The Roles of BTLA, LIGHT and HVEM Signaling Axes in Modulating CD4 T Cell and Airway Smooth Muscle Cell Phenotypes

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Abstract

Background: T cells are known to infiltrate the airway smooth muscle (ASM) layer in asthma to induce ASM hyperplasia. Previous studies have shown that antigen specific CD4 T cells mediate airway remodeling, and direct interaction between CD4 T cells and ASM cells *in vitro* promotes myocyte proliferation as well as the formation of lymphocyte-derived membrane conduits for the transfer of anti-apoptotic proteins. We wished to determine the pro-inflammatory profiles of CD4 T cells and ASM cells when they were in contact. B and T lymphocyte attenuator (BTLA), LIGHT and their interacting partner herpesvirus entry mediator (HVEM), have been implicated in the development and maintenance of airway inflammation and airway remodeling, both of which are key characteristics of asthma. Therefore, we examined the roles that BTLA, LIGHT, HVEM, and their respective downstream signaling pathways play in the context of CD4 T and ASM cell co-culture.

Methods: CD4 T cells isolated from human peripheral blood and ASM cells from healthy donors were co-cultured and their pro-inflammatory profiles were examined. ASM cells were treated with recombinant human interferon-gamma (IFN-γ) and LIGHT to study the interplay between IFN-γ and LIGHT-HVEM signaling. We transduced ASM cells with a lentiviral vector system expressing a short hairpin RNA (shRNA) to knockdown HVEM to determine the role of HVEM signaling in mediating the expression of pro-inflammatory markers.

Results: Co-culture induced a T helper type 1 (Th1) differentiation (RT-qPCR) of CD4 T cells as reflected by increased IFN-γ secretion (ELISA), while also promoting a pro-inflammatory ASM phenotype. Co-cultured ASM cells showed increased expression of IFN-γ-inducible C-X-C motif chemokine ligands (CXCL) 9, 10, 11, intercellular adhesion molecule 1 (ICAM-1), as well as activation of the signal transducer and activator of transcription 1 (STAT1) signaling pathway

(RT-qPCR, flow cytometry and Western blot). Suppression of IFN-γ signaling in ASM cells with a neutralizing antibody against the IFN-γ receptor (IFNGR) reduced the upregulation of these pro-inflammatory mediators (RT-qPCR). LIGHT-HVEM signaling acted synergistically with the IFN-γ/STAT1 pathway in ASM cells to further enhance the expression of CXCL9, 10 and 11. Inhibition of the LIGHT-HVEM signaling axis by HVEM knockdown in ASM cells reduced activation of IFN-γ/STAT1 signaling pathway and expression of CXCL9, 10, 11 and ICAM-1 (RT-qPCR and Western blot).

Conclusion: CD4 T cells co-cultured with ASM cells show preferential differentiation towards the Th1 subset and enhanced secretion of IFN-γ. LIGHT-HVEM ligation acts synergistically with IFN-γ on ASM cells to promote a pro-inflammatory ASM phenotype characterized by upregulation of C-X-C motif chemokine receptor (CXCR) 3 ligands CXCL9, 10, 11 and adhesion molecule ICAM-1. This pathway potentially mediates T cell recruitment and airway inflammation and thus could be targeted to reduce the inflammatory microenvironment seen in asthmatic airways.

Résumé

Contexte : Les cellules T sont connues pour infiltrer la couche musculaire lisse des voies aériennes dans l'asthme afin d'induire une hyperplasie du muscle lisse des voies respiratoires (ASM, *airway smooth muscle*). Des études antérieures ont démontré que les cellules T CD4 antigène-spécifiques jouent un rôle dans le remodelage des voies aériennes, et l'interaction directe entre les cellules CD4 et les cellules musculaires lisses des voies aériennes *in vitro* favorise la prolifération des myocytes ainsi que la formation de conduits membranaires dérivés des lymphocytes pour le transfert des protéines anti-apoptotiques. Nous avons cherché à déterminer les profils pro-inflammatoires des cellules T CD4 et des cellules musculaires lisses des voies respiratoires lorsqu'elles sont en contact. L'atténuateur de lymphocytes B et T (BTLA, *B and T lymphocyte attenuator*), le LIGHT et leur partenaire d'interaction, le médiateur d'entrée des virus herpétiques (HVEM, *herpesvirus entry mediator*), a été impliqués dans le développement et le maintien de l'inflammation et du remodelage des voies aériennes, tous deux étant des caractéristiques clés de l'asthme. Donc, nous avons examiné les rôles que jouent les BTLA, LIGHT, HVEM, et leur voie de signalisation en aval respective, dans le contexte d'une co-culture de cellules T CD4 et ASM.

Méthodes : Des cellules T CD4 isolées du sang périphérique humain et des cellules ASM de donneurs sains ont été co-cultivées et leurs profils pro-inflammatoires ont été examinés. Des cellules ASM ont été traitées avec de l'interféron gamma (IFN-γ) humain recombinant et du LIGHT pour étudier l'interaction entre la signalisation de l'IFN-γ et celle du LIGHT-HVEM. Nous avons transduit des cellules ASM avec un système de vecteurs lentiviraux exprimant un petit ARN en épingle à cheveux (shRNA, *short hairpin RNA*) pour inactiver HVEM afin de déterminer le rôle de la signalisation de HVEM dans la médiation de l'expression de marqueurs pro-inflammatoires.

Résultats : La co-culture a induit une différenciation (RT-qPCR) des cellules T CD4 par des cellules T auxiliaires de type 1 (Th1), telle reflétée par une sécrétion accrue d'IFN-γ (ELISA), tout en promouvant aussi un phénotype ASM pro-inflammatoire. Des cellules ASM co-cultivées ont montré une expression accrue des ligands à motif C-X-C de chimiokine (CXCL, *C-X-C motif chemokine ligands*) 9, 10 et 11 inductibles au IFN-γ, de molécule d'adhésion intercellulaire 1 (ICAM-1, *intercellular adhesion molecule 1*), ainsi que l'activation de la voie de signalisation du transducteur de signal et activateur de transcription 1 (STAT1, *signal transducer and activator of transcription 1*) (RT-qPCR, cytométrie de flux et transfert de protéines (*Western blot*)). La suppression de la signalisation d'IFN-γ dans les cellules ASM avec un anticorps neutralisant contre le récepteur de l'IFN-γ (IFNGR, *IFN-γ receptor*) a réduit la régulation positive de ces médiateurs pro-inflammatoires (RT-qPCR). La signalisation LIGHT-HVEM a agi synergiquement avec la voie IFN-γ/STAT1 dans les cellules ASM, renforçant davantage l'expression des CXCL9, 10 et 11. L'inhibition de cette voie par l'inactivation de HVEM dans les cellules ASM a réduit l'activation de la voie de signalisation IFN-γ/STAT1 et l'expression des CXCL9, 10, 11 et ICAM-1 (RT-qPCR et *Western blot*).

Conclusion : Les cellules T CD4 co-cultivées avec les cellules ASM montrent une différenciation préférentielle vers le sous-ensemble Th1 et a amélioré la sécrétion de l'IFN-γ. La ligature de LIGHT-HVEM agit synergiquement avec l'IFN-γ sur les cellules ASM pour promouvoir un phénotype ASM pro-inflammatoire caractérisé par une régulation positive des ligands du récepteur de chimiokine à motif C-X-C (CXCR , *C-X-C motif chemokine receptor*) 3, CXCL9, 10, 11, et de la molécule d'adhésion ICAM-1. Cette voie régule potentiellement le recrutement des cellules T et l'inflammation des voies aériennes et pourrait donc être ciblée afin de réduire le microenvironnement inflammatoire observé dans les voies respiratoires asthmatiques.

List of Abbreviations

Airway hyperresponsiveness	AHR
Airway smooth muscle	ASM
Alpha-smooth muscle actin	α-SMA
Antigen-presenting cell	APC
Aryl hydrocarbon receptor	AhR
B and T lymphocyte attenuator	BTLA
Basic fibroblast growth factor	bFGF
Basic fibroblast growth factor 2	FGF2b
Bone-marrow derived lymphocytes	B lymphocytes
Bovine serum albumin	BSA
C-C motif chemokine ligand	CCL
C-C motif chemokine receptor	CCR
Cluster of differentiation	CD
c-Jun N-terminal kinase	JNK
Common gamma chain	gc
C-X-C motif chemokine ligand	CXCL
C-X-C motif chemokine receptor	CXCR
Dendritic cell	DC
Dulbecco's Modified Eagle's Medium	DMEM
Endoplasmic reticulum	ER
Enzyme-linked Immunosorbent Assay	ELISA
Eosinophilic esophagitis	EoE

Epidermal growth factor	EGF
Epidermal growth factor receptor	EGFR
Epithelial-mesenchymal transition	EMT
ETS (Erythroblast Transformation Specific) Like-1	Elk1
Extracellular matrix	ECM
Extracellular signal-regulated kinase	ERK
Fetal bovine serum	FBS
Fas-associated death domain	FADD
Follicular helper T cell	Tfh
Forced expiratory volume in one second	FEV_1
Forkhead box P3	FOXP3
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH
Granulocyte-macrophage colony-stimulating factor	GM-CSF
Growth factor independent-1	Gfi-1
Herpesvirus entry mediator	HVEM
IFN-α/β receptor	IFNAR
IFN-γ receptor	IFNGR
IFN-γ-activation site	GAS
IFN-stimulated gene factor	ISGF
IFN-stimulated response element	ISRE
Immunoglobulin	Ig
Immunoglobulin superfamily	IgSF
Immunoreactive B cell lymphoma 2	Bcl-2

Immunoreceptor tyrosine-based inhibitory motif	ITIM
Immunoreceptor tyrosine-based switch motif	ITSM
Induced myeloid leukemia cell differentiation protein	Mcl-1
Indirect acute lung injury	iALI
Inducible T cell costimulator	ICOS
Inhibitor of κB-kinase	IKK
Intercellular adhesion molecule 1	ICAM-1
Interferon	IFN
Interferon regulatory factor	IRF
Interleukin	IL
Janus family of kinase	JAK
Knockdown	KD
Lymphocyte-derived membrane conduit	LMC
Lymphotoxin-alpha	LT-α
Lymphotoxin, exhibits inducible expression and competes with HSV glycoprotein D for binding to herpesvirus entry mediator, a receptor expressed on T lymphocytes	LIGHT
Major histocompatibility complex	MHC
Matrix metalloproteinase	MMP
Mesenchymal stromal cell	MSC
Mitogen-activated protein kinase	MAPK
Monoclonal antibody	mAb
Monocyte chemoattractant protein	MCP
Mucin-5AC	MUC5AC

Myocardin	MYOCD
Myosin light chain kinase	MLCK
Natural killer cell	NK cell
Natural killer T cell	NKT cell
Nuclear factor kappa-light-chain-enhancer of activated B cells	NF-κB
Ovalbumin	OVA
Pattern recognition receptor	PRR
Peripheral blood mononuclear cell	PBMC
Phosphate-buffered saline	PBS
Platelet-derived growth factor	PDGF
Provocative concentration or dose required to achieve a 20% reduction in FEV_1	PC_{20} or PD_{20}
Retinoic acid receptor-related orphan receptor gamma-t	RORγt
Real-time quantitative reverse transcription polymerase chain reaction	RT-qPCR
Receptor interacting protein 1	RIP
Regulatory T cell	Treg
Regulatory type 1 cell	Tr1
Serum response factor	SRF
Short hairpin RNA	shRNA
Signal transducer and activator of transcription	STAT
Smooth muscle myosin heavy chain	sm-MHC
Src homology	SH
Suppressor of cytokine signaling	SOCS
T-box transcription factor	T-bet

T cell receptor	TCR
T helper	Th
Thymus-derived lymphocytes	T lymphocytes
Tissue inhibitor of metalloproteinase	TIMP
TNF-converting enzyme	TACE
TNF receptor	TNFR
TNFR-associated death domain	TRADD
TNFR-associated factor	TRAF
TNF receptor superfamily	TNFRSF
TNF-related activation-induced cytokine	TRANCE
Transforming growth factor	TGF
Tris-buffered saline with 0.1% Tween	TBS-T
Tris(2- carboxyethyl) phosphine hydrochloride	TCEP
Tumor necrosis factor	TNF
Vascular cell adhesion molecule 1	VCAM-1
Vascular endothelial growth factor	VEGF

Chapter 1. Introduction

Section 1. Asthma

1.1 Introduction to Asthma

Asthma is a chronic respiratory disease that is characterized by airway inflammation and bronchial hyperresponsiveness, as well as structural changes to the airway walls that occur in response to repeated allergen exposures. Other stimuli such as exercise, viral infection, and cold air evoke bronchoconstriction in persons with airway hyperresponsiveness. Asthma is a heterogeneous condition in terms of its clinical manifestations, severity, etiology, and prevalence. Its symptoms range from mild, occasional wheezing to severe, life-threatening airway narrowing and closure. Both genetic predisposition and environmental exposure to allergenic and/or inflammatory substances can contribute to disease development and progression [1]. Asthma affects around 300 million people worldwide, and this number is projected to increase by another 100 million by 2025 [2]. The disease affects around 15% to 20% of people in developed, affluent countries and around 2% to 4% in developing countries. Nevertheless, most asthma-related mortalities occur in low-middle income countries. Asthma prevalence is significantly higher in children. Up to 40% of children develop a wheeze or some form of airway obstruction. On the other hand, asthma-related healthcare use and mortality are higher in adults [2].

Most of the currently available asthma treatments focus on attenuation of airway inflammation and bronchoconstriction through the actions of corticosteroids and β-adrenergic agonists, respectively [3, 4]. Although conventional treatment regimens provide rapid symptom relief and are effective for most asthmatics, a small subpopulation of patients is refractory to or develops resistance towards these medications. Curative therapeutic interventions have yet to be developed and various aspects of asthma pathophysiology remain not well understood.

1.2 Asthma Pathophysiology

Asthma triggered by allergen exposure can be classified into two phases: the early phase and late phase. The bronchoconstriction of both phases is mediated by cysteinyl leukotrienes, synthesized by the 5-lipoxygenase pathway expressed in cells such as mast cells, basophils, dendritic cells and macrophages.

1.2.1 Early Phase Response

The early phase response is triggered by the crosslinking of immunoglobulin E (IgE) antibodies on the surface of mast cells. These antibodies are synthesized following sensitized sensitization to foreign protein and are released by plasma cells. Prior sensitization occurs upon uptake of allergen by professional antigen-presenting cells (APCs) that present pieces of the allergenic protein to cluster of differentiation 4 (CD4) helper T cells and induce their differentiation into the T helper type 2 (Th2) subset. Interleukin (IL)-4 produced from Th2 cells coupled with the presence of allergen stimulates plasma cells to produce large amounts of IgE antibodies [5]. These antibodies in turn bind to high affinity Fcε receptors on the surface of mast cells and basophils to cause the secretion of cytokines, histamine, prostaglandins, leukotrienes and various other pro-inflammatory mediators, which result in smooth muscle contraction [4].

1.2.2 Late Phase response

Interleukins released by Th2 lymphocytes, including IL-4, IL-5, and IL-13, play an integral role during the late phase response of asthma [5]. IL-4 induces the synthesis and secretion of more IgE antibodies from plasma cells, IL-5 recruits and activates eosinophils, whereas IL-13 contributes to airway inflammation, fibrosis, and smooth muscle hyperplasia [6, 7]. IL-4 and IL-13 in conjunction cause class switching of immunoglobulin M (IgM) into IgE antibodies [8]. Mast cells and basophils are also considered important sources of IL-4 [7, 8]. All of these pro-inflammatory mediators cause

the recruitment and accumulation of inflammatory cells, including eosinophils, basophils, neutrophils, and helper and memory T cells, at the site of allergen exposure, which perpetuate inflammation and induce long-term structural changes of the airways known as remodeling [9].

1.3 Airway Hyperresponsiveness

Airway hyperresponsiveness (AHR) is a cardinal feature of asthma. It refers to the predisposition of asthmatic airways to excessive constriction in response to a contractile stimulus, such as methacholine, which would otherwise generate little to no response in healthy individuals [10]. Clinically, AHR is associated with increased decline in lung function, persistence of wheezing from childhood to adulthood, increased susceptibility to exacerbations, as well as higher level of treatment required to manage symptoms [11, 12]. Therefore, AHR is commonly assessed in the diagnosis and classification of the severity of asthma.

Airway responsiveness is measured by having the patient inhale increasing concentrations of a contractile agonist while monitoring the percentage reduction in forced expiratory volume in one second (FEV_1) [13]. The provocative concentration (PC_{20}) or dose (PD_{20}) required to achieve a 20% reduction in FEV_1 is recorded [14]. Normal airway responsiveness is defined by a negative test result of PC_{20} greater than 16 mg/mL or PD_{20} greater than 400 μg. On the other hand, AHR can be categorized as borderline (4.0 – 16 mg/mL), mild (1.0 – 4.0 mg/mL) and moderate-to-severe (< 1.0 mg/mL) based on the range within which PC_{20} lies [15, 16]. AHR can be a result of hypersensitivity, which is represented by a left shift in the dose response curve and/or hyperreactivity, denoted by a steeper dose response curve [14]. In addition, the loss of a maximal response plateau is possible and signifies excessive and uninhibited bronchoconstriction leading to potentially life-threatening exacerbations [10]. Alternatively, the forced oscillation technique can be used, which imposes

sinusoidal pressure oscillations over normal tidal breathing and does not involve any respiratory maneuvers [17]. The resulting changes in pressure and flow are used to calculate the resistance and reactance of the respiratory system, as a measure of airway caliber and elastance, respectively [10].

AHR is clinically described by two distinct components: baseline and variable AHR [18]. The baseline AHR is relatively constant and persistent. The current paradigm is that baseline AHR is secondary to structural changes in the airway, termed remodeling, which occur as a result of chronic airway inflammation [18, 19]. As such, baseline AHR is considered a reflection of the chronicity of asthma, rather than its activity or acuity. Baseline persistent AHR is better measured via direct stimuli such as histamine and methacholine, which act on histamine receptors and muscarinic receptors on airway smooth muscle, respectively [18].

The causal relationship between chronic airway inflammation, remodeling and persistent AHR has recently been challenged. Studies have shown that airway remodeling and bronchial hyperresponsiveness may occur independently of inflammation in childhood asthma and are refractory to corticosteroid treatment [20, 21].

On the other hand, variable AHR is induced acutely by episodic exposures to allergens or environmental factors, such as sulfur dioxide, fog, and cold air [22]. These stimuli are considered indirect since they induce inflammation and AHR by stimulating the release of mediators from inflammatory cells rather than directly acting on structural cells [18]. The variable component of AHR is more clinically relevant as it reflects asthma severity and activity. Mild or episodic asthmatics may only exhibit this form of AHR [23]. The association between variable AHR and airway inflammation, specifically Th2-driven eosinophilia, has been well established [18]. Both allergen-induced AHR and eosinophilia are sensitive to anti-inflammatory treatments such as corticosteroids and anti-IL-4, IL-5, and IL-13 antibodies [24, 25, 26]. Neutrophilia does not appear to

be associated with AHR but may contribute to an alteration to the type of bronchoconstriction towards predominantly airway closure [27].

The molecular underpinnings connecting eosinophilic inflammation to the development of AHR are not quite clear at this point, but it is hypothesized that inflammation-induced alterations in smooth muscle neurohumoral control as well as intrinsic changes to the contractile machinery of the smooth muscle cells could contribute to the development of AHR [28, 29].

1.4 Airway Remodeling

Airway remodeling, which refers to structural changes that occur to the small and large airways, has been reported in asthmatics of all severity [30]. Airway remodeling commonly observed in asthmatics includes epithelial denudation [31], subepithelial fibrosis [32], goblet cell metaplasia [33], angiogenesis [34], airway wall thickening [19], as well as increased airway smooth muscle mass and contractility [35, 36]. It is hypothesized that airway remodeling is associated with chronic airway inflammation, specifically the activation of inflammatory cells including eosinophils, basophils, neutrophils, mast cells and naïve and memory lymphocytes [37].

1.4.1 Epithelial Denudation

Fresh biopsies from asthma patients have shown increased epithelial destruction and shedding at all levels of the airways [31, 38]. The damage may be severe enough to expose the intraepithelial nerves [38]. Ciliated columnar epithelial cells are the most destroyed cell type in the bronchial epithelium [39]. Epithelial cell clusters have been observed in bronchoalveolar lavage samples of asthma patients and contained a far greater ratio of columnar to basal cells than intact epithelium [39]. Destruction and breach of the airway epithelium compromise its protective effects and increase the susceptibility of the airways to further allergen insult, thus exacerbating asthma.

In addition to structural damage, the epithelial cells switch into a more activated, proliferative and pro-inflammatory phenotype via activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and expression of a number of epidermal growth factors (EGFs) and pro-inflammatory mediators, which results in a chronic and vicious cycle of inflammation and injury [40].

Epithelial damage and the ensuing reparative responses, which involve activation of the myofibroblasts in the underlying lamina reticularis and generalized overexpression of epidermal growth factor receptor (EGFR), initiates the airway remodeling process [41]. This also promotes myofibroblast proliferation into the epithelium and smooth muscle layer, resulting in subepithelial fibrosis [40, 41].

1.4.2 Subepithelial Fibrosis

Subepithelial fibrosis has been consistently observed in all types of asthma and appears to be correlated with disease severity [32]. It is a result of increased deposition and decreased degradation of extracellular matrix (ECM) proteins, manifesting as a thickening of the basement membrane and accumulation of excess collagen, tenascin and proteoglycans in the lamina reticularis [37]. The molecular underpinning of subepithelial fibrosis is likely an imbalance between the level of proteases and antiproteases. For example, asthmatic airway fibroblasts have an increased ratio of tissue inhibitor of metalloproteinase (TIMP)-2 to matrix metalloproteinase (MMP)-2, which translates to reduced ECM degradation [42]. Other MMPs such as MMP-3, MMP-8 and MMP-9 are also elevated in the bronchioalveolar lavage of asthma patients and correlate with disease severity and decline in lung function [43, 44, 45].

1.4.3 Goblet Cell Hyperplasia and Mucus Hypersecretion

Goblet cell and submucosal gland hyperplasia have been reported in both adult and childhood asthma [46, 47]. Asthmatics show a significantly higher number of goblet cells and elevated levels of mucin-5AC (MUC5AC) in the airway epithelium [47]. These abnormalities lead to clinical manifestations such as increased sputum production, which aggravates airway narrowing.

1.4.4 Angiogenesis

Neovascularization is observed in asthmatics and is associated with an increased expression of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and angiogenin in the airway epithelium [34, 48]. Increased airway vascularity promotes reduced airway caliber via airway wall edema, infiltration of inflammatory cells and delivery of pro-inflammatory and remodeling mediators into the site of allergen exposure [48].

1.4.5 Increased Smooth Muscle Mass and Altered ASM Phenotype

ASM remodeling has been long recognized as an important determinant of AHR and asthma severity [49, 50, 51]. Asthmatic airways show increased smooth muscle mass as a result of an increase in smooth muscle cell number, termed hyperplasia as well as an increase in cell size, termed hypertrophy [49], but the relative contribution of each mechanism varies among patients. In addition to aberrant proliferation of existing ASM cells, other cellular sources that potentially contribute to increased ASM mass include fibrocytes recruited from the circulation, differentiation of tissue-resident mesenchymal stromal cells (MSCs), as well as epithelial-mesenchymal transition (EMT). Asthmatic ASM cells switch into a more proliferative and secretory phenotype characterized by reduced expression of contractile genes and secretion of a variety of cytokines, chemokines, and ECM proteins, thus becoming active contributors to the airway inflammation and remodeling process [52, 53, 54]. Chemokines produced by ASM cells, such as eotaxin, also have the ability to

induce smooth muscle migration into the subepithelial space, which is a newly discovered feature of asthma [55].

1.5 Airway Smooth Muscle Cell Phenotype

Chamley-Campbell et al. were the first to distinguish between the contractile and proliferative phenotypes of vascular smooth muscle cells [56]. These two states define the ends of the smooth muscle phenotype continuum, and the position at which the smooth muscle cell lies on the continuum is dependent on a variety of extracellular signals [57].

On the molecular level, the phenotype of the smooth muscle cell is determined by competition between two transcription factors, myocardin (MYOCD) and ETS Like-1 (Elk1). MYOCD is a master regulator of smooth muscle differentiation. MYOCD binds to another transcription factor, serum response factor (SRF), which in turn binds to the CArG box, a common promoter region of smooth muscle contractile genes [58]. The current understanding is that a dimerization of MYOCD, each bound to a SRF homodimer, via a leucine zipper-like structure is required to engage multiple CarG boxes and fully activate smooth muscle contractile gene expression and differentiation [58]. In addition, dimerization may expose the TAD region of MYOCD and allow the assembly of a transcriptional complex with other promoter-specific cofactors to further induce contractile gene expression [58]. On the other hand, Elk-1 binding to SRF homodimer at a single CarG box promotes the proliferative phenotype of smooth muscle [59].

MYOCD and Elk-1 share structurally similar SRF-binding domains that compete for a common binding site on SRF, resulting in competitive binding between the two transcription factors [59]. Growth signals such as platelet-derived growth factor (PDGF) trigger the binding of SRF to Elk1 and displacement of MYOCD, suggesting that Elk1 has a higher affinity for the SRF binding site[59].

1.5.1 Contractile Phenotype

The contractile phenotype of smooth muscle is characterized by upregulated expression of smooth muscle contractile genes such as alpha-smooth muscle actin (α-SMA), smooth muscle myosin heavy chain (sm-MHC) and myosin light chain kinase (MLCK), as well as decreased capacity of the cells to proliferate. The ultrastructure of these cells as visualized by transmission electron microscopy is composed of tightly bundled myofilaments and minimal rough endoplasmic reticulum (ER), Golgi, or free ribosomes [56].

1.5.2 Proliferative and Secretory Phenotype

Smooth muscle cells of the proliferative phenotype adopt a long, spread out, spindle-like shape and functionally resemble fibroblasts. The ultrastructure of these cells shows minimal contractile filaments in the cytoplasm but abundant ER, Golgi and ribosomes that are utilized to produce and secrete ECM proteins [57]. The switching of smooth muscle cells from the contractile to the proliferative phenotype is observed after 5 days of primary culture and is marked by significantly decreased sm-MHC, α-SMA, calponin and desmin. Other proteins such as MLCK, h-caldesmon, and β-tropomyosin are also downregulated to a lesser extent. Interestingly, smooth muscle cells have the ability to restore contractile phenotype after reaching full confluence, suggesting that they are capable of phenotypic plasticity [60].

Proliferative smooth muscle cells are capable of secreting a variety of pro-inflammatory mediators. Tumor necrosis factor (TNF)-stimulated ASM cells upregulate expression of cytokines IL-6 and IL-8, C-C motif chemokine ligand 5 (CCL5 or RANTES), monocyte chemoattractant protein (MCP), eotaxin and granulocyte-macrophage colony-stimulating factor (GM-CSF), as well as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) [53]. These changes and the chronic inflammatory response in asthma are mediated by the transcription

factor NF-κB [61]. Atopic sensitized human ASM cells endogenously express T helper type 1 (Th1) cytokines IL-2, IL-12, and interferon-gamma (IFN-γ), Th2 cytokines IL-5 and GM-CSF, as well as their respective receptors [62]. Interaction with CD4 T cells also induces C-X-C motif chemokine ligand (CXCL) 9, 10, and 11 expression in both healthy and asthmatic ASM cells in an IFN-γ/STAT1-dependent manner, which serves to induce chemotaxis of activated CD4 T cells [63]. IFN-γ augments the chemoattractive role of CXCL10 and 11, and disruption of the IFN-γ/STAT1 signaling axis inhibits T cell migration [63].

1.6 T Cell Infiltration and Interaction with ASM in Asthma

Tissue-resident T cells in the airways of healthy individuals are normally localized within the epithelium, specifically in the proliferating basal stratum. In asthma subjects, T cells infiltrate the lamina propria and ASM layer [64]. The degree of T cell infiltration is correlated with asthma severity [64]. In addition, infiltration and subsequent direct cellular interaction between the T cells and ASM cells induce ASM cell proliferation. Similarly, adoptively transferred CD4 T cells from sensitized rats are localized in juxtaposition to ASM cells and drive an increase in smooth muscle mass via enhanced proliferation and reduced apoptosis of ASM cells [65]. Taken together, these results suggest that direct cell-to-cell interaction between CD4 T cells and ASM cells drives ASM remodeling in experimental asthma.

In vitro, CD4 T and ASM cells in co-culture establish a contact-dependent crosstalk with induced ASM cell DNA synthesis, proliferation and formation of lymphocyte-derived membrane conduits (LMCs) connecting the two cell types [65, 66]. LMCs facilitate the transfer of anti-apoptotic proteins immunoreactive B cell lymphoma 2 (Bcl-2) and induced myeloid leukemia cell differentiation protein (Mcl-1), thereby serving as a novel mechanism that is utilized by CD4 T cells to promote

their survival [66]. ASM cell-derived basic fibroblast growth factor 2 (FGF2b) is required for the formation of LMCs by CD4 T cells [67].

Section 2. CD4 T Cells and Subset Differentiation

2.1 Introduction to CD4 T Cells and Its Subsets

The human immune system consists of the ancient innate immune system and the more recently evolved adaptive immune system [68]. The innate immune system serves as a first line of defense that acts rapidly within a few hours following pathogen exposure and utilizes germline-encoded pattern recognition receptors (PRRs) to detect molecular patterns associated with common pathogens [69]. The adaptive immune system orchestrates an antigen-specific and much more potent response involving the elimination of pathogens via recognition of unique antigens, acquisition of immunological memory and development of tolerance to self-antigens [69]. Adaptive immunity is largely mediated by cells of the lymphoid lineage, namely thymus-derived lymphocytes (T lymphocytes or T cells), bone-marrow derived lymphocytes (B lymphocytes or B cells) and natural killer (NK) cells. B cells originate and mature in the bone marrow and their main role is to produce antibodies, which mediates humoral immunity. The effector function of antibodies include pathogen and toxin neutralization, classical complement activation and opsonization [70]. On the other hand, T cells mature in the thymus and make up an important part of cellular immunity.

T cells can be broadly divided into CD4 and CD8 T cells based on the expression of CD4 or CD8 glycoprotein on their surface. CD8 T cells, also known as cytotoxic T cells, directly target and eliminate infected or malignant cells via the release of cytolytic granules or expression of ligands for the death receptors such as Fas ligand [71]. CD4 T cells or T helper (Th) cells serve a special role in coordinating adaptive immunity by bridging antigen presentation and downstream immune

responses. CD4 T cells are capable of differentiating into multiple phenotypically and functionally distinct lineages that secrete different sets of cytokines and tailor the immune response to the specific antigen encountered. Currently, five major CD4 T cell subsets have been identified, namely Th1, Th2, Th17, regulatory T (Treg), and follicular helper T (Tfh) cells, each characterized by its own unique cytokine profile [72]. Others such as Th9, Th22, and regulatory type 1 cells (Tr1) have been postulated to exist as distinct subsets but further evidence is required to support these classifications [68].

2.2 CD4 T Cell Activation and Differentiation

Full activation of naïve CD4 T cells requires the presence of two signals. Signal 1 is generated when the T cell receptor (TCR) coupled with CD3 interacts with antigen-major histocompatibility complex (MHC) II complex on the surface of professional APCs [68, 73]. The exact differentiation that the CD4 T cells pursue is dependent on the microenvironment and cytokine milieu, as well as costimulatory molecules, antigen concentration and the type of APCs that CD4 T cells encounter [74]. Signal 2 refers to the activation of costimulatory receptors such as CD28 by their interacting partners, which augments TCR signaling and promotes T cell proliferation, activation, and differentiation by activation of IL-2 production and entering the cell cycle [68, 73, 75]. Activation of signal 1 through the TCR complex in the absence of costimulatory signal 2 results in premature T cell apoptosis or anergy, whereas the presence of both signals promotes T cell proliferation without inducing early cell death [76]. CD28 on the surface of CD4 T cells engages with its ligand CD80 (B7-1) and CD86 (B7-2) on APCs such as dendritic cells (DCs) [68]. Co-stimulation of CD4 T cells via CD28 induces IL-2 gene transcription and expression through activation of NF-κB [77]. Magnetic microbeads coated with anti-CD3 and anti-CD28 antibodies have been used for in vitro activation

of CD4 T cells and boosting T cell immunity in immunosuppressed cancer patients [78, 79]. Other less potent costimulatory molecules include CD28 homolog inducible T cell co-stimulator (ICOS), as well as members of the TNF receptor family.

During the process of activation, a pattern of cytokine expression is established within the microenvironment, which induces the corresponding intracellular signaling pathways and mediators that coordinate the differentiation of CD4 T cells with the appropriate effector function. The affinity or strength of interaction between peptide ligands on MHCII and TCR also determines cell fate. High affinity binding of peptide by TCR results in Th1 differentiation whereas low affinity binding favors Th2 differentiation [80]. The duration of signal 1 could also influence differentiation, with Th1 subset being favored in the presence of short TCR stimulation with IL-12, whereas Th2 differentiation requires prolonged TCR activation [81].

2.3 T helper type 1 (Th1) Cells

T helper type 1 (Th1) cells play a central role in orchestrating cellular immunity against intracellular bacteria, protozoa and viral infections. IL-12 and IFN-γ are the major cytokines that promote Th1 differentiation [82]. APCs serve as the primary source of IL-12 following their activation through PRRs, and IL-12 in turn stimulates NK cells to produce IFN-γ [83, 84]. T-box transcription factor (T-bet) is the master transcriptional regulator that mediates Th1 differentiation. IFN-γ signaling through its surface receptor on naïve CD4 T cells induces the phosphorylation of signal transducer and activator of transcription 1 (STAT1), which in turn upregulates T-bet expression and further IFN-γ production by the differentiating cells. The abundant IL-12 produced by APCs also serves as a selective pressure to favor Th1 differentiation [85]. IL-12 induces the expression of STAT4, which is another transcription factor that potentiates IFN-γ production [86]. Aside from its role in supporting Th1 differentiation, T-bet also suppresses the development of

opposing cell lineages. T-bet silences IL-4 gene expression and interferes with transcription factor GATA3 binding to its target DNA, thereby inhibiting Th2 differentiation [87]. Interaction between T-bet and the transcription factor Runx1 blocks the transactivation of Rorc, which encodes the Th17 lineage master transcription regulator Retinoic acid receptor-related orphan receptor gamma-t (RORγt) [88].

Members of the tumor necrosis factor (TNF) receptor/ligand families act as costimulatory signals to promote Th1 differentiation. For example, CD40 ligand (CD40L or CD154) expressed on activated T cells binds to CD40 on APCs to augment production of IL-12 and other costimulatory factors [89]. TNF-related activation-induced cytokine (TRANCE) and its receptor augment IL-12 production by DCs and IFN-γ response during an *in vivo* viral infection model [90]. OX40 also supports Th1-type immune responses. Deficiency of OX40 in mice results in impaired CD4 T cell proliferation and reduced number of IFN-γ positive CD4 T cells. [91, 92].

Effector cytokines synthesized by Th1 cells include IFN-γ, TNF, lymphotoxin-alpha (LT-α) along with IL-2 to further potentiate expansion of the Th1 cell population.

2.3.1 Interferon-gamma (IFN-γ)

Interferons (IFNs) were given their names based on their ability to interfere with viral replication. Interferons are typically classified into type I, II and III based on the receptors through which they signal. Type I IFNs bind to the cell surface complex IFN-α/β receptor (IFNAR) that consist of the IFNAR1 and IFNAR2 chains [93]. Type III interferons are more recently discovered and serve as a first line of defense against viral infections [94].

Type II interferon, which consists of a single member IFN-γ, is of prime importance for Th1 differentiation as aforementioned. NK cells and T lymphocytes, specifically CD4 Th1 cells and cytotoxic CD8 T cells, are the major sources of IFN-γ [95]. Recently, a number of other cells types,

such as B cells, NKT cells, professional APCs including monocytes, macrophages and DCs, have been reported to also synthesize and secrete IFN-γ [96]. IFN-γ synthesis is stimulated by a number of cytokines produced by APCs, most notably IL-12 and IL-18 [96]. Activation of macrophages through PRRs induces IL-12 and chemokine secretion, which attracts NK and T cells to the site of inflammation [97, 98]. IL-12 and IL-18 in turn induce IFN-γ production from those lymphocytes. Negative regulators of IFN-γ expression include IL-4, IL-10, transforming growth factor-beta (TGF-β) and glucocorticoids [99].

IFN-γ signals through its receptor IFNGR, which is composed of two ligand-binding IFNR1 chains, two signal-transducing IFNR2 and associated intracellular signaling machinery [96]. Upon ligand binding, the intracellular domain of IFNGR acts as a docking site for the binding of downstream signaling components, which activates the JAK-STAT pathway, which involves a series of inter-correlated sequential recruitment and phosphorylation of members of the Janus family of kinases (JAKs) and STATs to regulate binding of response elements and transcription of target genes. IFNGR activation results in the autophosphorylation of JAK2 and subsequent transphosphorylation of JAK1 by JAK2 [100]. Activated JAK1 in turn phosphorylates tyrosine residue 440 on the each of the two IFNR1 chains and allows them to act as adjacent docking sites for the Src homology 2 (SH2) domains of the STAT1 homodimer [100, 101]. STAT1 is phosphorylated by JAK2 near its C terminus at Y701 [102]. In addition, STAT1 phosphorylation at S727 is essential for maximal transcriptional activity [103, 104]. Phosphorylation induces the dissociation of STAT1 from IFNGR and allows it to translocate into the nucleus to bind to promoter regions such as IFN-γ-activation sites (GAS) and IFN-stimulated response elements (ISRE) to activate or suppress the transcription of IFN-γ-regulated genes [105]. Alternatively, STAT1 may also form heterodimers with STAT2 and interferon regulatory factor (IRF)-9 to form the IFN-stimulated gene factor (ISGF) 3

complex [106]. Many of the IFN-γ-regulated gene products act as transcription factors on their own and are able to drive the next wave of transcription.

Numerous *in vivo* and *in vitro* studies have documented the ability of IFN-γ to regulate chemokine synthesis. Among the chemokines studied, C-C motif chemokine receptor (CCR) 1/2/3 ligands CCL2 [107, 108, 109, 110] and CCL7 [109], CCR1/5 ligands CCL3 [109] and CCL5 [109, 111], as well as C-X-C motif chemokine receptor (CXCR) 3 ligands CXCL9 [109, 112, 113], 10 [109, 114] and 11 [115, 116], are consistently reported to be regulated by IFN-γ. CCL2, 3, 5 and 7 are chemoattractants for immune cells of the myeloid lineage, including neutrophils, inflammatory macrophages and DCs, whereas CXCL9, 10 and 11 predominantly mediate lymphocytic recruitment and infiltration to the focal sites of inflammation. STAT1 deficiency causes changes, usually a downregulation, in the synthesis and secretion of these chemokines. It is difficult to determine whether those changes are a result of direct actions of STAT1 and ISGF3 or indirect effects. Nevertheless, promoter regions of several of the IFN-γ-regulated chemokines, including CCL2, CCL5, CXCL9, and CXCL10, contain binding sites for STAT1 and/or ISGF3, thus satisfying the criterion to be considered as direct target genes of IFN-γ signaling [107, 110, 111, 114]. These chemokines also respond to signals from classical pro-inflammatory pathways, most notably the NF-κB pathway [112, 113, 117]. Functional cooperation between STAT1, IRFs and NF-κB is not uncommon, and there is evidence suggesting that IFN/STAT and NF-κb pathways sequentially regulate the expression of CCL2 [118].

IFN-γ signaling also upregulates the expression of cell surface adhesion molecules, such as ICAM-1 and VCAM-1 [116]. IFN-γ treatment induces a robust, time-dependent upregulation of ICAM-1 expression on the apical membrane of epithelial cells [119]. This upregulation is dependent on the JAK-STAT signaling pathway. The promoter region of ICAM-1 contains binding sites for STAT1

and ets-1 [120]. Both transcription factors are required for full sensitivity of ICAM-1 expression to IFN-γ stimulation.

Chronic allergic inflammation and airway hyperresponsiveness (AHR) associated with asthma have been commonly attributed to the actions of type 2 cytokines, namely IL-4, IL-5 and IL-13, as well as infiltrating eosinophils. Th1 cells and their respective cytokines, such as IFN-γ, have also been identified in asthmatic airways, but their function remain controversial [121, 122]. On the one hand, IFN-γ has been shown to inhibit Th2 cell proliferation [123], AHR [124], mucus hypersecretion [125], as well as lymphocyte and eosinophil recruitment [126]. IFN-γ treatment also blocks the expansion of type 2 innate lymphoid cells and IL-13 expression in mice [127]. On the other hand, Kanda et al. reported that IFN-γ-deficient eosinophils failed to induce AHR in Balb/c mice, suggesting that eosinophil-derived IFN-γ mediates the development of AHR [128]. IFN-γ may also play a role in activating eosinophils with delayed onset of action [129]. In addition, Sun et al. showed that IFN-γ is important for the recruitment and migration of activated CD4 T cells to ASM cells [63]. Understanding the various signaling mechanisms of IFN-γ that mediate its paradoxical roles in asthma is crucial for potentially targeting it in different asthmatic contexts.

2.3.2 Tumor necrosis factor (TNF; TNF-a)

Tumor necrosis factor (TNF; also referred to as TNF-α) was first isolated in 1984 and characterized by its cytotoxicity to tumour cells and its capacity to induce tumour regression in mice [130]. Since its discovery, TNF has been implicated in various physiological and pathological processes including tumorigenesis, inflammation, immunodeficiency, graft rejection, rheumatoid arthritis, and septic shock. The past three decades of research have elucidated some conflicting and complicating roles of TNF. On the one hand, the anti-cancer property of TNF stems from its

ability to induce cancer cell death. On the other hand, TNF-resistant cancer cells have been shown to upregulate survival, proliferation, migration, and angiogenesis in response to TNF stimulation. TNF is a protein composed of 157 amino acids and synthesized as a type II transmembrane protein, pro-TNF. TNF-converting enzyme (TACE) induces the cleavage of TNF into its soluble form. TNF can interact with its two receptors, TNF receptor (TNFR) 1 and 2. The two receptors have their own unique structural and functional characteristics. TNFR1 is ubiquitously expressed whereas TNFR2 is mainly expressed on immune and endothelial cells. TNFR1 has a strong binding affinity for soluble TNF, while TNFR2 has a bias of associating with the membrane-bound form. Upon ligand binding, TNFR1 recruits the TNFR-associated death domain (TRADD). TRADD in turns serves as a docking site for the binding of other downstream adaptor proteins and signal transducers. These include TNFR-associated factor 2 (TRAF2), which activates the NF-κB pathway, receptor interacting protein 1 (RIP), which leads to c-Jun N-terminal kinase (JNK) activation, and Fas-associated death domain (FADD), which leads to caspase 3 and 7 activation and apoptosis. TNFR2, on the other hand, lacks a death domain but may still associate with signal transducers such as TRAF to induce apoptosis as well as activation of the NF-κB and mitogen-activated protein kinase (MAPK) pathways.

2.4 T-helper 2 (Th2) Cells

Cells of the Th2 subset are induced in response to extracellular pathogens and allergens. They predominantly mediate type II immune responses, which eliminate helminth infections and facilitate tissue repair, but also contribute to chronic inflammatory diseases, such as asthma and allergy. Th2 differentiation is orchestrated by the cytokines IL-4 and IL-2. IL-4 signaling results in the intracellular phosphorylation and activation of the transcription factor STAT6, which in turn

upregulates expression of the master transcription regulator of the Th2 phenotype, GATA3. GATA3 induces Th2 differentiation through 3 distinct mechanisms, including upregulation of Th2 cytokine production, selective proliferation of existing Th2 cells through recruitment of Growth factor independent-1 (Gfi-1), as well as inhibition of the Th1 master transcription regulator T-bet. Multiple studies have shown that several other signal transducers and transcription factors in addition to STAT6 and GATA3 are required for full Th2 lineage commitment. For example, STAT5 binds to the second intron on the IL-4 locus and acts cooperatively along with GATA3 to induce the synthesis of IL-4. On the other hand, coordinated activity between STAT3 and STAT6 is necessary for STAT6 to gain access to the relevant gene loci in differentiating T cells, as STAT3 deficiency results in normal STAT6 activation but impaired gene binding activity. Gfi-1 is another transcription factor induced by the IL-4/STAT6 signaling pathway and promotes the selective proliferation of GATA3-high cells for Th2 lineage commitment. In Gfi-1 deficient mice, Th2 cell expansion was significantly reduced.

Characteristic cytokines produced by Th2 cells include IL-4, IL-5, IL-9, IL-10, IL-13, IL-25 and amphiregulin. The following sections focus mainly on the respective functions of IL-4, IL-5 and IL-13.

2.4.1 Interleukin-4 (IL-4) and Interleukin-13 (IL-13)

The prototypical Th2 cytokines interleukin-4 (IL-4) and interleukin-13 (IL-13) are both implicated in asthma pathogenesis [68, 131]. They share a number of characteristics, including being encoded on adjacent genes, being regulated by regulatory elements such as GATA3, and signaling through a common receptor unit IL-4Rα [131]. IL-4 plays a role in mediating allergic inflammation, specifically the differentiation, proliferation, and survival of Th2 cells, as well as IgE synthesis [132, 133, 134], whereas IL-13 is responsible for the development of AHR and several aspects of airway

remodeling, including mucus hypersecretion, subepithelial fibrosis and ASM hyperplasia and hypertrophy [135, 136].

The receptors for IL-4 and IL-13 are composed of two of the four receptor chains: IL-4Rα, common gamma chain (gc), IL-13Rα1 and IL-13Rα2. The association between IL-4Rα and gc form the functional IL-4 receptor, also known as type I receptor, which exclusively binds IL-4. On the other hand, type II receptor consists of IL-4Rα and IL-13Rα1 and is capable of binding both IL-4 and IL-13. IL-13 also binds to IL-13Rα2, which was originally classified as a decoy receptor to sequester IL-13 activity but has been recently shown to mediate TGF-β production in macrophages and the development of pulmonary fibrosis [131, 137]. IL-4 and IL-13 signaling through both type I and type II receptor converge on STAT6 phosphorylation due to the common receptor subunit IL-4Rα that they share [138, 139]. Bronchial biopsies of asthmatics show increased IL-4Rα expression, suggesting their involvement in asthma [140].

IL-4 predominantly mediates allergic inflammation. It facilitates the differentiation of naïve CD4 T cells into the Th2 subset. IL-4 also induces the expression of low-affinity IgE receptor (FcεRI) on the surfaces of B cells, monocytes and macrophages, as well as high-affinity IgE receptor (FceRII) on the surfaces of mast cells and basophils, which facilitates their degranulation in response to IgE [141]. In B cells, IL-4 mediates immunoglobulin class switching from IgM into IgG1 and IgE [142, 143]. IL-13 is able to induce similar biological effects but to a lesser extent. Furthermore, IL-4 induces the chemotaxis, adherence and extravasation of circulating eosinophils via upregulated expression of eosinophil-binding adhesion molecule VCAM-1 on the surface of endothelial cells [144, 145, 146].

Similar to IL-4, IL-13 mediates trafficking and extravasation of eosinophils to the site of allergic inflammation in the airways via upregulated VCAM-1 on endothelial cells and eotaxin secretion

from airway epithelial cells [147, 148]. In addition, IL-13 is also involved in airway remodeling. Its effects seem to be independent of IL-4, as the selective inhibition of IL-13 ameliorates asthma phenotype, including goblet cell differentiation, mucus hypersecretion and AHR [135].

2.4.2 Interleukin-5 (IL-5)

Interleukin-5 (IL-5) acts on eosinophils and their precursors due to the high expression of IL-5R on their surfaces [149]. IL-5 is responsible for the development and maintenance of eosinophilia and its effects are evident throughout all stages of the eosinophil life cycle [150]. Recombinant IL-5 treatment results in increased infiltration, accumulation, and activation of eosinophils in asthmatic airways [151]. IL-5 signaling results in the expression of CD11b and inhibition of apoptosis in eosinophils [149].

2.4.3 T-helper 9 (Th9) Cells

Th9 cells were originally characterized as Th2 cells but are now increasingly recognized as a distinct subset characterized by IL-9 secretion. TGF-β acting synergistically with IL-4 induces differentiation towards Th9 subset. In addition, IRF4 was found to bind to the IL-9 promoter and promotes IL-9 expression.

2.5 Regulatory T Cells (Tregs)

Regulatory T cells (Tregs) can be categorized into two subpopulations, natural and induced Tregs (nTregs and iTregs), based on their origin. nTregs mature in the thymus and are released as a distinct lineage with its master transcription regulator forkhead box P3 (FOXP3) already expressed [152]. FOXP3-positive iTregs, on the other hand, are derived from naïve CD4+CD25+ T cells in the peripheral lymphoid organs following antigen exposure in the presence of TNF-β and IL-2 [152]. Multiple subpopulations of iTregs also exist within the subset, among which Tr1 cells are the best

characterized. Tr1 produce the main effector cytokine for Tregs, IL-10, which suppresses inflammation and autoimmune processes.

It is important that the immune system response occurs while minimizing host damage. IL-10 orchestrates one such anti-inflammatory pathway. IL-10 inhibits the production of IFN-γ and TNF by Th1 cells indirectly through its actions on professional APCs. IL-10 inhibits the antigen-presenting capacity of macrophages and monocytes by downregulating MHC II expression [153]. It also deactivates macrophages and DCs and inhibits their cytokine production, including IL-1, IL-6, TNF-α, and m phi [154, 155]. Other members of the IL-10 superfamily are important for maintaining the integrity and homeostasis of tissue epithelial layers [156].

2.6 T-helper 17 (Th17) Cells

The differentiation of Th17 cells is unique due to the fact that it is achieved through three distinct stages: the initiation and differentiation stage mediated by TGF-β and IL-6, the self-expansion stage induced by IL-21 and finally the stabilization stage involving IL-23 [68].

TGF-β induces lineage commitment to either the iTreg or Th17 phenotype based on its concentration and surrounding cytokines that act synergistically to specify lineage commitment. TGF-β on its own at high concentrations along with IL-2 induces differentiation to the iTreg subset through activation of STAT5 and induction of FOXP3 expression [152]. FOXP3 inhibits RORγt, the master transcription regulator of Th17 cells. On the other hand, at low concentrations, TGF-β synergizes with IL-6 and leads to the activation of RORγt, production of IL-21, as well as increased expression of IL-23R [152, 157].

STAT3 is one of the main intracellular signal transducers involved in promoting RORγt expression. In addition, STAT3 deficiency also results in the upregulation of T-bet, which suggests that

STAT3 may functionally inhibit STAT1 activation [158]. The aryl hydrocarbon receptor (AhR) also promotes Th17 differentiation, and this is hypothesized to be mediated by its ability to inhibit STAT1 and STAT5, which are intracellular mediators responsible for the differentiation of opposing cell lineages [159].

The main effector cytokines of the Th17 subset include IL-17A, IL-17F, IL-21 and IL-22. IL-17A and IL-17F signal through the common receptor IL-17RA. IL-17A signaling leads to the intracellular activation of the NF-κB and MAPK pathways and subsequent induction of cytokines such as IL-6, IL-1 and TNF-α [160]. IL-21 is the amplifying cytokine responsible for mediating the self-amplification stage but is also shown to have pleotropic abilities, including stimulating T cells, inducing B cells to differentiate into plasmocytes and memory cells, and activating NK cells [161, 162]. IL-22 is important in mediating inflammatory responses, such as acute liver inflammation, as well as tissue protective properties [163, 164].

Section 3. BTLA, LIGHT, HVEM and Their Signaling Pathways

3.1 The TNF Superfamily (TNFSF) of Cytokines

Members of the TNF superfamily (TNFSF) of cytokines are known for their ability to orchestrate inflammatory and immune responses. The superfamily is composed of more than 20 distinct ligand-receptor systems [165]. Nevertheless, their structures and intracellular signaling pathways share many similarities. Ligands of the TNF-SF are classified as type II trans-membrane proteins that assemble as compact trimers [165, 166]. On the other hand, members of the TNF receptor superfamily (TNFRSF) are typically type I transmembrane glycoproteins characterized by a cysteine-rich motif in their ligand-binding extracellular domain [167]. A cluster of four closely related ligands, namely TNF, LT-α, LT-β and LIGHT, along with their four cognate receptors form the

immediate TNF family due to their high degree of amino acid sequence similarity. Overlapping receptor binding of these immediate TNF family members suggests their redundant functions, but recent gene-knockout studies in mice showed that each ligand-receptor pair serves several unique and cooperative roles [168].

3.2 LIGHT/HVEM/LTβR Signaling Axis

3.2.1 LIGHT

LIGHT (lymphotoxin, exhibits inducible expression and competes with HSV glycoprotein D for binding to herpesvirus entry mediator, a receptor expressed on T lymphocytes), also known as TNFSF14 and CD258, is the most recently identified member of the TNF immediate family [169]. As the name suggests, LIGHT is known for its role in competing with the gD protein from HSV for binding of herpesvirus entry mediator (HVEM). HVEM is a member of the TNFRSF and originally identified as a receptor for the viral glycoprotein gD, which utilizes HVEM to mediate entry into and infection of activated T cell and now increasing recognized as an inhibitory modulator of LIGHT-HVEM signaling due to its competitive role [170]. LIGHT also binds to the LTβR, as well as a soluble immunomodulatory decoy receptor of the TNFRSF, DcR3 [171]. LIGHT is predominantly a cell surface molecule that is transiently expressed by T cells upon activation, although there is evidence of a secreted form of LIGHT [172]. Other cell types that express LIGHT include NK cells and T and B cells lymphoid progenitor cells [173]. Constitutive LIGHT expression elicits activation and a powerful inflammatory response in T cells [174]. However, its expression is regulated by immunomodulation by a number of transcription factors at the transcript level [175]. HVEM is widely expressed on all lymphocyte populations, including naïve CD4 and CD8 T and B cell subsets and in some endothelial, epithelial, and fibroblastic reticular cells in barrier organs,

suggesting that LIGHT-HVEM play an important immunostimulatory role in many contexts. In contrast, LTβR seems to be restricted to stromal and myeloid cells but absent in lymphocytes [166]. It is hypothesized that LIGHT-LTβR is more relevant in modifying tissue microenvironment at the site of inflammation and in the lymph nodes.

LIGHT engagement with HVEM leads to the recruitment of TRAF2 on the cytosolic side and the formation of a ubiquitin E3 ligase TRAF2/cIAP complex, which in turn activates a number of downstream inflammatory, proliferative and survival pathways for B and T cell activation, such as the canonical NF-κB RelA (p65) and JNK/AP-1 [176, 177]. LIGHT interacting with LTβR forms a docking site for the intracellular signaling complex TRAF2, TRAF3 and inhibitor of κB-kinase (IKK) complex, which also activates the NF-κB and JNK/AP-1 transcription factors and expression of prototypical TNF cytokines, TNF, IL-6, IL-8, and MMP9 [178]. LIGHT-LTβR signaling also activates apoptosis via TRAF3 but not TRAF2 [179]. Recent evidence suggests in the context of asthma, LIGHT signaling through LTβR also activates the non-canonical NF-κB RelB pathway and is responsible for mediating airway remodeling [180].

LIGHT is widely implicated in disease pathogenesis, including viral infection, intestinal inflammation, eosinophilic esophagitis (EoE), and asthma. The viral gD disrupts LIGHT-HVEM interaction, thereby suppressing the production of antigen-specific IgG2α, which is required for viral clearance via cytotoxicity and phagocytosis [181]. Another study suggests that LIGHT, and members of the IgSF BTLA and CD160 act as equivalent activating ligands of HVEM for evoking immunopathogenesis in ocular HSV-1 infection [182]. Constitutive expression of LIGHT on T cells in transgenic mice results in multi-organ inflammation, particularly mediating severe pro-inflammatory responses in the intestines [174, 183]. This is reminiscent of TNF-transgenic mice [184]. Such findings are also mirrored clinically, as LIGHT was found constitutively expressed on the

surface of human lamina propria T cells, as well as NK cells, in the small intestine of patients with inflammatory bowel disease, and more intensively upregulated upon further activation [185]. LIGHT mRNA is also upregulated in the inflamed intestines of patients with colitis and Crohn's disease [185, 186]. LIGHT was initially found in esophageal tissue of eosinophilic esophagitis affected (EoE) patients and later pinpointed to a Th2 cell subset that accumulated in the epithelium and lamina propria of those patients [187]. These LIGHT molecules activated esophageal fibroblasts, which allowed them to adhere to and cluster with eosinophils, a cardinal immunological feature of EoE [188]. Interestingly, numerous recent clinical studies have established a correlation between serum LIGHT levels and disease severity and mortality in COVID-19 patients [189, 190, 191, 192, 193].

LIGHT is linked to severity of lung inflammation. LIGHT expression was first shown in lung lavages of systemic sclerosis patients with pulmonary fibrosis [194]. Similarly, soluble LIGHT levels as well as LIGHT-expressing cells correlate with disease severity, particularly lower lung function, in asthmatics [195]. Recently, Doherty et al. reported that LIGHT-deficient mice, as well as those treated with a LTβR blocker show reduced collagen deposition, bronchial smooth muscle mass, and airway hyperreactivity in allergic asthma and bleomycin-derived IPF models [196, 197]. Injection of recombinant LIGHT restored these characteristic airway remodeling features. Croft et al. showed that LIGHT promoted contractility, hypertrophy and hyperplasia of human ASM cells, suggesting that LIGHT secreted by other cell types is acting on ASM cells; this is thought to be mediated by LTβR [180].

3.2.2 Herpes Virus Entry Mediator (HVEM)

HVEM was originally recognized by its role in binding to the gD protein on the surface of herpesvirus and facilitating viral entry, but is now classified as a member of the tumor necrosis factor receptor family (TNFRSF). Expression of HVEM has been broadly identified on

hematopoietic and non-hematopoietic cells. Endogenously, HVEM binds to LIGHT, BTLA and weakly to LT-α to serve a variety of contrasting roles in inflammation. HVEM expression was elevated in a murine model of indirect acute lung injury (iALI). HVEM knockdown by administration of siRNA reduced the upregulation in chemokines MCP-1, MIP-2, KC and cytokine IL-6, as well as MPO activity (as an indicator of neutrophil presence), BAL cell count and BALF protein concentration (as an indicator of vascular and pulmonary permeability and lung injury) [198]. HVEM signaling also causes intestinal epithelial cells to produce pro-inflammatory cytokines and provide host defense against enteric bacterial infection. In a mouse model for enteropathogenic Escherichia coli infection, HVEM-deficient mice showed decreased STAT3 activation, impaired responses in the colon, increased colonic epithelial permeability and bacterial dissemination, higher bacterial burdens and increased mortality. CD160-HVEM signaling on innate-like intraepithelial lymphocytes is important for mediating inflammation in the colonic epithelium. HVEM knockdown reduced STAT3 phosphorylation, as well as expression of pro-inflammatory cytokines IL-6, IFN-γ and chemokines CXCL1, CXCL2, CCL20. Stimulation of HVEM signaling in lung epithelial cells *in vitro* with BTLA-Ig induces these pro-inflammatory mediators, but to a lesser extent than *in vivo* [199]. HVEM mRNA expression is significantly higher in patients with severe and moderate persistent asthma, and this upregulation is positively correlated with other inflammatory parameters such as fractional exhaled nitric oxide and total lymphocyte count in peripheral blood [200]. Therefore, it is hypothesized that HVEM expression could serve as a biomarker for evaluating the severity of asthma.

3.3 BTLA/CD160/HVEM Signaling Axis

TNFR1 and 2, LTβR DcR3 and HVEM and their ligands form a costimulatory and co-inhibitory network characterized by shared utilization. Engagement of HVEM with members of the immunoglobulin superfamily (IgSF) further complicates these mechanisms. In addition to binding members of the TNFSF, LIGHT and LT-α, HVEM also interacts with IgSF family members BTLA and CD160. BTLA and CD160 bind to HVEM at a topically distinct sites CRD2 and CRD3, but engagement with any of the four ligands triggers TRAF2/3 E3 ligase pathway and pro-inflammatory responses in the cell expressing HVEM [201]. In contrast, BTLA ligation by HVEM delivers a co-inhibitory signal.

3.3.1 B and T lymphocyte attenuator (BTLA)

B and T lymphocyte attenuator (BTLA) is a member of the CD28 superfamily of proteins. Contrary to CD28, whose ligation delivers a costimulatory signal required for T cell activation and survival, BTLA is a co-inhibitory receptor mainly expressed on B and T cells [202]. Interaction between BTLA and its ligand, HVEM, dampens B and T cell responses, hence is classified as an inhibitory immune checkpoint. BTLA ligation by HVEM leads to the phosphorylation of immunoreceptor tyrosine-based inhibitory motif (ITIM) and immunoreceptor tyrosine-based switch motif (ITSM) on the cytoplasmic tail of the receptor and downstream recruitment of SHP1/2, which are protein-tyrosine phosphatases that limit antigen receptor activation [203, 204]. A functional dichotomy is also present with respect to BTLA-HVEM signaling. BTLA is expressed on resting B and T cells and is able to form a cis-heterodimeric complex with HVEM expressed on the same cell [205]. This interaction blocks BTLA-HVEM engagement in trans and is hypothesized to be important for preventing HVEM co-stimulation and maintaining T cells in their resting state [205]. However, Battin et al recently showed that this cis-complex does not impair the inhibitory effects of BTLA signaling,

suggesting that BTLA co-inhibition plays a dominant role [206]. In addition, Battin et al. also utilized a T cell reporter system to elucidate the effects of LIGHT and CD160 co-expression with HVEM. In contrast to the BTLA-HVEM cis-complex, LIGHT and CD160 co-expressed with HVEM constitutively activates and augments HVEM signaling [206].

Upregulated expression of both BTLA and HVEM have been observed in cancer and are associated with increased disease progression and poorer prognosis [207]. Checkpoint inhibitors that disrupt the BTLA-HVEM engagement have been a heavy focus of recent studies as potential immunotherapies by activating anti-tumor immunity. Interestingly, BTLA has been implicated in downregulating immune responses and inflammation in murine models of allergic asthma [202, 208]. Deppong et al. reported that BTLA is required for the termination of acute allergic airway inflammation, as BTLA-deficient mice demonstrated prolonged inflammatory responses up to 15 days post-ovalbumin (OVA) challenge, compared to wild-type mice whose inflammation resolved by day 10 [209]. T cells isolated from BTLA-deficient mice also exhibited enhanced survival and hyperresponsiveness in response to allergen challenge and TCR-mediated activation [210, 211]. On the other hand, crosslinking BTLA with an agonistic monoclonal antibody (mAb) suppresses T cell proliferation and secretion of pro-inflammatory cytokines IFN-γ and IL-10 upon anti-CD3 stimulation [212].

Section 4. Objectives and Hypothesis

Crosstalk between CD4 T cells and ASM cells mediates ASM proliferation, exchange of anti-apoptotic proteins, as well as other features of airway remodeling. The inflammatory profiles of these two cell types when in contact with each other have not been extensively studied. It is hypothesized that cytokines secreted by activated CD4 T cells induce a pro-inflammatory ASM

cell phenotype. IFN-γ signaling in ASM cells may be of prime importance given that *in vitro* transient engagement of TCR and CD28 on CD4 T cells favors Th1 polarization. IFN-γ-inducible pro-inflammatory molecules, such as CXCL9, 10, 11 and ICAM-1 may be upregulated in co-cultured ASM cells.

BTLA is involved in dampening the T cell response in murine models of asthma [210, 211, 212]. LIGHT has been implicated in the development of asthmatic characteristics, such as subepithelial fibrosis, smooth muscle hyperplasia, and AHR [180, 196]. In addition, HVEM ligation induces a pro-inflammatory pathway that activates NF-κB, which may serve to potentiate inflammation in ASM cells [177]. We wished to examine the role of BTLA-HVEM and LIGHT-HVEM signaling in the context of CD4 T – ASM cell co-culture, specifically focusing on the ability of BTLA to modify cytokine synthesis in CD4 T cells and the effects of HVEM signaling on the secretory phenotype of ASM cells.

The specific objectives pertaining to this thesis are to:

1. Characterize the inflammatory profiles of CD4 T cells and ASM cells when placed in co-culture.
2. Examine the immunomodulatory effects of HVEM as a ligand for BTLA on CD4 T cells and a receptor on ASM cells in co-culture via a HVEM knockdown model and recombinant LIGHT treatment, respectively.

Chapter 2. Materials and Methods

Section 1. Antibodies and Chemicals

Neutralizing antibody against IFNGR was purchased from R&D Systems and reconstituted in phosphate-buffered saline (PBS). Recombinant human IFN-γ was purchased from Stemcell and reconstituted in water. Active recombinant human LIGHT was purchased from Abcam and reconstituted in PBS.

Section 2. CD4 T – ASM Cell Co-culture

2.1 CD4 T Cell Isolation and Activation

Following informed consent, peripheral blood was collected from healthy volunteers in heparin-coated tubes and diluted with PBS at a 1:1 ratio. Peripheral blood mononuclear cells (PBMCs) were separated from total blood via density gradient centrifugation using the Lymphoprep Density Gradient Medium (Stemcell). CD4 T cells were then isolated from PBMCs by immunomagnetic positive selection using human CD4 microbeads (Miltenyi). Five million CD4 T cells were activated in each well of a 6-well plate by incubating with a 1:1 ratio of CD3/28 Dynabeads for 72 hours in complete RPMI (Wisent) supplemented with 10% fetal bovine serum (FBS, Wisent) and 1% antibiotic-antimycotic solution (Wisent). Activated CD4 T cells were separated from the Dynabeads by magnetic separation immediately before co-culture.

2.2 ASM Cell Culture

ASM cells were isolated from transplant-grade control lungs of matching donors that were procured by the International Institute for the Advancement of Medicine (IIAM, USA) and the National Disease Research Interchange (NDRI, USA). ASM tissue was dissected from a section of bronchus and incubated in 0.4mg/mL collagenase IV diluted in Dulbecco's Modified Eagle's

Medium (DMEM, Wisent) overnight to dissociate cells. Large fragments of tissue were removed, and the remaining cell suspension was centrifuged at 300g for 5 min. The pellet of ASM cells was re-suspended in growth medium (DMEM supplemented with 10% FBS (Wisent) and 1% antibiotic-antimycotic solution (Wisent)) and kept in a humidified atmosphere of 95% air - 5% CO2 at 37°C. Growth medium was changed every 2–3 days until cells were approximately 80–90% confluent before being passaged for experimentation. All ASM cells used for experimentation were between passages 2–6.

2.3 Co-culture

100,000 ASM cells were plated in each well of a 6-well plate one day prior to co-culture. 500,000 activated CD4 T cells were co-cultured with ASM cells in a 1:1 mixture of RPMI and DMEM supplemented with 10% FBS (Wisent) and 1% antibiotic-antimycotic solution (Wisent) for 24 hours. After co-culture, CD4 T cells were separated from ASM cells by gentle washing with PBS.

Section 3. Generation of HVEM Knockdown ASM Cells

To generate recombinant retrovirus, 450,000 HEK293T cells were transfected in each well of a 6-well plate using the Lenti-vpak Lentiviral Packaging Kit and human short hairpin (shRNA) constructs in lentiviral GFP vectors (Origene). Pseudoviral particles were recovered from the growth medium, and their concentration was determined by ELISA. 100,000 ASM cells were plated in antibiotic-antimycotic-free medium one day prior to transduction. Lentiviral particles were added at a multiplicity of infection (MOI) of 60 for 20 hours. Successfully transduced cells were selected by culturing in DMEM containing 2 μg/mL puromycin (Sigma-Aldrich) for 1 week. Culture medium was then replaced with growth medium, and the remaining ASM cells were allowed to expand for another week before being used for experimentation.

Section 4. Analysis Methods

4.1 RNA Extraction and RT-qPCR

Following co-culture, CD4 T cells were separated from ASM cells by collecting culture supernatant and multiple washes of the wells using PBS.

Total RNA from both CD4 T cells and ASM cells were extracted using RNAeasy Minikit (Qiagen) and 400ng was retrotranscribed using the Lunascript RT SuperMix Kit (NEB). Real-time quantitative PCR (RT-qPCR) was performed using CFX96 (Bio-Rad) with SYBR Select Master Mix to quantify mRNA expression levels. Relative mRNA expression was calculated using the "ΔΔCt" method. Individual data were normalized against the housekeeping gene ribosomal S9. Results were expressed as a fold change in mRNA expression compared to a calibrator sample. The primers were purchased from Thermo Fisher Scientific, with sequences as follows:

Table 1. Primer sequences for RT-qPCR

Gene	Forward Primer (5'–3')	Reverse Primer (5'–3')
CXCL9	ACTATCCACCTACAATCCTTGAAAGAC	TCACATCTGCTGAATCTGGGTTTAG
CXCL10	CTTCCAAGGATGGACCACACA	CCTTCCTACAGGAGTAGTAGCAG
CXCL11	AGAGGACGCTGTCTTTGCAT	TGGGATTTAGGCATCGTTGT
ICAM-1	ATGGCAACGACTCCTTCTCG	CGCCGGAAAGCTGTAGATGG
BTLA	TGGGTCATACCGCTGTTCTG	TCTGCTTGCCATTTCGTCCT
LIGHT	GGTCTCTTGCTGTTGCTGATGG	TTGACCTCGTGAGACCTTCGCT
HVEM	AGCCTCGTCATCGTCATTGT	GACTACATCACCCCTTGGCT
S9	CTGCTGACGCTTGATGAGAA	CAGCTTCATCTTGCCCTCA

4.2 Flow Cytometry

For surface staining of BTLA and LIGHT on CD4 T cells, as well as HVEM and ICAM-1 on ASM cells, both cell types were harvested together following co-culture. Cells were stained with

eBioscience Fixable Viability Dye eFluor 780 (Invitrogen) diluted 1:1000 in FACS buffer (1% bovine serum albumin (BSA) in PBS) for 30 minutes, and then with the following 1:100 diluted surface antibodies: BV510-conjugated anti-CD4 (BD Biosciences), BV786-conjugated anti-CD45 (BD Biosciences), BV421-conjugated anti-BTLA (BioLegend), PE/Cy7-conjugated anti-LIGHT (BioLegend), APC-conjugated anti-HVEM (BioLegend) and AF488-conjugated anti-ICAM-1 (BioLegend). Cells were then treated with fixation reagent from FOXP3/Transcription Factor Staining Buffer Set (Invitrogen) and stored at 4^{o}C until analysis.

For intracellular staining of CXCR3 ligands CXCL9, 10 and 11, ASM cells were separated from CD4 T cells via repeated PBS washes and immediately treated with BD GolgiStop (BD Biosciences) for 5 hours. Cells were stained with eBioscience Fixable Viability Dye eFluor 780 (Thermo Fisher Scientific) diluted 1:1000 in FACS buffer for 30 minutes, and then with the following 1:100 diluted surface antibodies: BV510-conjugated anti-CD4 (BD Biosciences) and BV786-conjugated anti-CD45 (BD Biosciences). Cells were then treated with fixation reagent from FOXP3/Transcription Factor Staining Buffer Set (Invitrogen). To assess chemokine synthesis, ASM cells were treated with permeabilization buffer and stained with 1:100 diluted APC-conjugated anti-CXCL9 (BioLegend), PE-conjugated anti-CXCL10 (BioLegend) and FITC-conjugated anti-CXCL11 (LifeSpan Biosciences) for 30 minutes. Stained cells were analyzed on BD LSRFortessa Cell Analyzer (BD Biosciences) and data was analyzed using FlowJo software (BD Biosciences). Appropriate isotype control antibodies were used for gating purposes.

4.3 Western Blot

Following co-culture, ASM cells were separated from CD4 T cells via repeated PBS washes. ASM cells were lysed using NP40 Cell lysis buffer (Life Technologies) supplemented with protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). Cell lysates were centrifuged at 13,000g

for 10 minutes, and the supernatant was evaluated for protein concentration by Bradford assay (Bio-Rad).

Five μg of protein re-suspended in loading buffer (Bio-Rad) containing Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) was incubated at 95°C for 5 minutes. Denatured proteins were separated on a precast Tris/glycine gel (Bio-Rad) and then transferred onto a PVDF membrane (Bio-Rad) for immunoblotting. The membrane was blocked for 1 hour at room temperature with 2% ECL Prime blocking agent (Cytiva) in Tris-buffered saline with 0.1% Tween (TBS-T). Membranes were incubated with 1:1000 diluted primary antibodies against phospho/total NF-kB p65 and phospho/total STAT1 (Cell Signaling) overnight at 4°C. After washing three times with TBS-T, membranes were incubated with a 1:2000 diluted HRP-linked secondary antibody (Cell Signaling) for 1 hour at room temperature and then visualized using the ChemiDocTM MP Imaging System (Bio-Rad) via ECL Plus Western blotting detection (Bio-Rad). Densitometric analysis was performed with Image Lab (Bio-Rad). Protein expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

4.4 Enzyme-linked Immunosorbent Assay (ELISA)

For quantification of cytokine secretion, supernatant of the CD4 T – ASM cell co-culture was collected. ELISA plates (96-well high binding microplates, Corning) were coated with mouse anti-human IFN-γ or IL-17 capture antibody (both from R&D Systems) and incubated overnight at room temperature. Plates were washed and were blocked with 1% BSA in PBS for 1 hour. Supernatant samples diluted to the appropriate concentrations were added and plates were incubated for 2 hours at room temperature. Biotinylated mouse anti-human IFN-γ or goat antihuman IL-17 detection antibody (both from R&D Systems) were added, and plates were incubated for 2 hours at room temperature. Reactions were developed with Streptavidin-HRP

(R&D Systems) for 20 minutes, halted using a sulfuric acid stop solution (R&D Systems), and read at 450 nm absorbance on a plate reader.

4.5 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 9 software. Data are presented as the mean ± SEM, unless otherwise specified. Paired Student's t-test or 2-way repeated measures ANOVA with *a post hoc* Bonferroni test were applied as appropriate. Level of significance is classified as follows: *, **, ***, **** correspond to $P < 0.05$, 0.01, 0.001, 0.0001, respectively. Exact P-values were given whenever possible.

Chapter 3. Results

Co-culture induces Th1 differentiation of CD4 T cells and increased secretion of IFN-γ

CD4 T cells isolated from human peripheral blood were activated anti-CD3/28 Dynabeads for 72 hours and subsequently put in co-culture with ASM cells from healthy donors at a 5:1 CD4 T–ASM ratio for 24 hours. Following co-culture, CD4 T cell subset differentiation and secretion of characteristic cytokines were examined via RT-qPCR and flow cytometry.

Co-cultured CD4 T cells showed upregulated expression of the transcription factor T-bet, which is the master transcription regulator mediating Th1 differentiation (Figure 1A). On the other hand, GATA3 and FOXP3, master transcription regulators that promote differentiation towards the Th2 and Treg subsets, were downregulated at the transcript level in response to co-culture, suggesting an inhibition of differentiation towards those subsets (Figure 1A). Expression of the transcription factor mediating Th17 subset differentiation, RORγt, did not show any changes (Figure 1A).

Coincident with the transcriptional increase of T-bet, secretion of the characteristic Th1 cytokine, IFN-γ, was increased in the co-culture medium (Figure 1B), which led to the hypothesis that IFN-γ signaling may play an important role in this CD4 T – ASM cell co-culture context. Secretion of the characteristic Th17 cytokine, IL-17, was also increased in the co-culture medium (Figure 1B).

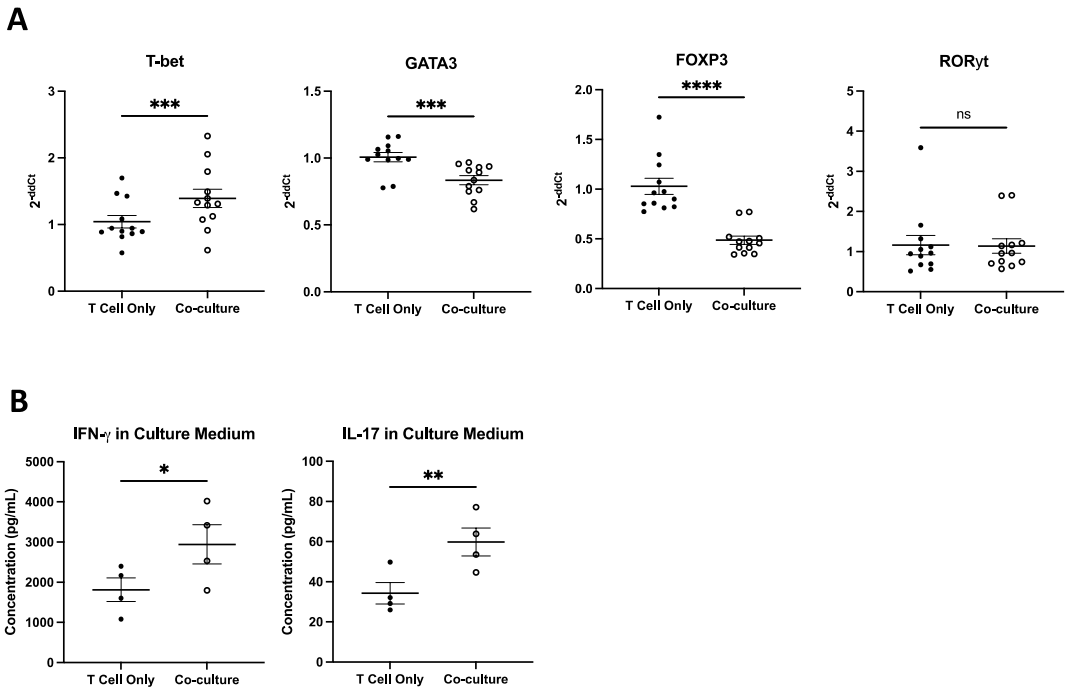

Figure 1. Upregulated T-bet expression and IFN-γ secretion in CD4 T cells co-cultured with ASM cells for 24 hours.

(A) Relative RNA levels of CD4 T cell subset-specific transcription factor T-bet, GATA3, FOXP3 and RORγt analyzed by RT-qPCR. Data are mean ± SEM (n = 12), ns = not significant, ***P < .001, ****P < .0001, 2 tailed, paired Student's t-test. (B) ELISA quantification of IFN-γ and IL-17 in the culture medium of control CD4 T cells and CD4 T – ASM co-culture. Data are mean ± SEM (n = 4), *P < .05, **P < .01, 2 tailed, paired Student's t-test.

Co-cultured ASM cells activate the STAT1 signaling pathway and upregulate expression of CXCR3 ligands and ICAM-1

Following co-culture, the pro-inflammatory phenotype of the ASM cells was examined, specifically focusing on quantifying activation of the IFN-γ signaling pathway and expression of classical IFN-γ-inducible chemokines and adhesion molecules. Co-cultured ASM cells showed upregulated gene transcription of CXCR3 ligands CXCL9, 10, 11, as well as the cell surface adhesion molecule ICAM-1 (Figure 2A). These transcriptional changes were confirmed at the protein level. Co-cultured ASM cells displayed higher mean fluorescence intensity (MFI) when stained with fluorophore-conjugated antibodies against CXCL9, 10, 11 and ICAM-1, indicating that these pro-inflammatory markers are also upregulated at the protein level (Figure 2B). ASM cells that have been co-cultured with CD4 T cells also showed increased STAT1 phosphorylation, compared to cells of the non-co-cultured control group for which little or no STAT1 phosphorylation was detected (Figure 2C), indicating increased activation of the IFN-γ/STAT1 signaling pathway in the co-cultured cells. We did not observe a statistically significant activation of the canonical NF-κB pathway, as represented by phosphorylation of its main mediator p65, albeit an increasing trend (Figure 2C).

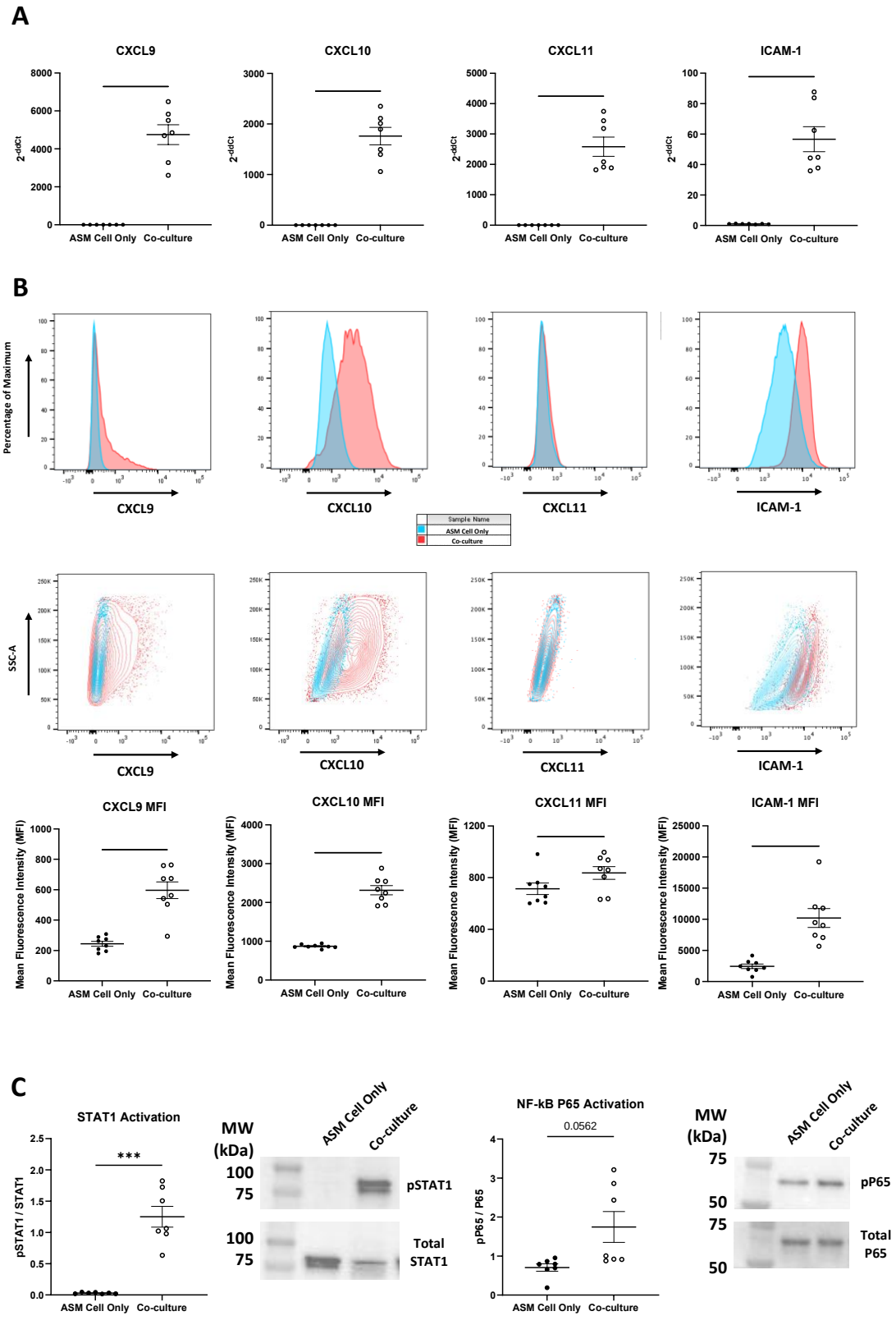
A
CXCL9
CXCL10
CXCL11
ICAM-1
2-ddCt
ASM Cell Only
Co-culture
B
Percentage of Maximum
CXCL9
CXCL10
CXCL11
ICAM-1
Sample Name
ASM Cell Only
Co-culture
SSC-A
CXCL9 MFI
CXCL10 MFI
CXCL11 MFI
ICAM-1 MFI
Mean Fluorescence Intensity (MFI)
C
STAT1 Activation
pSTAT1 / STAT1
***
MW (kDa)
100
75
pSTAT1
Total STAT1
NF-kB P65 Activation
0.0562
pP65 / P65
pP65
Total P65
50

Figure 2. Co-cultured ASM cells activate STAT1 and upregulate expression of CXCR3 ligands and ICAM-1

(A) Relative RNA levels of CXCR3 ligands CXCL9, 10, 11 and adhesion molecule ICAM-1 in naïve and co-cultured ASM cells quantified by RT-qPCR. Data are mean ± SEM (n = 7), ***P < .001, ****P < .0001, 2 tailed, paired Student's t-test. (B) Representative flow cytometry plots and quantification of MFI of intracellular CXCL9, 10, 11 and ICAM-1 comparing control ASM cells and co-cultured ASM cells. Data are mean ± SEM (n = 8), *P < .05, **P < .01, ****P < .0001, 2 tailed, paired Student's t-test. (C). Representative Western blots and quantification of STAT1 and p65 activation comparing control and co-cultured ASM cells. Data are mean ± SEM (n = 7), ns = not significant, ***P < .001, 2 tailed, paired Student's t-test.

Inhibition of IFN-γ signaling in co-cultured ASM cells reduces the expression of CXCR3 ligands and ICAM-1

ASM cells were pre-incubated with neutralizing antibodies (1μg/mL) against the alpha chain of IFN-γ receptor (IFNGR) or control IgG antibodies for 30 minutes and subsequently put into co-culture with activated CD4 T cells for 24 hours. Inhibition of the IFN-γ signaling pathway via anti-IFNGR antibody inhibited the upregulation of CXCL9, 10, 11 and ICAM-1 transcription in co-cultured ASM cells (Figure 3). Nevertheless, their transcript levels were still higher when compared to those of control, non-co-cultured ASM cells.

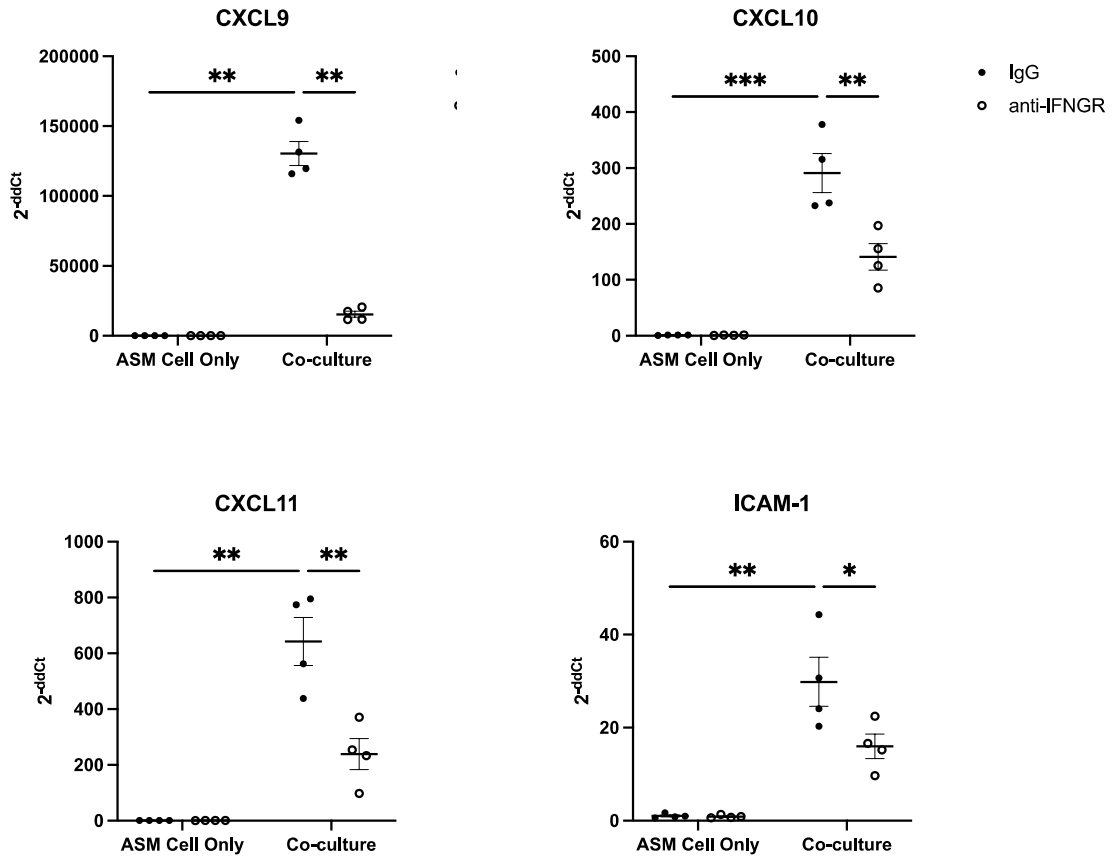

Figure 3. Inhibition of IFN-γ signaling in ASM cells via IFNGR neutralization reduces expression of CXCR3 ligands and ICAM-1

Relative RNA levels of CXCR3 ligands CXCL9, 10, 11 and adhesion molecule ICAM-1 in naïve and co-cultured ASM cells pretreated with control IgG or anti-IFNGR antibody quantified by RT-qPCR. Data are mean ± SEM (n = 4), *P < .05, **P < .01, ***P < .001, 2-way repeated measures ANOVA with a *post hoc* Bonferroni test.

Co-cultured ASM cells upregulate HVEM. CD4 T cells express BTLA and LIGHT

Transcription of BTLA and LIGHT in CD4 T cells, as well as HVEM in ASM cells, was measured by RT-qPCR. Both BTLA and HVEM mRNA expression was upregulated in CD4 T cells and ASM cells, respectively, compared to that of non-co-cultured cells (Figure 4A). The transcript level of LIGHT was not altered in response to co-culture (Figure 4A). The MFI of BTLA from co-cultured CD4 T cells was comparable to that of the control group, indicating that the transcriptional upregulation of BTLA was not accompanied by similar changes at the protein level (Figure 4B). The MFI of LIGHT was reduced on CD4 T cells in co-culture (Figure 4B). On the other hand, HVEM protein expression was elevated in co-cultured ASM cells, as evidenced by their higher MFI and fluorescence intensity as an entire population when stained with fluorophore-conjugated antibody against HVEM (Figure 4B).

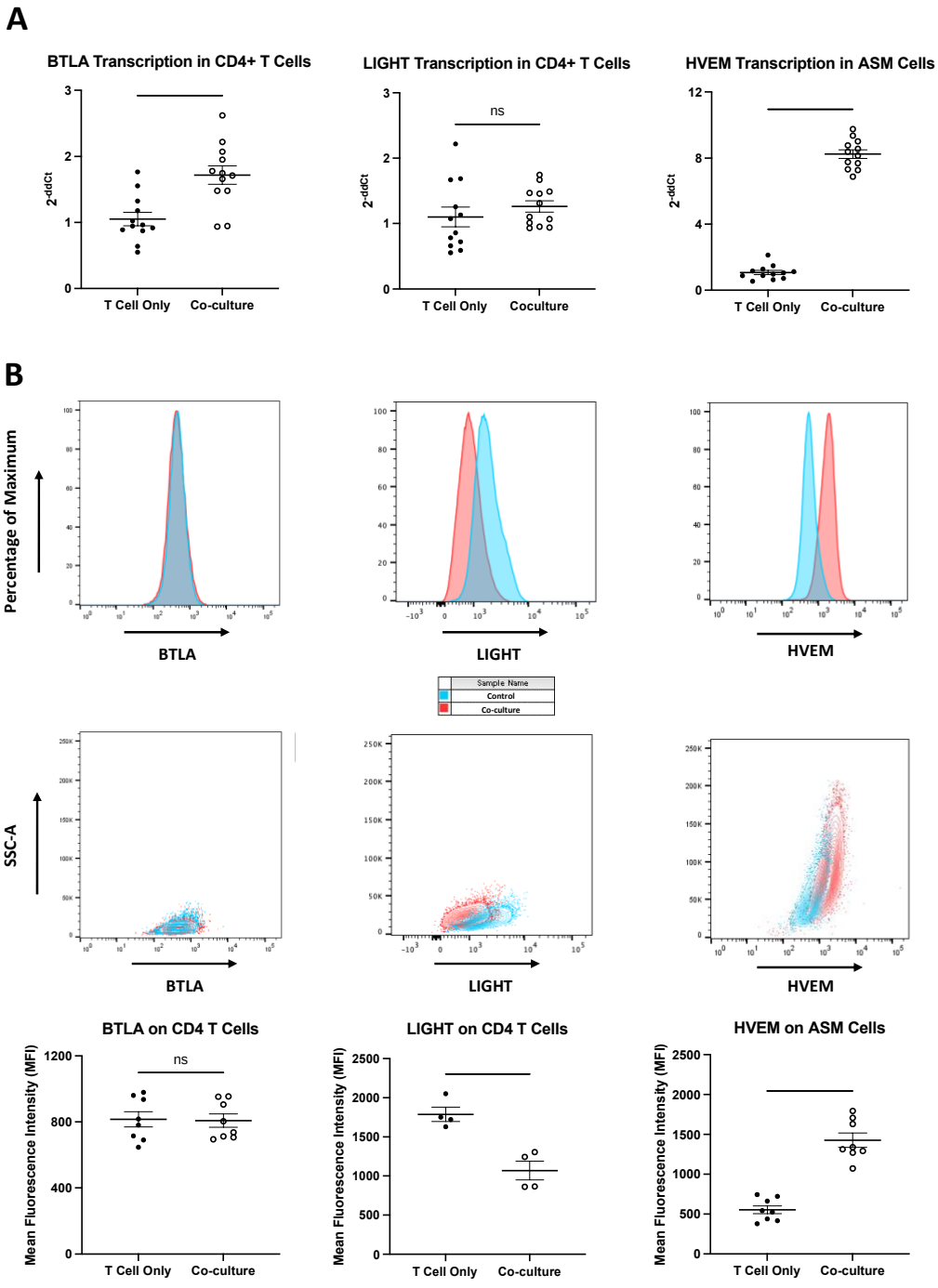
A
BTLA Transcription in CD4+ T Cells
LIGHT Transcription in CD4+ T Cells
HVEM Transcription in ASM Cells
2-ddCt
ns
T Cell Only
Co-culture
Coculture
B
Percentage of Maximum
BTLA
LIGHT
HVEM
Sample Name
Control
Co-culture
SSC-A
BTLA on CD4 T Cells
LIGHT on CD4 T Cells
HVEM on ASM Cells
Mean Fluorescence Intensity (MFI)

Figure 4. Transcript and protein levels of BTLA and LIGHT in CD4 T cells and HVEM in ASM cells

(A) Relative RNA levels of BTLA and LIGHT in CD4 T cells, as well as HVEM in ASM cells quantified by RT-qPCR. Data are mean ± SEM (n = 12), ns = not significant, ****P < .0001, 2 tailed, paired Student's t-test. (B) Representative flow cytometry plots and quantification of MFI of surface BTLA and LIGHT on CD4 T cells, as well as surface HVEM on ASM cells, comparing non-co-cultured control and co-cultured cells. Data are mean ± SEM (n = 4 or 8), ns = not significant, **P < .01, ****P < .0001, 2 tailed, paired Student's t-test.

IFN-γ and LIGHT synergistically upregulate IFN-γ-inducible CXCR3 ligands CXCL9, 10 and 11

ASM cells plated in a 6-well plate were treated with 5 ng/mL recombinant human IFN-γ and/or 10 ng/mL recombinant human LIGHT. Consistent with previous observations, IFN-γ stimulated the upregulation of CXCR3 ligands, CXCL9, 10 and 11, as well as the adhesion molecule ICAM-1. LIGHT treatment alone did not result in any change in the expression of these pro-inflammatory molecules. Combined administration of IFN-γ and LIGHT synergistically induced the expression of CXCL9, 10 and 11 to a greater extent than IFN-γ alone, LIGHT alone, or the predicted sum of those two combined. ICAM-1 also showed an increasing trend in response to combined IFN-γ and LIGHT treatment that did not reach statistical significance, compared to IFN-γ alone.

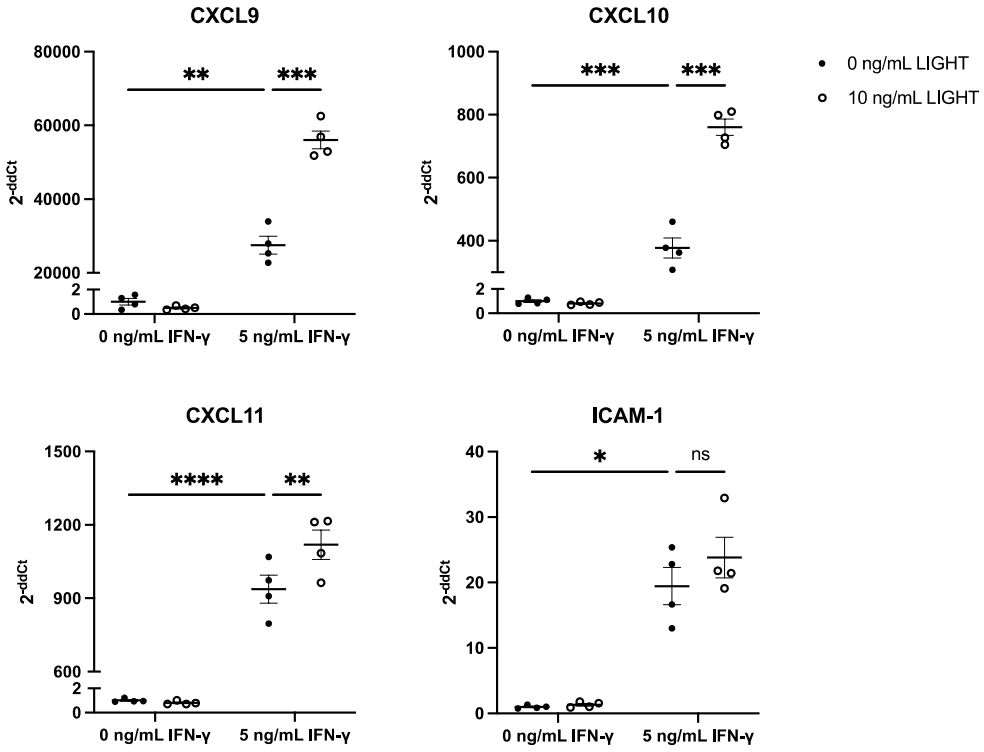

Figure 5. Recombinant human IFN-γ and LIGHT treatment synergistically upregulate CXCL9, 10 and 11

Relative RNA levels of CXCR3 ligands CXCL9, 10, 11 and adhesion molecule ICAM-1 in ASM cells treated with 5 ng/mL IFN-γ and/or 10 ng/mL LIGHT quantified by RT-qPCR. Data are mean ± SEM (n = 4), ns = not significant, *P < .05, **P < .01, ***P < .001, ****P < .0001. 2-way repeated measures ANOVA with a *post hoc* Bonferroni test.

Successful knockdown of HVEM in ASM cells

Briefly, HEK293T cells were transfected with a lentivirus vector system expressing shRNA against HVEM or a scrambled control construct. Normal, wild type ASM cells were transduced with these pseudoviral particles at a multiplicity of infection (MOI) of 60 for 20 hours. Successfully transduced ASM cells were selected by puromycin for 1 week and expanded for another week before being used for experimentation.

RT-qPCR and flow cytometry analysis confirmed a successful knockdown of HVEM both at the transcript and the protein level. In accordance with previous results, co-culture with activated CD4 T cells induced transcription and expression of HVEM in ASM cells (Figure 6). Transduction of ASM cells with the lentivirus shRNA vector system prevented HVEM upregulation and reduced HVEM expression in co-cultured ASM cells to a level comparable to baseline (Figure 6).

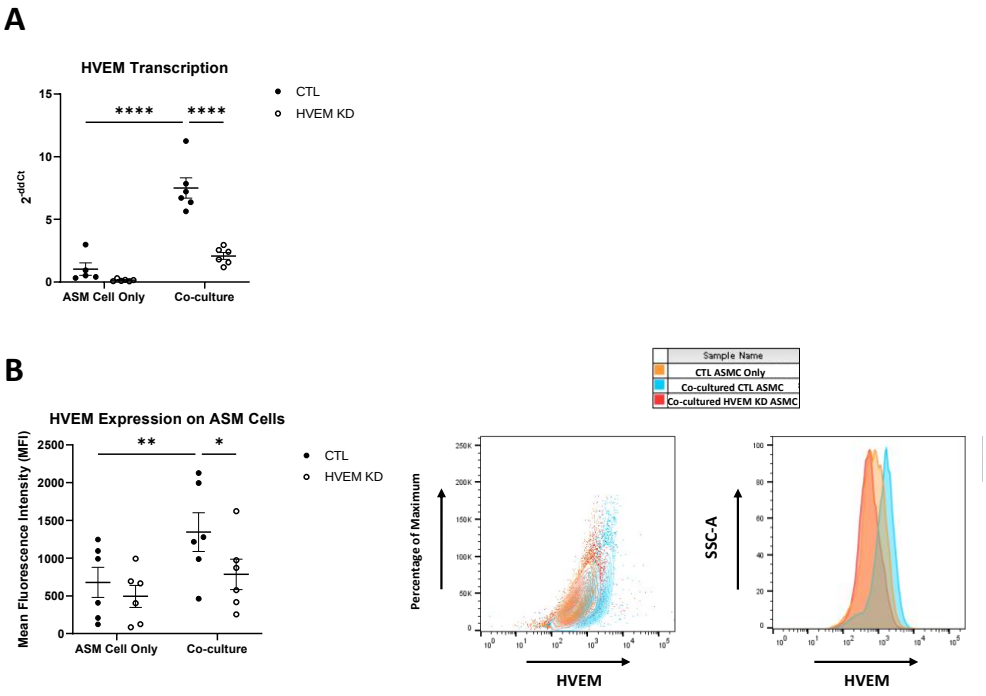

Figure 6. Successful knockdown of HVEM at the transcript and protein level

(A) Relative RNA levels of HVEM in naïve and co-cultured ASM cells transduced via a scrambled control (CTL) or HVEM shRNA-containing (HVEM KD) lentivirus vector. Data are mean ± SEM (n = 6), ****P < .0001, 2-way repeated measures ANOVA with a *post hoc* Bonferroni test. (B) Representative flow cytometry plots and quantification of MFI of surface HVEM on naïve and co-cultured ASM cells transduced via CTL or HVEM KD lentivirus vector. Data are mean ± SEM (n = 6), *P <.05, **P < .01, 2-way repeated measures ANOVA with a *post hoc* Bonferroni test.

HVEM knockdown in ASM cells reduced their ability to express IFN-γ-inducible CXCR3 ligands CXCL9 and 11, as well as adhesion molecule ICAM-1

ASM cells transduced with the lentivirus HVEM shRNA system, otherwise referred to as HVEM knockdown (KD) ASM cells, or control (CTL) ASM cells transduced with the scrambled control vector were put into co-culture with activated CD4 T cells. RT-qPCR analysis confirmed transcriptional upregulation of IFN-γ-inducible pro-inflammatory mediators CXCL9, 10, 11 and ICAM-1, as well as activation of the STAT1 signaling pathway, in ASM cells in response to co-culture (Figure 7A, B). Lentiviral transduction with the control vector did not alter the expression of chemokines and adhesion molecules, nor the activation of IFN-γ/STAT1 pathway (Figure 7A, B). HVEM knockdown partially reduced the elevated CXCL9, 11 and ICAM-1 transcript levels, as well as STAT1 phosphorylation (Figure 7A, B).

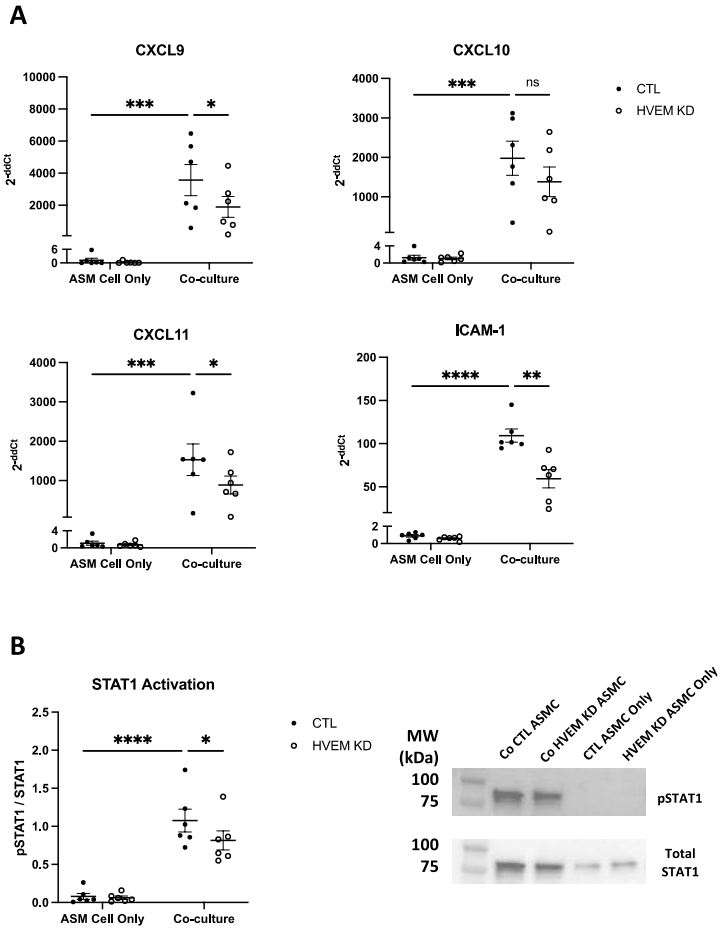

Figure 7. HVEM knockdown in ASM cells reduced their ability to express IFN-γ-inducible pro-inflammatory mediators CXCL9, 11, as well as adhesion molecule ICAM-1

(A) Relative RNA levels of CXCR3 ligands CXCL9, 10, 11, as well as ICAM-1, comparing naïve and co-cultured CTL and HVEM KD ASM cells. Data are mean ± SEM (n = 6), ns = not significant, *P <.05, **P < .01, ***P < .001, **** < .0001. 2-way repeated measures ANOVA with a *post hoc* Bonferroni test. (B) Representative Western blots and quantification of STAT1 activation in naïve and co-cultured CTL and HVEM KD ASM cells. Data are mean ± SEM (n = 6), ns = not significant, *P <.05, **** < .0001. 2-way repeated measures ANOVA with a *post hoc* Bonferroni test.

Upregulation of IFN-γ and IL-17 secretion was not affected by HVEM knockdown

Absence of HVEM in the interaction between activated CD4 T cells and ASM cell in co-culture was insufficient to block the upregulated secretion of IFN-γ (Figure 8). In addition, IL-17 secretion was also maintained in co-cultured HVEM knockdown ASM cells (Figure 8). Both of these findings suggest that BTLA-HVEM interaction plays a minimal role in mediating cytokine synthesis and secretion from CD4 T cells in co-culture.

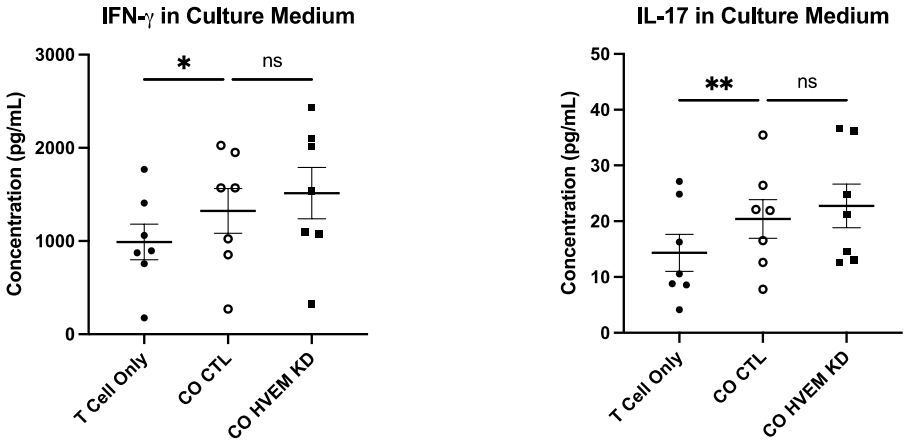

Figure 8. HVEM knockdown does not affect increased IFN-γ and IL-17 secretion from co-cultured CD4 T cells

ELISA quantification of IFN-γ and IL-17 secretion into the culture medium comparing naïve CD4 T cells, CD4 T co-cultured with CTL ASM cells and HVEM KD ASM cells. Data are mean ± SEM (n = 7), ns = not significant, *P < .05, **P < .01, 1-way repeated measures ANOVA with a *post hoc* Bonferroni test.

Chapter 4. Discussion

Upregulated T-bet expression in co-cultured CD4 T cells and increased IFN-γ level in co-culture medium

The CD4 T – ASM cell serves as a model to investigate the possible consequences of T cell infiltration into the ASM layer in asthmatic airways [64]. Our findings indicate that co-culture of activated CD4 T cells with ASM cells results in upregulated IFN-γ in the co-culture medium. IFN-γ acts on its receptor IFNGR, activates STAT1 and consequently induces T-bet expression in CD4 T cells [213]. Indeed, we observed increased T-bet expression in CD4 T cells following 24-hour co-culture with ASM cells. T-bet is known to enhance the differentiation of CD4 T cells towards the Th1 subset and increase secretion of IFN-γ from those Th1 cells. Therefore, an elevated IFN-γ level in the CD4 T–ASM cell co-culture medium facilitates a positive feedback loop involving selective expansion of Th1 cells and IFN-γ production. ASM cells sensitized by atopic asthmatic serum are able to express IFN-γ, hence could also account for the enhanced cytokine secretion [62]. The relative contribution of ASM cells to IFN-γ in the co-culture medium could be explored by quantifying the population of IFN-γ expressing ASM cells and inhibiting ASM cell-derived IFN-γ via siRNA knockdown.

Consistent with the current paradigm, which postulates a mutual antagonism between Th1 and Th2 subsets, the upregulated T-bet expression was accompanied by a reduction in the transcript level of GATA3 in co-cultured CD4 T cells. This indicates that differentiation towards the Th2 subset was inhibited. Secretion of the characteristic Th2 cytokine, IL-4, could be examined to confirm the inhibitory effects of T-bet on Th2 differentiation and GATA3 binding to its target genes [88].

Overacre-Delgoffe et al. have shown that IFN-γ inhibits Treg cell function, promotes Treg functional fragility and enhances anti-tumor immunity [214]. Our observation that FOXP3 is

downregulated in co-cultured CD4 T cells suggests that IFN-γ signaling and STAT1 activation may interfere with FOXP3 expression.

IL-17 secretion into the culture medium was elevated, albeit the lack of evidence of a preferential differentiation towards the Th17 subset. Chang et al. reported that IL-17 promotes ASM cell proliferation and migration via the MAPK pathways [215, 216]. Proliferative markers, as well as activation of MAPK pathways and downstream target genes, such as IL-6 and IL-8, could be examined in ASM cells. The increase in IL-17 level in the co-culture could be attributable to the actions of ASM cell-derived IL-6, which can activate IL-17 transcription through the STAT3 and NF-κB signaling pathways independent of RORγt [217, 218]. In addition, the expression of RORγt is much lower (C_q = ~29) compared to the other T cell subset master transcription regulators (C_q = 24 ~ 25), suggesting that very few cells of the Th17 subset are present. Thus, any changes to the transcript level of RORγt may also be minimal and only quantifiable by ELISA, which is much more sensitive to changes at the protein level.

Pro-inflammatory phenotype of co-cultured ASM cells

Given the Th1 bias of co-cultured CD4 T cells and the observation that the IFN-γ level was two orders of magnitude greater than IL-17 in the culture medium, we speculate that IFN-γ signaling plays the most prominent role in stimulating ASM cells in the current co-culture context. The pro-inflammatory profile of ASM cells was examined, specifically focusing on IFN-γ-inducible chemokines CXCL9, 10, 11 and adhesion molecule ICAM-1.

Co-culture induced the transcription and expression of CXCL9, 10, 11 and ICAM-1, as well as STAT1 phosphorylation in ASM cells. Sun et al. reported that activated CD4 T cells upregulate C-X-C Motif Chemokine Receptor 3 (CXCR3), the receptor for CXCL9, 10, 11, forming a homogeneous population, which provides a signaling axis through which the chemokines can act

[63]. We confirmed the role of T cell-derived IFN-γ in driving CXCR3 ligand and ICAM-1 expression by ASM cells. Neutralization of IFN-γ signaling via a blocking antibody against IFNGR effectively inhibited the upregulated CXCL9, 10, 11 and ICAM-1 expression in co-cultured ASM cells. Nevertheless, inhibition of IFN-γ signaling in ASM cells does not completely suppress the upregulation of these pro-inflammatory mediators, suggesting that their gene expression may also be regulated by other cytokines secreted by CD4 T cells and corresponding downstream signaling pathways, such as NF-κB activation via TNF signaling [219, 220].

It is hypothesized that CD4 T – ASM cell communication in the context of asthma evokes a positive feedback loop, through which the signaling of IFN-γ and CXCR3 ligands on ASM cells and CD4 T cells, respectively, reinforces each other's expression, thereby promoting T cell recruitment and aggravating associated pathological changes [63]. This pro-inflammatory mechanism may be induced or potentiated during respiratory conditions featuring Th1 immune responses, such as virally-induced asthma exacerbations. In addition, CXCL9, 10, 11 have the ability to induce Th1 polarization through the CXCR3 signaling axis [221, 222, 223], as well as the preferential recruitment of Th1 cells and blockage of Th2 migration by antagonizing the effects of eotaxins on CCR3 [224, 225, 226]. The relative contribution of CXCL9, 10 and 11 to T cell migration can be assessed by inhibiting each individually in co-culture, and their bioactivity can be compared via a Boyden chamber assay by quantifying the migration of CD4 T cells in response to different concentrations of each CXCR3 ligand. Aside from its involvement in leukocyte adhesion, studies have reported that ICAM-1 mediates the migration of Th1 cells *in vivo* and *in vitro* [227, 228]. LFA-1/ICAM-1 ligation promotes Th1 polarization by upregulating T-bet expression and IFN-γ production in CD4 T cells [229]. It would therefore be of interest to isolate and examine the CD4 T cells that are in direct contact with the ASM cells in co-culture and determine their subset

differentiation and cytokine profile. Taken together, activated CD4 T cells mediate the development and maintenance of a pro-inflammatory ASM phenotype characterized by upregulated expression of IFN-γ-inducible chemokines CXCL9, 10, 11 and adhesion molecule ICAM-1. These pro-inflammatory mediators serve to preferentially recruit Th1 cells and further induce Th1 polarization of CD4 T cells. Our findings suggest that IFN-γ and CXCR3 ligands play pivotal roles in the interaction between CD4 T cells and ASM cells. Whether IFN-γ and CXCR3 are involved in T cell homing to ASM *in vivo* remains to be confirmed. Inhibition of the IFN-γ and/or CXCR3 signaling axes in animal models of asthma could be explored for their ability to reduce T cell infiltration into the ASM layer and the associated airway remodeling features such as ASM cell proliferation.

BTLA–HVEM signaling in co-cultured ASM cells

BTLA is an immune checkpoint molecule that dampens B and T cell responses, specifically impairing their proliferation, survival, and ability to produce cytokines, such as IFN-γ and IL-10, when interacting with its partner HVEM [202]. In contrast to the classical notion of ligand-receptor pair, the BTLA-HVEM signaling axis is functionally bidirectional and activation of HVEM signaling induces the downstream pro-inflammatory NF-κB p65 pathway [177]. We hypothesized that BTLA signaling in CD4 T cells would limit IFN-γ production, while HVEM ligation on ASM cells would promote expression of pro-inflammatory molecules.

Therefore, we examined the role of BTLA-HVEM signaling in modulating the inflammatory profiles of CD4 T cells and ASM cells in co-culture. BTLA and HVEM gene expression were both upregulated by co-cultured CD4 T and ASM cells, respectively. Nevertheless, the transcriptional upregulation of BTLA did not translate to any changes at its protein level. This is potentially attributable to the progressive downregulation of BTLA expression on T cells upon activation [212].

Changes at the transcript level are likely difficult to detect given the minimal presence of surface BTLA on activated CD4 T cells. A model of BTLA overexpression on activated CD4 T cells could be utilized to explore the ability of BTLA to inhibit cytokine synthesis from co-cultured CD4 T cells in proximity to ASM cells.

On the other hand, HVEM protein expression was increased on the surface of ASM cells. The muscle cells appeared as a homogeneous population when subjected to flow cytometry analysis without any detectable separation in fluorescence intensity between cells expressing and not expressing HVEM. Therefore, we conclude that all ASM cells express and upregulate HVEM in response to co-culture with CD4 T cells. Disruption of the BTLA-HVEM signaling axis via the knockdown of HVEM expression in ASM cells did not induce any changes in the upregulated IFN-γ and IL-17 levels in co-culture, suggesting that BTLA has minimal immunosuppressive effects on co-cultured CD4 T cells and their cytokine profile. The upregulation of IFN-γ and IL-17 in co-culture that are independent of the BTLA-HVEM signaling pathway could be explained by the actions of ASM cell-derived IFN-γ and IL-6. These cytokines produced by ASM cells activate STAT1 and STAT3 signaling pathways in co-cultured CD4 T cells, which forms a positive feedback loop by further upregulating the expression of IFN-γ and IL-17 and in turn perpetuates the inflammatory ASM cell phenotype.

Regardless of the trends of BTLA expression, elevated HVEM expression on co-cultured ASM cells provided the basis for induced and upregulated HVEM signaling in ASM cells, which would be mediated by LIGHT from CD4 T cells and potentially promotes a pro-inflammatory ASM cell phenotype.

LIGHT-HVEM signaling synergizes with IFN-γ to augment expression of CXCR3 ligands and ICAM-1 in co-cultured ASM cells

LIGHT, whether in its membrane-bound or secreted form, is another molecule that interacts with HVEM. LIGHT has been extensively associated with the development of AHR and various features of airway remodeling, including subepithelial fibrosis, increased bronchial smooth muscle mass, as well as ASM contractility, hyperplasia, and hypertrophy [180, 196, 197]. In addition, LIGHT plays an augmentative role in human gingival fibroblasts by coordinately enhancing the production of IFN-γ-induced CXCL10 and 11 [230].

We examined the effects of LIGHT-HVEM signaling on the pro-inflammatory profile of ASM cells, as well as the interplay between the IFN-γ and LIGHT-HVEM signaling axes. Co-administration of recombinant human IFN-γ and LIGHT to ASM cells resulted in a synergistic upregulation of CXCL9, 10 and 11 to a greater extent than the effect of either molecule alone. LIGHT on its own did not induce any upregulation of the CXCR3 ligands, indicating that they are largely dependent on IFN-γ signaling. LIGHT, acting through surface HVEM on ASM cells, only serves to augment the expression of CXCL9, 10 and 11. ASM cells could also be treated with a set concentration of IFN-γ plus increasing concentrations of LIGHT to potentially establish a dose-response of IFN-γ-stimulated ASM cells in response to LIGHT.

To confirm that the augmentative role of LIGHT is mediated by HVEM signaling, we abolished this signaling axis in ASM cells. Based on our previous experience, CD4 T cells have significantly impaired viability following transduction, hence are not optimal targets for genetic manipulation. Therefore, we decided to knockdown HVEM expression in ASM cells via a lentivirus vector system, which contains a short hairpin RNA (shRNA) construct against HVEM. HVEM knockdown was confirmed at the transcript and protein level via RT-qPCR and flow cytometry, respectively. HVEM expression in co-cultured knockdown ASM cells was comparable to that of

baseline, non-co-cultured cells, indicating that the shRNA was effective at binding to and blocking the translation of most, if not all, transcripts of HVEM.

HVEM knockdown ASM cells were subsequently co-cultured with activated CD4 T cells. HVEM knockdown reduced the IFN-γ-induced upregulation of CXCL9, 11 and ICAM-1, as well as STAT1 activation in co-cultured ASM cells, suggesting that disruption of HVEM signaling impaired the STAT1 pathway. In other words, activation of the HVEM signaling pathway in ASM cells by LIGHT ligation increases their sensitivity to IFN-γ, thereby augmenting IFN-γ signaling, STAT1 phosphorylation and expression of CXCR3 ligands and ICAM-1. This observation led to the hypothesis that LIGHT-HVEM participates in and supports the positive feedback loop between IFN-γ and CXCR3 ligands in co-cultured CD4 T cells and ASM cells. Activation of IFN-γ and HVEM signaling in ASM cells in co-culture synergistically induces CXCR3 ligand expression and consequently the recruitment and migration of Th1 cells, which produce IFN-γ and LIGHT. Thus, co-cultured ASM cells are exposed to higher concentrations of IFN-γ and LIGHT, which serves to further upregulate HVEM signaling and potentiate the IFN-γ pathway and production of associated gene products, including CXCR3 ligands CXCL9, 10, 11 and the adhesion molecule ICAM-1. Multiple studies have reported that LIGHT stimulation induces the phosphorylation and subsequent degradation of IκBα, resulting in activation of the canonical NF-κB pathway [177, 178, 230, 231]. Furthermore, sc-514, an inhibitor of IκB kinase 2 and hence IκBα phosphorylation, effectively prevents the synergistic upregulation of CXCL9, 10 and 11 induced by IFN-γ and LIGHT co-treatment in human dermal fibroblasts [231]. The promoters of the CXCL9, 10, and 11 genes have all been shown to contain binding sites for STAT1 and NF-κB [232, 233, 234]. siRNA-mediated suppression of either pathway prevents the upregulation of CXCL10 synergistically induced by IFN-γ and IL-1β [233]. Therefore, the NF-κB pathway may act cooperatively with the

STAT1 pathway by binding to the promoters of CXCL9, 10, and 11 genes and synergistically activating their transcription in response to IFN-γ and LIGHT co-administration.

Such cooperation between members of the tumor necrosis factor (TNF) superfamily (TNFSF) and IFN-γ acting on ASM cells is not uncommon and has been reported by multiple studies. For example, TNF cooperates with IFN-γ to synergistically induce pro-inflammatory mediators such as CCL5/RANTES [235], CXCL10 [236], cyclooxygenase (COX)-2 [237], as well as toll-like receptors (TLR) 2 and 3 [238]. In the current CD4 T – ASM cell co-culture context, we established a functional cooperativity between IFN-γ and LIGHT-HVEM that promotes the expression of CXCR3 ligands and ICAM-1 in ASM cells (Figure 9).

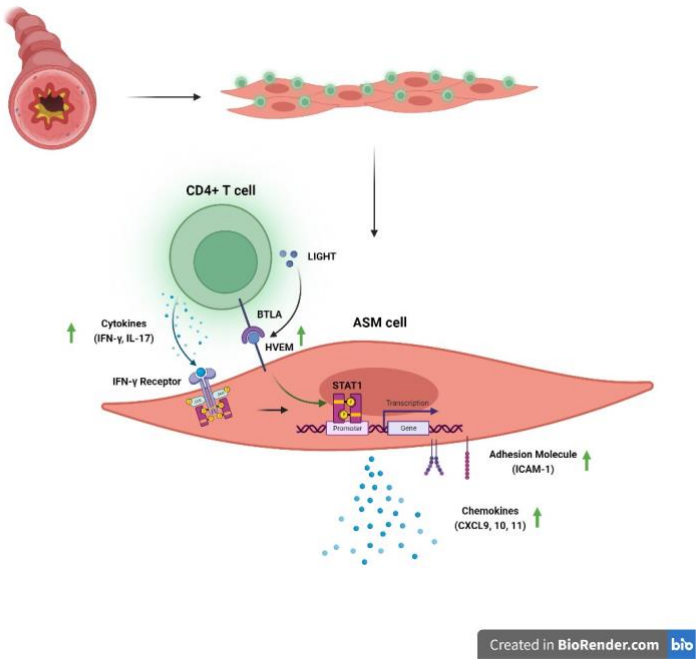

Figure 9. Proposed working model of synergism between IFN-γ/STAT1 and LIGHT-HVEM signaling pathways

Chapter 5. Conclusions and Future Directions

The co-culture between activated CD4 T cells and ASM cells led to respective changes in their pro-inflammatory profile. Activated CD4 T cells following co-culture showed a bias towards Th1 differentiation and increased secretion of IFN-γ, which serves to upregulate IFN-γ-inducible CXCR3 ligands CXCL9, 10 and 11, as well as adhesion molecule ICAM-1. The secreted chemokines signaling through CXCR3 and LFA-1/ICAM-1 ligation may in turn further recruitment and polarize CD4 T cells towards the Th1 subset, thereby creating a vicious cycle perpetuating the inflammatory microenvironment in the co-culture system. CD4 T cells in direct contact with ASM cells could be isolated and sampled for their subset markers and cytokine profile to confirm this hypothesis. Given our findings *in vitro*, it would also be of interest to sample CD4 T cells infiltrating ASM layers in animal models of asthma to determine if the same polarization and cytokine profile are present *in vivo*.

LIGHT-HVEM signaling increases the sensitivity of ASM cells to IFN-γ by augmenting activation of STAT1 pathway and expression of IFN-γ-induced CXCL9, 10, 11 and ICAM-1. The mechanism through which LIGHT-HVEM acts cooperatively and enhances IFN-γ/STAT1 signaling could be studied by determining the expression of IFNGR, JAK1/2 phosphorylation, negative feedback regulators of the JAK/STAT pathway, SOCS-1 and SOCS-3, as well as concurrent pro-inflammatory pathways in ASM cells, such as extracellular signal-regulated kinase (ERK) 1/2 and p38 MAPK. Based on studies done in other cell types, we also hypothesize that intracellular activation of the NF-kB pathway may be important for mediating the synergy between IFN-γ and LIGHT signaling. Suppression of said pathway via a small molecule inhibitor such as BAY11-7082 or NIK-SMI could be explored for its effects on IFN-γ and LIGHT synergy. On the other hand, lymphotoxin-beta receptor (LTβR) is another surface molecule expressed on ASM cells that

LIGHT can signal through [180]. LTβR signaling stimulates the non-canonical NF-κB pathway without inducing IκBα degradation, phosphorylates p65 at the Ser536 residue in the nucleus to mediate NF-κB-dependent gene activation [239]. A LTβR knockdown or knockout ASM cell model could be employed to determine whether LIGHT-LTβR signaling axis plays a role in mediating IFN-γ and LIGHT synergy and to compare the extent to which LIGHT-HVEM and LIGHT- LTβR each is responsible for the proinflammatory action of LIGHT.

Furthermore, type I and III interferons (IFNs) are also important inducers and mediators of Th1 type immunity. Binding of type I and III IFNs to the high affinity subunit of their cognate receptors, IFNAR2 and IFNLR1, respectively, recruits the low affinity receptor chains IFNAR1 and IL10Rb [240]. Receptor dimerization activates TYK2 and JAK1 kinases, which in turn phosphorylate STAT1 and STAT2 [240]. Phosphorylated STAT1 and STAT2 heterodimer ultimately associates with IRF9 to form the ternary complex ISGF3 [240]. Therefore, whether the synergism between IFN-γ and LIGHT-HVEM signaling may be generalized to all members of the IFN family is also of interest, given that type I and III IFN signaling also involves STAT1.

Other studies report that HVEM signaling leads to the downstream phosphorylation and activation of NF-κB. The effects of HVEM knockdown on the activation of the canonical NF-κB p65 pathway and expression of NF-κB associated genes, such as IL-6 and IL-8, could be examined in co-cultured ASM cells. However, elimination of HVEM, which only abolishes one of many intracellular NF-κB-activating pathways, may be insufficient to alter the overall upregulated NF-κB phosphorylation caused by signaling of other pro-inflammatory cytokines secreted by CD4 T cells.

The physiological relevance of the synergism between IFN-γ and LIGHT-HVEM signaling in the context of asthma may be explored by quantifying levels of LIGHT and HVEM in healthy and

asthmatic airways and examining correlations between LIGHT and HVEM expression and features of asthma, such as T cell infiltration, increase in ASM mass, as well as ASM hyperplasia and hypertrophy. HVEM-deficient animal models can be generated to study the effects of LIGHT-HVEM deficiency on CXCR3 receptor and ligand expression, T cell infiltration into the ASM and airway remodeling *in vivo*. Activated CD4 T cells could also be co-cultured with asthmatic ASM cells to determine if the cooperation between LIGHT-HVEM and IFN-γ signaling is applicable to asthmatics.

Another avenue of interest lies in examining the proliferation and contractility of ASM cells in response to co-culture, recombinant IFN-γ and LIGHT treatment, and HVEM knockdown. Abundant evidence supports the concept that the crosstalk between CD4 T cells and ASM cells mediates airway remodeling and increases airway smooth muscle mass, hence it is likely that co-cultured ASM cells will upregulate pro-proliferative transcription factors such as Elk1 and Klf4 and downregulate their contractile machinery, given the mutual antagonism between the proliferative and contractile phenotype of ASM cells. In addition, LIGHT is also involved in airway remodeling via the LIGHT-LTβR signaling axis *in vivo* [180, 196]. The mechanism through which LIGHT mediates ASM hyperplasia and hypertrophy *in vitro* has not yet been confirmed and LIGHT-HVEM may be a possibility.

We reported that BTLA-HVEM signaling did not affect the cytokine profile of CD4 T cells. The role of other immune checkpoints, such as PD-1/PD-L1 and CTLA-4, in this co-culture context could be explored to determine if they could be targeted to dampen the CD4 T cell response.

Lastly, HVEM expression on ASM cells provides the basis for mediated entry of herpesvirus HSV-1 and HSV-2 into ASM cells. It would be of interest to investigate the relative expression of HVEM on healthy versus asthmatic ASM cells to determine if asthma upregulates HVEM on airway

smooth muscle. The hypothesis is that HVEM expression allows latent HSV dormant in the nerves to infect ASM cells and possibly confers alterations in ASM properties that favour the expression of asthma, such as AHR. The association between HSV infection and features of asthma, such as airway remodeling and AHR, could be explored.

References

1. Mims, J.W. Asthma: definitions and pathophysiology. *Int Forum Allergy Rhinol* **5 Suppl 1**, S2-6 (2015).

2. Dharmage, S.C., Perret, J.L. & Custovic, A. Epidemiology of Asthma in Children and Adults. *Front Pediatr* **7**, 246 (2019).

3. McCracken, J.L., Veeranki, S.P., Ameredes, B.T. & Calhoun, W.J. Diagnosis and Management of Asthma in Adults: A Review. *JAMA* **318**, 279-290 (2017).

4. Quirt, J., Hildebrand, K.J., Mazza, J., Noya, F. & Kim, H. Asthma. *Allergy Asthma Clin Immunol* **14**, 50 (2018).

5. Fireman, P. Understanding asthma pathophysiology. *Allergy Asthma Proc* **24**, 79-83 (2003).

6. Holt, P.G. & Sly, P.D. Th2 cytokines in the asthma late-phase response. *Lancet* **370**, 1396-1398 (2007).

7. Lambrecht, B.N., Hammad, H. & Fahy, J.V. The Cytokines of Asthma. *Immunity* **50**, 975-991 (2019).

8. Liang, H.E. *et al.* Divergent expression patterns of IL-4 and IL-13 define unique functions in allergic immunity. *Nat Immunol* **13**, 58-66 (2011).

9. Sinyor, B. & Concepcion Perez, L. Pathophysiology Of Asthma. *StatPearls*: Treasure Island (FL), 2022.

10. Chapman, D.G. & Irvin, C.G. Mechanisms of airway hyper-responsiveness in asthma: the past, present and yet to come. *Clin Exp Allergy* **45**, 706-719 (2015).

11. Brutsche, M.H. *et al.* Bronchial hyperresponsiveness and the development of asthma and COPD in asymptomatic individuals: SAPALDIA cohort study. *Thorax* **61**, 671-677 (2006).

12. Sears, M.R. *et al.* A longitudinal, population-based, cohort study of childhood asthma followed to adulthood. *N Engl J Med* **349**, 1414-1422 (2003).

13. Sayeedi, I. & Widrich, J. Methacholine Challenge Test. *StatPearls*: Treasure Island (FL), 2022.

14. Lotvall, J., Inman, M. & O'Byrne, P. Measurement of airway hyperresponsiveness: new considerations. *Thorax* **53**, 419-424 (1998).

15. Coates, A.L. *et al.* ERS technical standard on bronchial challenge testing: general considerations and performance of methacholine challenge tests. *Eur Respir J* **49** (2017).

16. Hallstrand, T.S. *et al.* ERS technical standard on bronchial challenge testing: pathophysiology and methodology of indirect airway challenge testing. *Eur Respir J* **52** (2018).

17. Oostveen, E. *et al.* The forced oscillation technique in clinical practice: methodology, recommendations and future developments. *Eur Respir J* **22**, 1026-1041 (2003).

18. Cockcroft, D.W. & Davis, B.E. Mechanisms of airway hyperresponsiveness. *J Allergy Clin Immunol* **118**, 551-559; quiz 560-551 (2006).

19. Meurs, H., Gosens, R. & Zaagsma, J. Airway hyperresponsiveness in asthma: lessons from in vitro model systems and animal models. *Eur Respir J* **32**, 487-502 (2008).

20. Barbato, A. *et al.* Epithelial damage and angiogenesis in the airways of children with asthma. *Am J Respir Crit Care Med* **174**, 975-981 (2006).

21. Martinez, F.D. Asthma treatment and asthma prevention: a tale of 2 parallel pathways. *J Allergy Clin Immunol* **119**, 30-33 (2007).

22. Postma, D.S. & Kerstjens, H.A. Characteristics of airway hyperresponsiveness in asthma and chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* **158**, S187-192 (1998).

23. Cockcroft, D.W., Murdock, K.Y., Berscheid, B.A. & Gore, B.P. Sensitivity and specificity of histamine PC20 determination in a random selection of young college students. *J Allergy Clin Immunol* **89**, 23-30 (1992).

24. Hamelmann, E. *et al.* Anti-interleukin 5 but not anti-IgE prevents airway inflammation and airway hyperresponsiveness. *Am J Respir Crit Care Med* **160**, 934-941 (1999).

25. Gavett, S.H. *et al.* Interleukin-4 receptor blockade prevents airway responses induced by antigen challenge in mice. *Am J Physiol* **272**, L253-261 (1997).

26. Leigh, R., Ellis, R., Wattie, J., Donaldson, D.D. & Inman, M.D. Is interleukin-13 critical in maintaining airway hyperresponsiveness in allergen-challenged mice? *Am J Respir Crit Care Med* **170**, 851-856 (2004).

27. Porsbjerg, C.M. *et al.* Relationship between airway pathophysiology and airway inflammation in older asthmatics. *Respirology* **18**, 1128-1134 (2013).

28. Amrani, Y. *et al.* Bronchial hyperresponsiveness: insights into new signaling molecules. *Curr Opin Pharmacol* **4**, 230-234 (2004).

29. Barnes, P.J. Pharmacology of airway smooth muscle. *Am J Respir Crit Care Med* **158**, S123-132 (1998).

30. James, A.L., Maxwell, P.S., Pearce-Pinto, G., Elliot, J.G. & Carroll, N.G. The relationship of reticular basement membrane thickness to airway wall remodeling in asthma. *Am J Respir Crit Care Med* **166**, 1590-1595 (2002).

31. Carroll, N., Elliot, J., Morton, A. & James, A. The structure of large and small airways in nonfatal and fatal asthma. *Am Rev Respir Dis* **147**, 405-410 (1993).

32. Boulet, L.P. *et al.* Bronchial subepithelial fibrosis correlates with airway responsiveness to methacholine. *Chest* **112**, 45-52 (1997).

33. Jeffery, P.K. Remodeling in asthma and chronic obstructive lung disease. *Am J Respir Crit Care Med* **164**, S28-38 (2001).

34. Li, X. & Wilson, J.W. Increased vascularity of the bronchial mucosa in mild asthma. *Am J Respir Crit Care Med* **156**, 229-233 (1997).

35. Benayoun, L., Druilhe, A., Dombret, M.C., Aubier, M. & Pretolani, M. Airway structural alterations selectively associated with severe asthma. *Am J Respir Crit Care Med* **167**, 1360-1368 (2003).

36. Woodruff, P.G. *et al.* Hyperplasia of smooth muscle in mild to moderate asthma without changes in cell size or gene expression. *Am J Respir Crit Care Med* **169**, 1001-1006 (2004).

37. Bergeron, C., Tulic, M.K. & Hamid, Q. Airway remodelling in asthma: from benchside to clinical practice. *Can Respir J* **17**, e85-93 (2010).

38. Laitinen, L.A., Heino, M., Laitinen, A., Kava, T. & Haahtela, T. Damage of the airway epithelium and bronchial reactivity in patients with asthma. *Am Rev Respir Dis* **131**, 599-606 (1985).

39. Montefort, S., Roberts, J.A., Beasley, R., Holgate, S.T. & Roche, W.R. The site of disruption of the bronchial epithelium in asthmatic and non-asthmatic subjects. *Thorax* **47**, 499-503 (1992).

40. Hamilton, L.M. *et al.* The bronchial epithelium in asthma--much more than a passive barrier. *Monaldi Arch Chest Dis* **56**, 48-54 (2001).

41. Holgate, S.T. *et al.* Mechanisms of airway epithelial damage: epithelial-mesenchymal interactions in the pathogenesis of asthma. *Eur Respir J Suppl* **44**, 24s-29s (2003).

42. Bergeron, C. *et al.* Regulation of procollagen I (alpha1) by interleukin-4 in human bronchial fibroblasts: a possible role in airway remodelling in asthma. *Clin Exp Allergy* **33**, 1389-1397 (2003).

43. Lemjabbar, H. *et al.* Contribution of 92 kDa gelatinase/type IV collagenase in bronchial inflammation during status asthmaticus. *Am J Respir Crit Care Med* **159**, 1298-1307 (1999).

44. Prikk, K. *et al.* Airway obstruction correlates with collagenase-2 (MMP-8) expression and activation in bronchial asthma. *Lab Invest* **82**, 1535-1545 (2002).

45. Wenzel, S.E., Balzar, S., Cundall, M. & Chu, H.W. Subepithelial basement membrane immunoreactivity for matrix metalloproteinase 9: association with asthma severity, neutrophilic inflammation, and wound repair. *J Allergy Clin Immunol* **111**, 1345-1352 (2003).

46. Jenkins, H.A. *et al.* Histopathology of severe childhood asthma: a case series. *Chest* **124**, 32-41 (2003).

47. Ordonez, C.L. *et al.* Mild and moderate asthma is associated with airway goblet cell hyperplasia and abnormalities in mucin gene expression. *Am J Respir Crit Care Med* **163**, 517-523 (2001).

48. Hoshino, M., Takahashi, M. & Aoike, N. Expression of vascular endothelial growth factor, basic fibroblast growth factor, and angiogenin immunoreactivity in asthmatic airways and its relationship to angiogenesis. *J Allergy Clin Immunol* **107**, 295-301 (2001).

49. James, A.L. *et al.* Airway smooth muscle hypertrophy and hyperplasia in asthma. *Am J Respir Crit Care Med* **185**, 1058-1064 (2012).

50. Munakata, M. Airway remodeling and airway smooth muscle in asthma. *Allergol Int* **55**, 235-243 (2006).

51. Lambert, R.K., Wiggs, B.R., Kuwano, K., Hogg, J.C. & Pare, P.D. Functional significance of increased airway smooth muscle in asthma and COPD. *J Appl Physiol (1985)* **74**, 2771-2781 (1993).

52. Damera, G., Tliba, O. & Panettieri, R.A., Jr. Airway smooth muscle as an immunomodulatory cell. *Pulm Pharmacol Ther* **22**, 353-359 (2009).

53. Panettieri, R.A., Jr. Airway smooth muscle: an immunomodulatory cell. *J Allergy Clin Immunol* **110**, S269-274 (2002).

54. Johnson, P.R. Role of human airway smooth muscle in altered extracellular matrix production in asthma. *Clin Exp Pharmacol Physiol* **28**, 233-236 (2001).

55. Joubert, P. *et al.* CCR3 expression and function in asthmatic airway smooth muscle cells. *J Immunol* **175**, 2702-2708 (2005).

56. Chamley-Campbell, J., Campbell, G.R. & Ross, R. The smooth muscle cell in culture. *Physiol Rev* **59**, 1-61 (1979).

57. Beamish, J.A., He, P., Kottke-Marchant, K. & Marchant, R.E. Molecular regulation of contractile smooth muscle cell phenotype: implications for vascular tissue engineering. *Tissue Eng Part B Rev* **16**, 467-491 (2010).

58. Wang, Z., Wang, D.Z., Pipes, G.C. & Olson, E.N. Myocardin is a master regulator of smooth muscle gene expression. *Proc Natl Acad Sci U S A* **100**, 7129-7134 (2003).

59. Wang, Z. *et al.* Myocardin and ternary complex factors compete for SRF to control smooth muscle gene expression. *Nature* **428**, 185-189 (2004).

60. Halayko, A.J., Salari, H., Ma, X. & Stephens, N.L. Markers of airway smooth muscle cell phenotype. *Am J Physiol* **270**, L1040-1051 (1996).

61. Amrani, Y., Lazaar, A.L. & Panettieri, R.A., Jr. Up-regulation of ICAM-1 by cytokines in human tracheal smooth muscle cells involves an NF-kappa B-dependent signaling pathway that is only partially sensitive to dexamethasone. *J Immunol* **163**, 2128-2134 (1999).

62. Hakonarson, H., Maskeri, N., Carter, C. & Grunstein, M.M. Regulation of TH1- and TH2-type cytokine expression and action in atopic asthmatic sensitized airway smooth muscle. *J Clin Invest* **103**, 1077-1087 (1999).

63. Sun, R., Jang, J.H., Lauzon, A.M. & Martin, J.G. Interferon-gamma amplifies airway smooth muscle-mediated CD4+ T cell recruitment by promoting the secretion of C-X-C-motif chemokine receptor 3 ligands. *FASEB J* **35**, e21228 (2021).

64. Ramos-Barbon, D. *et al.* T Cells localize with proliferating smooth muscle alpha-actin+ cell compartments in asthma. *Am J Respir Crit Care Med* **182**, 317-324 (2010).

65. Ramos-Barbon, D., Presley, J.F., Hamid, Q.A., Fixman, E.D. & Martin, J.G. Antigen-specific CD4+ T cells drive airway smooth muscle remodeling in experimental asthma. *J Clin Invest* **115**, 1580-1589 (2005).

66. Al Heialy, S. *et al.* Nanotubes connect CD4+ T cells to airway smooth muscle cells: novel mechanism of T cell survival. *J Immunol* **194**, 5626-5634 (2015).

67. Farahnak, S. *et al.* Basic Fibroblast Growth Factor 2 Is a Determinant of CD4 T Cell-Airway Smooth Muscle Cell Communication through Membrane Conduits. *Journal of immunology (Baltimore, Md. : 1950)* **199**, 3086-3093 (2017).

68. Luckheeram, R.V., Zhou, R., Verma, A.D. & Xia, B. CD4(+)T cells: differentiation and functions. *Clin Dev Immunol* **2012**, 925135 (2012).

69. Dempsey, P.W., Vaidya, S.A. & Cheng, G. The art of war: Innate and adaptive immune responses. *Cell Mol Life Sci* **60**, 2604-2621 (2003).

70. Bonilla, F.A. & Oettgen, H.C. Adaptive immunity. *J Allergy Clin Immunol* **125**, S33-40 (2010).

71. Lieberman, J. The ABCs of granule-mediated cytotoxicity: new weapons in the arsenal. *Nat Rev Immunol* **3**, 361-370 (2003).

72. Lloyd, C.M. & Hessel, E.M. Functions of T cells in asthma: more than just T(H)2 cells. *Nat Rev Immunol* **10**, 838-848 (2010).

73. Bretscher, P.A. A two-step, two-signal model for the primary activation of precursor helper T cells. *Proc Natl Acad Sci U S A* **96**, 185-190 (1999).

74. Tao, X., Constant, S., Jorritsma, P. & Bottomly, K. Strength of TCR signal determines the costimulatory requirements for Th1 and Th2 CD4+ T cell differentiation. *J Immunol* **159**, 5956-5963 (1997).

75. Li, Y. & Kurlander, R.J. Comparison of anti-CD3 and anti-CD28-coated beads with soluble anti-CD3 for expanding human T cells: differing impact on CD8 T cell phenotype and responsiveness to restimulation. *J Transl Med* **8**, 104 (2010).

76. Levine, B.L. *et al.* Effects of CD28 costimulation on long-term proliferation of CD4+ T cells in the absence of exogenous feeder cells. *J Immunol* **159**, 5921-5930 (1997).

77. Verweij, C.L., Geerts, M. & Aarden, L.A. Activation of interleukin-2 gene transcription via the T-cell surface molecule CD28 is mediated through an NF-kB-like response element. *J Biol Chem* **266**, 14179-14182 (1991).

78. Rapoport, A.P. *et al.* Restoration of immunity in lymphopenic individuals with cancer by vaccination and adoptive T-cell transfer. *Nat Med* **11**, 1230-1237 (2005).

79. Laport, G.G. *et al.* Adoptive transfer of costimulated T cells induces lymphocytosis in patients with relapsed/refractory non-Hodgkin lymphoma following CD34+-selected hematopoietic cell transplantation. *Blood* **102**, 2004-2013 (2003).

80. Bhattacharyya, N.D. & Feng, C.G. Regulation of T Helper Cell Fate by TCR Signal Strength. *Front Immunol* **11**, 624 (2020).

81. Dong, C. & Flavell, R.A. Cell fate decision: T-helper 1 and 2 subsets in immune responses. *Arthritis Res* **2**, 179-188 (2000).

82. Trinchieri, G., Pflanz, S. & Kastelein, R.A. The IL-12 family of heterodimeric cytokines: new players in the regulation of T cell responses. *Immunity* **19**, 641-644 (2003).

83. Iwasaki, A. & Medzhitov, R. Toll-like receptor control of the adaptive immune responses. *Nat Immunol* **5**, 987-995 (2004).

84. Trinchieri, G. & Sher, A. Cooperation of Toll-like receptor signals in innate immune defence. *Nat Rev Immunol* **7**, 179-190 (2007).

85. Lazarevic, V. *et al.* T-bet represses T(H)17 differentiation by preventing Runx1-mediated activation of the gene encoding RORgammat. *Nat Immunol* **12**, 96-104 (2011).

86. Thierfelder, W.E. *et al.* Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. *Nature* **382**, 171-174 (1996).

87. Djuretic, I.M. *et al.* Transcription factors T-bet and Runx3 cooperate to activate Ifng and silence Il4 in T helper type 1 cells. *Nat Immunol* **8**, 145-153 (2007).

88. Hwang, E.S., Szabo, S.J., Schwartzberg, P.L. & Glimcher, L.H. T helper cell fate specified by kinase-mediated interaction of T-bet with GATA-3. *Science* **307**, 430-433 (2005).

89. Grewal, I.S. & Flavell, R.A. CD40 and CD154 in cell-mediated immunity. *Annu Rev Immunol* **16**, 111-135 (1998).

90. Bachmann, M.F. *et al.* TRANCE, a tumor necrosis factor family member critical for CD40 ligand-independent T helper cell activation. *J Exp Med* **189**, 1025-1031 (1999).

91. Kopf, M. *et al.* OX40-deficient mice are defective in Th cell proliferation but are competent in generating B cell and CTL Responses after virus infection. *Immunity* **11**, 699-708 (1999).

92. Chen, A.I. *et al.* Ox40-ligand has a critical costimulatory role in dendritic cell:T cell interactions. *Immunity* **11**, 689-698 (1999).

93. de Weerd, N.A., Samarajiwa, S.A. & Hertzog, P.J. Type I interferon receptors: biochemistry and biological functions. *J Biol Chem* **282**, 20053-20057 (2007).

94. Kotenko, S.V. & Durbin, J.E. Contribution of type III interferons to antiviral immunity: location, location, location. *J Biol Chem* **292**, 7295-7303 (2017).

95. Bach, E.A., Aguet, M. & Schreiber, R.D. The IFN gamma receptor: a paradigm for cytokine receptor signaling. *Annu Rev Immunol* **15**, 563-591 (1997).

96. Schroder, K., Hertzog, P.J., Ravasi, T. & Hume, D.A. Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol* **75**, 163-189 (2004).

97. Salazar-Mather, T.P., Hamilton, T.A. & Biron, C.A. A chemokine-to-cytokine-to-chemokine cascade critical in antiviral defense. *J Clin Invest* **105**, 985-993 (2000).

98. Pien, G.C., Satoskar, A.R., Takeda, K., Akira, S. & Biron, C.A. Cutting edge: selective IL-18 requirements for induction of compartmental IFN-gamma responses during viral infection. *J Immunol* **165**, 4787-4791 (2000).

99. Schindler, H., Lutz, M.B., Rollinghoff, M. & Bogdan, C. The production of IFN-gamma by IL-12/IL-18-activated macrophages requires STAT4 signaling and is inhibited by IL-4. *J Immunol* **166**, 3075-3082 (2001).

100. Briscoe, J. *et al.* Kinase-negative mutants of JAK1 can sustain interferon-gamma-inducible gene expression but not an antiviral state. *EMBO J* **15**, 799-809 (1996).

101. Igarashi, K. *et al.* Interferon-gamma induces tyrosine phosphorylation of interferon-gamma receptor and regulated association of protein tyrosine kinases, Jak1 and Jak2, with its receptor. *J Biol Chem* **269**, 14333-14336 (1994).

102. Greenlund, A.C. *et al.* Stat recruitment by tyrosine-phosphorylated cytokine receptors: an ordered reversible affinity-driven process. *Immunity* **2**, 677-687 (1995).

103. Goh, K.C., Haque, S.J. & Williams, B.R. p38 MAP kinase is required for STAT1 serine phosphorylation and transcriptional activation induced by interferons. *EMBO J* **18**, 5601-5608 (1999).

104. Wen, Z., Zhong, Z. & Darnell, J.E., Jr. Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation. *Cell* **82**, 241-250 (1995).

105. Darnell, J.E., Jr., Kerr, I.M. & Stark, G.R. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science* **264**, 1415-1421 (1994).

106. Schindler, C. & Darnell, J.E., Jr. Transcriptional responses to polypeptide ligands: the JAK-STAT pathway. *Annu Rev Biochem* **64**, 621-651 (1995).

107. Zhou, Z.H. *et al.* IFN-gamma induction of the human monocyte chemoattractant protein (hMCP)-1 gene in astrocytoma cells: functional interaction between an IFN-gamma-activated site and a GC-rich element. *J Immunol* **160**, 3908-3916 (1998).

108. Liu, J., Guan, X. & Ma, X. Interferon regulatory factor 1 is an essential and direct transcriptional activator for interferon gamma-induced RANTES/CCl5 expression in macrophages. *J Biol Chem* **280**, 24347-24355 (2005).

109. Lin, A.A., Tripathi, P.K., Sholl, A., Jordan, M.B. & Hildeman, D.A. Gamma interferon signaling in macrophage lineage cells regulates central nervous system inflammation and chemokine production. *J Virol* **83**, 8604-8615 (2009).

110. Conrady, C.D., Zheng, M., Mandal, N.A., van Rooijen, N. & Carr, D.J. IFN-alpha-driven CCL2 production recruits inflammatory monocytes to infection site in mice. *Mucosal Immunol* **6**, 45-55 (2013).

111. Kim, M.O., Suh, H.S., Brosnan, C.F. & Lee, S.C. Regulation of RANTES/CCL5 expression in human astrocytes by interleukin-1 and interferon-beta. *J Neurochem* **90**, 297-308 (2004).

112. Hiroi, M. & Ohmori, Y. The transcriptional coactivator CREB-binding protein cooperates with STAT1 and NF-kappa B for synergistic transcriptional activation of the CXC ligand 9/monokine induced by interferon-gamma gene. *J Biol Chem* **278**, 651-660 (2003).

113. Ohmori, Y. & Hamilton, T.A. The interferon-stimulated response element and a kappa B site mediate synergistic induction of murine IP-10 gene transcription by IFN-gamma and TNF-alpha. *J Immunol* **154**, 5235-5244 (1995).

114. Majumder, S. *et al.* p48/STAT-1alpha-containing complexes play a predominant role in induction of IFN-gamma-inducible protein, 10 kDa (IP-10) by IFN-gamma alone or in synergy with TNF-alpha. *J Immunol* **161**, 4736-4744 (1998).

115. Cole, K.E. *et al.* Interferon-inducible T cell alpha chemoattractant (I-TAC): a novel non-ELR CXC chemokine with potent activity on activated T cells through selective high affinity binding to CXCR3. *J Exp Med* **187**, 2009-2021 (1998).

116. Jaruga, B., Hong, F., Kim, W.H. & Gao, B. IFN-gamma/STAT1 acts as a proinflammatory signal in T cell-mediated hepatitis via induction of multiple chemokines and adhesion molecules: a critical role of IRF-1. *Am J Physiol Gastrointest Liver Physiol* **287**, G1044-1052 (2004).

117. Ohmori, Y., Schreiber, R.D. & Hamilton, T.A. Synergy between interferon-gamma and tumor necrosis factor-alpha in transcriptional activation is mediated by cooperation between signal transducer and activator of transcription 1 and nuclear factor kappaB. *J Biol Chem* **272**, 14899-14907 (1997).

118. Jia, T., Leiner, I., Dorothee, G., Brandl, K. & Pamer, E.G. MyD88 and Type I interferon receptor-mediated chemokine induction and monocyte recruitment during Listeria monocytogenes infection. *J Immunol* **183**, 1271-1278 (2009).

119. Sumagin, R., Robin, A.Z., Nusrat, A. & Parkos, C.A. Transmigrated neutrophils in the intestinal lumen engage ICAM-1 to regulate the epithelial barrier and neutrophil recruitment. *Mucosal Immunol* **7**, 905-915 (2014).

120. Yockell-Lelievre, J. *et al.* Functional cooperation between Stat-1 and ets-1 to optimize icam-1 gene transcription. *Biochem Cell Biol* **87**, 905-918 (2009).

121. Krug, N. *et al.* T-cell cytokine profile evaluated at the single cell level in BAL and blood in allergic asthma. *Am J Respir Cell Mol Biol* **14**, 319-326 (1996).

122. Corrigan, C.J., Hartnell, A. & Kay, A.B. T lymphocyte activation in acute severe asthma. *Lancet* **1**, 1129-1132 (1988).

123. Gajewski, T.F. & Fitch, F.W. Anti-proliferative effect of IFN-gamma in immune regulation. I. IFN-gamma inhibits the proliferation of Th2 but not Th1 murine helper T lymphocyte clones. *J Immunol* **140**, 4245-4252 (1988).

124. Huang, T.J. *et al.* Allergen-specific Th1 cells counteract efferent Th2 cell-dependent bronchial hyperresponsiveness and eosinophilic inflammation partly via IFN-gamma. *J Immunol* **166**, 207-217 (2001).

125. Cohn, L., Homer, R.J., Niu, N. & Bottomly, K. T helper 1 cells and interferon gamma regulate allergic airway inflammation and mucus production. *J Exp Med* **190**, 1309-1318 (1999).

126. Iwamoto, I., Nakajima, H., Endo, H. & Yoshida, S. Interferon gamma regulates antigen-induced eosinophil recruitment into the mouse airways by inhibiting the infiltration of CD4+ T cells. *J Exp Med* **177**, 573-576 (1993).

127. Han, M. *et al.* IFN-gamma Blocks Development of an Asthma Phenotype in Rhinovirus-Infected Baby Mice by Inhibiting Type 2 Innate Lymphoid Cells. *Am J Respir Cell Mol Biol* **56**, 242-251 (2017).

128. Kanda, A. *et al.* Eosinophil-derived IFN-gamma induces airway hyperresponsiveness and lung inflammation in the absence of lymphocytes. *J Allergy Clin Immunol* **124**, 573-582, 582 e571-579 (2009).

129. Valerius, T., Repp, R., Kalden, J.R. & Platzer, E. Effects of IFN on human eosinophils in comparison with other cytokines. A novel class of eosinophil activators with delayed onset of action. *J Immunol* **145**, 2950-2958 (1990).

130. Pennica, D. *et al.* Human tumour necrosis factor: precursor structure, expression and homology to lymphotoxin. *Nature* **312**, 724-729 (1984).

131. Gour, N. & Wills-Karp, M. IL-4 and IL-13 signaling in allergic airway disease. *Cytokine* **75**, 68-78 (2015).

132. Brusselle, G., Kips, J., Joos, G., Bluethmann, H. & Pauwels, R. Allergen-induced airway inflammation and bronchial responsiveness in wild-type and interleukin-4-deficient mice. *Am J Respir Cell Mol Biol* **12**, 254-259 (1995).

133. Corry, D.B. *et al.* Interleukin 4, but not interleukin 5 or eosinophils, is required in a murine model of acute airway hyperreactivity. *J Exp Med* **183**, 109-117 (1996).

134. Henderson, W.R., Jr., Chi, E.Y. & Maliszewski, C.R. Soluble IL-4 receptor inhibits airway inflammation following allergen challenge in a mouse model of asthma. *J Immunol* **164**, 1086-1095 (2000).

135. Grunig, G. *et al.* Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* **282**, 2261-2263 (1998).

136. Zhu, Z. *et al.* Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *J Clin Invest* **103**, 779-788 (1999).

137. Fichtner-Feigl, S., Strober, W., Kawakami, K., Puri, R.K. & Kitani, A. IL-13 signaling through the IL-13alpha2 receptor is involved in induction of TGF-beta1 production and fibrosis. *Nat Med* **12**, 99-106 (2006).

138. Kuperman, D.A. *et al.* Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. *Nat Med* **8**, 885-889 (2002).

139. Kuperman, D.A. & Schleimer, R.P. Interleukin-4, interleukin-13, signal transducer and activator of transcription factor 6, and allergic asthma. *Curr Mol Med* **8**, 384-392 (2008).

140. Siddiqui, S. *et al.* Airway wall expression of OX40/OX40L and interleukin-4 in asthma. *Chest* **137**, 797-804 (2010).

141. Steinke, J.W. & Borish, L. Th2 cytokines and asthma. Interleukin-4: its role in the pathogenesis of asthma, and targeting it for asthma treatment with interleukin-4 receptor antagonists. *Respir Res* **2**, 66-70 (2001).

142. Lebman, D.A. & Coffman, R.L. Interleukin 4 causes isotype switching to IgE in T cell-stimulated clonal B cell cultures. *J Exp Med* **168**, 853-862 (1988).

143. Junttila, I.S. Tuning the Cytokine Responses: An Update on Interleukin (IL)-4 and IL-13 Receptor Complexes. *Front Immunol* **9**, 888 (2018).

144. Dubois, G.R., Schweizer, R.C., Versluis, C., Bruijnzeel-Koomen, C.A. & Bruijnzeel, P.L. Human eosinophils constitutively express a functional interleukin-4 receptor: interleukin-4 -induced priming of chemotactic responses and induction of PI-3 kinase activity. *Am J Respir Cell Mol Biol* **19**, 691-699 (1998).

145. Dubois, G.R., Bruijnzeel-Koomen, C.A. & Bruijnzeel, P.L. IL-4 induces chemotaxis of blood eosinophils from atopic dermatitis patients, but not from normal individuals. *J Invest Dermatol* **102**, 843-846 (1994).

146. Schleimer, R.P. *et al.* IL-4 induces adherence of human eosinophils and basophils but not neutrophils to endothelium. Association with expression of VCAM-1. *J Immunol* **148**, 1086-1092 (1992).

147. Bochner, B.S., Klunk, D.A., Sterbinsky, S.A., Coffman, R.L. & Schleimer, R.P. IL-13 selectively induces vascular cell adhesion molecule-1 expression in human endothelial cells. *J Immunol* **154**, 799-803 (1995).

148. Li, L. *et al.* Effects of Th2 cytokines on chemokine expression in the lung: IL-13 potently induces eotaxin expression by airway epithelial cells. *J Immunol* **162**, 2477-2487 (1999).

149. Martinez-Moczygemba, M. & Huston, D.P. Biology of common beta receptor-signaling cytokines: IL-3, IL-5, and GM-CSF. *J Allergy Clin Immunol* **112**, 653-665; quiz 666 (2003).

150. McBrien, C.N. & Menzies-Gow, A. The Biology of Eosinophils and Their Role in Asthma. *Front Med (Lausanne)* **4**, 93 (2017).

151. Shi, H. *et al.* Infiltration of eosinophils into the asthmatic airways caused by interleukin 5. *Am J Respir Cell Mol Biol* **16**, 220-224 (1997).

152. Chen, W. *et al.* Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* **198**, 1875-1886 (2003).

153. Fiorentino, D.F. *et al.* IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *J Immunol* **146**, 3444-3451 (1991).

154. Bogdan, C., Vodovotz, Y. & Nathan, C. Macrophage deactivation by interleukin 10. *J Exp Med* **174**, 1549-1555 (1991).

155. Fiorentino, D.F., Zlotnik, A., Mosmann, T.R., Howard, M. & O'Garra, A. IL-10 inhibits cytokine production by activated macrophages. *J Immunol* **147**, 3815-3822 (1991).

156. Ouyang, W., Rutz, S., Crellin, N.K., Valdez, P.A. & Hymowitz, S.G. Regulation and functions of the IL-10 family of cytokines in inflammation and disease. *Annu Rev Immunol* **29**, 71-109 (2011).

157. Zhou, L. *et al.* TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function. *Nature* **453**, 236-240 (2008).

158. Yang, X.O. *et al.* STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. *J Biol Chem* **282**, 9358-9363 (2007).

159. Kimura, A., Naka, T., Nohara, K., Fujii-Kuriyama, Y. & Kishimoto, T. Aryl hydrocarbon receptor regulates Stat1 activation and participates in the development of Th17 cells. *Proc Natl Acad Sci U S A* **105**, 9721-9726 (2008).

160. Moseley, T.A., Haudenschild, D.R., Rose, L. & Reddi, A.H. Interleukin-17 family and IL-17 receptors. *Cytokine Growth Factor Rev* **14**, 155-174 (2003).

161. Korn, T. *et al.* IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. *Nature* **448**, 484-487 (2007).

162. Leonard, W.J. & Spolski, R. Interleukin-21: a modulator of lymphoid proliferation, apoptosis and differentiation. *Nat Rev Immunol* **5**, 688-698 (2005).

163. Aujla, S.J. *et al.* IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia. *Nat Med* **14**, 275-281 (2008).

164. Zenewicz, L.A. *et al.* Interleukin-22 but not interleukin-17 provides protection to hepatocytes during acute liver inflammation. *Immunity* **27**, 647-659 (2007).

165. Steinberg, M.W., Shui, J.W., Ware, C.F. & Kronenberg, M. Regulating the mucosal immune system: the contrasting roles of LIGHT, HVEM, and their various partners. *Semin Immunopathol* **31**, 207-221 (2009).

166. Ware, C.F., Croft, M. & Neil, G.A. Realigning the LIGHT signaling network to control dysregulated inflammation. *J Exp Med* **219** (2022).

167. Ward-Kavanagh, L.K., Lin, W.W., Sedy, J.R. & Ware, C.F. The TNF Receptor Superfamily in Co-stimulating and Co-inhibitory Responses. *Immunity* **44**, 1005-1019 (2016).

168. Schneider, K., Potter, K.G. & Ware, C.F. Lymphotoxin and LIGHT signaling pathways and target genes. *Immunol Rev* **202**, 49-66 (2004).

169. Mauri, D.N. *et al.* LIGHT, a new member of the TNF superfamily, and lymphotoxin alpha are ligands for herpesvirus entry mediator. *Immunity* **8**, 21-30 (1998).

170. Aschner, C.B. & Herold, B.C. Alphaherpesvirus Vaccines. *Curr Issues Mol Biol* **41**, 469-508 (2021).

171. Locksley, R.M., Killeen, N. & Lenardo, M.J. The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell* **104**, 487-501 (2001).

172. Granger, S.W., Butrovich, K.D., Houshmand, P., Edwards, W.R. & Ware, C.F. Genomic characterization of LIGHT reveals linkage to an immune response locus on chromosome 19p13.3 and distinct isoforms generated by alternate splicing or proteolysis. *J Immunol* **167**, 5122-5128 (2001).

173. Heng, T.S., Painter, M.W. & Immunological Genome Project, C. The Immunological Genome Project: networks of gene expression in immune cells. *Nat Immunol* **9**, 1091-1094 (2008).

174. Shaikh, R.B. *et al.* Constitutive expression of LIGHT on T cells leads to lymphocyte activation, inflammation, and tissue destruction. *J Immunol* **167**, 6330-6337 (2001).

175. Castellano, R. *et al.* Mechanisms regulating expression of the tumor necrosis factor-related light gene. Role of calcium-signaling pathway in the transcriptional control. *J Biol Chem* **277**, 42841-42851 (2002).

176. Hsu, H. *et al.* ATAR, a novel tumor necrosis factor receptor family member, signals through TRAF2 and TRAF5. *J Biol Chem* **272**, 13471-13474 (1997).

177. Marsters, S.A. *et al.* Herpesvirus entry mediator, a member of the tumor necrosis factor receptor (TNFR) family, interacts with members of the TNFR-associated factor family

and activates the transcription factors NF-kappaB and AP-1. *J Biol Chem* **272**, 14029-14032 (1997).

178. Kim, W.J. *et al.* LIGHT is involved in the pathogenesis of rheumatoid arthritis by inducing the expression of pro-inflammatory cytokines and MMP-9 in macrophages. *Immunology* **114**, 272-279 (2005).

179. Rooney, I.A. *et al.* The lymphotoxin-beta receptor is necessary and sufficient for LIGHT-mediated apoptosis of tumor cells. *J Biol Chem* **275**, 14307-14315 (2000).

180. Miki, H. *et al.* Lymphotoxin beta receptor signaling directly controls airway smooth muscle deregulation and asthmatic lung dysfunction. *J Allergy Clin Immunol* (2022).

181. Burn Aschner, C. *et al.* HVEM signaling promotes protective antibody-dependent cellular cytotoxicity (ADCC) vaccine responses to herpes simplex viruses. *Sci Immunol* **5** (2020).

182. Park, S.J. *et al.* Herpesvirus Entry Mediator Binding Partners Mediate Immunopathogenesis of Ocular Herpes Simplex Virus 1 Infection. *mBio* **11** (2020).

183. Wang, J. *et al.* The regulation of T cell homeostasis and autoimmunity by T cell-derived LIGHT. *J Clin Invest* **108**, 1771-1780 (2001).

184. Probert, L. *et al.* Wasting, ischemia, and lymphoid abnormalities in mice expressing T cell-targeted human tumor necrosis factor transgenes. *J Immunol* **151**, 1894-1906 (1993).

185. Cohavy, O., Zhou, J., Ware, C.F. & Targan, S.R. LIGHT is constitutively expressed on T and NK cells in the human gut and can be induced by CD2-mediated signaling. *J Immunol* **174**, 646-653 (2005).

186. Wang, J. *et al.* The critical role of LIGHT in promoting intestinal inflammation and Crohn's disease. *J Immunol* **174**, 8173-8182 (2005).

187. Zhang, Z., Sferra, T.J. & Eroglu, Y. T cell co-stimulatory molecules: a co-conspirator in the pathogenesis of eosinophilic esophagitis? *Dig Dis Sci* **58**, 1497-1506 (2013).

188. Manresa, M.C. *et al.* Increased Production of LIGHT by T Cells in Eosinophilic Esophagitis Promotes Differentiation of Esophageal Fibroblasts Toward an Inflammatory Phenotype. *Gastroenterology* **159**, 1778-1792 e1713 (2020).

189. Perlin, D.S. *et al.* Levels of the TNF-Related Cytokine LIGHT Increase in Hospitalized COVID-19 Patients with Cytokine Release Syndrome and ARDS. *mSphere* **5** (2020).

190. Qu, H.Q. *et al.* Circulating LIGHT (TNFSF14) and Interleukin-18 Levels in Sepsis-Induced Multi-Organ Injuries. *Biomedicines* **10** (2022).

191. Arunachalam, P.S. *et al.* Systems biological assessment of immunity to mild versus severe COVID-19 infection in humans. *Science* **369**, 1210-1220 (2020).

192. Haljasmagi, L. *et al.* Longitudinal proteomic profiling reveals increased early inflammation and sustained apoptosis proteins in severe COVID-19. *Sci Rep* **10**, 20533 (2020).

193. Tan, Y. *et al.* Integrating longitudinal clinical laboratory tests with targeted proteomic and transcriptomic analyses reveal the landscape of host responses in COVID-19. *Cell Discov* **7**, 42 (2021).

194. Luzina, I.G. *et al.* Occurrence of an activated, profibrotic pattern of gene expression in lung CD8+ T cells from scleroderma patients. *Arthritis Rheum* **48**, 2262-2274 (2003).

195. Hirano, T. *et al.* Abundant TNF-LIGHT expression in the airways of patients with asthma with persistent airflow limitation: Association with nitrative and inflammatory profiles. *Respir Investig* **59**, 651-660 (2021).

196. Doherty, T.A. *et al.* The tumor necrosis factor family member LIGHT is a target for asthmatic airway remodeling. *Nat Med* **17**, 596-603 (2011).

197. Herro, R., Antunes, R.D.S., Aguilera, A.R., Tamada, K. & Croft, M. The Tumor Necrosis Factor Superfamily Molecule LIGHT Promotes Keratinocyte Activity and Skin Fibrosis. *J Invest Dermatol* **135**, 2109-2118 (2015).

198. Cheng, T. *et al.* Herpes Virus Entry Mediator (HVEM) Expression Promotes Inflammation/ Organ Injury in Response to Experimental Indirect-Acute Lung Injury. *Shock* **51**, 487-494 (2019).

199. Shui, J.W. *et al.* HVEM signalling at mucosal barriers provides host defence against pathogenic bacteria. *Nature* **488**, 222-225 (2012).

200. Xiong, Y. *et al.* Expression of herpesvirus entry mediator gene as a potential biomarker for disease severity in patients with persistent asthma. *J Asthma* **58**, 717-724 (2021).

201. Cheung, T.C. *et al.* Unconventional ligand activation of herpesvirus entry mediator signals cell survival. *Proc Natl Acad Sci U S A* **106**, 6244-6249 (2009).

202. Ning, Z., Liu, K. & Xiong, H. Roles of BTLA in Immunity and Immune Disorders. *Front Immunol* **12**, 654960 (2021).

203. Murphy, T.L. & Murphy, K.M. Slow down and survive: Enigmatic immunoregulation by BTLA and HVEM. *Annu Rev Immunol* **28**, 389-411 (2010).

204. Sedy, J., Bekiaris, V. & Ware, C.F. Tumor necrosis factor superfamily in innate immunity and inflammation. *Cold Spring Harb Perspect Biol* **7**, a016279 (2014).

205. Cheung, T.C. *et al.* T cell intrinsic heterodimeric complexes between HVEM and BTLA determine receptivity to the surrounding microenvironment. *J Immunol* **183**, 7286-7296 (2009).

206. Battin, C. *et al.* BTLA inhibition has a dominant role in the cis-complex of BTLA and HVEM. *Front Immunol* **13**, 956694 (2022).

207. Demerle, C., Gorvel, L. & Olive, D. BTLA-HVEM Couple in Health and Diseases: Insights for Immunotherapy in Lung Cancer. *Front Oncol* **11**, 682007 (2021).

208. Yu, X., Zheng, Y., Mao, R., Su, Z. & Zhang, J. BTLA/HVEM Signaling: Milestones in Research and Role in Chronic Hepatitis B Virus Infection. *Frontiers in immunology* **10**, 617 (2019).

209. Deppong, C. *et al.* Cutting edge: B and T lymphocyte attenuator and programmed death receptor-1 inhibitory receptors are required for termination of acute allergic airway inflammation. *J Immunol* **176**, 3909-3913 (2006).

210. Han, P., Goularte, O.D., Rufner, K., Wilkinson, B. & Kaye, J. An inhibitory Ig superfamily protein expressed by lymphocytes and APCs is also an early marker of thymocyte positive selection. *J Immunol* **172**, 5931-5939 (2004).

211. Deppong, C., Degnan, J.M., Murphy, T.L., Murphy, K.M. & Green, J.M. B and T lymphocyte attenuator regulates T cell survival in the lung. *J Immunol* **181**, 2973-2979 (2008).

212. Otsuki, N., Kamimura, Y., Hashiguchi, M. & Azuma, M. Expression and function of the B and T lymphocyte attenuator (BTLA/CD272) on human T cells. *Biochem Biophys Res Commun* **344**, 1121-1127 (2006).

213. Afkarian, M. *et al.* T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4+ T cells. *Nat Immunol* **3**, 549-557 (2002).

214. Overacre-Delgoffe, A.E. *et al.* Interferon-gamma Drives T(reg) Fragility to Promote Anti-tumor Immunity. *Cell* **169**, 1130-1141 e1111 (2017).

215. Chang, Y. *et al.* Th17-associated cytokines promote human airway smooth muscle cell proliferation. *FASEB J* **26**, 5152-5160 (2012).

216. Chang, Y. *et al.* TH17 cytokines induce human airway smooth muscle cell migration. *J Allergy Clin Immunol* **127**, 1046-1053 e1041-1042 (2011).

217. Iwakura, Y., Ishigame, H., Saijo, S. & Nakae, S. Functional specialization of interleukin-17 family members. *Immunity* **34**, 149-162 (2011).

218. Chang, S.H. & Dong, C. Signaling of interleukin-17 family cytokines in immunity and inflammation. *Cell Signal* **23**, 1069-1075 (2011).

219. Ohmori, Y. *et al.* Tumor necrosis factor-alpha induces cell type and tissue-specific expression of chemoattractant cytokines in vivo. *Am J Pathol* **142**, 861-870 (1993).

220. Xia, J.B. *et al.* Hypoxia/ischemia promotes CXCL10 expression in cardiac microvascular endothelial cells by NFkB activation. *Cytokine* **81**, 63-70 (2016).

221. Yang, S. *et al.* Foxp3+IL-17+ T cells promote development of cancer-initiating cells in colorectal cancer. *J Leukoc Biol* **89**, 85-91 (2011).

222. Wildbaum, G., Netzer, N. & Karin, N. Plasmid DNA encoding IFN-gamma-inducible protein 10 redirects antigen-specific T cell polarization and suppresses experimental autoimmune encephalomyelitis. *J Immunol* **168**, 5885-5892 (2002).

223. Zohar, Y. *et al.* CXCL11-dependent induction of FOXP3-negative regulatory T cells suppresses autoimmune encephalomyelitis. *J Clin Invest* **124**, 2009-2022 (2014).

224. Qian, C., An, H., Yu, Y., Liu, S. & Cao, X. TLR agonists induce regulatory dendritic cells to recruit Th1 cells via preferential IP-10 secretion and inhibit Th1 proliferation. *Blood* **109**, 3308-3315 (2007).

225. Martin-Fontecha, A. *et al.* Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming. *Nat Immunol* **5**, 1260-1265 (2004).

226. Xanthou, G., Duchesnes, C.E., Williams, T.J. & Pease, J.E. CCR3 functional responses are regulated by both CXCR3 and its ligands CXCL9, CXCL10 and CXCL11. *Eur J Immunol* **33**, 2241-2250 (2003).

227. Haghayegh Jahromi, N. *et al.* Intercellular Adhesion Molecule-1 (ICAM-1) and ICAM-2 Differentially Contribute to Peripheral Activation and CNS Entry of Autoaggressive Th1 and Th17 Cells in Experimental Autoimmune Encephalomyelitis. *Front Immunol* **10**, 3056 (2019).

228. Bharadwaj, A.S., Schewitz-Bowers, L.P., Wei, L., Lee, R.W. & Smith, J.R. Intercellular adhesion molecule 1 mediates migration of Th1 and Th17 cells across human retinal vascular endothelium. *Invest Ophthalmol Vis Sci* **54**, 6917-6925 (2013).

229. Verma, N.K. *et al.* LFA-1/ICAM-1 Ligation in Human T Cells Promotes Th1 Polarization through a GSK3beta Signaling-Dependent Notch Pathway. *J Immunol* **197**, 108-118 (2016).

230. Hosokawa, Y., Hosokawa, I., Ozaki, K., Nakae, H. & Matsuo, T. TNFSF14 coordinately enhances CXCL10 and CXCL11 productions from IFN-gamma-stimulated human gingival fibroblasts. *Mol Immunol* **47**, 666-670 (2010).

231. Miyagaki, T. *et al.* Low herpesvirus entry mediator (HVEM) expression on dermal fibroblasts contributes to a Th2-dominant microenvironment in advanced cutaneous T-cell lymphoma. *J Invest Dermatol* **132**, 1280-1289 (2012).

232. Ding, Q. *et al.* CXCL9: evidence and contradictions for its role in tumor progression. *Cancer Med* **5**, 3246-3259 (2016).

233. Burke, S.J. *et al.* Synergistic expression of the CXCL10 gene in response to IL-1beta and IFN-gamma involves NF-kappaB, phosphorylation of STAT1 at Tyr701, and acetylation of histones H3 and H4. *J Immunol* **191**, 323-336 (2013).

234. Harris, D.P., Chandrasekharan, U.M., Bandyopadhyay, S., Willard, B. & DiCorleto, P.E. PRMT5-Mediated Methylation of NF-kappaB p65 at Arg174 Is Required for Endothelial CXCL11 Gene Induction in Response to TNF-alpha and IFN-gamma Costimulation. *PLoS One* **11**, e0148905 (2016).

235. John, M. *et al.* Human airway smooth muscle cells express and release RANTES in response to T helper 1 cytokines: regulation by T helper 2 cytokines and corticosteroids. *J Immunol* **158**, 1841-1847 (1997).

236. Hardaker, E.L. *et al.* Regulation of TNF-alpha- and IFN-gamma-induced CXCL10 expression: participation of the airway smooth muscle in the pulmonary inflammatory response in chronic obstructive pulmonary disease. *FASEB J* **18**, 191-193 (2004).

237. Singer, C.A. *et al.* p38 MAPK and NF-kappaB mediate COX-2 expression in human airway myocytes. *Am J Physiol Lung Cell Mol Physiol* **285**, L1087-1098 (2003).

238. Sukkar, M.B. *et al.* Toll-like receptor 2, 3, and 4 expression and function in human airway smooth muscle. *J Allergy Clin Immunol* **118**, 641-648 (2006).

239. Jiang, X., Takahashi, N., Matsui, N., Tetsuka, T. & Okamoto, T. The NF-kappa B activation in lymphotoxin beta receptor signaling depends on the phosphorylation of p65 at serine 536. *J Biol Chem* **278**, 919-926 (2003).

240. Lazear, H.M., Schoggins, J.W. & Diamond, M.S. Shared and Distinct Functions of Type I and Type III Interferons. *Immunity* **50**, 907-923 (2019).