin /FN1	Rabbit Monoclonal, CST, Cat No26836	1:1000
Tenascin C	Rabbit Monoclonal, CST, Cat No. 33352S	1:1000
Lumican	Rabbit Monoclonal, abcam, 168348	1:1000
MMP-2	Rabbit Monoclonal, CST, Cat No. 40994	1:1000
MMP-3	Rabbit Monoclonal, CST, Cat No. 14351	1:1000
MMP-7	Rabbit Monoclonal, CST, Cat No. 3801	1:1000
MMP-9	Rabbit Monoclonal, CST, Cat No.13667	1:500

TIMP-1	Rabbit Monoclonal, CST, Cat No. 8946	1:1000
TIMP-2	Rabbit Monoclonal, CST, Cat No. 5738	1:1000
Tubulin	Mouse Monoclonal, CST, Cat No, 2144	1:1000

2.7 Immunofluorescence

Healthy and severe asthmatic cells were seeded at 2 x 10^4 cell/ml and left to reach 70% confluency. Cells were harvested and washed twice with PBS, cells then were fixed using 4% paraformaldehyde for 20min at RT, fixative solution was washed with PBS and cells were permeabilized with 0.01% Triton-PBS for 10 min at RT. Blocking was done using 3%BSA-PBS for 1hr at RT. Cells were reacted against IL-5Rα (Cat: MAB253 R&D) and Vimentin (Cat: 5741 Cell Signaling Technology) primary antibodies at 1:100 dilution overnight at 4C'. Cells were then washed with 1X PBS and reacted with the Alexafluor®488-labeled secondary antibody (Cat: 150077 Abcam) for 1 h at RT; excess reagent was rinsed with 1X PBS. Genomic DNA was stained with 4′,6′-diamidino-2-phenylindole (DAPI) (Cat. No. D1306, Invitrogen) according to manufacturer's instructions. Slides were visualized by confocal microscopy using a Nikon Confocal Microscope (Nikon, Tokyo, Japan). ImageJ software was used to measure the mean florescence intensity.

2.8 Immunofluorescence for tissue sections

Paraffin slides of bronchial biopsy tissues obtained by fibreoptic bronchoscopy from non-asthmatic control subjects archived at the Biobank of the Quebec Respiratory Health Research Network Canada with MUHC REB number BMB-02-039-t were obtained. Severe asthmatic subjects, who fulfilled the American Thoracic Society (ATS) criteria and were taking treatments based on (GINA), were recruited by the treating physician and nurse who obtained written informed consent, from the Severe Asthma Clinic in the Pulmonary Medicine department at Rashid Hospital, Dubai, UAE. The endobronchial biopsies were performed in accordance with a study protocol approved by the Dubai Scientific Research Ethics Committee with approval number DSREC-11/2017_04. The biopsies were collected and embedded as previously described (Ichikawa et al., 2019). Sections from healthy and severe asthmatic individuals were prepared on positive-charged slides and sequentially used for routine hematoxylin and eosin (H&E) staining as previously described (Ichikawa et al., 2019) and for

immunofluorescence. Immunofluorescence staining was carried on after de-waxing and re-hydration to distilled water. Sections was subjected to heat-induced target retrieval using Sodium citrate buffer pH 6.0 for 5 minutes at 750 W followed by 5 minutes at 450 W in a microwave oven then kept cooling down for 1 hour. Permeabilization was carried on using 0.05% triton-PBS for 10 min at RT, slides were rinsed with PBS and blocked with 3% BSA-PBS-Tween for 1 hour at RT. Primary Antibody were diluted in the same blocking buffer, Anti-IL-5Rα (Cat: MAB253 R&D) and Vimentin (Cat: 5741 Cell Signaling Technology) were used as primary antibodies diluted at 1:100 overnight at 4C˚. Slides were then washed with 1X PBS and reacted with the Alexafluor®488-labeled secondary antibody (Abcam) for 1 h at RT; excess reagent was rinsed with 1X PBS. Genomic DNA was stained with 4′,6′-diamidino-2-phenylindole (DAPI) (Cat. No. D1306, Invitrogen) according to manufacturer's instructions. Slides were visualized by confocal microscopy using a Nikon Confocal Microscope (Nikon, Tokyo, Japan). ImageJ software was used to measure the mean florescence intensity.

2.9 Flow cytometric assays

2.7.1 Annexin V staining

Human lung fibroblasts, isolated from both asthmatic and healthy subjects, were seeded in 60 mm culture dishes at a density of $3x10^5$ cells per plate. The cells were placed in a CO_2-supplemented incubator (5% CO_2) for 24 hours to achieve 60%-80% confluency for starvation and treated with hr-IL5 for 48hrs. All plates were then placed on ice. After discarding the culture media, plates were washed twice with ice-cold PBS, followed by the addition of 700 µL of 1x trypsin to each plate. The plates were incubated at room temperature (RT) for 2-3 minutes, gently shaken to ensure even trypsin distribution, then the trypsin was discarded. The plates were further incubated in a 37°C incubator for 6-7 minutes, and cell detachment was confirmed under a microscope. Cells were collected and centrifuged at 1000 RPM at 4°C and washed twice with PBS to remove any remaining media. For apoptosis detection using the Annexin V-FITC Apoptosis Detection Kit (Abcam – ab14085), a master mix was prepared based on the number of samples.

In a 1.5 mL tube, the following components were combined: (5 µL x n) of Annexin V, (5 µL x n) of Propidium iodide (PI), and (190 µL x n) of binding buffer, where **n** represents the number of samples. The mixture was kept protected from light. In a separate 1.5 mL Eppendorf tube, an Annexin V and PI staining mix was prepared for the compensation tubes by adding 5 µL of

Annexin V to 195 µL of binding buffer in one tube, and 5 µL of PI to 195 µL of binding buffer in another. These tubes were kept on ice. Control cells were divided into four tubes for compensation: a US tube to detect autofluorescence, an Annexin V tube with 200 µL of Annexin V stain alone, a PI tube with 200 µL of PI stain alone, and a tube treated like the rest of the samples. For each of the previously collected cell samples, 200 µL of the master mix was added, and the cells were transferred to flow cytometry tubes, applying a gentle vortex to ensure uniform staining. The cells were incubated in the dark for 20 minutes at room temperature (RT). Immediately after, the cells were examined using the BD FACS III flow cytometer, and the data were processed with FlowJo software.

2.7.2 EdU Proliferation detection Assay

Human lung fibroblasts, isolated from both asthmatic and healthy subjects, were seeded in 60 mm culture dishes at a density of 3x10^5 cells per plate. The cells were placed in a CO2-supplemented incubator (5% CO2) for 24 hours to achieve 60%-80% confluency for starvation and treatment. Cell collection was carried on in a similar way as described in 2.7.1

Cell proliferation was measured using the EdU Proliferation Kit (ab219801, abcam) in accordance with the manufacturer's instruction. Post 48hrs of stimulation with 0.5ng hrIL-5 cells were incubated with 10nM of Edu reagent for 4 hrs before being processed. Cells were fixed with 4% formaldehyde and permeabilized as per the kit’s instructions. Afterwards, cells that had been incubated with EdU detection reagent were stained and examined using flow cytometry (BD FACS AriaTM III), while analysis was carried on using FlowJo software.

2.7.3 Intracellular Staining

Human lung fibroblasts, isolated from both asthmatic and healthy subjects, were seeded in 6 well plates at a density of 1x10^5 cells per plate. The cells were placed in a CO2-supplemented incubator (5% CO2) for 24 hours to achieve 60%-80% confluency for starvation and treatment, cells were stimulated with rh-IL-5 for 4hrs, and brefeldin was added one hour before harvesting the cells Cell collection was carried on in a similar way as described in **2.7.1.**

Cell fixation and permeabilization were performed using BD Fix/Perm reagent. The collected cell pellet was resuspended in 100 µL of the Fix/Perm reagent and incubated on ice for 15 minutes, kept in the dark. After incubation, the cells were washed with PBS to prepare them for staining.

Conjugated human antibodies were used, including Anti-TGFβ conjugated with PE, Anti-IL-6 conjugated with APC, and Anti-TNFα conjugated with AlexaFluor 488. The antibodies were diluted at a 1:25 ratio as follows: for each sample, 2 µL of each antibody was added to 46 µL of FACS staining buffer, making a total volume of 50 µL. The cell pellet was resuspended in 50 µL of the staining mix, vortexed thoroughly, and incubated on ice for 30 minutes. After incubation, the cells were spine down and washed with ice-cold PBS to remove any unbound antibodies and then resuspended in PBS. The cells were analysed using the BD FACS Aria III flow cytometer, and the data were processed using FlowJo software.

2.10 Enzyme Linked Immunosorbent Assay (ELISA)

The assays were using commercial kits (Abcam) using the cell culture media supernatent. Sensitivity of TGF-b assay was 18 pg/ml, IL-6 1.6 pg/ml, MMP3 levels 4.4pg/ml, and the MMP-3 activity < 50 µU.

2.11 Next Generation Sequencing (NGS)

2.9.1 RNA Extraction:

Human lung fibroblasts, isolated from both asthmatic and healthy subjects, were seeded in 60 mm cell culture plates at a density of $2x10^5$ cells per plate. The cells were placed in a CO_2-supplemented incubator (5% CO_2) for 24 hours to achieve 60%-80% confluency for starvation and treatment with hr-IL5 for 6 hrs. Cells were trypsinized and harvested for RNA extraction. RNA extraction was carried on using Qiagen RNeasy extraction kit (Germany, Cat: 74104), and quantified using Nano-drop.

2.9.2 Whole transcriptome

Genomic DNA removal from RNA samples was ensured by treating the RNA with Turbo DNase (ThermoFisher Scientific, USA). Next, whole transcriptome sequencing was performed using targeted RNA-Seq with Ion AmpliSeq™ whole transcriptome human gene expression kit (ThermoFisher Scientific, USA). Briefly, cDNA was synthesized using a SuperScript™ VILO™ cDNA Synthesis kit (ThermoFisher Scientific, USA) and amplified using Ion AmpliSeq gene expression core panel primers. The amplified products were subjected to

enzymatic shearing to get amplicons of ~200 bp, then ligated with the adapter and the unique barcodes. Next, the constructed library was purified using Agencourt AMPure XP Beads (Beckman Coulter, USA), quantified using an Ion Library TaqMan™ Quantitation Kit (Applied Biosystems, Waltham, USA), and further diluted to 100 pM, and pooled equally with 16 individual samples. The diluted library was amplified and enriched in Ion Chef System (ThermoFisher Scientific) according to the manufacturer's instructions. The prepared template libraries were sequenced on an Ion S5 XL Semiconductor sequencer using Ion 540 Chip.

2.9.3 RNAseq Data Analysis

As previously described (Alhosani et al., 2024), transcriptome data analysis was conducted using the Ion Torrent Software Suite (version 5.4). Raw sequencing reads were aligned to the hg19 reference genome (GRCh37 assembly) using the Torrent Mapping Alignment Program (TMAP), which is specifically tailored for Ion Torrent sequencing data. To ensure data reliability, a quality control check was performed on the expression of the selected genes including (*COL1A1, FN1, MMP3*). Differentially Expressed Genes (DEG) was performed with the DESeq2 package in R/Bioconductor using R (version 4.2), leveraging the raw RNA-seq data. To maintain specificity and sensitivity, two stage mapping approach were employed including BWA-short, BEA-long and SSAHA, super maximal exact matching as well as the Smith-Waterman algorithm for optimal mapping. Normalization to the raw read counts of the target genes were conducted using the Fragments Per Kilobase Million method. Genes with normalized counts below ten were excluded from subsequent analysis. DEGs were identified based on a significance threshold of $p < 0.05$ and were used for further downstream analysis in the gene set enrichment analysis (GSEA).

2.9.4 Gene Set Enrichment Analysis

The significant DEG were obtained and further analysed to identify the activated and enriched cellular pathways in response to IL-5 stimulation in both normal and asthmatic derived lung fibroblasts using the absolute GSEA (absGSEA) as previously described (Alhosani et al., 2024; Hamoudi et al., 2010). The absGSEA analysis was conducted on expression data using approximately 120,000 annotated cellular pathways from the Broad Institute's database (https://www.gsea-msigdb.org, accessed on 17 December 2024) along with custom-defined pathways from the gene set collections used (C1 to C8). In this study, C5 and C7 gene set

collections were the primary focus to obtain significantly enriched pathways. C5 and C7 represent the gene ontology (which includes biological processes, molecular functions and cellular processes) and the immunological pathways, respectively.

2.9.5 Functional Annotation and Enrichment Analysis

Functional clustering, annotation and pathway analysis was conducted using Metascape (Zhou et al., 2019) (http://metascape.org, accessed on 17 December 2024). The frequently occurring genes generated from the GSEA analysis were used in Metascape to further validate the pathways identified in C5 and C7 results.

2.9.6 CIBERSORTx – Immune cell characteristics exploration

Cell-Type Identification by Estimating Relative Subsets of RNA Transcripts (CIBERSORTx) (https://cibersort.stanford.edu, accessed on 17 December 2024), developed by Newman *et al.,* is a deconvolution algorithm utilizing RNA-seq data to precisely quantify the relative proportions of immune cell types within a complex gene expression dataset, and the averages from raw RNAseq data from each group were utilized to generate the CIBERSORTx results(Newman et al., 2015). The algorithm calculates a *p*-value that reflects the statistical significance of the deconvolution outcomes across all cell subsets, providing a measure of confidence in the accuracy of the results (Newman et al., 2015). The algorithm utilizes gene expression signatures composed of approximately 500 genes. In this study, we employed the original CIBERSORTx gene signature file, LM22, which includes 547 genes and can distinguish 22 human hematopoietic cell phenotypes. These phenotypes encompass seven T cell subtypes, naïve and memory B cells, plasma cells, NK cells, and various myeloid subsets.

2.12 Murine Fibroblast Isolation:

All experiments involving mice were approved by the Akten Zeichen in Germany (3_2024-01-09). Female Balb/c mice were maintained until 8 weeks of age, after which they were euthanized by cervical dislocation. The chest cavity was opened, and all five lung lobes were harvested. In a 6-well plate, the lungs were washed with 5 mL of pure RPMI-1640 medium to remove any residual blood. In another well, a mixture of 2.5 mL pure RPMI-1640, 2 mL Dispase enzyme (Cat: 07913, Stem cell Technology), 50 µL Liberase (Cat: 05989132001,

ROCHE), and 25 µL DNase (Cat: 05952077103, ROCHE) was prepared and used to prime a 70 µm cell strainer.

After washing, the lungs were placed in the strainer, chopped into small pieces, and incubated at 37°C for enzyme activation, with gentle shaking every 15 minutes. The tissues were then thoroughly minced using a 10 mL plunger, and the strainer was washed with 50 mL of complete RPMI-1640 medium, including an additional 25 µL of DNase. Cells were centrifuged at 3500 RPM for 5 minutes at 4°C, after which the supernatant was discarded. Red blood cells were lysed by adding 3 mL of sterile water for 30 seconds, followed by neutralization with 30 mL of PBS. Cells were then centrifuged again at 3500 RPM for 5 minutes at 4°C.

The cell pellet was resuspended in 6 mL of DMEM/F12 medium supplemented with 10% FBS and 1% penicillin-streptomycin. Cells were then seeded in a 6-well plate with 3 mL of the DMEM/F12 medium and incubated with 5% CO2 for 48 hours, without shaking. After 48 hours, media was changed and cells were kept till they reach 70%-80% confluency, then they were plated in a T75 plates for further experimental needs.

2.10.1 Cell Culture:

Murine primary lung fibroblasts isolated from female balb/c, Cells were maintained in Gibco Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) (Cat: 10565018 thermoFisher Scientific) supplemented with 10% fetal bovine serum (FBS, Sigma Aldrich), 1% Penicillin/Streptomycin (thermoFisher Scientific). Cells were grown in a 37°C humidified incubator (Thermo-Scientific HERA cell 150i Carbon Dioxide Incubator) containing 5% CO2 and 21% oxygen. For cellular detachment, plates were incubated with 2ml of Acutase for 5 minutes, then cells were collected with 5 ml of complete DMEM/F12 media and counted for seeding experimental needs.

2.10.2 Cell Treatment:

Murine Lung fibroblasts were seeded in different plates depending on the experiment needs. Cells were treated after reaching 70% confluency with or without the following cytokines: murine LIGHT 100 ng/ml, murine TGF-b 100 ng/ml, murine IL-5 0.5 ng/ml.

2.10.3 Proliferation/viability assay:

Murine lung fibroblasts were seeded in 24 well plate at a density of $20x10^4$ and left to reach 50% confluency, before being treated with the mentioned cytokines. Proliferation was then assessed using an InCucyte SX5 system (Sartorius). Such system is as an automated analysis system allowing for real-time and quantitative monitoring of cell cultures. Runs were performed for 96 hours with a 4 hours interval between each measurement. After image collections, and measures the confluence of each time point, the software calculated the phase area with a created mask. The confluence was evaluated as phase area and shown as a ratio or the percentage normalized to the starting time point of the experiment for comparison. The ratio or percentage shows the proliferation rate over time. Analysis was conducted using Area Under the curve (AUC) through prism.

2.10.4 RT-PCR:

Murine lung fibroblasts were seeded at 0.5-1 × 105 cells/mL in a 6 well cell culture plate and kept till ~70% confluency. Cells then were stimulated with mLIGHT, mTGF-b, m-IL5 for 24 hours, collected and washed with ice cold PBS for RNA extraction. RNA extraction was carried on using KIT. cDNA was synthesized from 1 µg of total RNA using the KIT. RT-PCR was performed using 100ng/µl of complementary DNA (cDNA), the sequence of the primers used is mentioned in **table 6**, Bio-Gener machine was used as a thermal cycler, and the following specific primers for the used genes are provided in the table below. 2x syber green qPCR enzyme was used, Quantstudio 1 was used for RT-PCR. Fold change analysis was done using 2^-ΔΔct.

Table 6 showed the sequence of mouse primers used in the qRT-PCR

Gene	**Forward**	**Reverse**
Col1A1	CCT CAG GGT ATT GCT GGA CAA C	CAG AAG GAC CTT GTT TGC CAG G
FN-1	CCC TAT CTC TGA TAC CGT TGT CC	TGC CGC AAC TAC TGT GAT TCG G
MMP2	CAA GGA TGG ACT CCT GGC ACA T	TAC TCG CCA TCA GCG TTC CCA T
MMP3	CTC TGG AAC CTG AGA CAT CAC C	AGG AGT CCT GAG AGA TTT GCG C
MMP9	TCC CAC TAT ACC TCC CAC GG	AGT CGA ATC TCC AGA CAC GC
TIMP1	TCT TGG TTC CCT GGC GTA CTC T	GTG AGT GTC ACT CTC CAG TTT GC

TIMP2	AGC CAA AGC AGT GAG CGA GAA G	GCC GTG TAG ATA AAC TCG ATG TC
UBC	GAG CCCA GTG TTA CCA CCAA	CAC ACC CAA GAA CAA GCA CA
S14	GAG GAG TCT GGA GAC GAC GA	TGG CAG ACA CCA AAC ACA TT

2.10.5 Flow cytometry:

Murine lung fibroblasts were seeded at a density of $2x10^5$ cells in a 6-well plate and allowed to grow until reaching 70% confluency. The cells were then treated with mLIGHT, mTGF-β, and mIL-5 for 24 hours, after which they were harvested for IL-5Ra expression analysis via flow cytometry.

Following cell harvesting, they were fixed using BioLegend's fixation buffer by resuspending the cells in 100 µL of the buffer and incubating on ice for 15 minutes, protected from light. The cells were then washed with ice-cold PBS and resuspended in the permeabilization buffer (BioLegend), followed by a 15-minute incubation. After washing with ice-cold PBS, the cells were prepared for staining.

anti-mouse CD125 (Biolegend, Cat: 153407) and Anti-Vimentin antibody (Biolegend, Cat: 677813) were diluted in 1x PBS-BSA at a 1:50 ratio, with 2 µL of each antibody added to 98 µL of dilution buffer. The cells were incubated with this antibody mixture on ice in the dark for 30 minutes. After incubation, the cells were washed, resuspended in 1x PBS-BSA buffer, and analyzed using the Attune NxT Flow Cytometer, with the data processed through FlowJo software.

2.13 Precision cut lung sections (PCLS)

Female Balb/c mice were kept until they reached 8 weeks of age, at which point they were euthanized via cervical dislocation. The trachea was exposed and opened for agarose infusion, and the chest was opened to allow room for the lungs to expand during the filling process. Low-melting-point agarose (0.15 g) was weighed and heated with 10 mL of PBS until fully melted, ensuring it did not boil to avoid damaging the lungs during the filling. An 18-gauge cannula was inserted into the trachea, and 1 mL of agarose was carefully injected into the lungs, with lung inflation visually monitored. Once the lungs were properly filled, they were removed from the chest and placed in 50 mL of ice-cold complete media until ready for cutting/slicing.

The vibratome was set up, and each lung lobe was mounted in the machine. Slices were cut at a speed of 0.40 mm/s with a thickness of 350 µm. The lung sections were then placed in a 24-well plate, with each well containing 1 mL of DMEM/F12 (ThermoFisher Scientific), and incubated overnight to allow recovery before treatment.

Precision-cut lung slices (PCLS) were treated for subsequent histological staining and qRT-PCR with the following conditions: mLIGHT (100 ng/mL), mIL-5 (0.5 ng/mL), Anti-IL-5 antibody (1.4 µg/mL), a combination of mLIGHT + mIL-5, and a combination of mIL-5 + Anti-IL-5 antibody (0.5 ng/mL + 1.4 µg/mL).

2.11.1 RNA extraction and qRT-PCR

PCLS were treated as mentioned above, and incubated for 24hrs for gene expression analysis, they were stored at -20C till the time of extraction.

For extraction, the tissues were grinded using tissue grinder, then 350µl of TRizol reagent was added for lysing purposes and incubated for 5 minutes, vortexing was done in between. Then 200µl of chloroform was added, and mixed for 15 seconds, then the whole mix was centrifuged for 15min at 12000xg at 4C, layer separation post centrifugation was observed. To a new 1.5ml Eppendorf tube, the aquas layer which contain the RNA was transferred, and an equal amount of 70% ethanol was added and the extraction was done following the instruction of the Qiagen Kit (Germany, Cat: 74104), and quantified using nano-drop machine, cDNA synthesis was done and qPCR was done using 100ng/µl of the cDNA material, the sequence of the primers used is mention in table 6 amplification was done using syber green enzyme and quantification was done using Quant-studio 1. Analysis was done by calculation the fold change through the $2^{-\Delta\Delta Ct}$, and normalization was done using the S14 as a housekeeping gene.

2.11.2 PCLS processing:

After 48, 72 hours of incubating the tissues with the specified treatment as described in Tissues were collected and kept in 4% formalin as a fixation step for at least overnight, the tissues were transferred to increasing concentrations of Isopropanol starting from 70% isopropanol for at least 2 hours as in the following table 7:

Table 7 Showed the PCLS processing steps prior to the embedding step

Reagent	Percentage	Duration
Isopropanol I	70%	2 hours
Isopropanol II	70%	2 hours
Isopropanol III	70%	Overnight
Isopropanol I	80%	2 hours
Isopropanol I	96%	2 hours
Isopropanol I	100%	Overnight
Isopropanol II	100%	2 hours
Isopropanol III	100%	2 hours
Isopropanol + Paraffin	1 to 1 ratio	Overnight
Paraffin I	100%	2 hours
Paraffin II	100%	2 hours
Paraffin III	100%	Up to the embedding

Tissues were then embedded in paraffin loaded with cassettes and stored in +4C for further cutting, cutting was done at thickness of 3 um using charged slides and stored in the incubator at 37C for further staining procedures.

2.11.3 Tissue staining:

Haematoxylin and Eosin Staining:

Slides were assembled in a rack for de-paraffnaiziation (Re-hydration step), the following steps were carried on for both staining assays, the **table 8** below is summarizing the reagents used:

Table 8 showed the steps of deparaffinization prior to staining

Reagent	Percentage	Duration
Xylene I	100%	5 minutes
Xylene II	100%	5 minutes
Xylene III	100%	5 minutes
Ethanol I	100%	3 minutes
Ethanol II	100%	3 minutes

Ethanol	96%	3 minutes
Ethanol	80%	3 minutes
Ethanol I	70%	3 minutes
Ethanol II	70%	3 minutes
Distilled water		Dipping

After the rehydration steps were done, the rack loaded with slides was dipped in the haematoxylin reagent container for exactly **1 minute**, then the rack was transferred to the distilled water container, then the rack was left with a running tap water for 10 minutes, then the rack was placed in the Eosin container for 30 second that was activated with absolute acetic acid prior to the staining process, slides were kept in distilled visualized under the microscope observing orange reddish colour before heading to de-hydration steps. Rehydration steps were carried on as the following table 9:

Table 9 showing the de-hydration steps prior to mounting

Reagent	Percentage	Duration
Ethanol I	70%	15 dips
Ethanol II	70%	15 dips
Ethanol	80%	15 dips
Ethanol	96%	15 dips
Ethanol	100%	15 dips
Ethanol	100%	15 dips
Xylene I	100%	5 min
Xylene II	100%	5 min
Xylene III	100%	up to 1 hour till the mounting step is done

After that slides were mounted using mounting media (sigma) and left under the chemical hood till they dry out.

Masson Goldner Trichrome staining:

Trichrome staining kit is used to evaluate the connective tissues, and it was used to assess the level of collagen deposition in the airways. The staining was done following the Masson Goldner Trichrome Staining kit (Cat#: 3459, ROTH) instructions. Briefly, all the provided reagents were filtered using filter paper prior to use. De-paraffinization steps were carried on as mentioned in **2.13.3.1.**

Staining was started with Iron haematoxylin solution (the mix of A+B reagent in 1:1 ratio) for 3 minutes, then the slides were washed in a flowing tap water for 10 minutes, then stain with Goldner's stain I for 10 minutes, then the slides were rinsed with 1% acetic acid for 30 second, then slides were stained with Goldner's stain II for 8-10 minutes, one more rinse with a fresh 1% acetic acid for 30 seconds was done, then counterstaining was done with Goldner's stain III for 10 minutes, Then the slides were washed with 1% acetic acid solution by dipping the slides into the container and take it out. Dehydrating was done using an ascending alcohol series including 70%, 96% and 100% of Ethanol, then mounting was done with mounting media from (Sigma) using a proper coverslip and kept under the fume hood till drying,

Slides were visualized under the microscope and the blue-greenish colour is what represents collagen deposition (this color comes from the Goldner's stain III).

2.14 Statistical Analysis

Statistical analysis was carried on using Graph-pad Prism 8.0. After ensuring a normal data distribution, the study includes unpaired two tailed t-test, paired student t-test and One-way ANOVA test was, in addition to the multiple comparison between each group was carried on using Tukey's Multiple comparison test, p-value < 0.05 was statistically significant. Microsoft office excel was utilized to obtain the log2FC and *p*-values from the conducted unpaired t-test on the RNA seq raw data to further validate the output from GSEA and gene frequency.

Chapter III.

Results

IL-5 has a potential role in the airway remodelling in asthma, which emerged as an interesting and effective target to control not only lung inflammation but also airway remodeling. In this thesis, we have investigated the baseline level of IL-5Ra in asthmatic fibroblasts and its role in remodeling. We have also examined the effect of anti-IL-5 and anti-IL5Ra in suppressing this process. Our study employed two approaches: *in-vitro* using human primary airway fibroblasts from healthy and asthmatic patients, as well as murine primary lung fibroblasts from healthy mice; and *ex-vivo* using precision-cut lung slices from mice.

3.1 Elevated baseline expression of IL-5Ra in severe asthmatic fibroblasts

The basal expression of IL-5Ra was evaluated in fibroblasts obtained from both normal and asthmatic subjects by qRT-PCR. Data revealed that IL-5Ra was expressed in both cells but in a significantly higher levels in asthma derived cells when compared to their normal counterparts (Figure 3.1A). For further validation of the qRT-PCR, western blot were performed and showed the level of IL-5Ra at the protein level in both asthmatic and normal derived fibroblasts, with the level of IL-5Ra protein significantly higher in asthma cells when compared to their normal counterparts (Figure 3.1B).

Cellular localization of IL-5Ra expression was assessed through immunofluorescence, using Vimentin as a marker for fibroblasts. The mean fluorescence intensity (MFI) indicated significantly higher IL-5Ra expression in asthmatic fibroblasts compared to normal cells (Figure 3.1C and D).

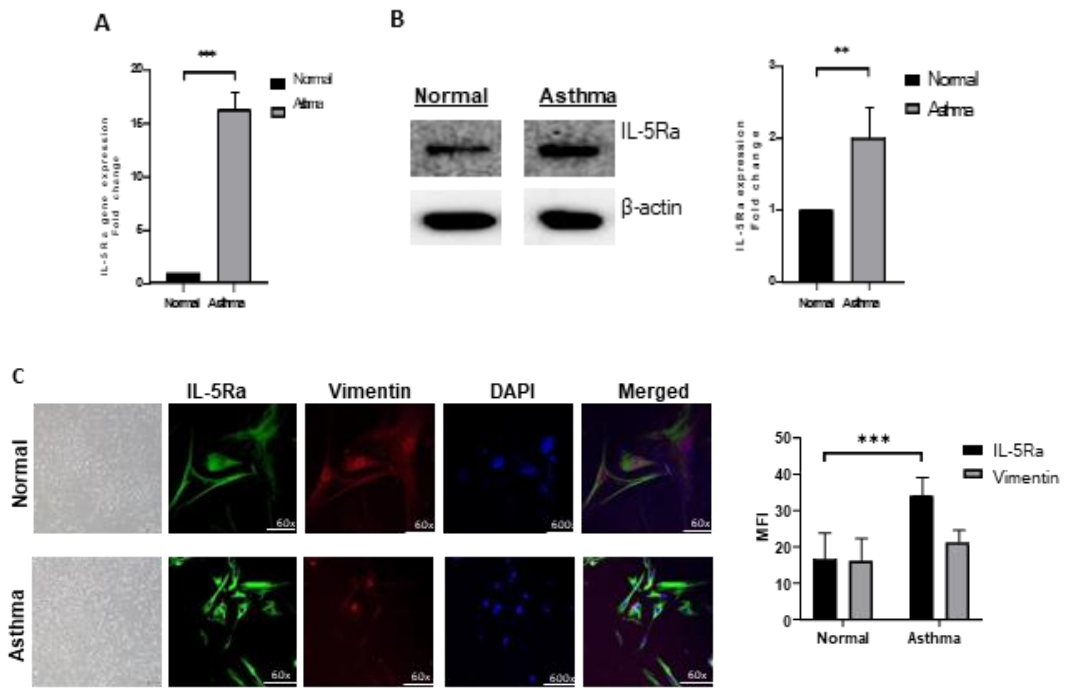


Figure 3.1 Elevated baseline expression of IL-5Ra in severe asthmatic fibroblasts (A) mRNA analysis of IL-5Rα levels extracted from normal and asthmatic lung fibroblasts. Fold change Ct values were calculated after normalization against GAPDH. Significance was determined using unpaired two-tailed Student's t-test from three independent experiments. (B) Western Blot assay was conducted using cell lysate from normal and asthmatic fibroblasts to measure the basal expression of IL-5Rα protein, β-actin was used as a loading control. Graphical data represent calculated mean ± SD fold change in expression levels in normal and asthma fibroblasts based on three separate experiments. Significance was determined using unpaired two-tailed Student's t-test from three independent experiments. (C) Left panel: Cultured fibroblast images with representative images taken at 60X magnification showing immunofluorescence staining of IL-5Rα (green), vimentin (red), DAPI (blue) in normal and asthmatic fibroblasts. Right panel: Mean fluorescent intensity (MFI) of and IL-5Rα and vimentin expression in lung fibroblasts. *Represents statistically significant change $p < 0.05$, **Represents statistically significant change ($p < .01$), ***Statistically significant change $p < 0.001$,

3.2 Higher IL-5Ra expression in lung biopsies obtained from severe asthmatics:

We then analysed IL-5Ra expression in human lung biopsies from severe asthmatic patients (n=3) and healthy controls (n=3) using histochemical and immunofluorescence techniques. Our findings showed positive IL-5Ra expression in the submucosal tissue of both groups. A co-

localization study with Vimentin confirmed that fibroblasts in tissue biopsies, particularly those from asthmatic patients, exhibited higher IL-5Ra expression compared to the healthy controls **(Figure 3.2A and B).**

Our observations revealed that airway fibroblasts express IL-5Ra, with significantly higher levels in asthmatic fibroblasts compared to healthy ones.

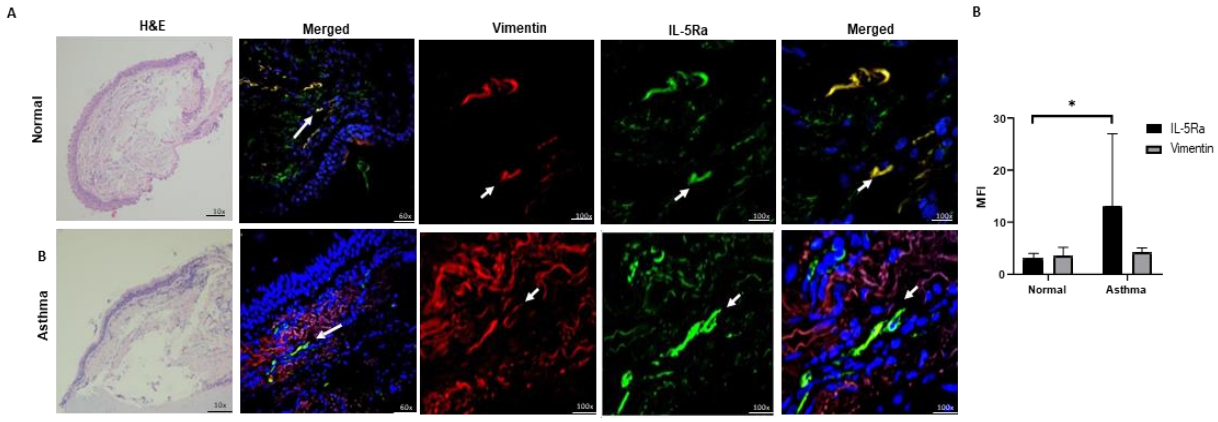
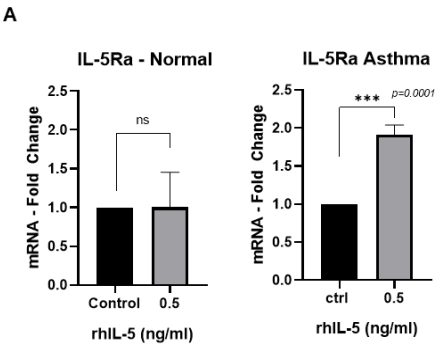


Figure 3.2 Higher IL-5Ra expression in lung biopsies obtained from severe asthmatics (A)Left panel represents images for H&E staining of normal and asthmatic bronchial biopsy tissues (n = 3) taken at 10× magnification and right panel represents images taken at 10× and 60×, and 100× magnification showing Immunofluorescence staining of IL-5Rα (green), vimentin (red), DAPI (Blue) in normal and asthmatic bronchial biopsy tissues, white arrows indicate IL-5Ra-positive fibroblasts. (B) Mean fluorescent intensity (MFI) of IL-5Ra and vimentin expression in bronchial biopsy tissues. *Represents statistically significant change ($p < 0.05$), **Represents statistically significant change ($p < 0.01$), ***Statistically significant change ($p < 0.001$), determined using unpaired two-tailed Student's t-test between IL-5Ra from asthmatic and normal fibroblasts from three independent experiments.

3.3 Expression of IL-5Ra is induced in lung derived fibroblasts upon IL-5 stimulation

To assess the functionality of IL-5Rα, fibroblasts were treated with IL-5 to investigate its regulatory effect on IL-5Rα expression. The literature presents conflicting evidence regarding the role of IL-5 in the regulatory mechanism of IL-5Rα expression, with reports indicating that

it may either upregulate or downregulate its expression in eosinophiles (HELLMAN et al., 2003; Liu et al., 2002a; Tavernier et al., 2000). Limkar and his colleagues, showed that stimulating eosinophils with reduced IL-5 concentration (0.5ng/mL) resulted in a significant increase in IL-5Rα expression (Limkar et al., 2020). To explore whether IL-5 exerts a similar effect on IL-5Rα expression in lung-derived fibroblasts, we assessed IL-5Rα mRNA levels after 6 hours of stimulation by qRT-PCR analysis. Data revealed no effect on IL-5Rα expression in normal lung fibroblasts (**Figure 3.3 A**). However, in asthmatic fibroblasts, IL-5Rα is significantly overexpressed in response to IL-5 stimulation (**Figure 3.3 A**). To validate these findings, an immunofluorescence assay was performed to evaluate IL-5Rα protein levels by measuring the mean fluorescence intensity (MFI) after 24 hours of IL-5 stimulation. Consistent with the mRNA data, normal fibroblasts did not show an increase in IL-5Rα expression, whereas asthmatic fibroblasts exhibited a significant increased expression of IL-5Rα (**Figure 3.3 B-C)**.



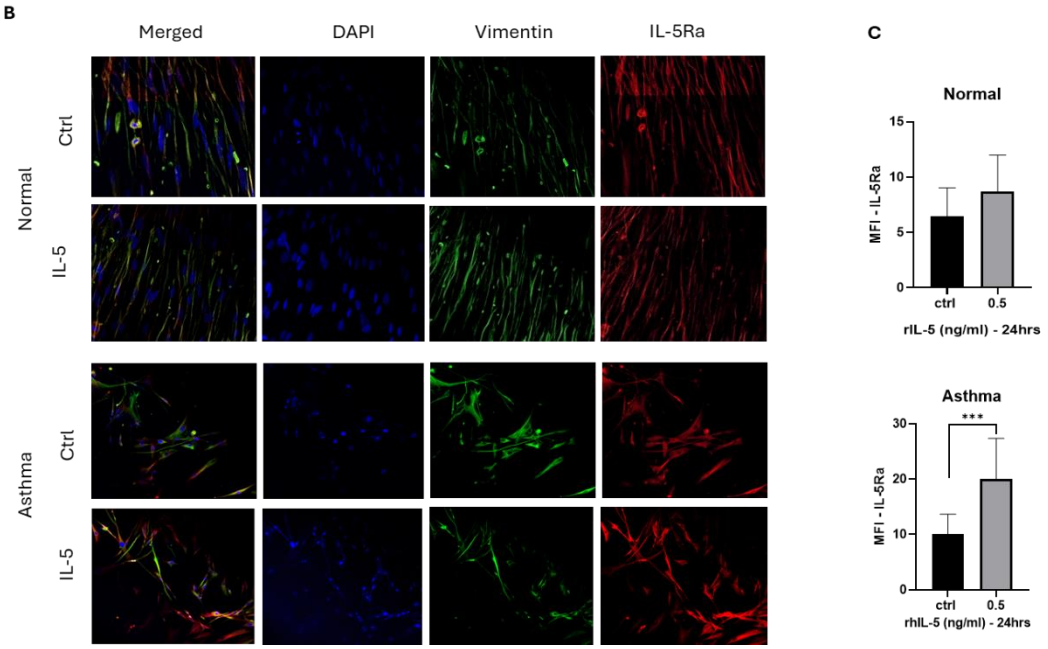


Figure 3.3 The induction of IL-5Ra expression in lung derived fibroblasts upon IL-5 stimulation. (A) mRNA levels of IL-5Rα in both normal and asthmatic derived lung fibroblasts, 18s was used as a housekeeping gene for fold change calculation, Fold change was calculated by 2^-ΔΔCt, data shown is the mean ± SD of four separate experiment; t-Test statistical analysis was done * $p < 0.05$, ** $p < 0.01$. **(B)** Cultured normal and asthmatic fibroblasts, captured at 40X magnification, display immunofluorescence staining for IL-5Rα (green), vimentin (red), and DAPI (blue) in both cell types. **(C)** The mean fluorescence intensity (MFI) of IL-5Rα in lung fibroblasts was quantified following 24 hours of IL-5 stimulation. Statistical significance was indicated as follows: *$p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, determined using an unpaired two-tailed Student's t-test based on data from three independent experiments.

3.4 Stimulation with IL-5 induces distinct transcriptional profiles in lung derived fibroblasts

To assess the landscape-wide effects of IL-5 stimulation on lung-derived fibroblasts, we sought to examine the impact of IL-5 on the gene expression profiles of lung fibroblasts derived from both healthy and asthmatic individuals. To achieve this, RNAseq was performed on RNA extracted from both IL-5-stimulated and unstimulated fibroblasts obtained from both normal and asthmatic subjects.

The differential gene expression analysis revealed distinct transcriptional profiles between IL-5-stimulated fibroblasts from asthmatic versus normal subjects, as well as between unstimulated fibroblasts from both groups. After normalization and filtering, we identified 498

differentially expressed genes between unstimulated fibroblasts isolated from normal and asthmatic fibroblasts, supporting the fact that asthmatic derived cells do exhibit different genetic profile when compared to their healthy counter parts. While 472 differentially expressed genes were identified between normal and asthmatic lung fibroblasts stimulated with IL-5. Among these genes 213 were common between the two conditions, while 284 genes were unique to the unstimulated fibroblasts, and 259 were distinct to the stimulated fibroblasts as shown in **figure 3.4A**. for the comparisons of IL-5-stimulated and unstimulated fibroblasts, respectively, between asthmatic and normal conditions. However, when we compared the number of DEGs of stimulated asthma cells to its unstimulated control there was only 39 DEG while stimulated normal controls to its unstimulated control was giving only 11 DEG, due to the low number of DEGs, these two study groups were excluded from the rest of the downstream analysis. The DEGs are annotated in the volcano plots as shown in **figure 3.4 B** and genes were shown to be significantly differentially expressed in stimulated asthma vs stimulated normal as shown in **figure 3.4 C**, those genes were selected based on the log2FC and gene frequency.

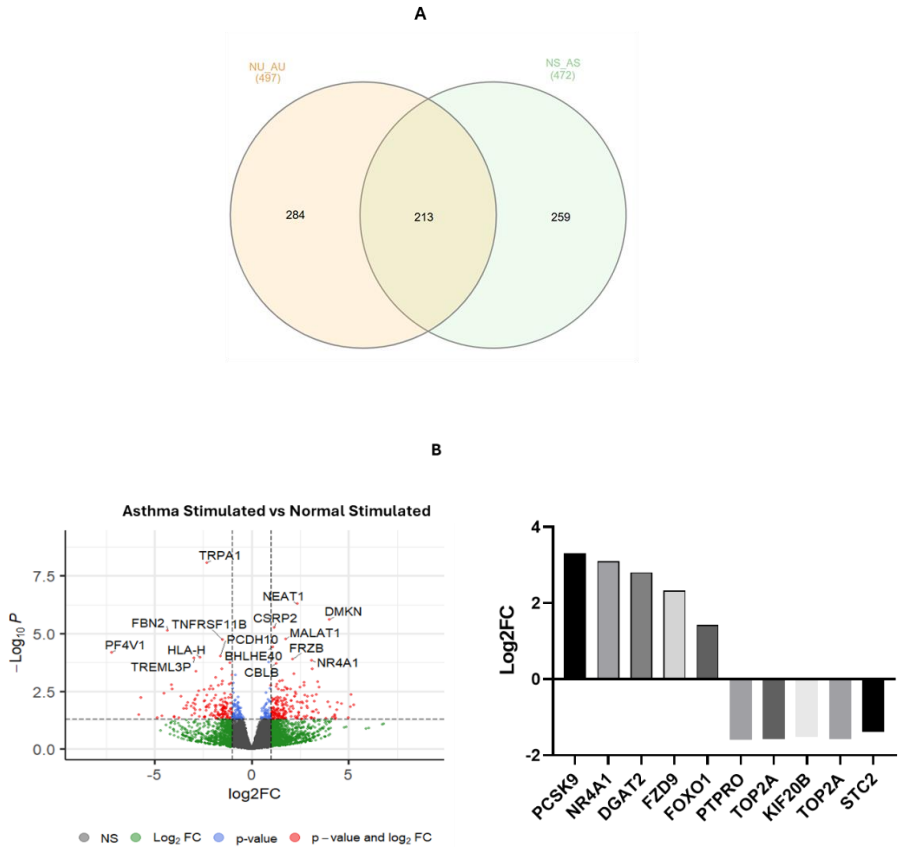


Figure 3.4 Lung derived fibroblasts stimulated with IL-5 exhibit distinct transcriptional Profiles (A)The Venn Diagram figure represents the overlap of the DEGs between the unstimulated lung fibroblasts

and the IL-5 stimulated fibroblasts, highlighting 213 shared genes, and 284 and 259 unique genes to each condition, respectively. **NU**, Normal Unstimulated fibroblasts, **AU**, Asthma Unstimulated fibroblasts, **NS**, Normal Stimulated Fibroblasts **AS**, Asthma Stimulated Fibroblasts. **(B)** Volcano blots of differentially expressed genes between asthmatic and normal derived fibroblasts stimulated with IL-5. Genes that are either differentially expressed in response to IL-5 stimulation based on the obtained Log2FC.

3.5 Gene set enrichment analysis of fibroblasts stimulated with IL-5 revealed an increasing sensitivity to the surrounding stimuli and extracellular matrix organization

To investigate the biological pathways potentially enriched following IL-5 stimulation in asthmatic and normal derived fibroblasts, Absolute gene set enrichment analysis (absGSEA) was performed on the generated data. **Figure 3.5** demonstrates the process of gene filtering and processing. The analysis revealed enrichment of pathways associated with an enhanced response to cytokine and extracellular matrix stimuli. In addition to heightened sensitivity of fibroblasts to environmental signals, the analysis indicated increased extracellular matrix organization in IL-5-stimulated asthmatic fibroblasts, a hallmark characteristic of activated fibroblasts, as illustrated in **Figure 3.6 A**. Furthermore, the upregulated pathways in IL-5-stimulated asthmatic fibroblasts were assessed. As shown in **Figure 3.6 B**, the MAPK signaling pathway was prominently upregulated, accompanied by enhanced cellular migration in these fibroblasts post-stimulation with IL-5, alongside degradation of extracellular matrix, which could explain to a point the increased turnover rate of the ECM element in the pathogenesis of remodelling in asthma. On the other hand, multiple pathways were downregulated in asthmatic fibroblasts post IL-5 stimulation, including regulation of canonical NF-kb signal transduction as well as extracellular matrix organization as highlighted with red arrows in **figure 3.6 C**.

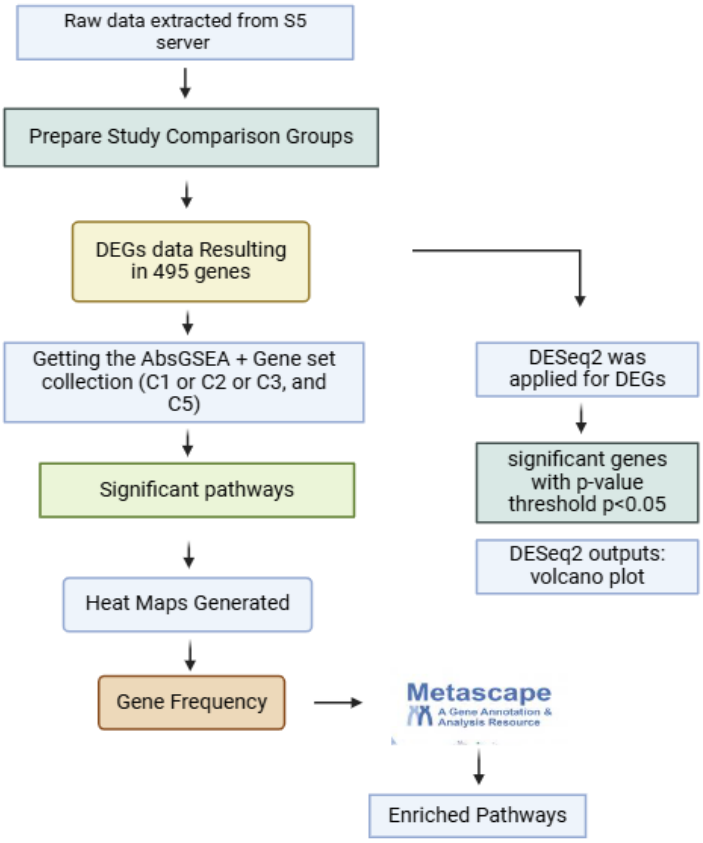


Figure 3.5 showing the process of filtering, processing and analysis of the genes using the raw data generated from S5 server.

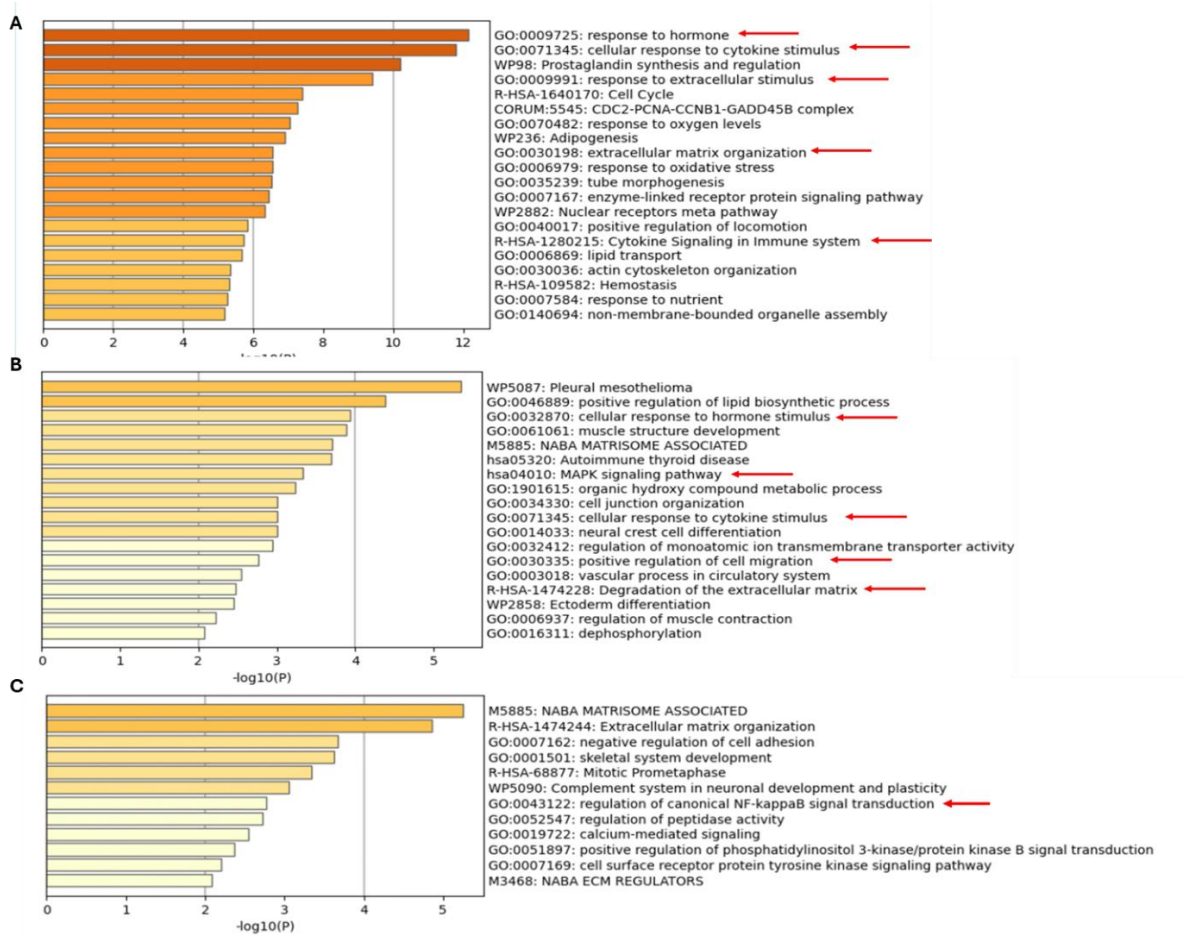


Figure 3.6 Significant enrichment pathways based on frequency in IL-5 stimulated asthmatic fibroblasts vs IL-5 stimulated normal fibroblasts (A) Enriched pathways in asthmatic derived fibroblasts post IL-5 stimulation **(B)** Upregulated pathways in asthma Stimulated based on log2FC >2 **(C)** Downregulated Pathways in asthma Stimulated based log2FC <2.

Furthermore, the absGSEA analysis extends to encompass biological processes and immune responses, covering significantly identified pathways from C2, C4, C5, and C8. Among the C5 significantly activated pathways listed in **Table 10**, the primary focus was directed towards the programmed cell death pathway due to its relevance in the pathophysiology of tissue remodelling, particularly fibrosis. It is well-documented that fibroblasts, which constitute the predominant cellular component of the fibrotic layer, exhibit increased rates of proliferation and differentiation. To explore this further, leading-edge analysis identified several upregulated genes associated with the inhibition of programmed cell death, including NR4A1 as shown in **figure 3.7**. The expression of NR4A-1 was validated using qRT-PCR, data revealed that IL-5 significantly enhances its expression in asthmatic derived fibroblasts as presented on **figure 3.8 C**.

Table 10 Demonstrate the list of C5 pathways obtained from absGSEA

GS	SIZE	SOURCE	ES	NES	Gene %	NOM p-val	Tag %	Signal
Gene Ontology: Biological Processes								
Cell junction organization	22	GO:0034330	0.36703	1.4228	0.146	<0.001	0.318	0.285
Positive regulation of catabolic process	16	GO:0009896	0.37578	1.4225	0.288	<0.001	0.438	0.322
Regulation of programmed cell death	21	GO:0043068	0.37859	1.2455	0.379	<0.001	0.619	0.402
Regulation of Hormone levels	21	GO:0010817	0.32879	1.1903	0.375	<0.001	0.571	0.374
Gene Ontology: Molecular Functions								
Protein homodimerization activity	22	GO:0042803	0.46241	1.695	0.322	<0.001	0.591	0.42
Protein dimerization activity	31	GO:0046983	0.37892	1.4633	0.322	<0.001	0.516	0.375
Gene Ontology: Cellular Processes								
Synapse	30	GO:0045202	0.41766	1.5681	0.29	<0.001	0.533	0.404
Perinuclear region of cytoplasm	26	GO:0048471	0.31735	1.3491	0.415	<0.001	0.577	0.357

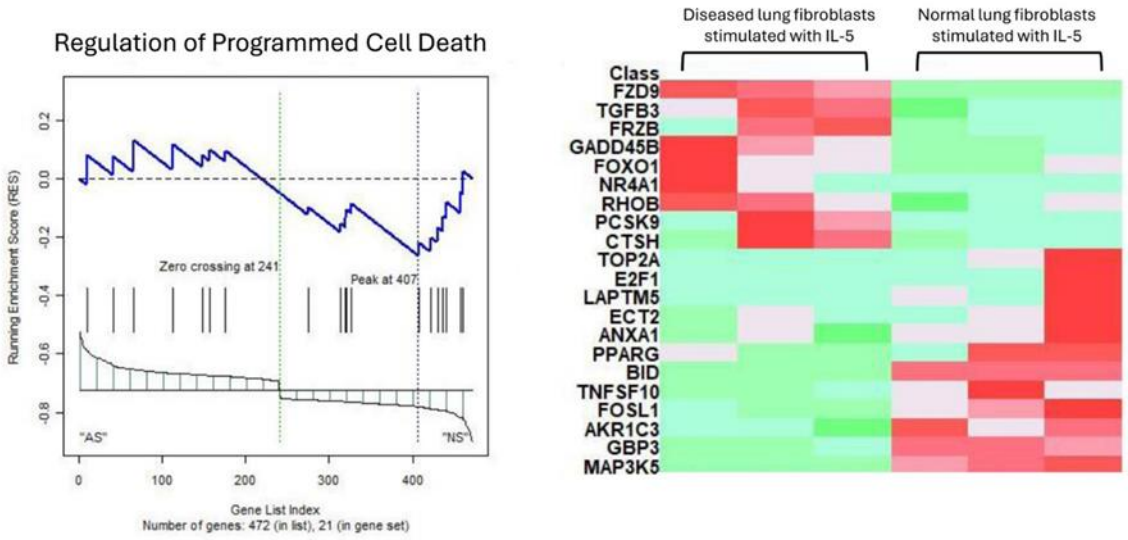


Figure 3.7 Heatmap for the selected enriched pathway: Regulation of Programmed Cell Death. Leading edge analysis showed a significant enrichment in the regulation of programmed cell death. The heatmap demonstrates the clustering of upregulated and down regulated genes, particularly NR4A1, FZD9 and FOXO1; upregulated genes presented in red, down regulated genes presented in green.

3.6 IL-5 stimulation enhances the cellular survival and increases the proliferation of asthmatic derived fibroblasts through the inhibition of programmed cell death

Based on our transcriptomic results that IL-5 stimulation resulted in the regulation of genes related to programmed cell death (**Figure 3.7)**, we started by evaluating the impact of IL-5 on viability and proliferation of lung fibroblast obtained from both normal and asthmatic subjects. An MTT assay was conducted to measure cell viability in response to varying concentrations of recombinant human IL-5 (rh-IL-5) at three points: 24, 48, and 72 hours. Results demonstrated that human lung fibroblasts maintained significant viability, particularly at a concentration of 0.5 ng/mL after 48 hours of exposure to IL-5, however, normal fibroblasts didn't respond similarly (**Figure 3.8 A**).

To further confirm this finding, EDU staining, was employed to measure DNA synthesis, which serves as an indicator of active cell proliferation. EDU staining results (**Figure 3.8 B**) revealed a significant increase in EDU-positive cells among asthmatic fibroblasts following stimulation with 0.1 and 0.5 ng/mL of IL-5, compared to untreated controls. In contrast, normal fibroblasts also showed an increase in EDU-positive cells, although the effect was less pronounced compared to their asthmatic counterparts. These findings suggest that IL-5 selectively enhances the proliferative capacity of asthmatic fibroblasts.

Then, we hypothesized that IL-5 enhances cell survival and promotes proliferation in asthmatic fibroblasts is through the inhibition of programmed cell death. Therefore, we performed an Annexin V assay to detect the percentage of apoptotic cells. We have shown that following IL-5 stimulation for 48 hours, the percentage of apoptotic cells in asthmatic fibroblasts was significantly lower than in the corresponding unstimulated controls as in **Figure 3.8 D**. To confirm these findings, we assessed the levels of caspase-3 via western blot analysis, as the cleavage of this caspase is a hallmark of active apoptosis. The results, presented in **Figure 3.8 E**, indicated a substantial reduction in cleaved caspase-3 levels following IL-5 stimulation, consistent with the leading-edge analysis and the upregulation of NR4A-1 results depicted in **Figure 3.7** and **Figure 3.8 A,** respectively. Together, these findings suggest that IL-5 may contribute to fibroblast survival by reducing the cellular apoptosis, potentially offering a mechanism through which IL-5 could promote apoptosis-resistant fibroblasts in the asthmatic lung.

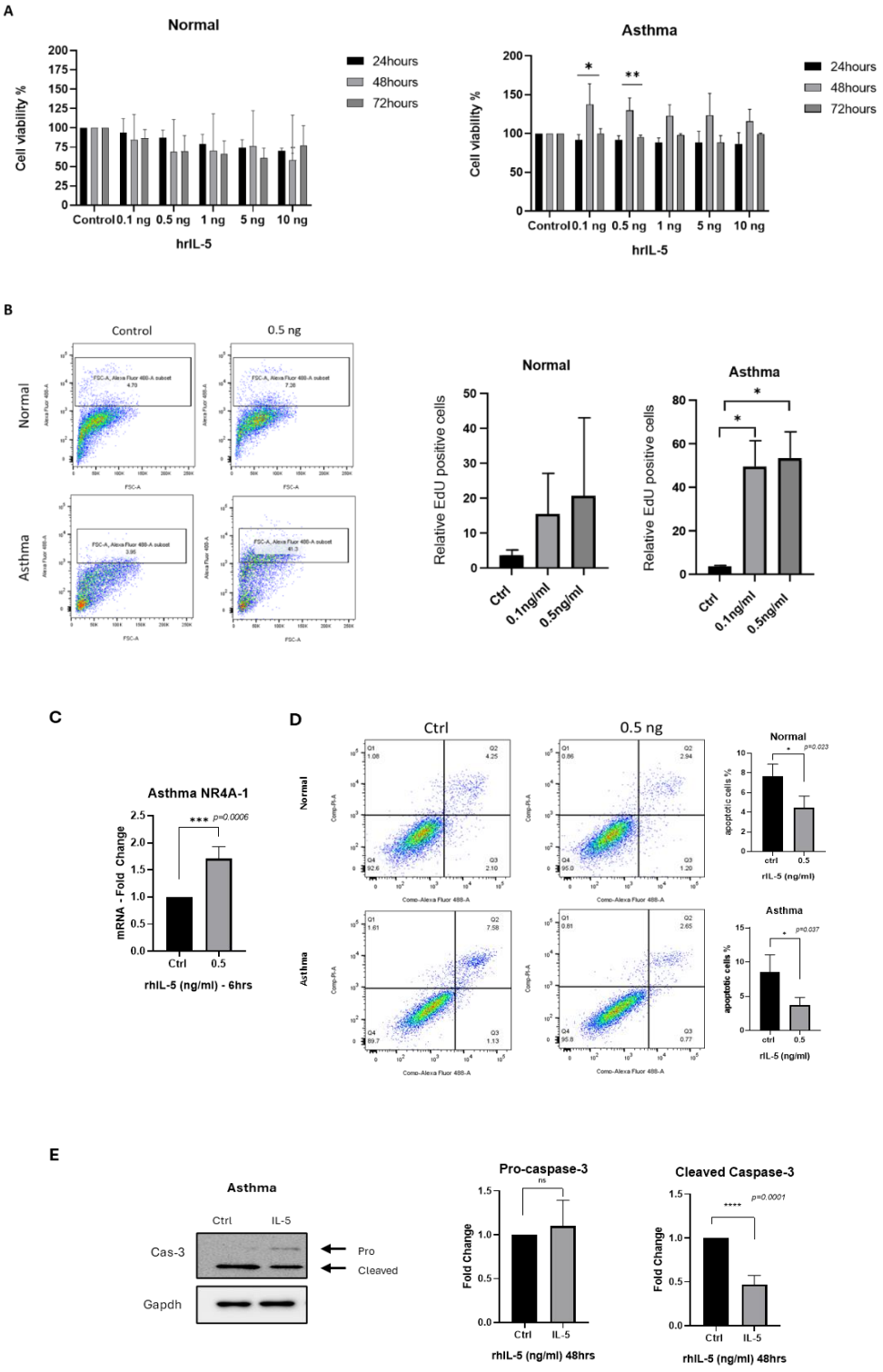


Figure 3.8 IL-5 stimulation enhances the cellular survival and increases the proliferation of asthmatic derived fibroblasts through the inhibition of programmed cell death (A) MTT assay analysis of normal and asthmatic lung fibroblasts after treatment with various rhIL-5 concentrations starting with as lowest as 0.1 ng/ml up to 10ng/ml as the highest concentration, across several time points; 24, 48, 72 hrs. **(B)** EDU assay of both normal and asthmatic cells post 0.1 and 0.5ng/ml of IL-5 for 48hrs. **(C)** The upregulation of the mRNA level of NR4A1 post IL-5 stimulation in asthmatic derived fibroblasts, 18s was used as a

housekeeping gene for fold change calculation, Fold change was calculated by 2^-ΔΔCt,. **(D)** left panel representative figure of Annexin V assay of both normal and asthma derived fibroblasts post 48 hours of IL-5 stimulation, right panel represents the bar graphs of the percentage of apoptotic cells post IL-5 stimulation. **(E)** representative immunoblots of Caspase-3 in asthma derived lung fibroblasts pre and post IL-5 stimulation, bar graps demonstrate the densitometric analysis of the immunoblots, proteins were normalized against GAPDH, data shown is the mean ± SD of four separate experiment; t-Test statistical analysis was done * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

3.7 IL-5Rα is functionally active in response to rh-IL-5 through ERK and AKT

The enrichment analysis data presented earlier revealed that IL-5 upregulated the MAP kinase signaling pathway. Western blotting was done post stimulating lung fibroblasts with 0.5ng/ml of rh-IL-5 for 24 hours, and the levels of total ERK, p-ERK, total AKT and p-AKT were measured to validate the outcomes of the enriched pathways shown in **figure 3.6 B**. IL-5 significantly induces the expression of both p-AKT and p-ERK at the protein level indicating an active signaling pathway when compared to the unstimulated cells, this observation was mainly pronounced in asthmatic fibroblast not in the healthy controls as in **figure 3.9 A**. The elevation in the p-ERK and p-AKT levels explained the increase in proliferation and the enhanced in the survival rate, respectively.

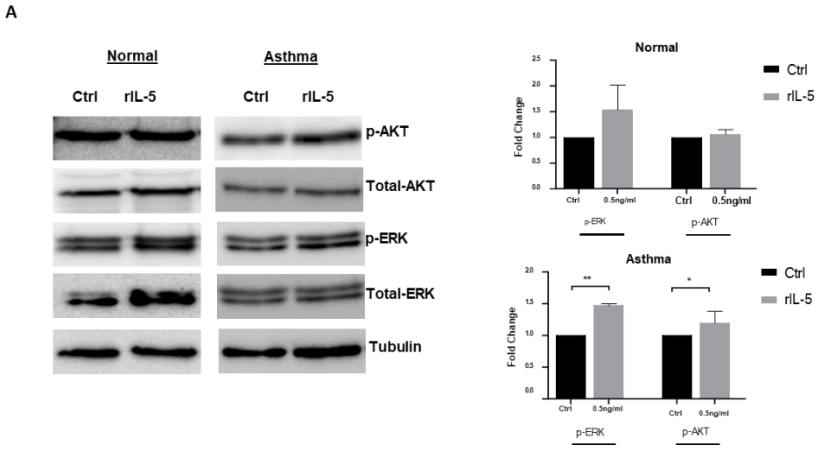
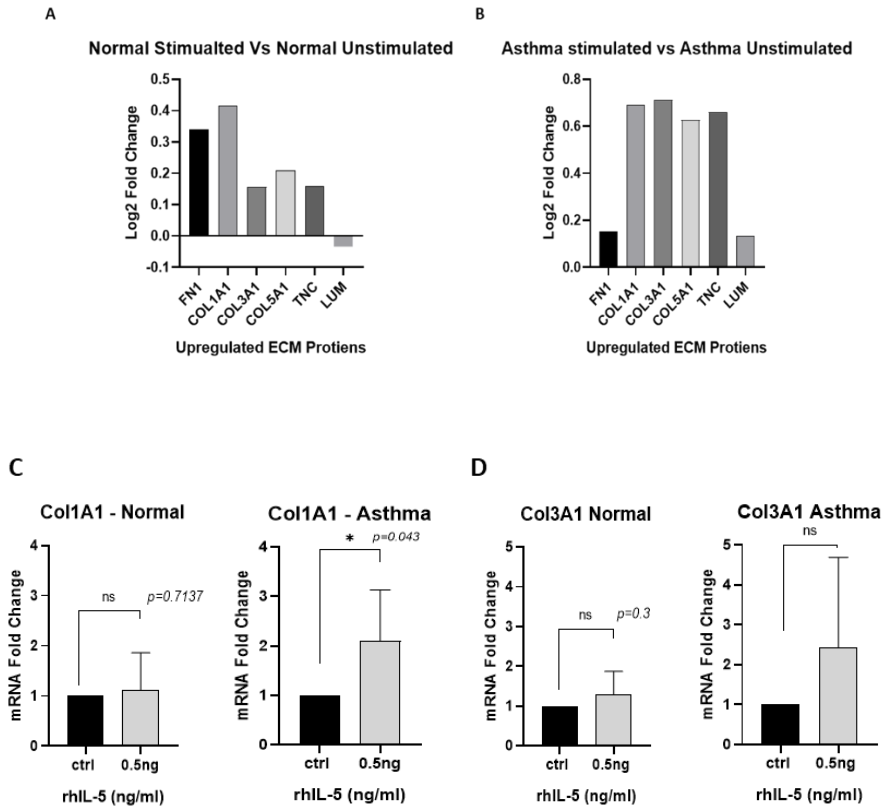


Figure 3.9 IL-5Rα is functionally active in response to rh-IL-5 through ERK and AKT (A) Western blot assay was conducted using cell lysate from normal and asthmatic fibroblasts to measure ERK and Akt phosphorylation levels after 48 h treatment with rIL-5 (0.5 ng/ml), tubulin was used as a loading control. Graphical data represent calculated mean ± SD fold change in expression levels in normal and asthma

fibroblasts based on three separate experiments. * $p < 0.05$ and, ** $p < 0.01$, determined using unpaired two-tailed Student's t-test from three independent experiments

3.8 Stimulation of human lung fibroblasts with IL-5 induces the release of ECM component.

The fibroblasts are the major cellular component of the fibrotic layer and key producers of ECM elements (Michalik et al., 2018a). The accumulation of ECM components is a key feature of fibrosis. To investigate the effect of IL-5 on fibrosis, we evaluated a comprehensive panel of ECM proteins on lung fibroblasts. This analysis was performed using RNA seq (**figure 3.10 A-B**), followed by validation of the results using qRT-PCT, pre and post stimulation with IL-5 for 6 hours in healthy control and asthmatic derived lung fibroblasts. The data presented in **Figure 3.10 C-H** showed a wide panel of ECM genes evaluated in both normal and asthmatic derived fibroblasts.



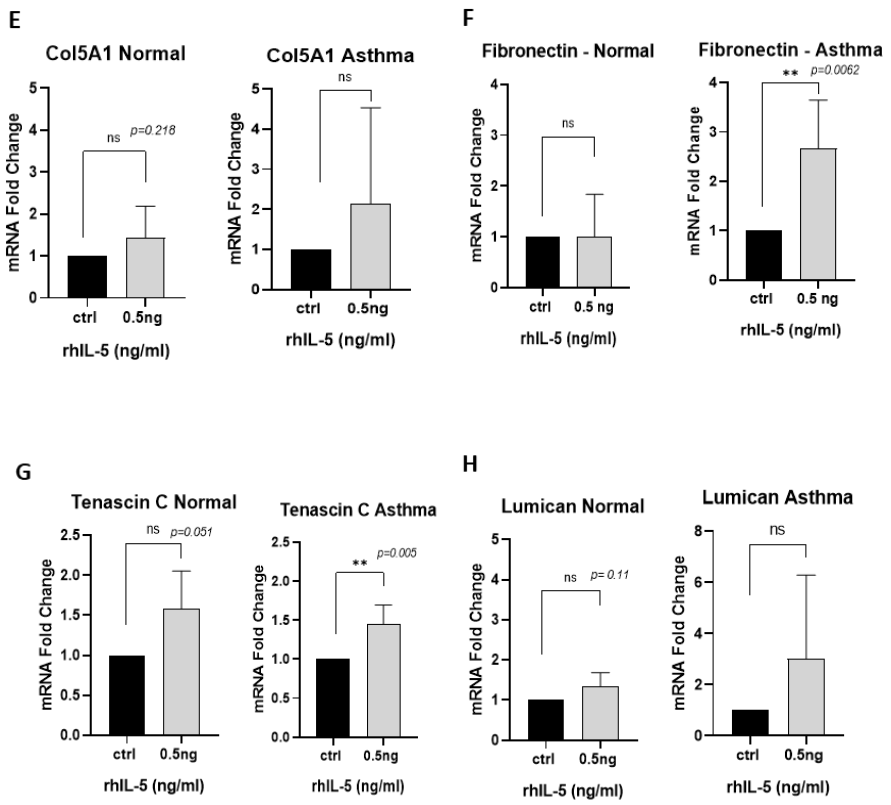


Figure 3.10 The upregulation of ECM genes upon stimulation with IL-5. (A-B) Showing the RNAseq analysis presented based on the Log2FC in normal and asthmatic derived fibroblasts post IL-5 stimulation. **(C)** mRNA levels of Col1A1. **(D)** mRNA levels of Col3A1. **(E)** mRNA levels of Col5A1. **(F)** mRNA levels of FN1. **(G)** mRNA levels of Tenascin C. **(H)** mRNA levels of Lumican. Data shown are representative for at least five different experiments; 18s was used as a housekeeping gene for fold change calculation, Fold change was calculated by $2^{-\Delta\Delta Ct}$, data shown is the mean ± SD of five separate experiment; t-Test statistical analysis was done * $p < 0.05$, ** $p < 0.01$. Fold expression change relative to the respective untreated control post normalization to housekeeping gene in each cell groups.

The qRT-PCR findings were further validated at the protein level through immunoblotting analysis, using cell lysates extracted from lung fibroblasts stimulated with IL-5 for 24, 48, and 72 hours to determine the optimal time point for stimulation (**Figure 3.11**). After 24 hours of IL-5 stimulation, no increase in Collagen 1A1 or Tenascin C protein levels was observed in either normal or asthmatic fibroblasts (**figure 3.11 A and C)**. However, Fibronectin levels were significantly elevated in asthmatic fibroblasts (**Figure 3.11 B**), whereas this increase was less pronounced in normal fibroblasts.

At 48 hours of stimulation, IL-5 demonstrated a more pronounced effect. Collagen 1A1 expression was significantly increased in both normal (p=0.0146) and asthmatic (p=0.017) lung derived fibroblasts (**Figure 3.11 D**). Similarly, Fibronectin levels showed significant elevation in normal (p = 0.03) and asthmatic (p = 0.007) fibroblasts (**Figure 3.11 E**). Despite significant

increases in Tenascin C mRNA expression, the protein levels were not different (**Figure 3.11 F**). Additionally, stimulation for 72 hours did not lead to any further enhancement in the expression of extracellular matrix components when compared to 48-hour stimulation (**figure 3.11 G-H)**, suggesting that the latter represents the optimal time point for observing IL-5-mediated effects on ECM protein expression.

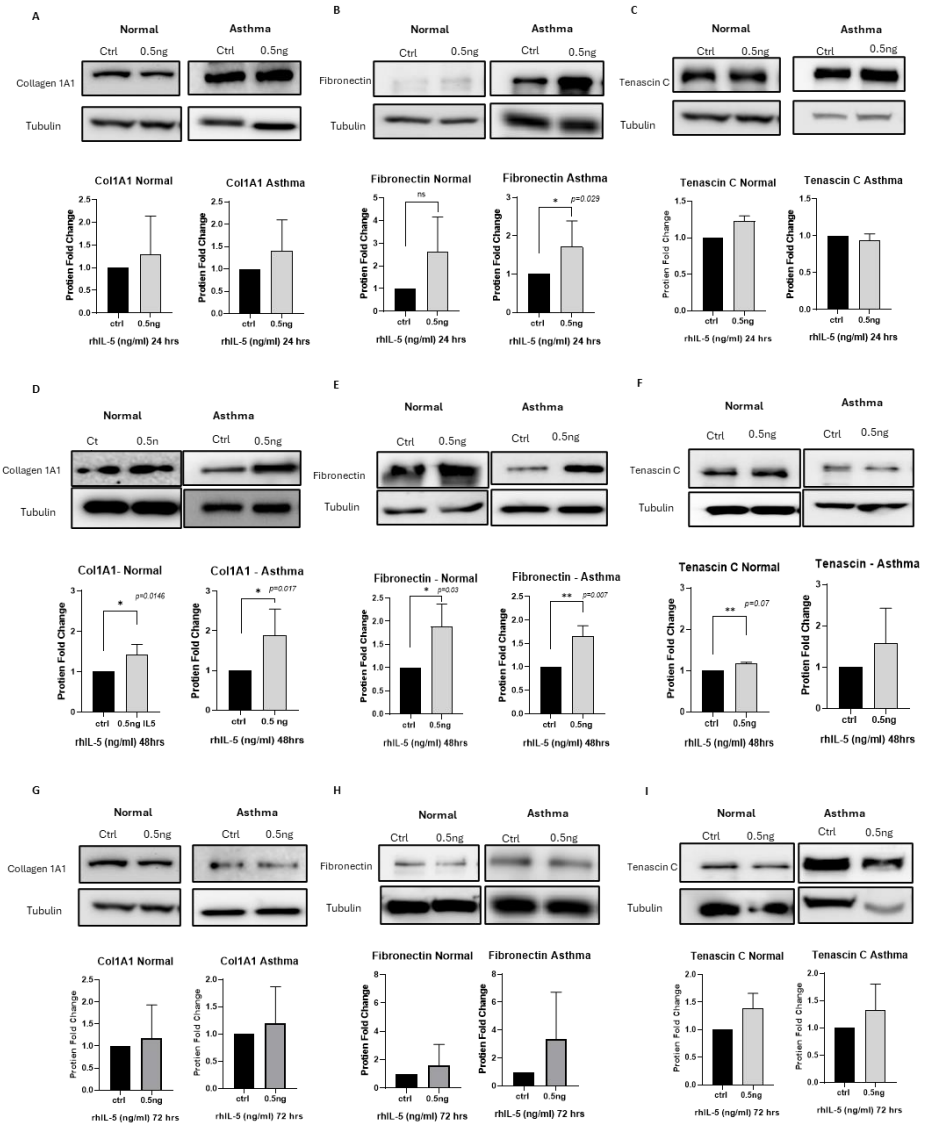
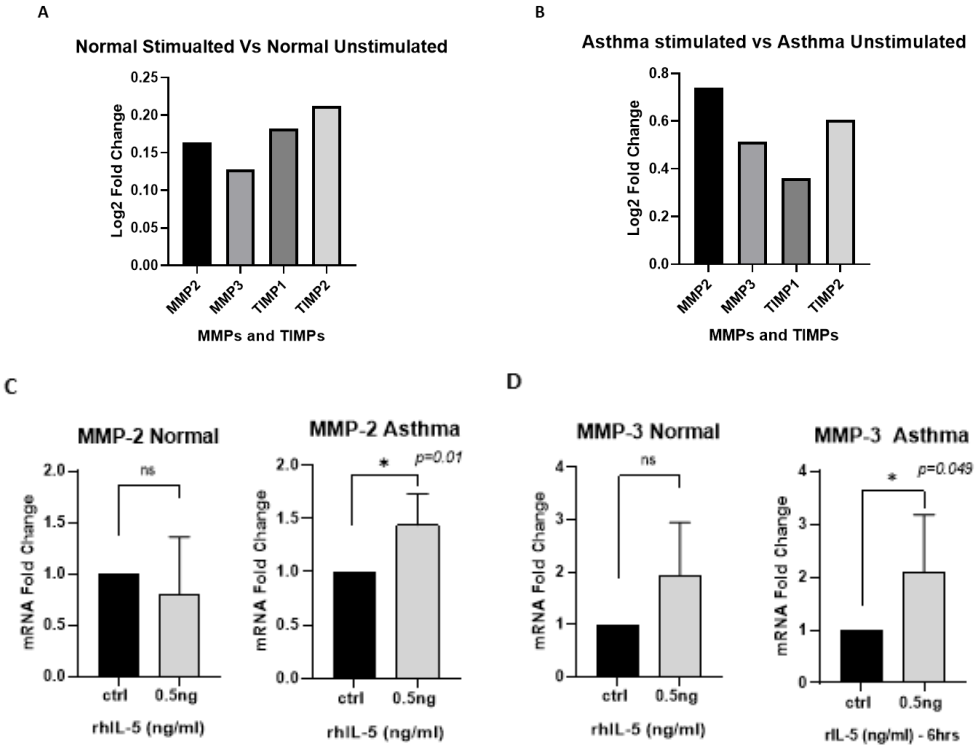


Figure 3.11 Elevating ECM protein levels upon IL-5 stimulation in Normal and Asthma derived lung fibroblasts Representative immunoblots and densitometric analysis of **(A)** Collagen I, **(B)** Fibronectin**,** and **(C)** Tenascin C in normal and asthma derived lung fibroblasts post stimulation with 24 hrs. Representative immunoblots and densitometric analysis of **(D)** Collagen I, **(E)** Fibronectin, and **(F)** Tenascin C in normal and asthma derived lung fibroblasts post stimulation with 48 hrs. Representative immunoblots and densitometric analysis of **(G)** Collagen I, **(H)** Fibronectin, and **(I)** Tenascin C in normal and asthma derived lung fibroblasts post stimulation with 72 hrs. Data shown is representative for at least four different

experiments, and presented as mean ± SD after normalization to the respective untreated control; Tubulin was used as loading control, t-Test was done as a statistical tool, * $p < 0.05$, **$p < 0.01$.

3.9 .IL-5 induces the release of MMP-2 and MMP-3 from asthmatic fibroblasts

The fibrotic layer, characteristic of airway remodelling, is predominantly shaped by the enzymatic activities of matrix metalloproteinases (MMPs) and their natural tissue inhibitors (TIMPs). These enzymes are synthesized by both immune and non-immune cell types, including fibroblasts (Bajbouj et al., 2021). To investigate the role of IL-5 in regulating MMPs and TIMPs expression and release, we first conducted an RNA seq analysis (**figure 3.12 A-B**) for the MMPs that are majorly implicated in asthma disease, this was followed by a qRT-PCR analysis for validation purposes. Results indicated that MMP-2, MMP-3, and MMP-7 mRNA were significantly upregulated in asthmatic fibroblasts following 6 hours of IL-5 stimulation (**Figure 3.12 C-E**), whereas MMP-9 expression remained unaffected (**Figure 3.12 F**). In contrast, IL-5 stimulation did not alter the mRNA expression of these MMPs in normal fibroblasts, suggesting a selective response in the asthmatic cell environment.



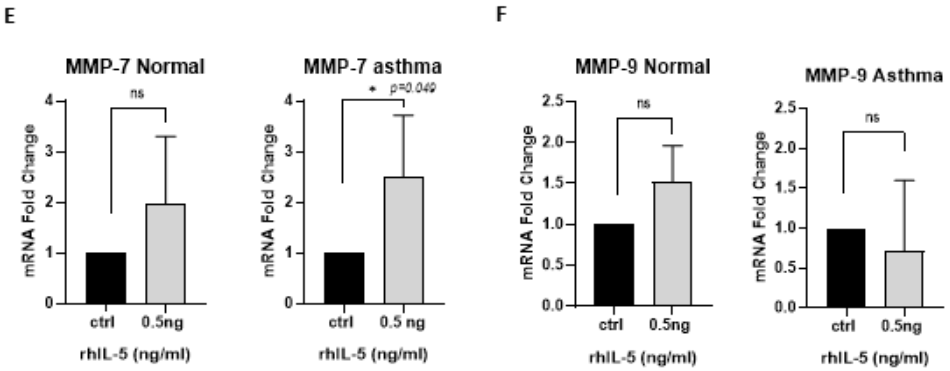


Figure 3.12 IL-5 induces the expression of MMP-2 and MMP-3 from asthmatic fibroblasts (A-B) Showing the RNAseq analysis presented based on the Log2FC in normal and asthmatic derived fibroblasts post IL-5 stimulation, **(C-F)** Showing the mRNA levels of the four MMP genes in Normal and asthmatic Derived Lung fibroblasts post stimulation with IL-5. Data shown is representative for at least four different experiments; 18s was used as a housekeeping gene for fold change calculation, Fold change was calculated by 2^-ΔΔCt, Data shown is representative for at least four different experiments, and presented as mean ± SD after normalization to the respective untreated control;, t-Test was done as a statistical tool, * $p < 0.05$.

To corroborate these mRNA findings, we examined MMP protein levels using immunoblotting after multiple time points including 24, 48 and 72 hours of IL-5 stimulation, using cell lysate from both healthy and asthmatic lung fibroblasts. **Figure 3.13 A-D** shows that 24 hours of IL-5 stimulation didn't induce any elevation in the protein level of any of the MMPs. However, stimulation for 48 hours with IL-5 induces a robust increase in MMP-2 (p=0.012) and MMP-3 (p=0.0023) protein levels in asthmatic fibroblasts (**Figure 3.13 E-F**), while normal fibroblasts did not exhibit comparable responses. In contrast, IL-5 stimulation did not alter MMP-7 or MMP-9 protein levels in either asthmatic or normal fibroblasts (**Figure 3.13 G-H**). The results at 48 hours align with the gene expression data. Finally, prolonged exposure to IL-5 (up to 72 h) didn't effectively induce a noticeable increase in any of the MMPs except of MMP-9 in normal fibroblasts as shown in **figure 3.13 M** .

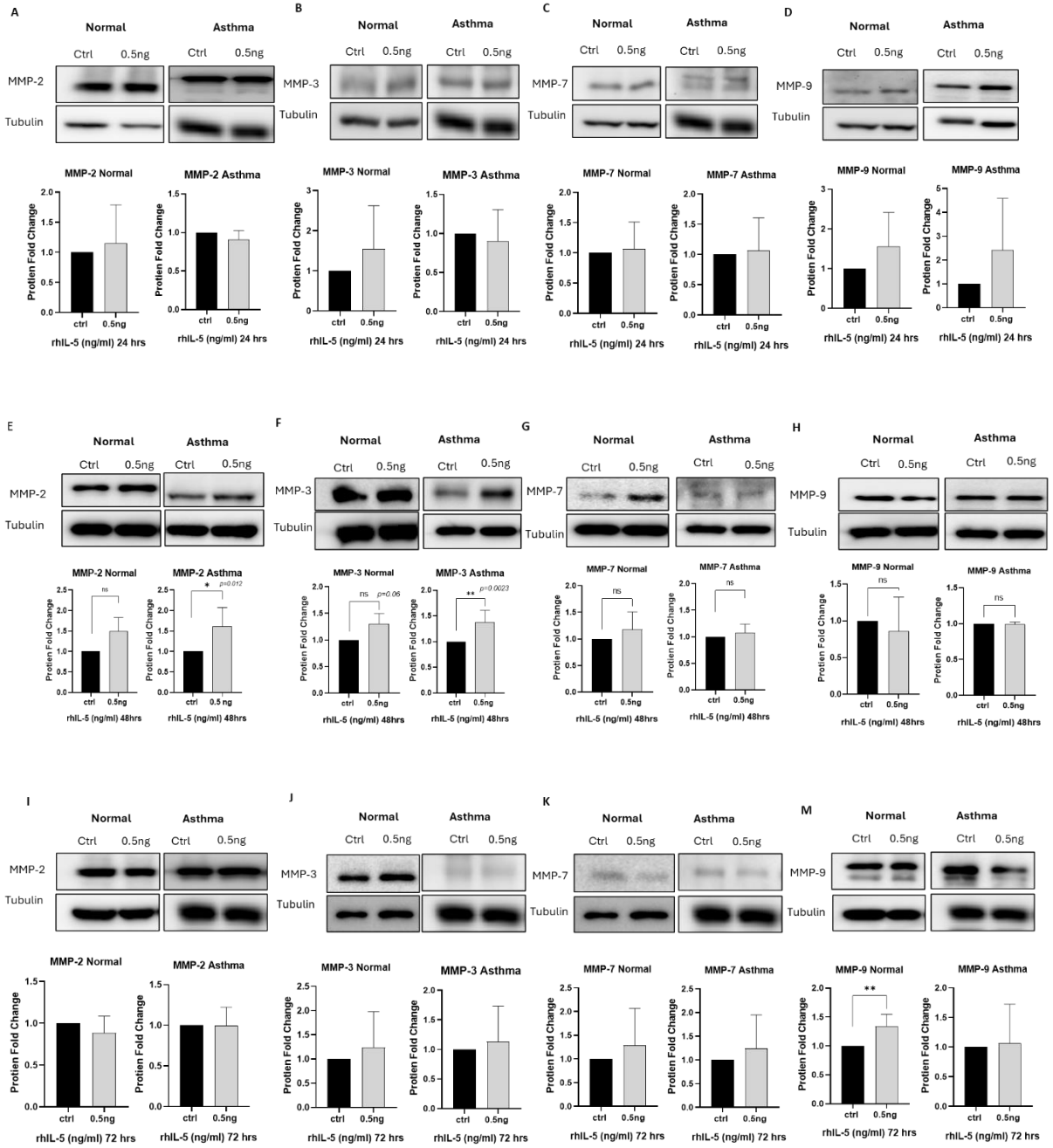


Figure 3.13 Elevating the MMPs levels in Normal and Asthma derived lung fibroblasts post 24, 48 and 72 hours of stimulation with IL-5 (A) Representative immunoblots and densitometric analysis of MMP-2, (B) MMP-3, (C) MMP-7, and (D) MMP-9 in normal and asthma derived lung fibroblasts post stimulation with 24 hrs, Representative immunoblots and densitometric analysis of (E) MMP-2, (F) MMP-3, (G) MMP-7 and (H) MMP-9 in normal and asthma derived lung fibroblasts post stimulation with 48 hrs Representative immunoblots and densitometric analysis of (I) MMP-2, (J) MMP-3, (K) MMP-7 and (M) MMP-9 in normal and asthma derived lung fibroblasts post stimulation with 72 hrs. Data shown is representative for at least four different experiments, and presented as mean ± SD after normalization to the respective untreated control; Tubulin was used as loading control, t-Test was done as a statistical tool, * $p < 0.05$, ** $p < 0.01$.

Additionally, we performed ELISA on the supernatant of IL-5-stimulated cells at 48 hours to assess MMP-3 secretion. Results revealed a significant increase in secreted MMP-3 in both asthmatic (p=0.0071) and normal fibroblast supernatants (p=0.039) (**Figure 3.14 A-B**). Furthermore, an enzymatic activity assay for MMP-3 was also conducted using the same supernatant, which showed an increase in MMP-3 activity in both cell types; however, this increase did not reach statistical significance (**Figure 3.14 C-D**).

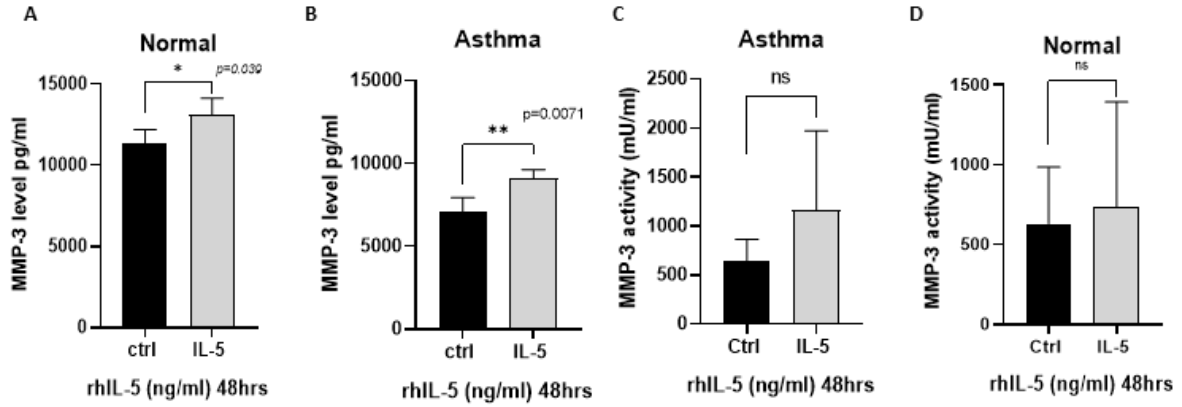
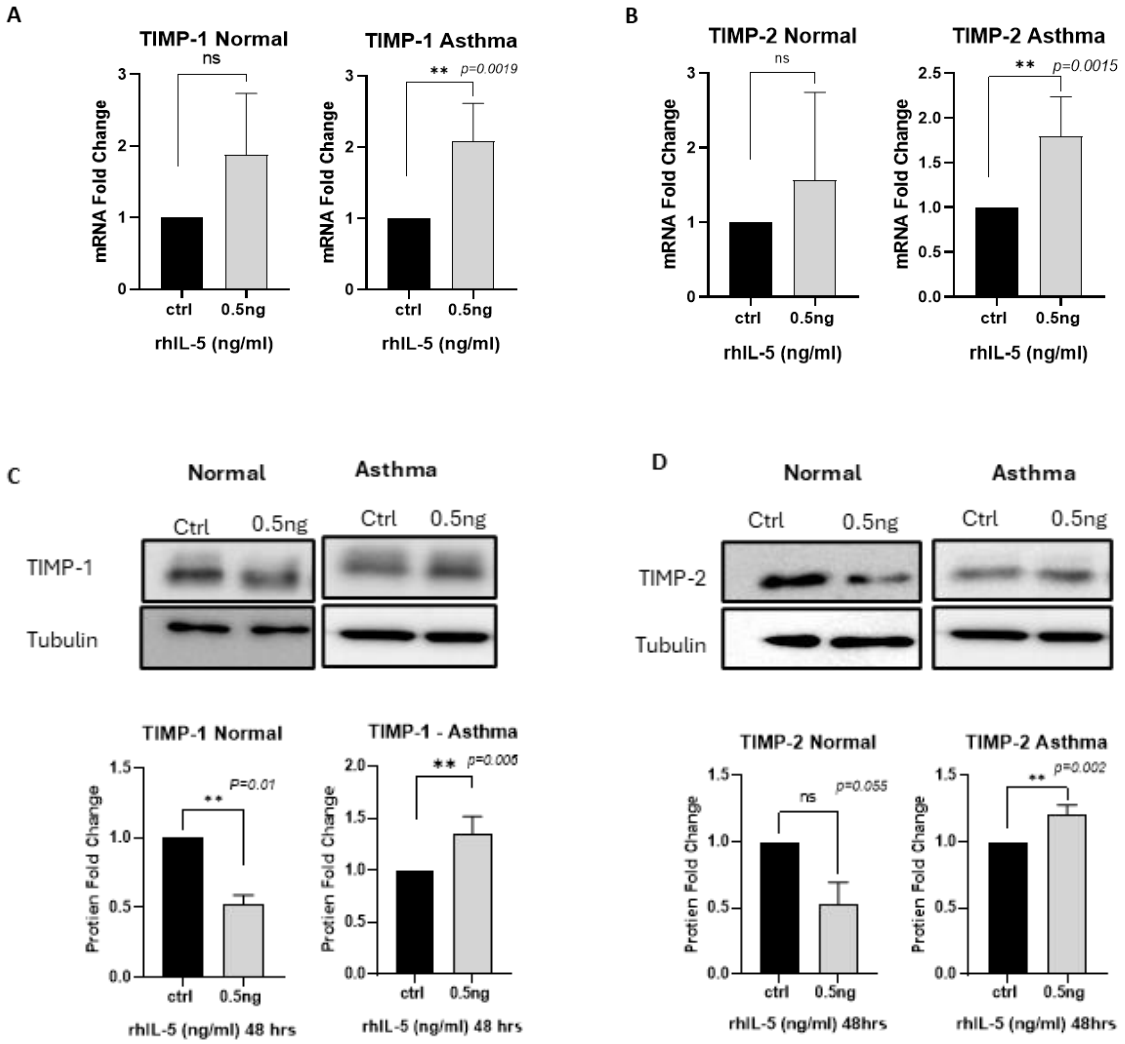


Figure 3.14 IL-5 induces the release of MMP-3 from asthmatic fibroblasts (A-B) Secreted levels of MMP-3 from Normal and asthma derived fibroblasts supernatant measured by ELISA. **(C-D)** Activity levels of MMP-3 in both normal and asthma derived fibroblasts respectively measured by ELISA in a mU/ml. Data shown is representative for at least four different experiments, and presented as mean ± SD after normalization to the respective untreated control; Tubulin was used as loading control, t-Test was done as a statistical tool, * $p < 0.05$, ** $p < 0.01$.

Following the MMP analysis, we explored the effect of IL-5 on the expression of TIMPs, TIMP-1 and TIMP-2, the primary inhibitors of MMPs. Starting with observing the RNAseq data as in **figure 3.12 A-B**. IL-5 stimulation significantly upregulated TIMP-1 and TIMP-2 mRNA levels in asthmatic fibroblasts relative to their unstimulated controls whereas there was no difference in normal fibroblasts (**Figure 3.15 A-B**). Similarly, while TIMP-1 protein levels increased significantly in asthmatic fibroblasts, it was not observed in normal fibroblasts (**Figure 3.15 C**). In contrast, TIMP-2 protein levels showed a significant increase following IL-5 stimulation in asthmatic fibroblasts ($p = 0.002$) (**Figure 3.15 D**), while TIMP-2 tended to be lower in normal fibroblasts post-stimulation ($p = 0.055$) (**Figure 3.15 D**).

To further understand the balance between MMPs and TIMPs and how critical they are for ECM turnover and fibrosis in asthma, we calculated the ratios of MMP-9 to TIMP-1 and MMP-2 to TIMP-2, mimicking the relative abundance of these enzymes in the asthmatic airway

environment. Despite the no significant increase observed in MMP-9 levels following IL-5 stimulation, the notable reduction in TIMP-1 skewed the MMP-9/TIMP-1 ratio significantly (p=0.0126) toward TIMP-1, indicating a disrupted balance (**Figure 3.15 E**). However, the MMP-2/TIMP-2 ratio remained stable (**Figure 3.15 F**). These data collectively suggest that IL-5 selectively modulates the expression of MMPs and TIMPs in asthmatic fibroblasts, potentially contributing to the fibrotic remodelling characteristic of asthma.



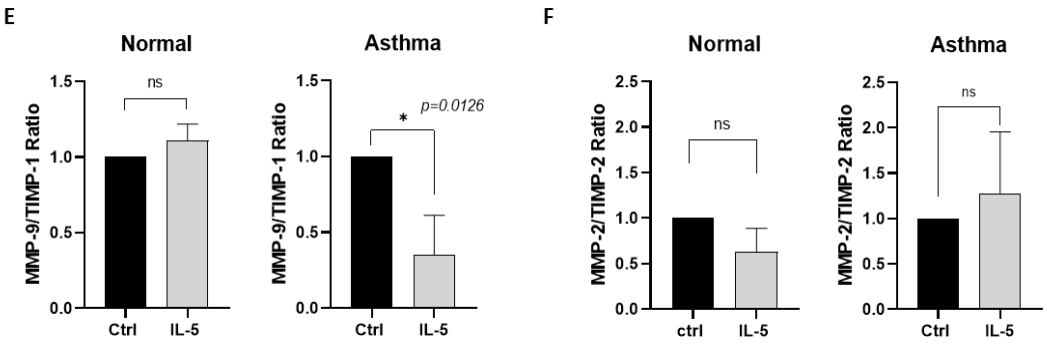


Figure 3.15 Lung fibroblasts stimulated with IL-5 have a higher level of TIMP-1 and TIMP-2 (A-B) mRNA levels of both TIMP-1 and TIMP-2 in both Normal and asthma derived lung fibroblasts, Data shown is representative for at least four different experiments; 18s was used as a housekeeping gene for fold change calculation, Fold change was calculated by 2^-ΔΔCt, data shown is the mean ± SD of five separate experiment; t-Test statistical analysis was used * $p < 0.05$, ** $p < 0.01$. **(C-D)** Densitometric analysis and representative immunoblots of TIMP-1 and TIMP-2 in normal and asthma derived lung fibroblasts. Data shown is representative for at least four different experiments, and presented as mean ± SD after normalization to the respective untreated control; Tubulin was used as loading control, t-Test was done as a statistical tool, * $p < 0.05$, ** $p < 0.01$, **(E-F)** Association between MMP-9/TIMP-1 and MMP-2/TIMP-2 ratios in normal and asthmatic fibroblasts, in the presence of IL-5.

3.10 IL-5 stimulation enhances the release of IL-6 and TGF-b from human lung derived fibroblasts.

Fibroblasts play an active role in maintaining the inflammatory environment of the airways in asthma through the production of various pro-inflammatory cytokines and growth factors, including tumor necrosis factor-alpha (TNFα), IL-6, transforming growth factor-beta (TGFβ), and IL-11. These cytokines are known to play a key role in the progression of airway remodelling and inflammation.

To investigate whether IL-5 impacts the expression of these cytokines in lung-derived fibroblasts, we used the RNAseq data to evaluate the expression of the above-mentioned genes (**Figure 3.16 A-B**). And qRT-PCR to measure their mRNA levels following IL-5 stimulation and validate the RNAseq outcome. One hour of IL-5 stimulation significantly increased the mRNA expression of TGFβ (p=0.0229) and IL-11 (p=0.019) in fibroblasts derived from asthmatic subjects (**Figure 3.16 C-D**), indicating that IL-5 may has a rapid effect on the fibrotic responses in the asthmatic environment. Additionally, IL-5 upregulated the expression of IL-6

(p=0.04) and TNFα (p=0.046), both mediators in inflammatory signaling, in asthmatic fibroblasts (**Figure 3.16 E-F**).

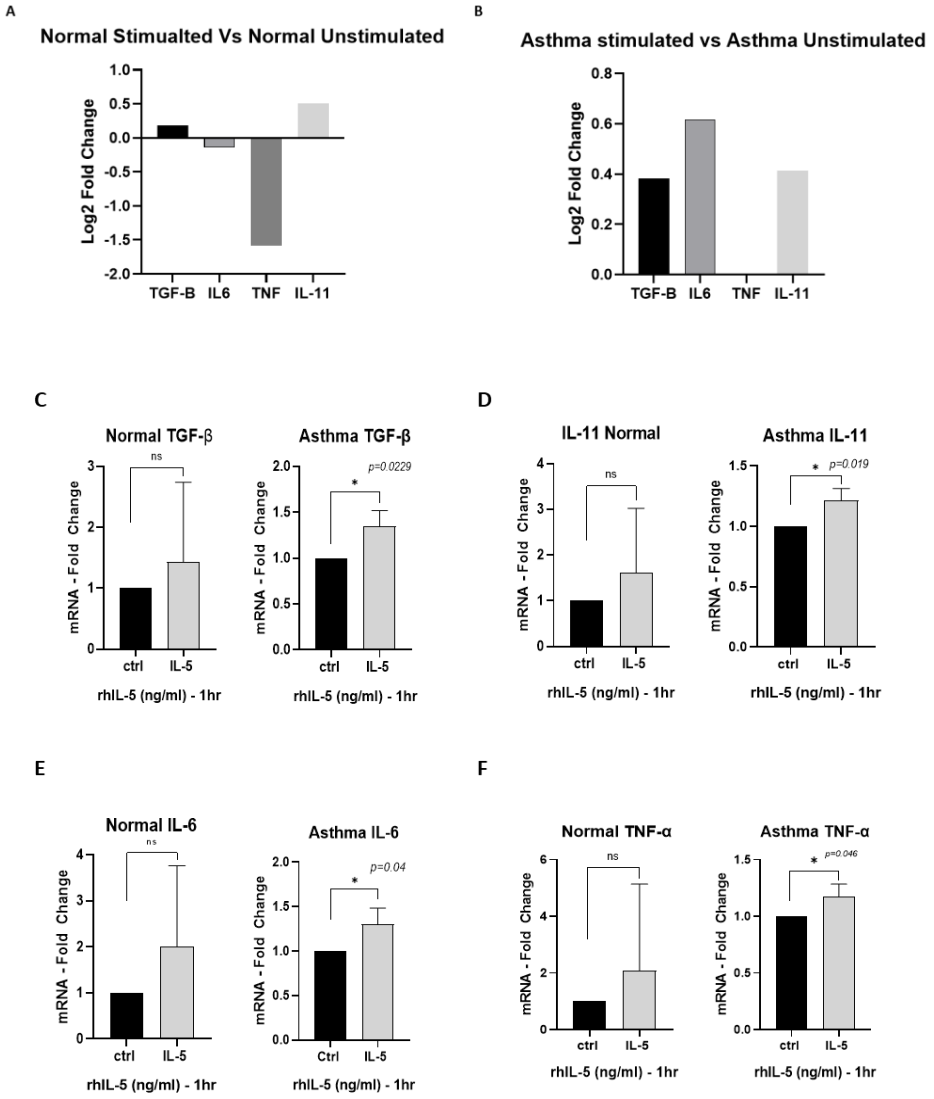
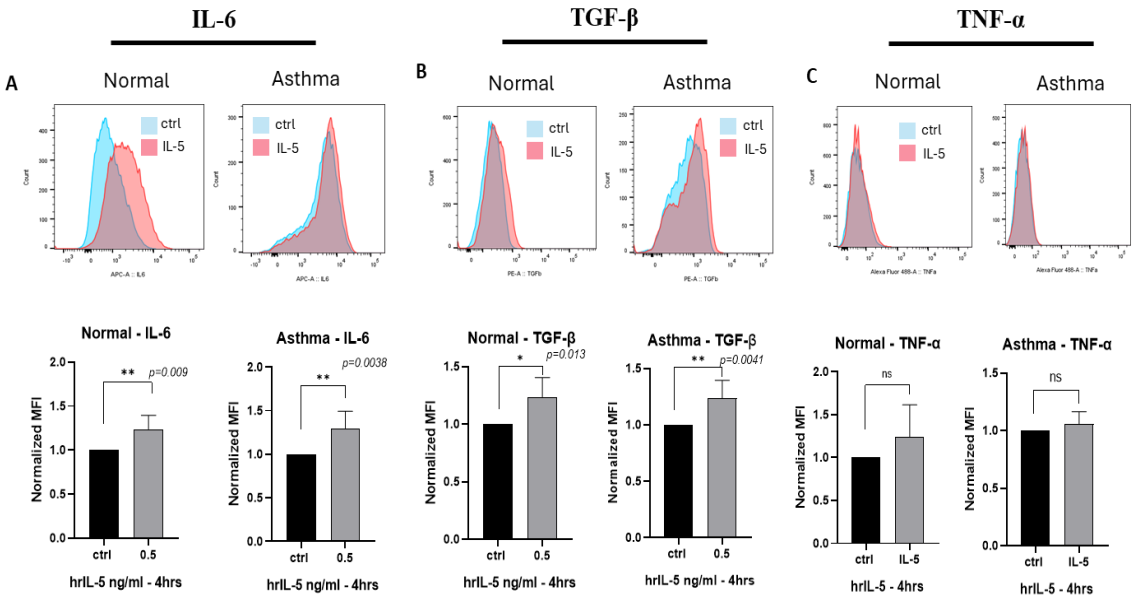


Figure 3.16 IL-5 stimulation increased mRNA expression of pro-inflammatory and pro-fibrotic cytokines (A-B) RNAseq analysis presented based on the Log2FC in normal and asthmatic derived fibroblasts post IL-5 stimulation, **(C)** mRNA levels of TGF-b **(D)** mRNA levels of IL-11 **(E)** mRNA levels of IL-6 **(F)** mRNA levels of IL-6 in both normal and asthma Derived Lung fibroblasts post stimulation with IL-5. Data shown is representative for three different experiments; 18s was used as a housekeeping gene for fold change calculation, Fold change was calculated by 2^-ΔΔCt, data shown is the mean ± SD of three separate experiment; t-Test statistical analysis was done * $p < 0.05$, ** $p < 0.01$

To validate the qRT-PCR findings, we conducted intracellular staining for IL-6, TGFβ, and TNFα after 4-hour IL-5 stimulation period, measuring protein expression levels. In line with the mRNA results, IL-6 ($p = 0.0038$) and TGFβ ($p = 0.0041$) protein levels were significantly elevated in asthmatic fibroblasts (**Figure 3.17 A-B**). Interestingly, while the mRNA levels did

not show a comparable increase in normal fibroblasts, the intracellular protein levels of IL-6 and TGFβ were similarly elevated in both normal and asthmatic cells (**Figure 3.17 A-B**), suggesting that IL-5 may modulate fibroblast cytokine release at a translational or post-translational level.

To further confirm these findings, we assessed the secreted cytokine levels in the supernatant from IL-5-stimulated fibroblasts using ELISA. Consistent with the intracellular staining results, the supernatant of asthmatic fibroblasts showed a significant increase in both IL-6 (p=0.0304) and TGFβ (p=0.049) compared to unstimulated controls (**Figure 3.17 D-E**). Interestingly, while the normal fibroblasts also secreted elevated levels of these cytokines (**Figure 3.17 D-E**), the effect was more pronounced in asthmatic cells, supporting the hypothesis that asthmatic fibroblasts may have an enhanced response to IL-5. These findings collectively indicate that IL-5 stimulation may drive fibroblast activation and inflammatory cytokine production, suggesting a potential mechanism by which IL-5 contributes to persistent inflammation and tissue remodeling in asthma.



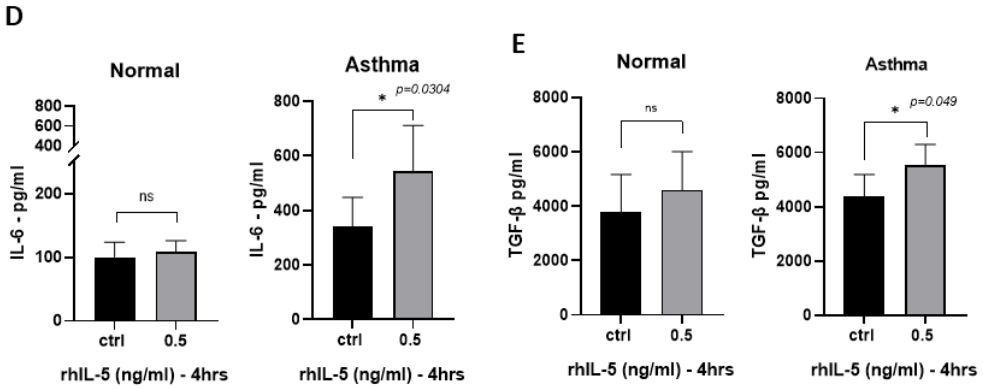


Figure 3.17 IL-5 stimulation induces the elevation in IL-6 and TGF-β cytokines levels and secretion in asthmatic fibroblasts. (A) Representative histogram of the intracellular expression of IL-6, TGF-β and TNF-α in normal derived lung fibroblasts, for the overlap graph: Blue is the control (Unstimulated), Red is the IL-5 stimulated cells. **(B)** The corresponding normalized MFI of the expression of the mentioned cytokines, Normalization was done against the control sample of each run. **(C)** Representative histogram of the intracellular expression of IL-6, TGF-β and TNF-α in asthmatic derived lung fibroblasts, for the overlap graph: Blue is the control (Unstimulated), Red is the IL-5 stimulated cells. **(D)** The corresponding normalized MFI of the expression of the mentioned cytokines, Normalization was done against the control sample of each run. **(E-F)** Showing the secreted levels of IL-6 and TGF-b from normal derived fibroblasts measured by ELISA, **(G-H)** Showing the secreted levels of IL-6 and TGF-b from asthmatic derived fibroblasts measured by ELISA The data is a representative of at least three different experiments, t-test was done as statistical tool, * $p < 0.05$, ** $p < 0.01$.

3.11 TGFβ is a potential inducer of IL-5Rα in human lung derived fibroblast

As shown in the above results, stimulation with IL-5 significantly promote the expression and the release of TGFβ (**Figure 3.17**). Due to its potent role in activation of lung derived fibroblasts, we examined whether TGF-β have any regulatory role on the IL-5/IL5Ra signalling, following 50ng/ml of rh-TGFβ for 6 hours the expression of IL-5Rα was evaluated for mRNA related studies, and for 24 hours for protein measurements. **Figure 3.18 A-B**, showed an increase in the mRNA levels of IL-5Rα in both normal and asthmatic lung fibroblasts, but this elevation didn't reach a statistical significance, However, immunoblotting analysis, showed that in both normal (p=0.005) and asthmatic (p=0.022) derived fibroblasts the expression of the receptor is significantly upregulated (**Figure 3.18 C-D)**, These results suggest a potential positive regulatory loop between TGF-b signalling and IL-5 signalling in lung derived fibroblasts.

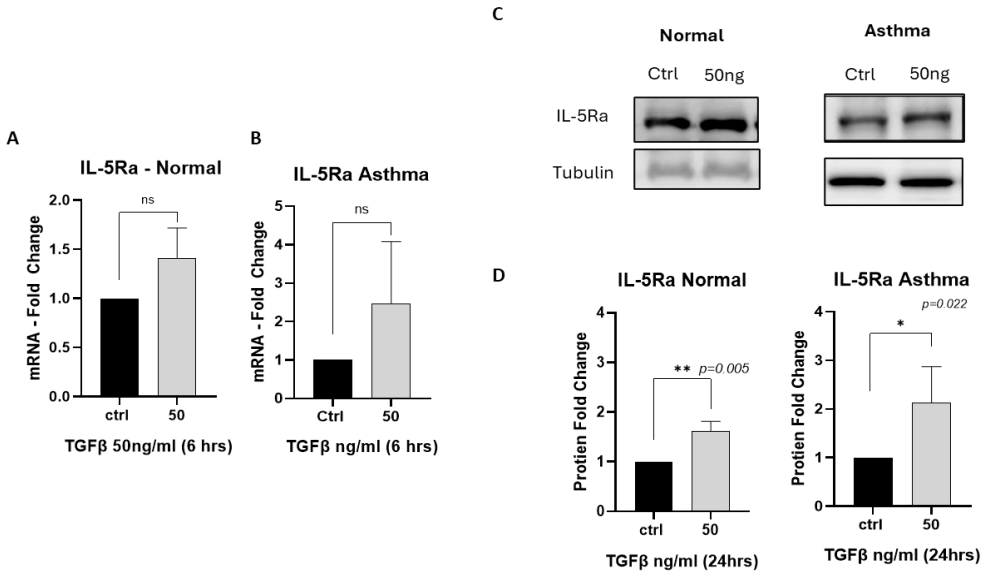


Figure 3.18 Lung fibroblasts stimulated with TGF-β increase their expression of IL-5Rα (A-B) showing the mRNA levels of IL-5Rα in both normal and asthmatic derived lung fibroblasts, Data shown is representative of two different experiments; 18s was used as a housekeeping gene for fold change calculation, Fold change was calculated by 2^-ΔΔCt, data shown is the mean ± SEM of two separate experiment; t-Test statistical analysis was done * $p < 0.05$, ** $p < 0.01$. **(C-D)** Representative immunoblots and Densitometric analysis of IL-5Rα in Normal and Asthma derived lung fibroblasts. Data shown is representative for at least two different experiments, and presented as mean ± SD after normalization to the respective untreated control; Tubulin was used as loading control, t-Test was done as a statistical tool, * $p < 0.05$, ** $p < 0.01$

All together our data sheds an important light on a new functional role of IL-5 in the pathogenesis of asthma, specifically in airway remodelling by acting directly on lung fibroblasts. Then, we inhibit IL-5 using anti IL-5 or anti-IL-5Rα to reverse the effect of IL-5 stimulation.

3.12 Anti IL-5 didn't reverse the proliferation rate but induces apoptosis in severe asthmatic fibroblasts.

As previously described, IL-5 significantly enhances fibroblast proliferation after 48 hours of stimulation. To determine if anti-IL-5 could mitigate this proliferative effect, fibroblasts were treated with IL-5, anti-IL-5 alone, or a combination of IL-5 and anti-IL-5 for 48 and 72 hours. EDU assay results indicated that anti-IL-5 did not reduce proliferation rates in either normal or asthmatic lung fibroblasts (**Figure 3.19 A)**.

Further, we evaluated whether anti-IL-5 treatment could induce apoptosis in lung fibroblasts. Cells were incubated with anti-IL-5, with or without IL-5, and analysed at 72 hours. Anti-IL-5 treatment alone resulted in a significant increase in apoptotic cell percentage compared to untreated controls, whereas co-incubation of IL-5 and anti-IL-5 did not show a difference in apoptosis relative to anti-IL-5 alone (**Figure 3.19 B**).

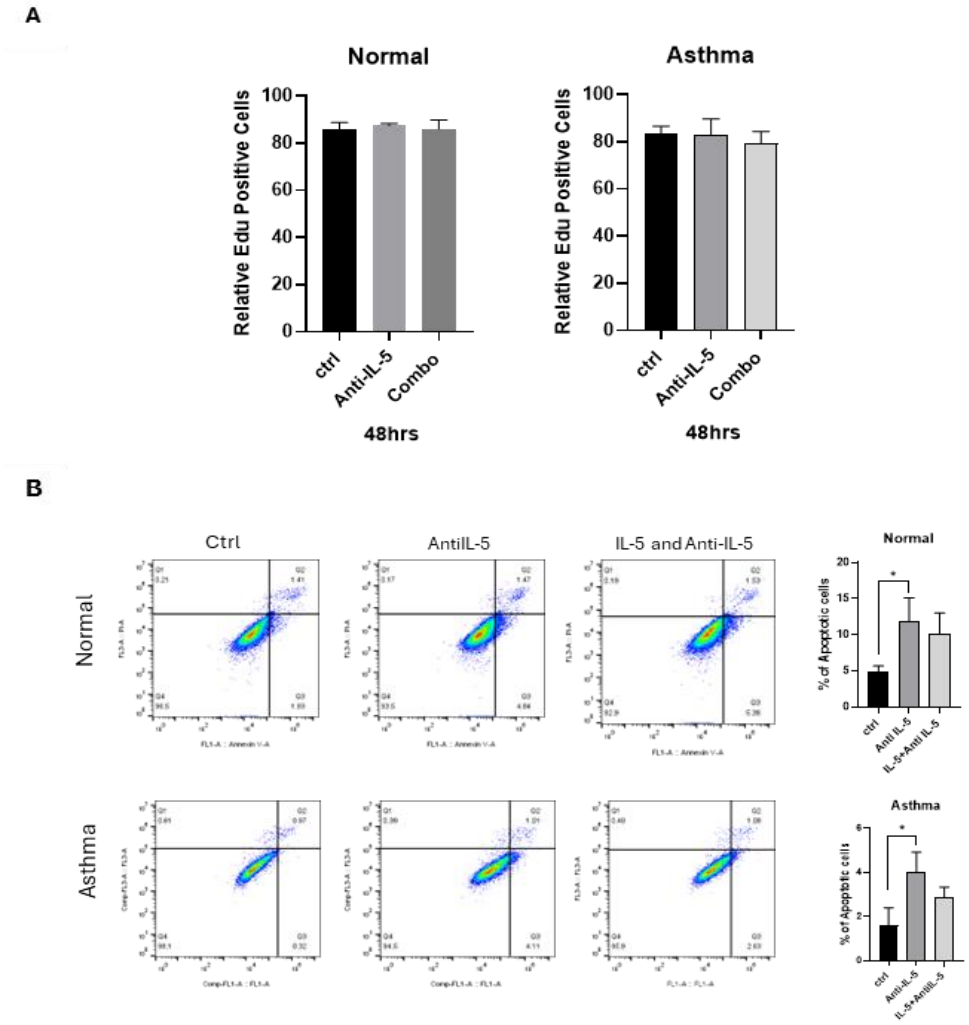


Figure 3.19 Anti-IL-5 didn't affect cellular proliferation, but it induces cellular apoptosis (A-B) Edu Proliferation assay of both normal and asthmatic fibroblasts post 48 hours inhibition with Anti-IL-5 with and without IL-5, **(C)** Representative image of Annexin V assay to measure early and late apoptosis of both normal and asthmatic fibroblasts post 72 hours of inhibition with anti-IL-5 and both in the presence and absence of IL-5. **(D)** The total percentage of early and late apoptotic cells post inhibition with Anti-IL-5 with and without IL-5. Data shown is representative for at least two different experiments, and presented as mean ± SD after normalization to the respective untreated control; Tubulin was used as loading control, t-Test was done as a statistical tool, * $p < 0.05$, ** $p < 0.01$.

3.13 Anti IL-5 potentially reverses the deposition rate of extracellular matrix proteins in asthmatic derived fibroblasts.

The previously presented findings demonstrated that IL-5 significantly upregulates the expression of extracellular matrix (ECM) components, including COL1A1, Fibronectin, and, to a lesser extent, Tenascin C. To further validate the role of IL-5 in this process, we explored its antagonism by treating fibroblasts derived from asthmatic patients with an anti-IL-5 monoclonal antibody, both in the presence and absence of IL-5. Cell lysates from these treatments were analysed via immunoblotting to evaluate the protein levels of ECM components that responded significantly to IL-5 stimulation. Results showed a substantial reduction in collagen 1A1 levels following treatment with anti-IL-5 alone compared to IL-5 stimulation. Furthermore, co-treatment with IL-5 and anti-IL-5 resulted in decreased collagen 1A1 expression relative to IL-5 stimulation (**figure 3.20 A)**. Similarly, fibronectin expression was downregulated with anti-IL-5 treatment compared to IL-5 stimulation (**figure 3.20 B)**. However, while tenascin C exhibited a similar trend, the inhibitory effect of anti-IL-5 on its expression was less pronounced compared to its impact on collagen (**figure 3.20 C**).

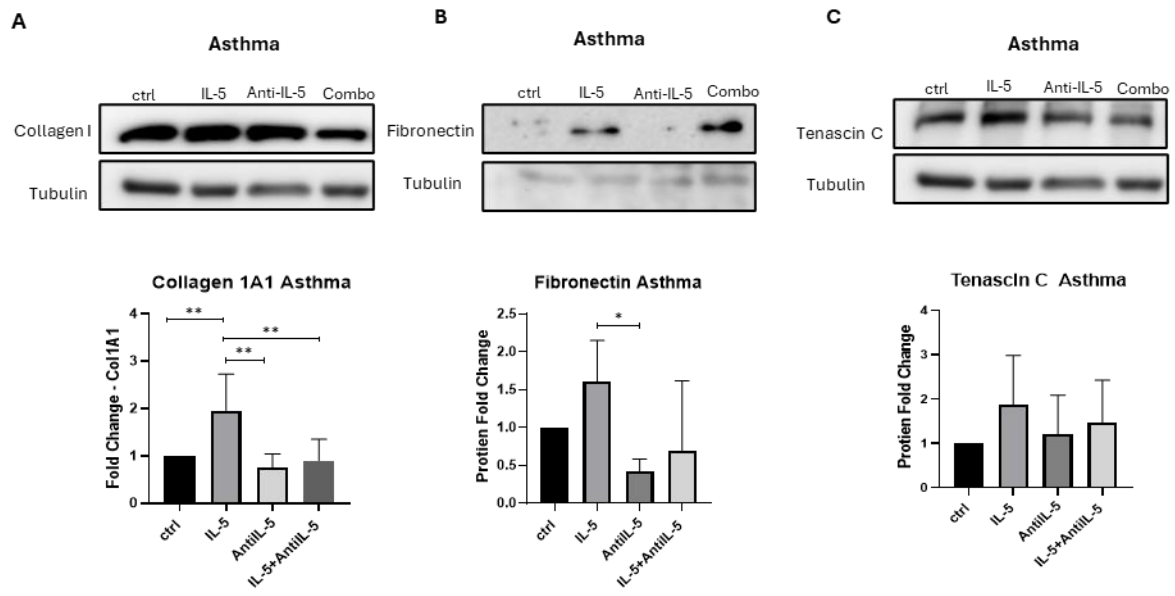


Figure 3.20 Anti-IL5 reduces the expression of Collagen and Fibronectin. (A) Representative immunoblots and Densitometric analysis of Collagen 1A1 in Asthma derived lung fibroblasts treated with anti-IL5 (0.4µg/ml) with and without IL-5 (0.5ng/ml) for 72 hours. **(B)** Representative immunoblots and Densitometric analysis of Fibronectin in Asthma derived lung fibroblasts treated with anti-IL5 (1.4µg/ml) with and without IL-5 (0.5ng/ml) for 72 hours. **(C)** Representative immunoblots and Densitometric analysis of Collagen 1A1 in Asthma derived lung fibroblasts treated with anti-IL5 (1.4µg/ml) with and without IL-5

(0.5ng/ml) for 72 hours. Data shown is representative for at least three different experiments and presented as mean ± SD after normalization to the respective untreated control; Tubulin was used as loading control, One way ANOVA was done as a statistical tool, * $p < 0.05$, ** $p < 0.01$.

3.14 Anti-IL5 reduces the expression of MMP-3 levels in asthmatic lung derived fibroblasts

In the previously presented data, IL-5 stimulation was shown to upregulate the expression of several MMPs, particularly MMP-2 and MMP-3, in fibroblasts derived from asthmatic lungs. To determine whether anti-IL-5 treatment could counteract this stimulatory effect, asthmatic fibroblasts were treated with anti-IL-5, with and without IL-5, for 72 hours. Cellular lysates were prepared and analysed via immunoblotting to assess the protein levels of MMP-2 and MMP-3. As depicted in **Figure 3.21 A**, treatment with anti-IL-5 with or without IL-5 didn't reduce MMP-2 protein levels, however solo treatment with anti-IL-5 significantly reduced MMP-3 protein levels compared to cells stimulated with IL-5. Additionally, anti-IL-5 was observed to mitigate the stimulatory effect of IL-5 in co-treatment conditions, leading to reduced MMP-3 levels compared to IL-5-stimulated cells, though not statistically when compared to the untreated controls (**figure 3.21 B)**. We further measured the protein level of TIMPs post treatment with anti-IL5, data in **figure 3.21 D** showed that anti-IL-5 reduces the expression level of TIMP-2 when compared to IL-5 stimulation, The MMP-2/TIMP-2 ratio was also measured, but unfortunately no significant change was reported as in **figure 3.21 E**.

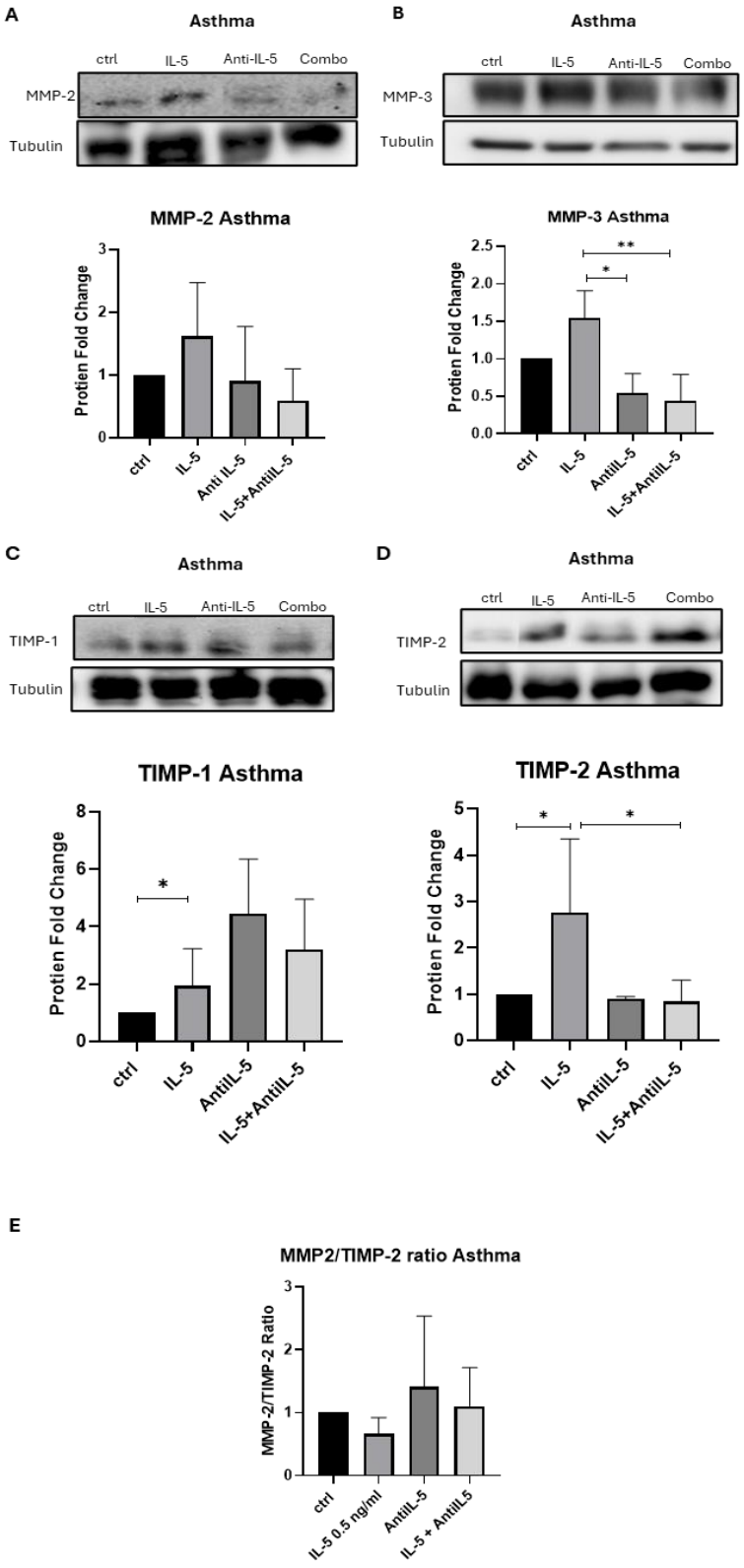


Figure 3.21 Anti-IL5 reduces the expression of MMP-3 levels in asthmatic lung derived fibroblasts. Asthma derived lung fibroblasts treated with anti-IL5 (0.4 µg/ml) with and without IL-5 (0.5ng/ml) for 72 hours. **(A)** Representative immunoblots and densitometric analysis of MMP-2. **(B)** Representative immunoblots and densitometric analysis of MMP-3. **(C)** Representative immunoblots and densitometric

analysis of TIMP-1. **(D)** Representative immunoblots and densitometric analysis of TIMP-2. **(E)** Calculated MMP2/TIMP1 ratio in asthmatic derived lung fibroblasts post treatment with anti-IL-5 with and without IL-5. Data shown is representative for at least three different experiments and presented as mean ± SD after normalization to the respective untreated control; Tubulin was used as loading control, one-way ANOVA test was done as a statistical tool, * $p < 0.05$, ** $p < 0.01$.

3.15 Anti-IL-5 reduces the secretion of IL-6 and TGF-β cytokines.

Our results demonstrated that IL-5 stimulation induces the expression of profibrotic and pro-inflammatory cytokines (**Figure 3.16**). To evaluate whether anti-IL-5 treatment could counteract these effects, we treated asthmatic lung-derived fibroblasts with anti-IL-5, and in combination with IL-5. The data in **Figure 3.22** indicates that a four-hour treatment with anti-IL-5 was sufficient to mitigate the effects of IL-5 stimulation. Specifically, anti-IL-5 treatment significantly reduced IL-6 protein level in cells treated with anti-IL-5 and those treated with a combination of both in comparison to IL-5 stimulated fibroblasts (**figure 3.22 A)**. Similar observations were noticed in the levels of fibrotic growth factor; TGF-β, where anti-IL-5 treatment sufficiently reduced its protein level in cells treated with anti-IL-5 and those treated with a combination of both when compared to the cells stimulated with IL-5 **figure 3.22 B**. This suggests that anti-IL-5 effectively reverses IL-5-mediated pro-inflammatory responses in asthmatic fibroblasts.

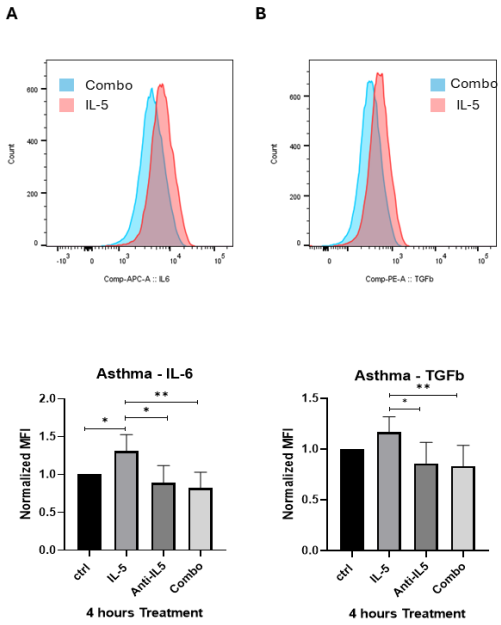


Figure 3.22 Anti-IL-5 reduces the secretion of IL-6 and TGF-β cytokines. (A) Representative histogram and normalized MFI of the expression of the IL-6 measured in asthmatic derived fibroblasts. Normalization

was done against the control sample of each run. **(B)** Representative histogram and the normalized MFI of the expression of the TGFβ measured in asthmatic derived fibroblasts. Normalization was done against the control sample of each run. Data shown is representative for at least three different experiments and presented as mean ± SD after normalization to the respective untreated control, one-way ANOVA test was done as a statistical tool, * $p < 0.05$, ** $p < 0.01$

The impact of IL-5 on human airway fibroblasts was comprehensively studied using an in vitro experimental model, highlighting its novel and direct fibrotic role on lung fibroblasts. Furthermore, the potential of anti-IL-5 therapy to ameliorate the fibrotic effects of IL-5 on airway fibroblasts was demonstrated. However, further validation in a more relevant physiological system is essential. Therefore, we will explore the role of IL-5 in an ex-vivo murine system.

3.16 Murine lung fibroblasts positively express IL-5Rα

To validate the fibrotic role of IL-5 in a physiological system such as murine asthmatic model, we first aimed to confirm the expression of IL-5 receptor alpha (IL-5Rα) in lung fibroblasts derived from murine models. Primary fibroblasts were isolated from murine lung tissue and initially assessed for morphological characteristics. As shown in **Figure 3.23 A**, these cells exhibited the typical elongated, spindle-shaped morphology characteristic of fibroblasts. To further confirm cell identity and homogeneity, we evaluated the expression of Vimentin, a well-established fibroblast marker. Immunostaining for Vimentin demonstrated that 98.3% of the isolated cells stained positively (**figure 3.23 B)**. Following this, we proceeded to assess IL-5Rα expression. Baseline IL-5Rα expression was observed in the murine lung fibroblasts (**figure 3.23 B)**, confirming the presence of this receptor in the isolated cells. For further validation of the IL-5Rα expression, we stained a murine lung isolated from healthy and asthmatic models induced by either HDM or OVA, stained sections showed a positive staining of IL-5Rα, where higher levels of the receptor expression where observed in HDM induced asthmatic mouse model when compared to their healthy controls as in **figure 3.23 C**, consistent with what we observed earlier in human bronchial tissue sections in **figure 3.2**

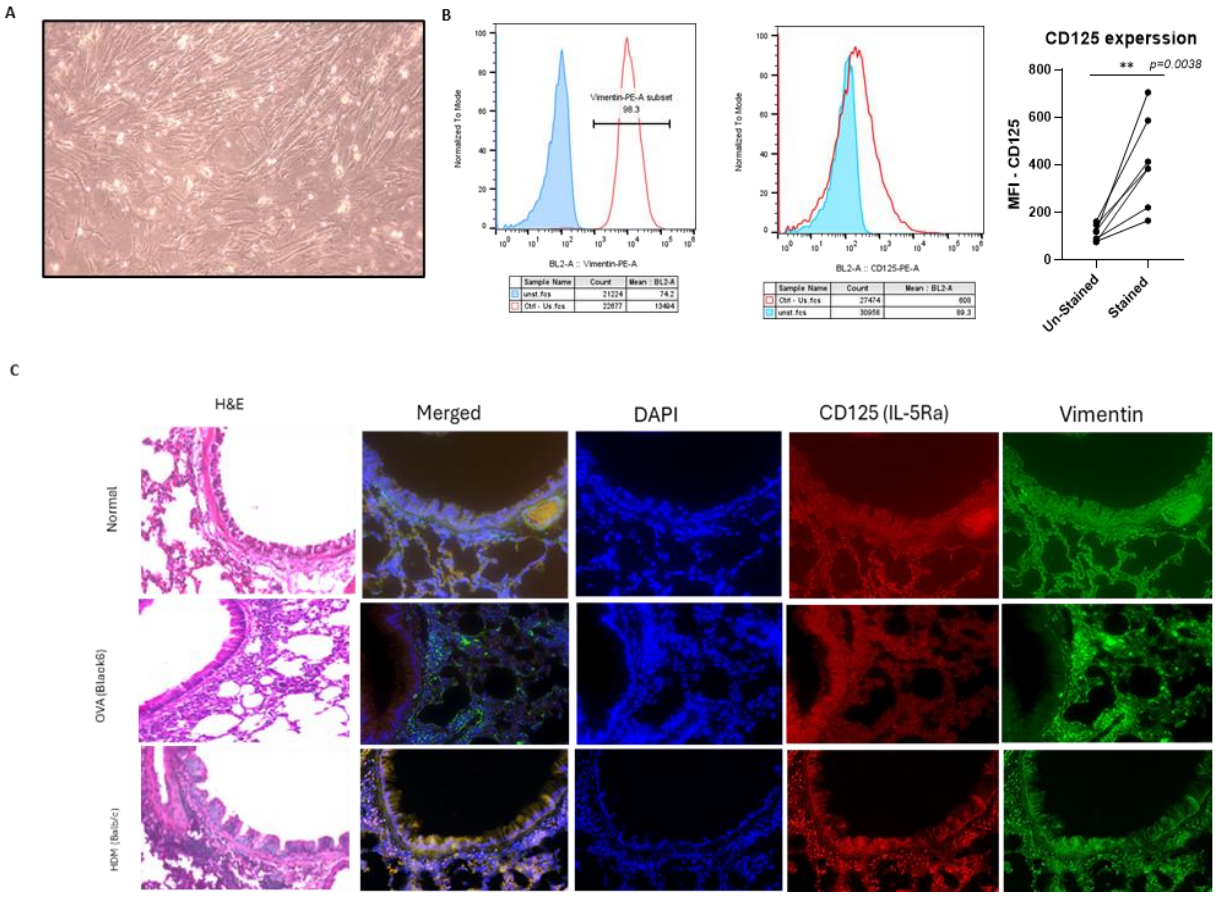


Figure 3.23 Figure Murine lung fibroblasts positively express IL-5Rα (CD125) (A) Phase contrast microscopy image showing the morphological characteristics of cultured Isolated murine lung fibroblasts. **(B)** The expression of Vimentin (left) and CD125 (right) using Flowcytometry analysis of the isolated cells. **(C)** Left panel represents images for H&E staining of normal and asthmatic murine lung tissue, right panel represents the Immunofluorescence staining of IL-5Rα (red), vimentin (green), DAPI (Blue) in normal and asthmatic murine lung tissue. Data representative of 6 separate independent experiments, and presented as mean ± SD, paired student t-test was conducted, * $p < 0.05$, **$p < 0.01$.

3.17 mrIL-5 successfully induces the proliferation of murine lung fibroblasts

In order to explore the role of IL-5Rα in terms of viability in the isolated lung fibroblasts. Cells were exposed to multiple cytokines that are known to induce fibroblasts proliferation including mTGFβ (50ng/ml), mLIGHT (100ng/ml) and mIL-5 (0.5ng/ml) and left in the incucyte for up to 96 hours which measures the cell confluency every 4 hours. **Figure 3.24 A-B** showing the exponential increase in the cell confluency in the treated cells when compared to the starting point. Then we measured the Area under the curve (AUC) to statistically measure the

confluency rate. Then IL-5 induced the proliferation rate as significant as the LIGHT cytokine which is known beside TGFβ for its role in promoting fibroblasts viability.

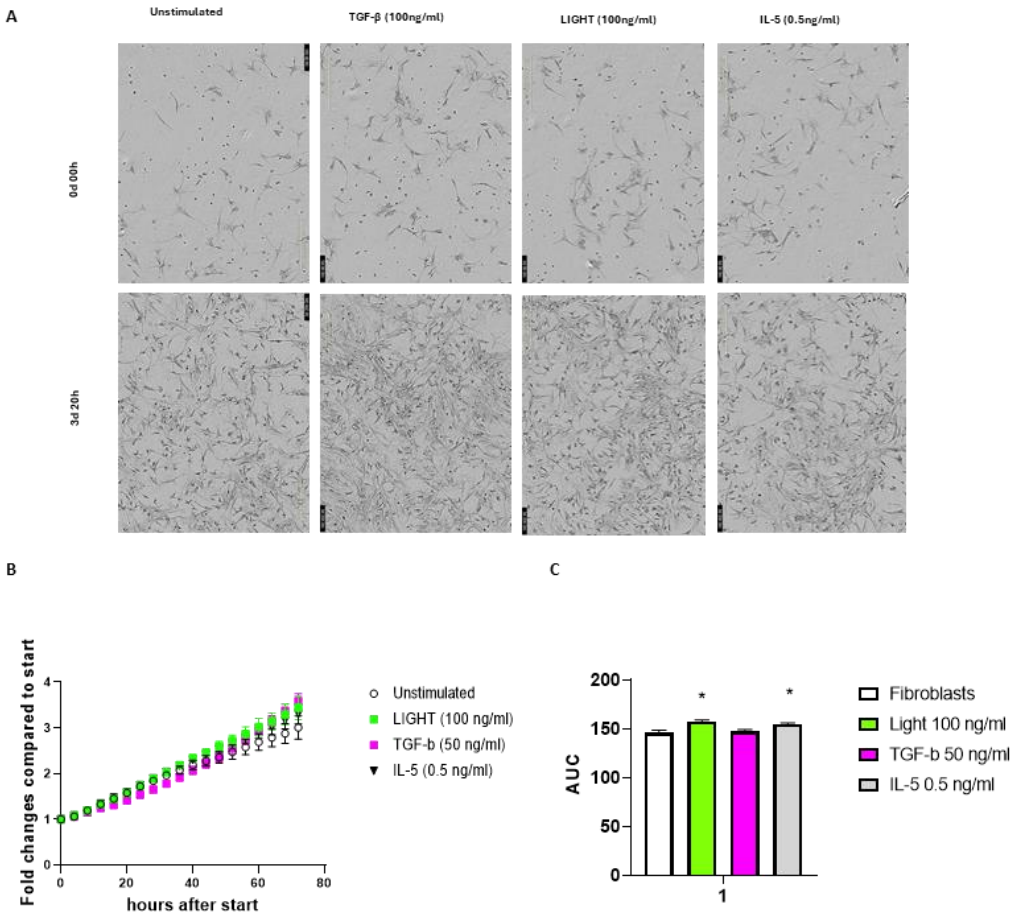


Figure 3.24 mrIL-5 successfully induces the proliferation of murine lung fibroblasts (A) Representative phase-contrast images captured using Incucyte live-cell imaging microscopy, illustrating cell confluency at 0 hours and after 72 hours of stimulation with LIGHT, TGF-β, and IL-5. **(B)** The observed exponential increase in cell growth over a 96-hour incubation period, with or without stimulation by LIGHT, TGF-β, and IL-5. **(C)** Area under the curve (AUC) analysis of numerical data generated by Incucyte, comparing cell responses in the presence and absence of LIGHT, TGF-β, and IL-5 stimulation. Data representative of 3 separate independent experiments, and presented as mean ± SD, one way ANOVA test was conducted, * $p > 0.05$.

3.18 Stimulation of murine lung fibroblasts with mrIL-5 results in elevated levels of Fibronectin expression.

Activated fibroblasts play a crucial role in extracellular matrix (ECM) deposition, a key process in fibrosis development. Our previous findings in human lung fibroblasts have shown that IL-

5 stimulation leads to increased expression of fibronectin (*FN-1*) and collagen type I alpha 1 (*COL1A1*), two major ECM proteins associated with fibrotic progression. To extend these findings to a murine model, isolated murine lung fibroblasts were stimulated with 0.5 ng/ml IL-5 Following 24-hour stimulation, qRT-PCR analysis was conducted to assess FN-1 and Col1A1 expression levels. Results demonstrated that IL-5 at 0.5 ng/ml has no effect on the mRNA levels of either Col1A1 **(figure 3.25 A**) or FN-1 **(figure 3.25 B)**.

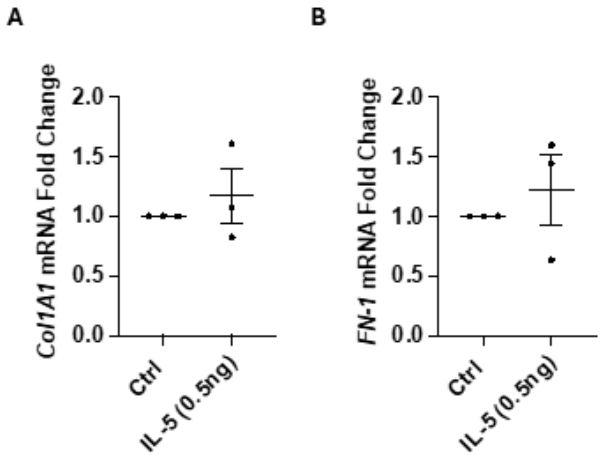


Figure 3.25 Stimulation of murine lung fibroblasts with mrIL-5 results in elevated levels of Fibronectin expression. (A) Showing the mRNA level of Col1A1 in murine Lung derived fibroblasts post 24hrs stimulation with IL-5, **(B)** showing the mRNA level of FN-1 in murine lung derived fibroblasts post 24hr of IL-5 stimulation. Data shown is a representation of three different independent experiments, UBC was used as a housekeeping gene for fold change calculation, Fold change was calculated by 2^-ΔΔCt, data shown is the mean ± SD of three separate experiment; student t-Test statistical analysis was done.

3.19 mrIL-5 induces the expression of MMP-2 from isolated lung fibroblasts

To assess the capacity of IL-5 to induce the release of key matrix metalloproteinases (MMPs) involved in asthma pathogenesis, specifically MMP-2 and MMP-9, as well as their natural inhibitors TIMP-1 and TIMP-2, isolated fibroblasts were treated with 0.5 ng/ml IL-5. Expression levels of mRNA encoding *MMP-2, MMP-9, TIMP-1*, and *TIMP-2* were then analysed by qRT-PCR. As shown in **figure 3.26 A**, MMP-2 expression was significantly upregulated following IL-5 stimulation, while MMP-9 expression remained unaffected (**figure 3.26 B)**. Conversely, IL-5 stimulation led to no change in *TIMP-1*, TIMP-2 levels (**figure 3.26 C-D)**.

These findings imply that IL-5 may have a species-consistent effect in terms of MMP-2 expression in lung fibroblasts and support its role in ECM remodelling.

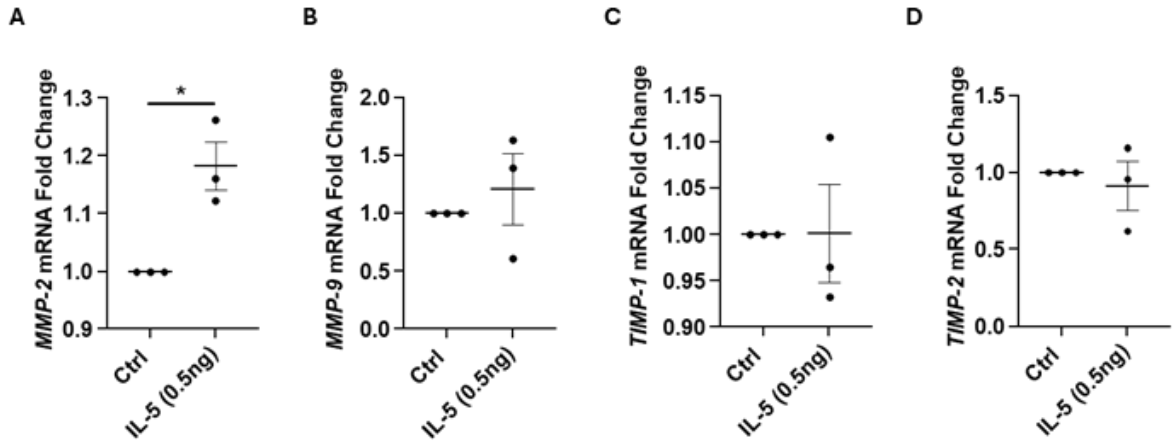


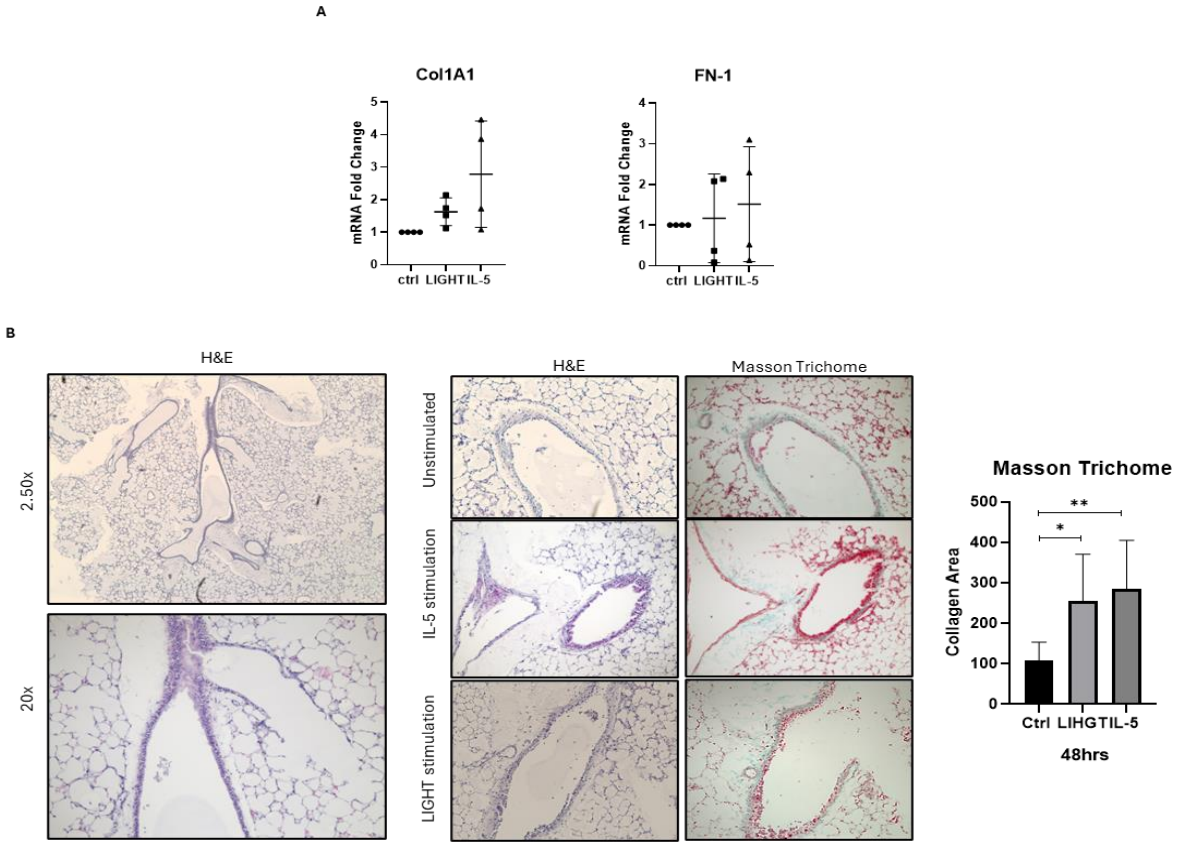
Figure 3.26 mrIL-5 induces the release expression of *MMP-2* in isolated lung fibroblasts
Murine lung derived fibroblasts were stimulated for 24hrs with IL-5, before cells were harvest and mRNA expression analysed by RT-PCR. **(A-B)** mRNA levels of *MMP-2* and *MMP-9*. **(C-D)** mRNA levels of *TIMP-1* and *TIMP-2*. Data are the fold change data shown is the mean ± SD from three different independent mice; UBC was used as a housekeeping gene for normalization. Fold change was calculated by 2^-ΔΔCt. t-Test statistical analysis was done * $p < 0.05$.

3.20 IL-5 induces collagen secretion in PCLS ex-vivo system

To validate the preceding findings, we transitioned to an ex vivo experimental model, which offers a more intricate system compared to in vitro approaches. Lung tissues were sectioned into slices with a thickness of 350 µm to create precision-cut lung slices (PCLS). The structural integrity of these sections was assessed post-cytokine stimulation using qRT-PCR analysis to evaluate the two major ECM RNA levels, *COL1A1* and *FN-1* (**figure 3.27 A**), a trend of elevation was observed post IL-5 stimulation, but the finding didn't reach a statistical significance, might be explained due to the heterogeneity of the extracted samples. haematoxylin and eosin (H&E) staining was conducted, which allowed for the identification of structural features such as small and large airways **(figure 3.27 B)**. To specifically evaluate collagen deposition, the same sections were stained using Masson's trichrome (MT), a collagen-selective staining method where collagen-rich areas appear blue. The PCLS were stimulated with recombinant murine IL-5 (mr-IL-5) at a concentration of 0.5 ng/mL and mrLIGHT at 100 ng/mL for 48 hours. MT staining revealed a significant increase in collagen

deposition in the cytokine-stimulated sections compared to the unstimulated controls (**Figure 3.27 B**).

Building on supplementary data showing that LIGHT stimulation induces IL-5 receptor alpha (IL-5Rα) expression (**Figure 6.2** – Appendix I), we further tested the combined effects of LIGHT and IL-5 on collagen deposition. PCLS were first stimulated with LIGHT for 24 hours, followed by IL-5 for an additional 48 hours, resulting in a total stimulation period of 72 hours. Co-stimulation with LIGHT and IL-5 demonstrated a marked increase in collagen-rich areas compared to stimulation with LIGHT alone (**Figure 3.27 C**), supporting the fact that LIGHT is a potential inducer of IL-5Rα in lung airways.



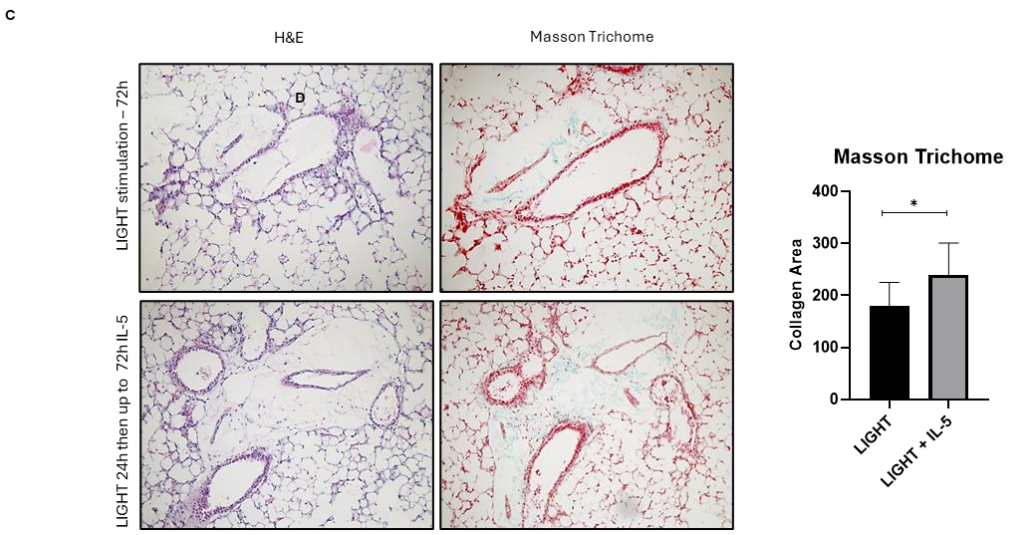


Figure 3.27 IL-5 induces collagen secretion in PCLS ex-vivo system (A) Displays the mRNA expression levels of Col1A1 and FN-1 in PCLS sections following 24 hours of stimulation with LIGHT (100 ng/ml) and IL-5 (0.5 ng/ml), compared to unstimulated controls. UBC was used as a housekeeping gene for fold change calculation, Fold change was calculated by 2^-ΔΔCt, data shown is the mean ± SD of the fold expression change relative to the respective untreated control post normalization to housekeeping gene of four separate experiment **(B)** The left panel displays H&E staining of the precision-cut lung slices (PCLS), highlighting lung section characteristics at two magnifications, 25x and 200x.The middle panel presents a representative H&E-stained section alongside its corresponding Masson's trichrome-stained sections following 48 hours of stimulation with LIGHT (100 ng/ml) and IL-5 (0.5 ng/ml), compared to unstimulated control sections. The right panel depicts the quantification of collagen-enriched areas within potential airways. The data is a representative of ± SD from at least three different independent mice, student t-test was done * $p < 0.05$, ** $p < 0.01$. **(C)** The left panel features H&E-stained images of sections stimulated with LIGHT, both with and without IL-5, for up to 72 hours, while the right panel shows the corresponding Masson's trichrome-stained sections at 200x magnification, right graph illustrates the quantification of collagen-enriched areas within potential airways. The data is a representative of ± SD from at least three different independent mice, One way ANOVA test was done, * $p < 0.05$.

3.21 IL-5 stimulation promotes the expression of MMP-2 and MMP-9 while reducing TIMP-2 expression in PCLS ex-vivo system

We showed that IL-5 stimulation increases collagen positive areas, and a trend increase in the mRNA expression of *collagen 1A1*, however MMPs and TIMPs are highly critical enzymes that play a significant role remodelling process as discussed earlier, their dysregulated levels are as important as the increased deposition of the ECM elements in the progression of fibrosis.

To further investigate the parameters changed in PCLS post IL-5 and LIGHT stimulation, we moved to measure the mRNA expression of the *MMP-2, MMP-3,* and *MMP-9* along with *TIMP-1* and *TMP-2* in the PCLS sections stimulated for 24h with IL-5. The qRT-PCR data revealed that IL-5 elevated the expression of *MMP-2* (p=0.045) and *MMP-9* (p=0.023) but not *MMP-3* when compared to unstimulated and LIGHT stimulated sections (**figure 3.28 A-C)**. In addition, IL-5 stimulation significantly reduces the *TIMP-2* level (p=0.0032) compared to the control and in compared to LIGHT stimulation (*p=0.0031*), while *TIMP-1* expression showed no noticeable effect pre and post IL-5 stimulation, overall, IL-5 stimulation disrupted the balance between the MMPs and TIMPs levels in the PCLS (**figure 3.28 D-E)**.

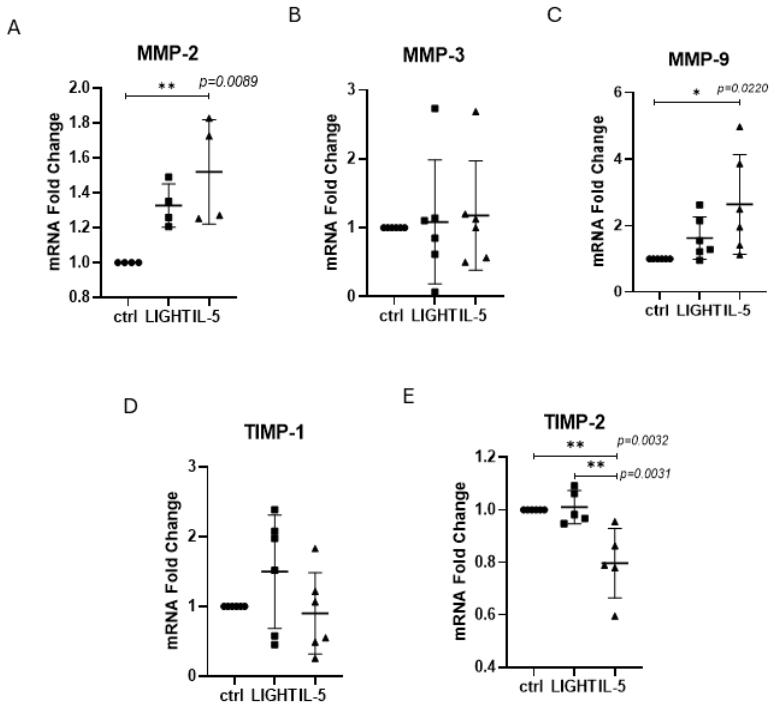


Figure 3.28 IL-5 stimulation promotes the expression of MMP-2 and MMP-9 while reducing TIMP-2 expression in PCLS ex-vivo system. After 24 hours stimulation with either LIGHT (100 ng/ml) or IL-5 (0.5 ng/ml) in fibroblasts derived from murine lung tissue cells were harvest and mRNA content analysed. **(A-C)** mRNA expression levels of *MMP-2, MMP-3* and *MMP-9* **(D-E)** mRNA level of *TIMP-1* and *TIM-2.* UBC was used as a housekeeping gene for fold change calculation, Fold change was calculated by 2^-ΔΔCt, data shown is the mean ± SD of four separate experiment; one way ANOVA statistical analysis test was done * $p < 0.05$, **$p < 0.01$. Fold expression change relative to the respective untreated control post normalization to housekeeping gene

Chapter IV.

Discussion

In this study, we were the first to demonstrate that i) human lung-derived fibroblasts expressed IL-5Rα, and ii) that the expression was significantly higher in fibroblasts from asthmatic patients compared to those from healthy controls. Notably, human lung fibroblasts responded to IL-5 stimulation with an increased proliferation rate. Furthermore, IL-5 stimulation significantly influenced remodeling markers, including elevated protein levels of Collagen I, fibronectin, MMP-2, and MMP-3. Additionally, IL-5 enhanced the secretion of profibrotic and proinflammatory cytokines, such as TGF-β and IL-6, respectively. Interestingly, these effects were more pronounced in fibroblasts derived from asthmatic patients than in those from non-asthmatic individuals. However, although treatment with anti-IL-5 reduced MMP-3 levels, it only partially inhibited ECM component deposition. iii) murine lung fibroblasts were also positive to the expression of IL-5Ra, and its expression was significantly induced in response to LIGHT stimulus. In addition, IL-5 stimulation also induces the collagen deposition in a comparable levels to LIGHT. Overall, these findings suggest that IL-5 plays a critical role in the remodelling process by directly stimulating lung-derived fibroblasts, an effect that was partially mitigated by anti-IL-5 treatment.

4. 1 Lung derived fibroblasts express functional IL-5Rα

Our major finding demonstrated the expression of IL-5Rα on human lung derived fibroblasts. Interestingly, the baseline expression of IL-5Rα was significantly higher in asthmatic derived fibroblasts when compared to their normal counterparts, in line with the findings obtained from human cells, murine lung derived fibroblasts were positively expressing IL-5Rα as well.

IL-5 plays a central role in mediating eosinophil-driven inflammation and is found at elevated levels in the blood and airways of individuals with asthma. It is crucial for the maturation, activation, migration, and survival of eosinophils (Hamid et al., 1991; Lamkhioued et al., 1997). It is well documented that IL-5 acts on its target cells through the expression of IL-5Rα that was known to be exclusively expressed on eosinophiles (Matucci et al., 2019) and to a certain extent on basophile (Elena-Pérez et al., 2020). However, neutrophiles also shown

to express IL-5Rα (Gorski et al., 2019) . IL-5 along with IL-3 and GM-CSF facilitates the differentiation, activation, and prolonged survival of eosinophils, which are key inflammatory cells involved in asthma. In response to IL-5, eosinophils migrate to the airways contributing to airway inflammation, tissue damage, and remodelling (Siddiqui et al., 2023). However, excessive recruitment of eosinophils, driven by elevated levels of IL-5, underscores the critical role of this cytokine in the pathogenesis of asthma (Pelaia et al., 2019). Recently non-immune cells such as AEC have shown to express functional IL-5Rα (Pelaia et al., 2019). There, the cognate binding of IL-5 to its receptor leads to the activation of both ERK and PI3K pathways, which induces cellular proliferation as well as increase their survival (Barretto et al., 2020). The expression of IL-5Rα on AEC could further signify a direct role of IL-5 in inflammation and remodelling by sustaining an inflammatory milue in the airways through their secretion of alamrins (Barretto et al., 2020), however, further investigations are required in this area. IL-5 was observed to enhance the proliferation rate of human fibroblasts derived from both asthmatic and non-asthmatic sources. Notably, the proliferative response was more pronounced in fibroblasts obtained from asthmatic patients compared to their non-asthmatic counterparts, the elevated proliferation capacity of these cells considered to be a pathophysiological feature of fibrosis. Moreover, the enhanced proliferation was mediated through the ERK and AKT/PI3K signaling pathways, mirroring the pathways activated in eosinophils and AEC (Pelaia et al., 2019). However, its role in basophiles was more toward activation to secrete their inflammatory mediators such as histamine and LTC4 (Bischoff et al., 1990)In line with the finding observed in the human derived cells, IL-5 stimulation was inducing murine fibroblasts proliferation as much as other potent fibrotic cytokines including LIGHT and TGFβ.

4. 2 IL-5 stimulation induces fibrotic markers in severe asthmatic fibroblasts

Our study revealed that stimulation with IL-5 enhances the deposition of Fibronectin, collagen I, and Tenascin C when compared to another ECM components in human lung fibroblasts, although these could not be confirmed in murine derived fibroblasts. This is in line with the fact that fibroblasts as major cellular component in the subepithelial layer, and the fibrotic process. It is characterized by an increased turnover rate of the ECM which is defined by the increase deposition and reduced degradation of the ECM component including Collagen I, III and V, as well as Fibronectin, Tenascin C and Lumican by mainly fibroblasts as well as myofibroblasts (Thiam et al., 2023). The increased turnover rate of these proteins is known to

contribute to thickening of the fibrotic layer thus narrowing the airway lumen (J. T. Ito et al., 2019a).

In our study we used Mepolizumab to investigate the effect of this therapeutic approach on tackling airway remodelling. Our finding demonstrated that the effect of IL-5 was partially attenuated following the exposure of fibroblasts to Mepolizumab. These observations align with previously reported findings, wherein Mepolizumab not only reduced inflammatory markers in blood and sputum but also led to a decreased deposition of pro-collagen III and Tenascin C in treated patients with eosinophilic asthma (Flood-Page et al., 2003). This is of importance, because recent studies have demonstrated the effectiveness of anti-IL-5 therapy in managing severe asthma, both in clinical trial settings and real-world applications. Anti-IL-5 biologic treatments do not only reduce eosinophil levels in both blood and tissue, decrease the annual incidence of moderate to severe exacerbations, and lower both the need for and the dosage of oral corticosteroids (Bel et al., 2014). In this context, the FDA approved a range of three biologics that tackle the IL-5/IL-5Rα signaling axis, these are Mepolizumab (Menzella et al., 2015), Benralizumab (Ricciardi et al., 2022) and Reslizumab (Varricchi et al., 2017). However, the impact of these biologics in remodelling remains unclear and mostly ineffective. Our data provide evidence that Mepolizumab could interfere with remodeling process.

Another key factor that contributes to fibrosis is the disruption of the homeostatic balance between two group of enzymes that normally shape the airways, MMPs and their natural inhibitors TIMPs. MMPs belongs to a large family of calcium-dependent and zinc containing proteases known to degrade the ECM proteins during remodelling process. Their activity is regulated by TIMPs, which inhibit their activity. In addition to ECM degradation, MMPs play a crucial role in facilitating extra and intravasation of the immune cells to the site of inflammation. In our current study, we emphasized the significant roles of MMP-3 and MMP-2, both of which were upregulated following stimulation with IL-5, in contrast the levels of MMP-9 were not affected under similar conditions. Under pathological conditions, the normal 1:1 enzymatic ratio is disrupted resulting in an aberrant tissue repair (Löffek et al., 2011; Ohbayashi & Shimokata, 2005b; Takahashi et al., 2019). In asthma, MMP-9 is the most reported enzyme to contribute to the disease, its level in the sputum and the lung tissue correlates with the severity of the disease condition (Farhat et al., 2014; Ohbayashi & Shimokata, 2005b). Additionally, both MMP-2 and MMP-9 mediates eosinophiles infiltration (Kumagai et al., 1999).. Notably, studies have shown a correlation between MMP-3 levels

and collagen I, highlighting a negative association with FEV1 among asthma patients (Ingram & Kraft, 2015; Todorova et al., 2010), Furthermore, MMP-3 levels have been linked to a higher migratory rate of ASM cells (I. Ito et al., 2009), all of which is contributing to the remodelling progress. These findings were also consistent in murine lung fibroblasts stimulated with IL-5, as we observed an elevation in the mRNA levels of *MMP-2*. Although, the level of MMP-9 didn't show to be highly affected upon IL-5 stimulation in both human and murine fibroblasts, the increasing levels of TIMP-1 could explain a skewing of the ratio toward high TIMP-1 levels which also adds into the complex fibrotic mechanism. After assessing the impact of IL-5 stimulation on the levels of MMPs and TIMPs, we sought to determine whether targeting IL-5 could reverse its effects on these markers. Interestingly, our findings revealed that neutralizing IL-5 in cultured fibroblasts significantly reduced the protein levels of MMPs, particularly MMP-3, thereby mitigating the effects induced by IL-5.

In the current study, we highlighted that IL-5 stimulation does enhance the secretion level of both IL-6 and TGF-β from lung derived fibroblasts, but not TNFα this elevation was reversed when the cells were exposed to Anti IL-5. Although fibroblasts are majorly a structural cell in the airways that maintain the subepithelial layer, they also add to the inflammatory environment in the airways. This is mainly due to their capacity to secrete a potent group of cytokines and growth factors such as IL-6, TNFα, TGFβ, and IL-1B (Kendall & Feghali-Bostwick, 2014). This dual role of fibroblasts could explain the steroid resistance in severe asthmatic cases, and why airway remodelling couldn't be managed by conventional steroid therapy. IL-6 is known as an effector cytokine due to its role in determining the fate of Th cells, as its major role is promoting IL-4 secretion in Th2 CD4 cells during type 2 immune response (Rincon & Irvin, 2012). Thus, the induction of IL-6 expression in response to IL-5, will sustain the inflammatory condition and the persistent recruitment and activation of immune cells to the airways, this prolonged inflammation is one of the major factors that contribute to the remodelling and abnormal tissue repair process.

The regulatory mechanism exerted by IL-5 on its receptor remains contradictory and unclear. In the human lung-derived fibroblasts, IL-5 appears to upregulate its receptor specifically in asthmatic fibroblasts, maintaining a positive feedback loop, whereas no significant effect is observed in their normal counterparts. This phenomenon may be attributed to the distinctive pathological traits of asthmatic fibroblasts, which deviate from the typical regulatory mechanisms that operate in healthy eosinophils. However Previously, it was reported that asthmatic derived eosinophiles express higher levels of IL-5Rα that was thoroughly explained

due to the persistent allergen exposure, (Sehmi et al., 1997). While others reported a downregulatory effect of IL-5 on IL-5Ra expressed on eosinophiles explained mainly due to the effect of MMPs (L. Y. Liu et al., 2002b). Furthermore, others demonstrated that this effect is changing depending on the maturation stage of eosinophiles as well as localization of eosinophils and their progenitors (HELLMAN et al., 2003).. In the context of IL-5Ra regulation, our study showed asthmatic fibroblasts exhibit distinct phenotypic and molecular differences compared to their normal counterparts, including higher levels of TGF-β receptors (Michalik et al., 2018a). This receptor upregulation suggests a novel function for TGF-β in enhancing the expression of IL-5Rα, thereby increasing airway fibroblast sensitivity to IL-5. This heightened sensitivity creates a potential positive feedback loop, further amplifying the fibrotic process and contributing to the progression of airway remodelling in asthma. In murine lung fibroblasts, IL-5 did not enhance the expression of IL-5Rα. This could potentially be attributed to the fact that these cells are considered normal, reflecting a condition like what has been observed in normal human lung fibroblasts. In addition, we examined the effect TGF-β and LIGHT on the expression of IL-5Rα, showing that both were able to induce IL-5Rα expression, but the effect was more pronounced in LIGHT stimulated cells when compared to TGFβ stimulated cells. Highlighting that both LIGHT and TGFβ are known as potent pro-fibrotic cytokines in the context of airway remodelling in asthma (Doherty et al., 2011; Saito et al., 2018).

4. 3 The Functional role of IL-5 in Bronchial Fibroblasts in severe Asthma

Gene set enrichment analysis revealed multiple pathways influenced by IL-5 stimulation, many of which are directly or indirectly associated with the pathophysiology of asthma.

Asthmatic fibroblasts stimulated with IL-5 resulted in the identification of 39 DEGs, although the number of genes is relatively low. A noteworthy observation was the upregulation of *CDK11b* the coding gene of the cyclin-Dependent Kinase 11 (CDK11), a critical kinase that facilitate and regulate cell cycle progression(Loyer & Trembley, 2020). The upregulation of *CDK11b* gene mostly correlates with high proliferative rate, aligning with our initial findings. In cancer, this gene is a key target for therapeutic purposes (Zhou et al., 2016), but in asthma, bronchial epithelial cells shown to have dysregulated cell cycle proteins (Puddicombe et al., 2003), but in the context of airway fibroblasts, less is known. We further conducted a focused analysis of the upregulated pathways in fibroblasts derived from asthmatic patients. Consistent with our previously discussed findings , MAP kinase signalling pathway was

notably upregulated following IL-5 stimulation. This activation was associated with the promotion of fibroblast proliferation, a critical process contributing to the development of fibrosis. Many genes related to programmed cell death were upregulated post IL-5 stimulation in asthmatic derived fibroblasts that are potentially involved in fibroblasts survival, among them was Nuclear receptor subfamily 4, group A, member 1 *(NR4A1)*. In the context of apoptosis, this protein reported to have contradictory roles. Studies have shown that it acts as a pro-apoptotic protein through disrupting the mitochondrial membrane and inducing the released of the cytochrome C various cell types including cardiocytes and melanoma cells (Cheng et al., 2011; Yu et al., 2007). While others showed that it acts as an anti-apoptotic protein in B cells and in Normal umbilical cord fibroblast (Shimizu et al., 2015). In our study, the transcriptomic data revealed an overexpression in *NR4A-1* gene in asthmatic stimulated fibroblasts, and this was further validated using qRT-PCR. In line with this report (Shimizu et al., 2015), the percentage of apoptotic cells were significantly regulated post IL-5 stimulation in both normal and asthmatic fibroblasts and were exhibiting a lower level of the cleaved caspase-3 indicating an inhibition of extrinsic apoptosis. Although, Normal cells didn't show to have upregulated NR4A-1 expression but instead BID expression was upregulated as per the heat map suggesting that IL-5 was inhibiting normal fibroblast death using different mechanism. Collectively, IL-5 is a potential activator of asthmatic lung fibroblasts, due to the activation of MAP kinase signalling pathway, in addition it enhances the survival of these cells through the upregulation of the *NR4A-1*.

Fibroblasts are the major cells that are responsible for normal ECM production and turnover (J. T. Ito et al., 2019b). The latter is defined as the degradation of the old ECM proteins to be replaced by newly synthesized elements all of which to maintain the integrity of the airway structure (Sand et al., 2015). Under pathological conditions, the deposition and the turnover rate will be accelerated leading to a defective repair mechanism, The degraded ECM components serve as cellular stimuli, promoting cellular activity by binding to integrins and tyrosine kinase receptors on both fibroblasts and airway smooth muscle cells. (Dzobo & Dandara, 2023; Huang & Qiu, 2022), This interaction potentially fosters remodelling and fibrosis. This accelerated turnover is mostly facilitated by MMPs that are mainly secreted from activated fibroblasts (Bajbouj et al., 2021). Among the upregulated pathways was the enhanced ECM degradation post IL-5 stimulation which could be explained based on the fact that IL-5 is a potential activator of fibroblasts, and this activation might lead to an increase in the MMPs secretion thus enhancing ECM degradation. This effect mimics the impact of other

established pro-fibrotic cytokines, such as TGFβ and periostin (Kanaoka et al., 2018; Sidhu et al., 2010), which are known to drive similar cellular responses. These findings underscore the multifaceted role of IL-5, not only as a mediator of inflammation but also as a contributor to the pro-fibrotic environment in asthma. This suggests that IL-5 may act synergistically with other cytokines to exacerbate airway remodelling, highlighting its potential as a therapeutic target to mitigate fibrosis-related complications in asthma.

The enrichment analysis revealed several pathways intricately linked to cytokine signalling and immune response. Among these, notable pathways include "cellular response to cytokine stimulus," "response to extracellular stimulus," and "cytokine signalling in the immune system." These findings highlight the fundamental role of the immune system, particularly its cellular components and mediators, which are well-documented in scientific literature. To correlate the above with asthma, the disease is characterized by the pivotal involvement of diverse cytokines that drive its pathogenesis (Kips, 2001; Lambrecht et al., 2019). Our transcriptomic data provides further insights into this dynamic, indicating a significant role for IL-5. Specifically, these results suggest that IL-5 may play a critical role in amplifying the responsiveness of airway fibroblasts to the surrounding cytokines, particularly during inflammatory conditions. This enhanced sensitivity could contribute more to the complex inflammatory landscape observed in asthma particularly T2 high where they are characterized with high levels of IL-5 (Ricciardolo et al., 2021), further underscoring the multifaceted interplay between immune mediators and structural cells in the airway microenvironment.

However, one of the most notably downregulated pathways is the regulation of canonical NF-κB signalling pathway. Further, the DEG showed the gene *IKBIP* coding the Inhibitor of nuclear factor kappa-B kinase-interacting protein was downregulated in asthmatic stimulated fibroblasts, which is well known to inhibit the activity of this pathway (Hu et al., 2024). Extensive evidence highlights the pivotal role of NF-κB in the pathogenesis of asthma, as it regulates the expression of cytokines, chemokines, and adhesion molecules, all of which are crucial in the allergic response (Edwards et al., 2009; Levine, 2003; Schuliga, 2015), NF-κB, in particular, is activated in response to cytokine stimulation, emphasizing its central role in mediating inflammatory processes in asthma (Schuliga, 2015). However, this pathway is greatly complex and being regulated with various regulatory proteins adds to its complexity (Schuliga, 2015), what needs further to be investigated is the specific mechanism of action of how IL-5 could potentially disrupt the regulatory process of this pathway in lung derived fibroblasts.

Chapter V.

Conclusion

The present investigation highlights the importance of the IL-5/IL-5Rα signalling axis by examining a novel pathogenic role for IL-5 and its receptor in human lung fibroblasts. The findings underscored the direct role of this axis in airway remodeling and fibrosis, particularly in severe asthma. IL-5 promotes lung fibroblast survival by inhibiting programmed cell death and deregulation of important genes such as NR4A-1 and FOXO-1. It also increases the synthesis of fibronectin, collagen 1A1, IL-6, and TGF-β. In addition, this interaction alters the MMP/TIMP balance, which contributes to tissue remodelling. Moreover, IL-5 induces the expression of its cognate receptor making it a potent fibrotic cytokine. Collectively, our findings place the IL-5/IL-5Rα signalling axis as a potential therapeutic target to address both eosinophilia and the fibrotic elements of asthma.

Study limitations:

Our study has various limitations that should be considered. One of the limitations is the small number of asthmatic patient's cells used in the study. Due to this constraint, we used three biological replicates from each donor to validate and reinforce our findings.

The study was extensively conducted in an in-vitro experimental setup, however generation of a chronic mouse model with a severe airway remodelling considered to be challenging, due to the need of a prolonged exposure to allergens, and some mice would survive this kind of experiments.

The resulting enriched pathways, need further validation with wider functional assays to be implemented, especially pathways like response to hormones.

Future work

The study focuses on investigating the role of IL-5 in airway remodelling in asthma beside its well-known role in airway inflammation. We could investigate more and validate the enriched pathways that results from the transcriptomic data, to further highlight the role of fibroblasts in both airway inflammation and remodelling.

Validate the DEGs that was picked up from the Transcriptomic data, on human bronchial tissues isolated from mild, moderate and severe asthmatic cases and link the expression of the DEGs with the severity of the disease.

Generate a mouse model that lacks the eosinophilic lineage, to eliminate their role in airway remodelling in response to IL-5, focusing on the role of IL-5 directly on fibroblasts in remodeling.

Abstract

Role of IL-5 in Airway Remodeling in Severe Asthma

Rola AY Abujabal

Background: Asthma is a chronic disorder of the lower respiratory tract. The chronicity of the condition derives a distinguished feature of the disease called airway remodelling, including fibrosis. which strongly correlates with the severity of the disease. A key contributor to this process is interleukin-5 (IL-5), mainly associated with airway eosinophilia through their expressed IL-5Rα, contributing to remodelling. This study aims to identify the expression of IL-5Rα and the direct impact of IL-5 in remodeling **Methodology:** Human lung fibroblasts obtained from healthy and severe asthmatic subjects were utilized to characterize the baseline expression of IL-5Rα and assess recombinant human h-IL-5's direct role on lung fibroblasts, Transcriptomic analysis along with various functional and mechanistic assays were employed in this work. To assess the role of IL-5 in remodelling from various aspects including cellular proliferation, ECM deposition, matrix metalloproteinases (MMPs) and tissue tissue inhibitors of metalloproteinases (TIMPs) expression alongside with inflammatory and fibrotic cytokines release, all the above were further confirmed by inhibiting IL-5 or its receptor using available monoclonal antibodies. **Results:** this work characterizes, at the molecular and cellular level, the elevated levels of IL-5Rα expression in asthmatic fibroblasts. In addition, it identifies a novel role of IL-5 in directly influencing lung fibroblast function. Transcriptomic analysis revealed that IL-5 enhances fibroblast survival through upregulation of *NR4A1*, which inhibits apoptosis in asthmatic fibroblasts. Additionally, Gene Set Enrichment Analysis highlighted significant dysregulation in pathways such as MAP kinase, Wnt signalling, and NF-κB. Furthermore, IL-5 exposure triggered an upregulation of extracellular matrix (ECM) components, including collagen 1A1 (COL1A1) and fibronectin (FN1), as well as MMP-2, MMP-3 and TIMP-1. This disruption in the MMP/TIMP balance promotes fibrosis. IL-5 also induced the release of pro-inflammatory (IL-6) and pro-fibrotic (TGF-β) cytokines, with these effects being more pronounced in asthmatic fibroblasts than in healthy controls. The effect of IL-5 was significantly reversed when inhibiting the cells with IL-5/IL-5Rα inhibitors. **Conclusion:** These findings highlight a previously un-investigated pro-fibrotic role of IL-5 in asthma, mediated by its direct action on lung fibroblasts. This study underscores the therapeutic

potential of targeting IL-5 or its downstream pathways to mitigate or reverse airway remodelling in asthma.

Zusammenfassung

Hintergrund: Asthma ist eine chronische Erkrankung der unteren Atemwege. Die chronische Ausprägung der Erkrankung führt zu einem besonderen Merkmal, dem Umbau der Atemwegsschleimhaut, einschließlich Fibrose, der stark mit dem Schweregrad der Erkrankung korreliert. Ein wichtiger Faktor in diesem Prozess ist Interleukin-5 (IL-5), das über die Expression von IL-5Rα hauptsächlich mit der Eosinophilie der Atemwege in Verbindung gebracht wird und so zum Umbau beiträgt. Ziel dieser Studie war es, die Expression von IL-5Rα und die direkte Auswirkung von IL-5 auf den Gewebsumbau zu identifizieren. Methodik: Humane Lungenfibroblasten von Gesunden und schwer asthmatischen Patienten wurden verwendet, um die basale Expression von IL-5Rα zu charakterisieren und die direkte Rolle von rekombinantem humanem-IL-5 auf Lungenfibroblasten zu untersuchen. Dazu wurden eine Transkriptomanalyse sowie verschiedene funktionelle und mechanistische Experimente duchgeführt. Um die Rolle von IL-5 beim Umbau unter verschiedenen Aspekten wie Zellproliferation, Ablagerung von extrazellulärer Matrix (EZM), Expression von Matrix-Metalloproteinasen (MMPs) und deren Inhibitoren (TIMPs) sowie Freisetzung von entzündlichen und fibrotischen Zytokinen zu analysieren, wurden alle oben genannten Aspekte durch Hemmung von IL-5 oder seines Rezeptors mit spezifischen monoklonalen Antikörpern bestätigt. Ergebnisse: Diese Arbeit charakterisiert die erhöhte Expression von IL-5Rα in asthmatischen Fibroblasten auf molekularer und zellulärer Ebene. Darüber hinaus wird eine neue Rolle von IL-5 in der direkten Beeinflussung der Lungenfibroblastenfunktion identifiziert. Die Transkriptomanalyse ergab, dass IL-5 das Überleben der Fibroblasten durch die Hochregulierung von *NR4A1* verbessert, das die Apoptose in asthmatischen

Fibroblasten hemmt. Darüber hinaus zeigte die Gene Set Enrichment Analysis eine signifikante Dysregulation in Signalwegen wie dem MAP-Kinase-Weg, dem Wnt-Signalweg und NF-κB. Darüber hinaus führte die Stimulation mit IL-5 zu einer Hochregulierung von Komponenten der EZM, einschließlich Kollagen 1A1 (COL1A1) und Fibronektin (FN1), sowie von MMP-2 und MMP-3, während die Expression von TIMP-1 reduziert wurde. Diese Verschiebung des MMP/TIMP-Gleichgewichts fördert die Fibrose. IL-5 induzierte auch die Freisetzung von pro-inflammatorischen (IL-6) und pro-fibrotischen (TGF-β) Zytokinen, wobei diese Effekte bei asthmatischen Fibroblasten stärker ausgeprägt waren als bei gesunden Kontrollen. Die Wirkung von IL-5 wurde durch die Stimulation der Zellen mit IL-5/IL-5Rα-Inhibitoren aufgehoben. Schlussfolgerung: Die Ergebnisse dieser Arbeit weisen auf eine bisher nicht untersuchte pro-fibrotische Rolle von IL-5 bei Asthma hin, die durch eine direkte Wirkung auf Lungenfibroblasten vermittelt wird. Diese Studie hebt das therapeutische Potenzial einer gezielten Beeinflussung von IL-5 oder seiner nachgeschalteten Signalwege hervor, um den Gewebsumbau der Atemwege bei Asthma abzuschwächen oder umzukehren.

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APPENDICES VI.

Appendix I.

6.1 Identification of Immune Cell Types in Asthmatic fibroblasts with and without InterLeukin-5.

Investigation of Immune cell distribution profile between normal and asthmatic fibroblasts with and without IL-5. This was done by applying the cibrosortex analysis to the transcriptomic data, which shows some variation of the immune cell response related to fibroblasts in asthma disease as in **figure 6.1 A**.

In Normal fibroblasts stimulated with IL-5, the results showed mainly an increase of the CD4 naïve T cells when compared to its unstimulated control, While in the asthmatic fibroblasts with and without IL-5, the results showed an increase in the un polarized macrophages M0 in the stimulated when compared to its unstimulated control, similarly, a slight increase was noticed in the monocytes from 0% to 2% , this could be attributed to the observation that monocytes are activated in asthmatics and great number of these cells are sequestered in the lung (TOMITA et al., 1995). On the other hand, a reduction in the naïve b cells post stimulation with IL-5 was observed in asthmatic fibroblasts, this could be explained based on the fact that naïve B cells will differentiate into IgE secretory plasma B cells during developmental phases of the disease (Lipińska-Opałka et al., 2024) . When comparing the normal to asthma immune cell response, the resting NK cells decreased in asthmatic fibroblasts when compared to the normal fibroblasts regardless of the presence or absence of IL-5 stimulus. The major cellular component spotted was macrophages from 4.6% in unstimulated fibroblasts up to 12% post stimulation with IL-5.

To link the increase in the macrophage, levels post IL-5 stimulation in asthmatic fibroblasts, we went back to the DEG, among the genes found, FOXO-1 is significantly upregulated as shown in **figure 6.1 B** explaining the CIBERSORTx findings. To validate the DEGs, qRT-PCR analysis was used to assess the expression of FOXO-1 in asthmatic derived fibroblasts with and without IL-5 stimulation, and data revealed a significant upregulation of FOXO-1 in response to IL-5.

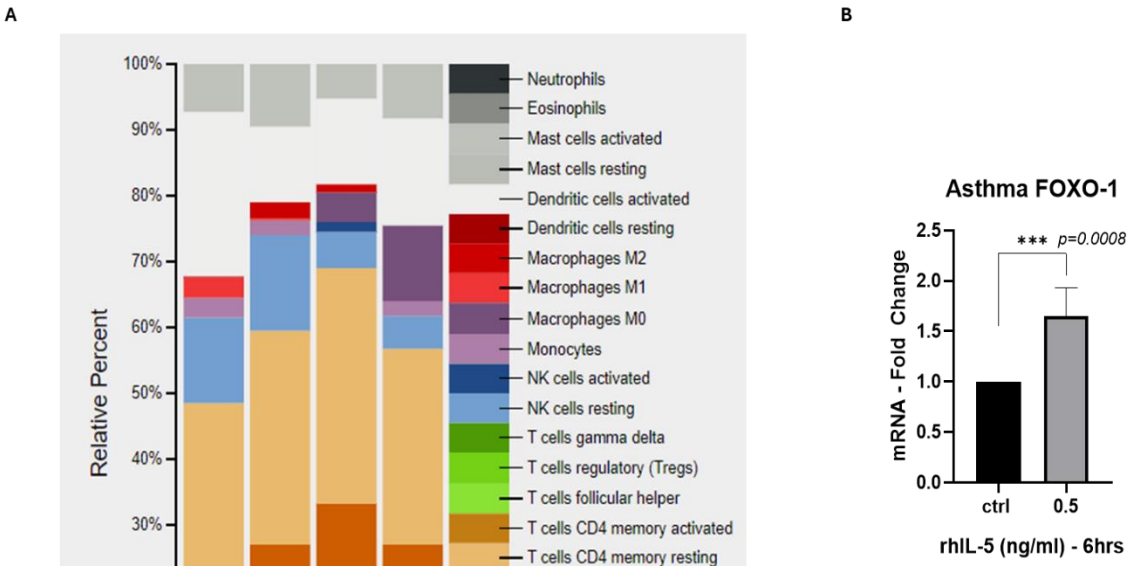

Figure 6.1 Comparison of CIBERSORTX immune cell fractions between normal and asthmatic derived lung fibroblasts stimulated or unstimulated with IL-5 (A)CIBERSORTx figure indicating the immune cell signature in both normal and asthmatic derived lung fibroblasts, stimulated or unstimulated with IL-5. **(B)** the mRNA levels of FOXO-1 gene post stimulating asthmatic lung fibroblasts with IL-5, 18s was used as a housekeeping gene for fold change calculation, Fold change was calculated by 2^-ΔΔCt, data shown is the mean ± SD of four separate experiment; t-Test statistical analysis was done * P≤0.05, **P≤0.01, ***P≤0.001.

6.2 LIGHT induces the expression of IL-5Ra (CD125)

In the above results, we noticed a notable response to TGF-b and LIGHT in terms of cellular growth. To further to evaluate the effect of fibrotic growth factors on TGF-β and LIHGT on the expression of IL-5Ra (CD125). Isolated fibroblasts were stimulated with either TGF-β (100ng/ml) or LIGHT (100ng/ml) for 24 hours, then cells were fixed and stained with anti-CD125 and analysed with flowcytometry. **Figure 6.2** showing The MFI post staining with a significant increase in the CD125 post LIHGT stimulation (p=0.040). Then we evaluated the effect of IL-5 om its cognate receptor expression, we found that post IL-5 stimulation, the receptor expression didn't show any increase.

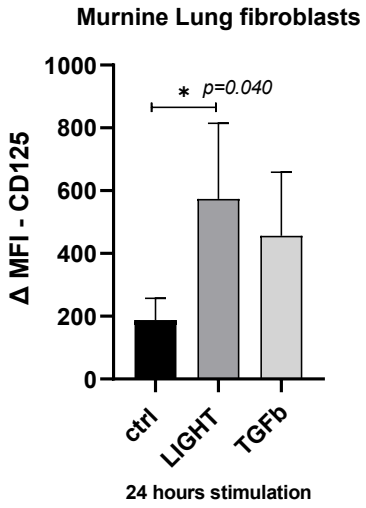

Figure 6.2 LIGHT induces the expression of CD125 in murine derived lung fibroblasts. Expression of CD125 post 24 hours stimulation with LIGHT 100 ng / ml, TGF-β 100 ng / ml, measured by flowcytometric analysis. Data shown are representative of four independent experiments, as mean ± SD of mean fluorescence intensity normalized to unstained (DeltaMFI); statistical analysis was done using one-way ANOVA. Significance is shown as * P≤0.05, **P≤0.01

6.3 LIGHT is an inducer of fibrotic characteristics in fibroblasts

As demonstrated in the results above, TGF-β did not elicit the anticipated fibrotic response. This may be due, in part, to the normal characteristics of the isolated cells, which could limit their ability to respond similarly to asthmatic fibroblasts. Recent studies, however, have identified LIGHT as a highly potent fibrotic cytokine capable of inducing airway remodelling within as short as four days in asthmatic murine model (Doherty et al., 2011; Herro et al., 2015). So, we aimed to test the effect of LIGHT on the isolated fibroblasts based on the above findings, where it was shown that LIGHT could induce the proliferation of these cells (**Figure 3.23 C)** as well as inducing the expression of IL-5Ra (**Figure 6.2**). Using qRT-PCR the same panel of genes were evaluated including FN-1, Col1A1, MMP-2, MMP-9, TIMP-1 and TIMP-2. LIGHT was able to significantly increase the FN-1 levels when compared to the control (p=0.0009) and to TGF-b stimulated cells (p=0.0012) (**Figure 6.3A)**, however although Col1A1 showed not to be effective in response to either LIGHT or TGFβ (**figure 6.3B)**. Interestingly, LIGHT stimulation was significantly sufficient to induce MMP-2 in compared to the unstimulated cells (p=0.0054) and compared to the TGF-β stimulation (p=0.0084). However. MMP-9 was significantly upregulated in cells stimulated with LIGHT when compared to their unstimulated control (p=0.0387), but not compared to TGF-β. In addition, isolated fibroblasts stimulated with LIGHT was significantly overexpressing TIMP-2 when

compared to the unstimulated controls (p=0.0062) and to the TGF-β Stimulated cells (p=0.0058). Overall, these findings indicate that LIGHT may exert a more pronounced fibrotic effect compared to TGF-β, potentially enhancing extracellular matrix deposition and promoting airway remodelling more effectively. This suggests that LIGHT could play a pivotal role in fibrosis, highlighting its potential as a potent mediator in comparison to TGF-β within the context of fibrotic signaling pathways.

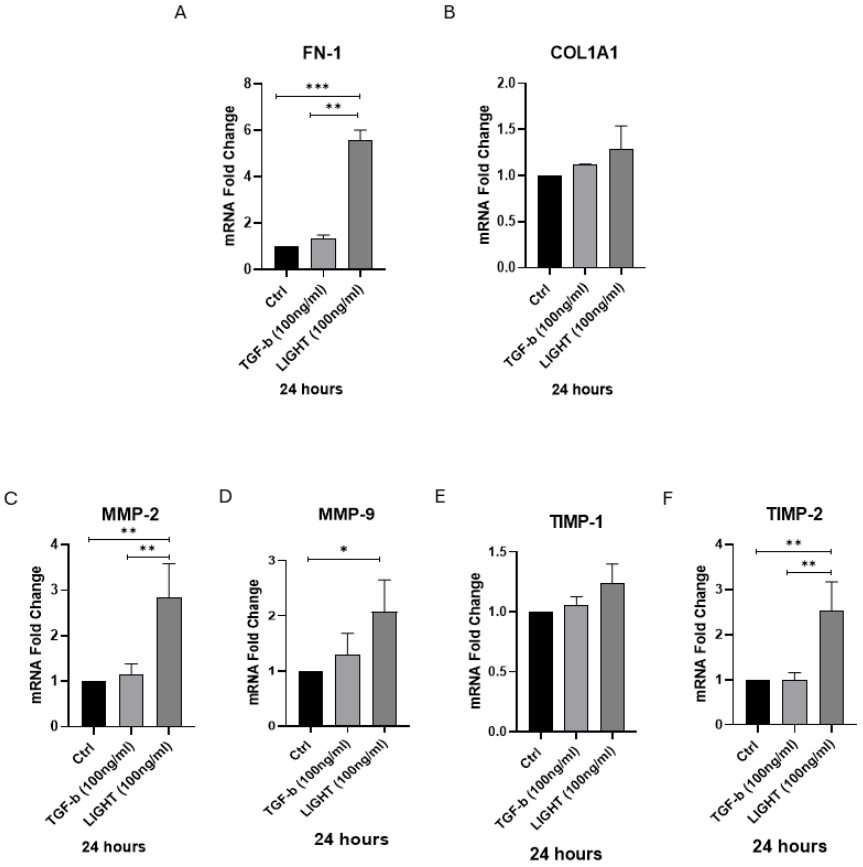

Figure 6.3 Evaluating the effect of TGFβ and LIGHT on the mRNA levels of genes involved in remodeling in murine lung derived fibroblasts. (A-B) Depicting the mRNA expression levels of ECM components FN-1 and Col1A1 following 24 hours of stimulation with either TGF-β (100 ng/ml) or LIGHT (100 ng/ml) in fibroblasts derived from murine lung tissue. **(C-D)** Evaluating the mRNA expression levels of MMP-2 and MMP-9 following 24 hours of stimulation with either TGF-β (100 ng/ml) or LIGHT (100 ng/ml) in fibroblasts derived from murine lung tissue. **(E-F)** Evaluating the mRNA expression levels of MMP-2 and MMP-9 following 24 hours of stimulation with either TGF-β (100 ng/ml) or LIGHT (100 ng/ml) in fibroblasts derived from murine lung tissue. Data shown is representative of four different experiments, the mean ± SD of four separate experiment; one-way ANOVA statistical analysis was done * $p < 0.05$, ** $p < 0.01$.