Sphingosine Kinase-1 Regulates

Neutrophil Recruitment During Allergic Inflammation

By

Wai Yan (Kiwi) Sun

B.Biotech(Hons), B.Pharm

Vascular Biology and Cell Trafficking Laboratory,
Centre for Cancer Biology
SA Pathology

Department of Medicine,
Faculty of Health Sciences,
University of Adelaide

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Thesis abstract

Current health costs for allergy are more than US$300 billion annually worldwide. Although anti-histamines and steroids are the mainstay treatment for allergic inflammation, their effectiveness is varied. Rapid recruitment of neutrophils to sites of inflammation is associated with allergic diseases, such as dermatitis and anaphylaxis, yet they have been largely ignored as a treatment target. Thus, a better understanding of neutrophils in allergic inflammation is required to develop new therapeutic options.

Leukocyte infiltration to a site of inflammation is an important process for the development of allergic diseases and the roles of eosinophils and mast cells have been well described in allergic inflammation. By contrast, neutrophil infiltration has not been fully examined even though they are one of the first responders to be recruited to the inflammatory site(s) and become a dominant producer of histamine over time to enhance the recruitment of other inflammatory cells. Herein, this thesis focuses on the better understanding of neutrophil infiltration during allergic inflammation.

The classical paradigm of leukocyte recruitment suggests that this localization of cells, including neutrophils, from the circulation to a site of inflammation occurs via specific interactions of adhesion molecules. During allergic inflammation, the adhesion molecule P-selectin, which is pre-formed and pre-stored in Weibel Palade

bodies of vascular endothelial cells (ECs), is rapidly exocytosed to the EC surface to recruit neutrophils. The mechanisms underpinning rapid P-selectin exocytosis by ECs are not fully understood.

This study investigated the hypothesis that sphingosine kinase (SK) is a regulator for P-selectin-induced neutrophil recruitment during allergic inflammation. SK is a highly conserved lipid kinase, which is ubiquitously expressed at varying levels in different cell types, and catalyses the phosphorylation of sphingosine to form the bioactive molecule sphingosine-1-phosphate (S1P). Fingolimod (Gilenya) is an FDA approved oral drug currently used to treat multiple sclerosis via its effects on SK and S1P axis. Herein, I reveal that Fingolimod can inhibit histamine-induced neutrophil recruitment *in vitro* and *in vivo*. In addition, I show that topical application of Fingolimod can attenuate the production of inflammatory chemoattractants and ear swelling in two mouse models of allergic inflammation. Finally, I demonstrate that Fingolimod blocks calcium influx in histamine-treated ECs. Taken together, I have begun to unravel previously unknown processes underpinning neutrophil recruitment during allergic inflammation.

Overall, this study provided evidence that SK can be a therapeutic target to prevent and treat allergic inflammation via regulation of excessive neutrophil recruitment. Our findings also reveal a new indication for Fingolimod wherein it can

be applied topically to treat allergic-associated diseases. By doing so we may well provide a new treatment option for acute allergic inflammation and possibly fatal anaphylaxis.

Key words: neutrophil, endothelial cells, allergic inflammation, sphingosine kinase, Fingolimod, P-selectin, histamine

Declaration

This body of work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Miss Wai Yan Sun. To the best of my knowledge and belief, this work contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in any university or other tertiary institution to Miss Wai Yan Sun without the prior approval of the University of Adelaide.

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This thesis began with a loose collection of ideas when I was working as a research assistant in A/Prof Claudine Bonder's laboratory, and then developed into a more solid plan as my PhD project. During my PhD candidature, I undertook a concurrent degree of B.Pharmacy and aimed to incorporate the pharmacy knowledge into my research. I admit that the past few years were very busy but extremely enjoyable and fruitful, and it could never be possible without the kind support of my principal supervisor, A/Prof Claudine Bonder.

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Awards

International:

2014 International Vascular Biology Meeting Travel Award- AUD 2000
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2013 The Best Student Publication- AUD 750
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2012 The Best of The Best Student Poster Presentation- AUD 1000
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2012 ASI Postgraduate International travel award- AUD 2000
Australasian Society for Immunology, Australia

2012 The Best Poster Presentation- AUD 500
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The 41st Australasian Society for Immunology Annual Meeting, Australasian Society for Immunology, Adelaide, Australia

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The 5th Barossa Meeting: Cell Signalling and Molecular Medicine, Adelaide, Australia

2011 The D R Stranks Travelling Fellowships- AUD 3,000
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16. Sun WY, Pitson SM, Grimbaldeston M and Bonder CS. Epicutaneous application of Fingolimod attenuates allergic inflammation. *The 18th International Vascular Biology Meeting*, *hosted by International Vascular Biology Society,* Kyoto, Japan, 14-17th April 2014
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-Oral presentation

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-*Poster presentation*

Abbreviations

$[Ca^{2+}]i$	intracellular calcium
ABC transporter	ATP-binding cassette transporter
ACD	citrate-dextrose solution
ACK	Ammonium-Chloride-Potassium
ADAM	A disintegrin and metallopeptidase
Ang	angiopoietin
APC	antigen presenting cell
ATF	activating transcription factor
BCR	B cell receptor
BMMC	bone marrow-derived mast cell
BSA	bovine serum albumin
CAM	cellular adhesion molecule
cAMP	cyclic adenosine monophosphate
CCL	chemokine ligand
CIA	collagen-induced arthritis
CIB	calcium and integrin binding protein
COPD	chronic obstructive pulmonary disease
CSR	class switch recombination

CXCL	C-X-C motif ligand
DHS	dihydro-sphingosine
DMS	d-*erythro*-*N*,*N*-dimethylsphingosine
DNP	2,4-dinitrophenol
EC	endothelial cell
ECM	extracellular matrix
EGF	epidermal growth factor
EPH	ethanol/propylene glycol/water
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FCS	fetal calf serum
FDA	Food and Drug Administration
Fn	fibronectin
FOV	field of view
G-CSF	granulocyte colony-stimulating factor
GDP	guanosine diphosphate
GF	growth factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
GTP	guanosine-5'-triphosphate

HAEC	human aortic endothelial cell
HDAC	histone deacetylases
HDL	high density lipopolysaccharide
HSA	human serum albumin
HUVEC	human umbilical vein endothelial cell
ICAM	intercellular adhesion molecule
IFN	interferon
IL	interleukin
IRF1	interferon-regulatory factor 1
LAD	leukocyte adhesion deficiency
LPS	lipopolysaccharides
LT	leukotriene
MadCAM	mucosal vascular addressin cell adhesion molecule
MetOH	methanol
MCP	monocyte chemoattractant protein
MHC	major histocompatibility complex
MS	multiple sclerosis
Naf	Nef associated factor
NSF	*N*-ethylmaleimide-sensitive factor

OVA	ovalbumin
PAR	proteinase-activated receptor
PCA	passive cutaneous anaphylaxis
PDMC	peritoneal-derived mast cells
PHB	prohibitin
PI3K	phosphatidylinositol 3-kinase
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PP2A	protein phosphatase 2A
PPAR	peroxisome proliferator-activated receptors
PPS	pentosan polysulfate sodium
PSGL	P-selectin glycoprotein ligand
PTX	pentraxin
RT	room temperature
S1P	sphingosine-1-phosphate
siRNA	small interfering RNA
SK	sphingosine kinase
SK2L	sphingosine kinase 2 long
SNAP	soluble NSF attachment protein

TGA	Therapeutic Goods Administration
Th	T helper lymphocyte
TNF	tumour necrosis factor
tPA	tissue plasminogen activator
TRAF	TNF receptor-associated factor
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
VSMC	vascular smooth muscle cell
vWF	von Willebrand factor
WPB	Weibel-Palade bodies
WT	wildtype

Chapter 1: Introduction

1.1 Allergic inflammation

Allergic inflammation is a response of both adaptive and innate immune system, which is triggered by the presence of foreign allergen(s) in the host body. Uncontrolled and excessive immune reactions against the foreign allergen(s) often result in pathophysiological disabilities and medical conditions, such as asthma, atopic dermatitis, allergic rhinitis and anaphylaxis [1]. The development of allergic diseases is related to the tolerance and sensitivity of the host's immunity to the particular allergen, including but not limited to pollen, animal dander, grass, mould and various food allergens [reviewed in 2]. Notably, allergic diseases are patient specific and can occur either alone or in combination at any time [reviewed in 2]. Furthermore, a correlation between asthma, allergic rhinitis and/or atopic dermatitis has been shown in children and teenagers, wherein those suffering from asthma are more likely to also suffer from the other two allergic diseases [3].

In 2007, The Australian Society of Clinical Immunology and Allergy (ASCIA) published that approximately 20% of the Australian population suffers from at least one type of allergic response and that the prevalence continues to increase every decade in Australia [1, 4]. These allergic and immune diseases can become chronic in nature, which results in a profound impact on health, quality of life, co-morbidities

and thereby a financial burden on our society [1]. The medical cost incurred from all types of allergic inflammation is more than A$7 billion per year in Australia [4]. The mainstay of preventative strategy is avoidance of the allergen but that is not always feasible given their ubiquitous nature [1]. Moreover, the current treatment strategies (Table 1.1a) are insufficient in their control of allergic symptoms in all patients with a continual rise in allergic inflammation across all ages, race and sex [4]. Clearly, better treatment options are needed.

Table 1.1a Current medical treatment options for allergic inflammation in Australia [5, 6]

Drugs	Indications	Limitations	Common adverse effects
Anti-histamines	Itch, rash, allergic rhinitis	No role in acute anaphylaxis	Dry mouth, dry eyes, sedation
Steroids	Asthmatic management, chronic dermatitis	Delayed effects (onset at 4-6 hours)	Throat irritation, candidiasis infection, hoarseness
β_2 agonists	Relieve brochospasm, Asthmatic management	symptomatic relief only	Headache, tremor, palpitations
Adrenaline	Anaphylactic reaction	Often require hospitalization	

1.2 Development of allergic inflammation

Allergic inflammation can occur immediately (within seconds to minutes; type I hypersensitivity), or delayed (days to months; type IV hypersensitivity) via the classical inflammatory immune reactions [reviewed in 7]. Briefly, antigen presenting cells (APC; eg macrophages and dendritic cells) present a processed allergen via major histocompatibility complex II (MHCII) to naïve T helper cells (Th0), which then differentiate into Th1, Th2 or Th17 cells [reviewed in 2]. Th1 and Th2 cells are responsible for the production of different cytokines that determine the development of allergic inflammation [reviewed in 2, 8]. It has been established that Th1 and Th2 cells can execute opposing roles. For example, while Th2 cells release interleukin (IL)-4 and IL-13 to promote B cell activation and specific IgE antibody production that can then sensitize mast cells, Th1 cells release interferon (IFN)-γ to inhibit the inflammatory properties of Th2 cells (Figure 1.2a) [reviewed in 2]. Th17 cells are a subset of CD4 T-cells that release IL-17A and IL-17F to specifically induce neutrophilic inflammation [9], and have a role in the early and chronic phase of inflammation which will be discussed later in this chapter. In the presence of foreign allergen, activated T cells up-regulate the gene expression for the production of IL-3, IL-4, IL-5, IL-9, IL-13 and granulocyte-macrophage colony-stimulating factor (GM-CSF), which drive a range of allergic immune responses (Table 1.2a).

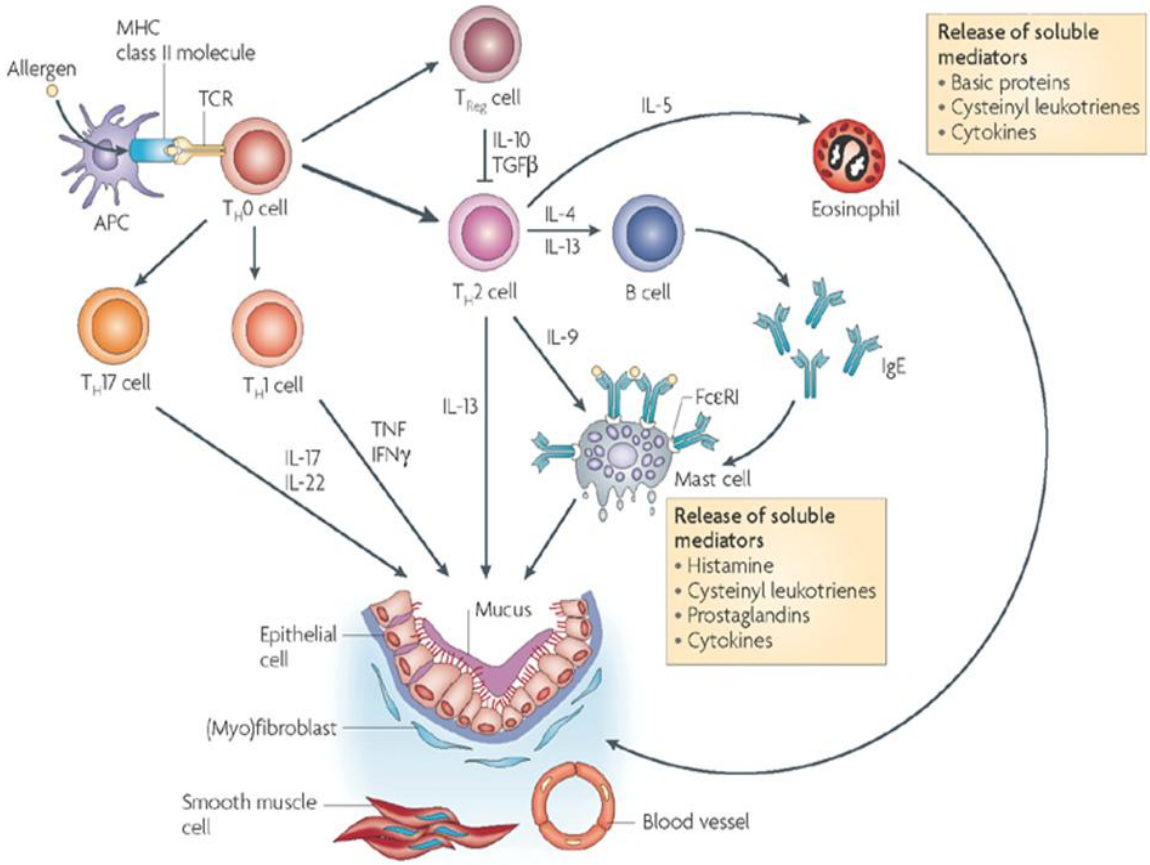


Figure 1.2a The development of immune reactions during allergic inflammation.

The presence of allergen is detected and processed by APCs prior to presentation to Th cells. Th cell subsets differentiate to release cell specific cytokines which facilitate the development of an allergic response. The early phase of allergic inflammation is driven by Th2 cytokines (IL-4 and IL-13), which stimulate IgE production by B cells and the degranulation of mast cells to release pro-inflammatory mediators (eg histamine).

(Figure adapted from Holgate et al [2])

Table 1.2a Cytokine-driven cellular responses during allergic inflammation [2, 10].

Allergic immune response	**Cytokine-involved**
Ig class switching of B cells	IL-4 and IL-13
Maturation of eosinophils	IL-3, IL-5 and GM-CSF
Maturation of basophils	IL-3 and IL-4
Recruitment of mast cells	IL-4, IL-9 and IL-13
Recruitment of neutrophils	IL-17A
Activation of Th17 cells	IL-1β, IL-6 and TGF-β

In different tissues, allergic sensitization is further enhanced by their APC subsets, such as airway mucosal DCs, macrophages and B cells, ensuring that even the smallest amount of foreign allergen can be detected by the host's immune system [11]. Notably, B cells have a dual role in the development of allergic inflammation. First, they contribute to the allergic sensitization by capturing, processing and presentation of foreign allergen via B cell receptor (BCR) and MHCII, with or without the involvement of CD23, to facilitate the presentation to the local T cells (Figure 1.2b) [11, 12]. Second, B cells are responsible for specific immunoglobulin (Ig)-E antibody production via a process of Ig class switching, which occurs in both non-germinal centre and germinal centre of the lymphoid tissue [reviewed in 13]. Early IgE-mediated responses are generated from short-lived plasma cells which are derived from naïve B cells in non-germinal centre [reviewed in 13]. By contrast, late IgE responses have a higher affinity to the particular allergen and arise from those plasma cells that are differentiated in the germinal centre (Figure 1.2c) [reviewed in 13]. Importantly, B cells express FcεRII (or known as CD23), which binds to IgE at low affinity, to induce signalling pathways of B cells for IgE production [14, 15]. The levels of IgE are low under normal circumstances, with the concentrations of only 50-200 ng/mL of blood when compared to 1-10 mg/mL of blood for other Ig isotypes in humans [14]. Also, the half-life of IgE is the shortest amongst all Ig isotypes, with

approximately 2 days when compared to 20 days for IgG [14].

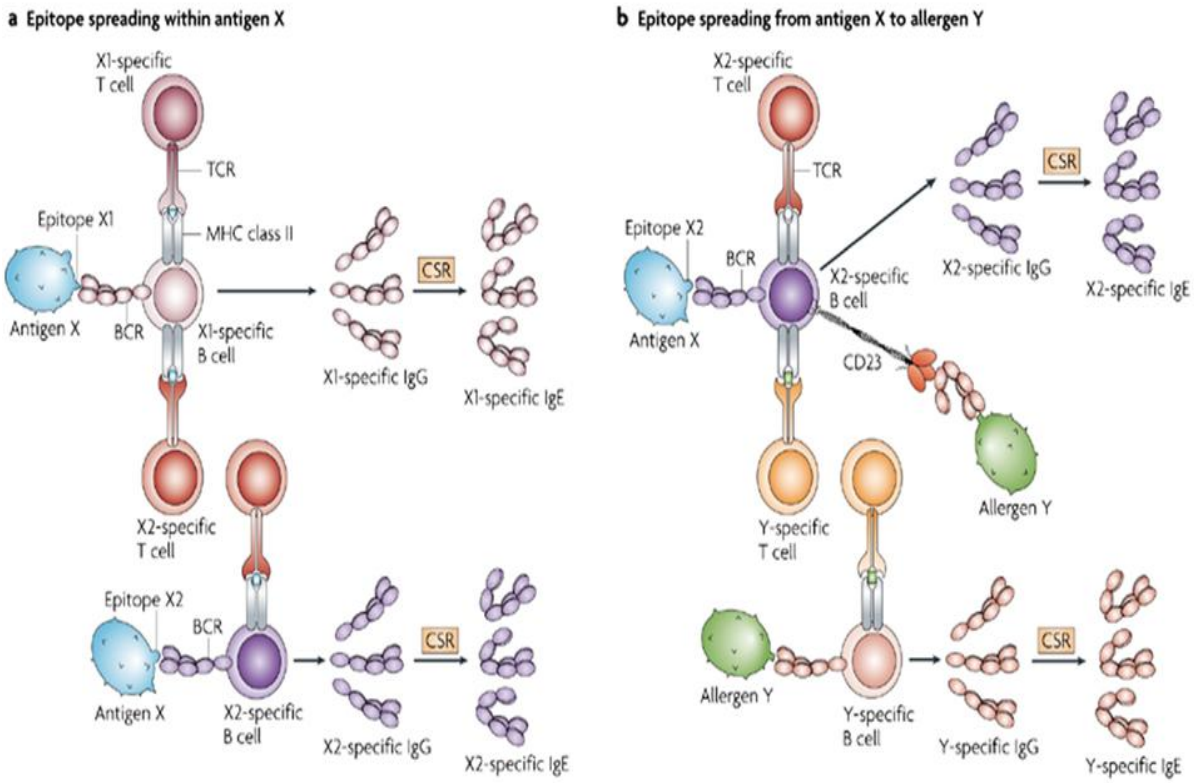


Figure 1.2b Activation of B cells for specific immunoglobulin production. (a) B cells act as APCs to present and process different epitopes of the antigen via BCR and then present to T cells via MHCII following specific Ig production. The specific IgE is generated by a process of class switch recombination (CSR) at the variable region of the Ig in the presence of IL-4 and IL-13 secreted by Th2. (b) Furthermore, B cells can sensitize multiple foreign allergens via BCR and CD23 followed by specific Ig production.

(Adapted from Gould et al [16])

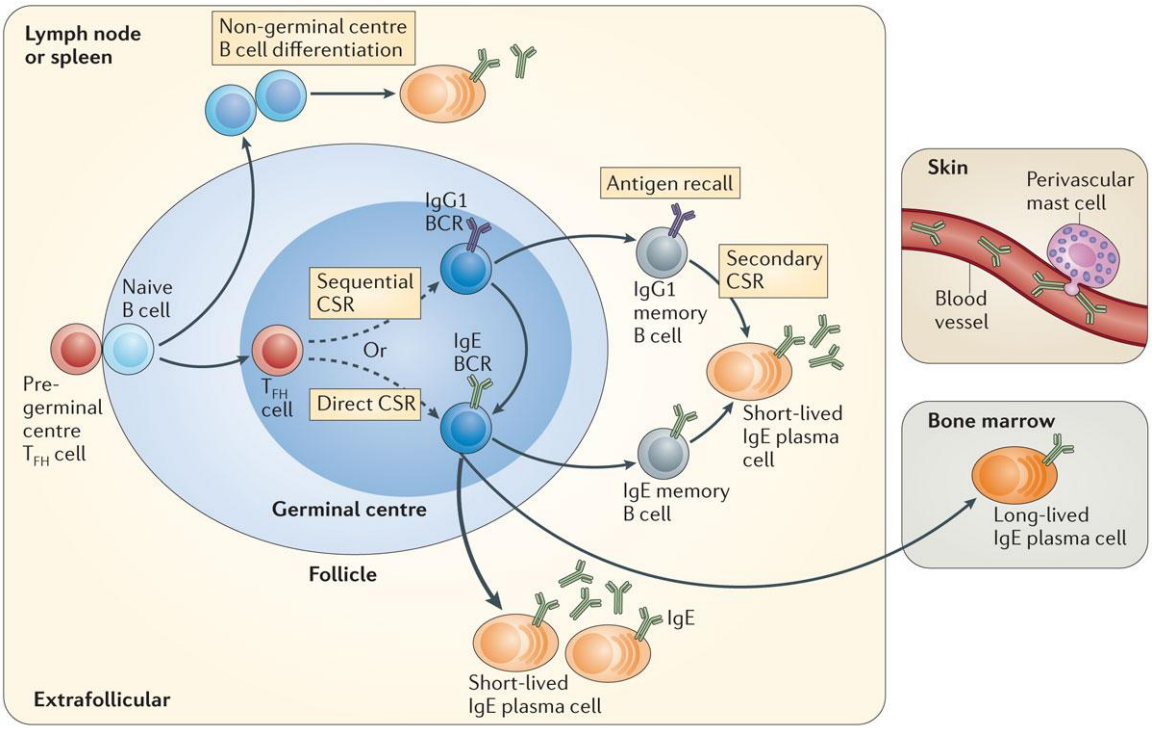


Figure 1.2c IgE production via Immunoglobulin class switching in lymphoid tissue. Early and low affinity IgE responses are generated from the short-lived IgE plasma cells that differentiate in the non-germinal centre. Late and high affinity IgE responses are generated from germinal centre reactions, where activated B cells undergo class-switch recombination to IgE. Germinal centre B cells can differentiate into short-lived IgE producing plasma cells that produce high-affinity IgE, or long-lived IgE producing plasma cells that migrate to the bone marrow, or IgE producing memory B cells.

(Adapted from Wu et al [13])

During the sensitization phase, the allergen specific IgE is produced and released into the bloodstream and tissue so that the Fc region can bind to mast cells and basophils via their surface expressed FcεRI [17]. Upon re-exposure to the same allergen, the IgE pre-bound mast cells or basophils cross-link with the allergen for rapid degranulation of the cells, which releases a number of cytokines and pro-inflammatory mediators to induce an allergic immune response (Table 1.2b) [17]. As these allergic reactions can occur within minutes of exposure to the allergen, this is known as the acute/early phase of allergic inflammation [18]. The cytokines and chemokines continuously drive the recruitment of leukocytes, such as macrophages and eosinophils that then drive the late phase of allergic inflammation (after ~6 hours) (Figure 1.2d) [19]. Chemokines, including CC chemokine ligand (CCL)-2, CCL3, CCL5 (also known as RANTES), CCL7, CCL8, CCL13 (also known as monocyte chemotactic proteins (MCP)1-4), CCL24 and CCL26 (also known as eotaxin 1-3), are secreted by mast cells and other immune cells to promote the release of more pro-inflammatory mediators and further enhance the recruitment of leukocytes [20]. In the late phase of allergic inflammation, toll-like receptor-4 is activated on leukocytes to induce the gene expression encoding pro-inflammatory factors, such as tumor necrosis factors (TNF)-α, which can further develop and maintain the pro-inflammatory responses [21].

Table 1.2b Mast cell derived cytokines, chemokines and inflammatory mediators

	Cellular function	**Reference**
Cytokines		
TNFα	-Activation of EC -Recruitment of inflammatory cells -Enhance DC function	[22]
IL-1β	-Upregulation of CAM expression -Recruitment of inflammatory cells	[23]
IL-3	-Cell survival and differentiation	[24]
IL-4	-B cell activation for IgE production -Upregulation of VCAM expression -Recruitment of eosinophils	[25]
IL-5	-Cell survival -IgA synthesis	[26]
IL-6	-Increase vascular permeability -EC activation -Recruitment of leukocytes	[22]
IL-13	-B cell activation for IgE production -Upregulation of VCAM expression	[27]
IFNγ	-Increase vascular permeability -EC activation -Recruitment of leukocytes	[22]

	Cellular function	Reference
Chemokines		
CCL5	-Chemoattractant for leukocyte recruitment	[28]
CCL2	-Chemoattractant for leukocyte recruitment	[29]
CCL11	-Chemoattractant for leukocyte recruitment	[30]
CCL24	-Chemoattractant for leukocyte recruitment	[30]
Inflammatory mediators		
Histamine	-Activation of EC -Increase vascular permeability	[31]
Leukotriene (LT)-B4	-Smooth muscle contraction -Increase vascular permeability	[32]
LTC(4), LTD(4), and LTE(4)	-Bronchoconstriction	[reviewed in 33]

Note: CAM: Cell adhesion molecules; VCAM: Vascular cell adhesion molecules; CCL5 is also known as RANTES; CCL2 is also known as MCP; CCL11 and CCL24 are also known as Eotaxins

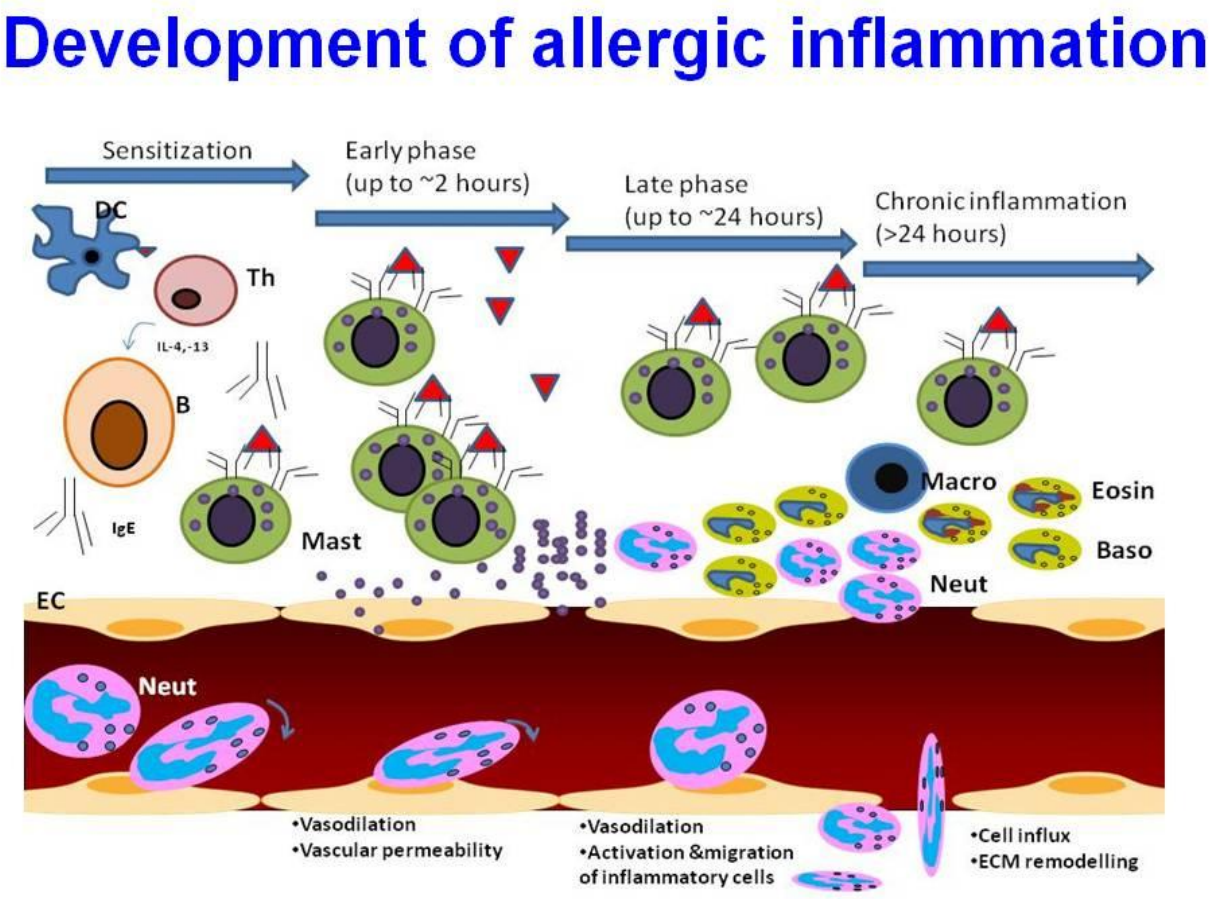


Figure 1.2d The development of allergic inflammation. Allergic inflammation firstly begins with a sensitization phase, where an APC presents the processed allergen to Th cells for activation of the plasma B cells and IgE production. Upon re-exposure to the allergen, IgE pre-loaded mast cells are rapidly activated by cross-linking of the allergen to IgE. Mast cell activation and degranulation are important during the early phase of allergic inflammation as the cytokines and inflammatory mediators induce vascular permeability and recruitment of leukocytes. If this immune reaction persists, a late and chronic phase of allergic inflammation

occurs. With the mast cell-secreted cytokines and chemokines, the recruitment of pro-inflammatory cells, such as macrophages and eosinophils, is further enhanced and the allergic reaction continues.

As mentioned above, the naïve Th0 cells can differentiate into Th17 after being activated by the APCs with the processed allergen [9]. Although Th17 cells have only recently been identified, it is understood that they are the main producer of IL-17 [34], which promotes the production of other cytokines and chemokines (eg IL-8) to recruit eosinophils and neutrophils. [34, 35]. Based on this, Th17 is described as the mediator for neutrophilic inflammatory events, especially in psoriasis, allergic contact dermatitis and atopic dermatitis [36]. In the late and chronic phase of allergic inflammation, the number of Th17 cells expands significantly and correlates with the severity of atopic dermatitis [35, 37].

1.3 Role of neutrophils during allergic inflammation

Neutrophils are the most abundant leukocyte, constituting ~56% of the total circulating leukocyte count in humans [38]. Furthermore, with a short half-life of six to eight hours they are continuously generated and mobilized from the bone marrow to the periphery [39, 40]. During allergic inflammation, neutrophils are one of the first responders to an inflammatory site in response to the chemoattractants, such as IL-8 (known as KC in mice), IFN-γ, complement C5a, or leukotriene B4 [41]. The levels of the circulating neutrophils are found to be increased by 10-fold as the mobilization of neutrophils from the bone marrow is significantly enhanced [42]. Once migrated to the inflammatory site(s), these mature neutrophils extend their lifespan for up to two days in tissues to further advance the development of allergic inflammation [43].

Neutrophils are recruited from the circulation to an inflammatory site via the classical cell recruitment adhesion molecule cascade [44]. The details of this multiple-step process will be discussed later in this Chapter and have been published by our group in a review article (Appendix 4, p.241) [45]. Interestingly, endothelial cells (ECs) not only up-regulate their adhesion molecules to interact with the circulating neutrophils, but also actively recruit the neutrophils by releasing chemoattractants, such as granulocyte colony stimulating factor (G-CSF) and IL-8 [46, 47]. Once neutrophils have arrived at the inflammatory site, they also act as a source

of chemokines, including IL-8, CCL3, chemokine (C-X-C motif) ligand (CXCL)-1, CXCL9 and CXCL10, to further promote the recruitment of neutrophils, monocytes, DCs and T lymphocytes [23, 48]. The levels of IL-8 have been shown to be significantly elevated in patients with allergic inflammatory diseases, such as psoriasis, chronic airway disease and sepsis [48].

Conversely and interestingly, neutrophils exhibit a "self-regulatory" role during inflammation, where neutrophils and other immune cells (macrophages and DCs) secrete the protein, pentraxin (PTX)-3, to inhibit the binding site of P-selectin in the inflamed vascular venules (Figure 1.3a) [49]. As a result, the rolling events of neutrophils on P-selectin are abolished [50]. However, the mechanism for this anti-inflammatory effect of PTX-3 is still unclear.

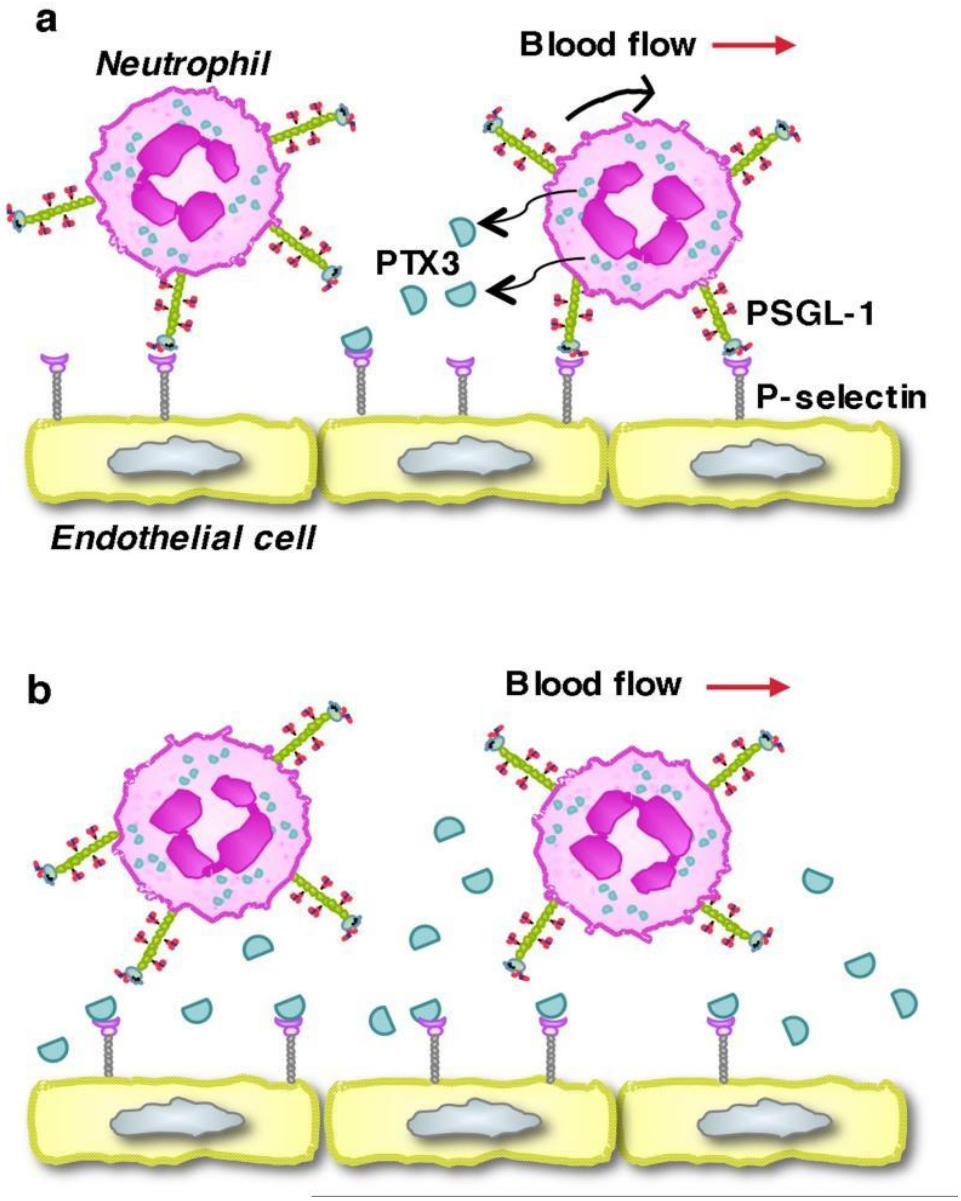


Figure 1.3a Inhibition of neutrophil rolling on P-selectin by pentraxin 3. (a) Circulating neutrophils roll slowly on the endothelium via the binding of P-selectin to PSGL-1. The recruited neutrophils release PTX3, which occupies the binding site of P-selectin. (b) As a result, the circulating neutrophils are unable to roll on the endothelium.

(Adapted from McEven et al [49])

1.4 Physiological roles of vascular endothelial cells

In addition to the inflammatory immune cells, vascular ECs are also important in the development of allergic inflammation. ECs are found as the inner monolayer of the blood vessels and form a tight physical barrier between the circulating blood and local parenchymal tissue (Figure 1.4a) [51]. The endothelium has a number of diverse functions, including regulation of vascular integrity, buffering of normal blood flow and facilitation of oxygen and nutrient exchange to tissues [51]. Compared to many other cell types, ECs have long life span with a turn-over rate from days to years, dependent on which vascular bed they reside and their exposure to detrimental factors [52, 53]. ECs can become activated by inflammatory stimuli during allergic inflammation, such as histamine and TNFα, which then promote the recruitment of leukocytes via cell-cell interactions of surface expressed adhesion molecules [54].

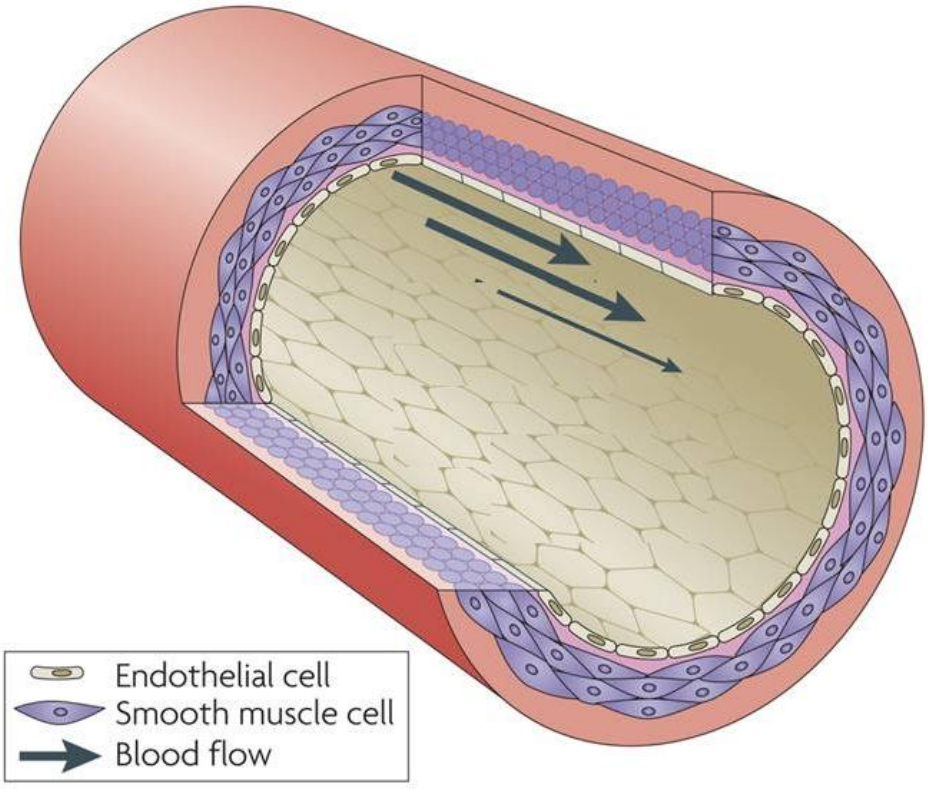


Figure 1.4a Structure of a vascular blood vessel. A section of blood vessel shows that endothelial cells form the inner monolayer aligning longitudinally with tight cell junction, and smooth muscle cells form the outer layers of the blood vessel.
(Adapted and modified from Hahn et al [55])

1.4.1 Endothelial cell adhesion molecules

In response to the foreign allergen, ECs are activated by inflammatory mediators and/or cytokines to upregulate their surface expression of adhesion molecules [56]. These EC-expressed adhesion molecules command the recruitment of leukocytes from the circulation to the inflammatory site(s) [56]. Notably, the classical cell recruitment paradigm involves multiple steps: tethering, rolling, slow rolling, arrest, adhesion spreading, intravascular crawling and transmigration, which are governed by different families of adhesion molecules [reviewed in 54]. The selectin family, especially P-selectin, is responsible for the initial slow rolling events of the leukocyte recruitment cascade [44]. Then, integrin and cellular adhesion molecule (CAM) families promote adhesion and spreading of the cells [57] (Figure 1.4.1a)

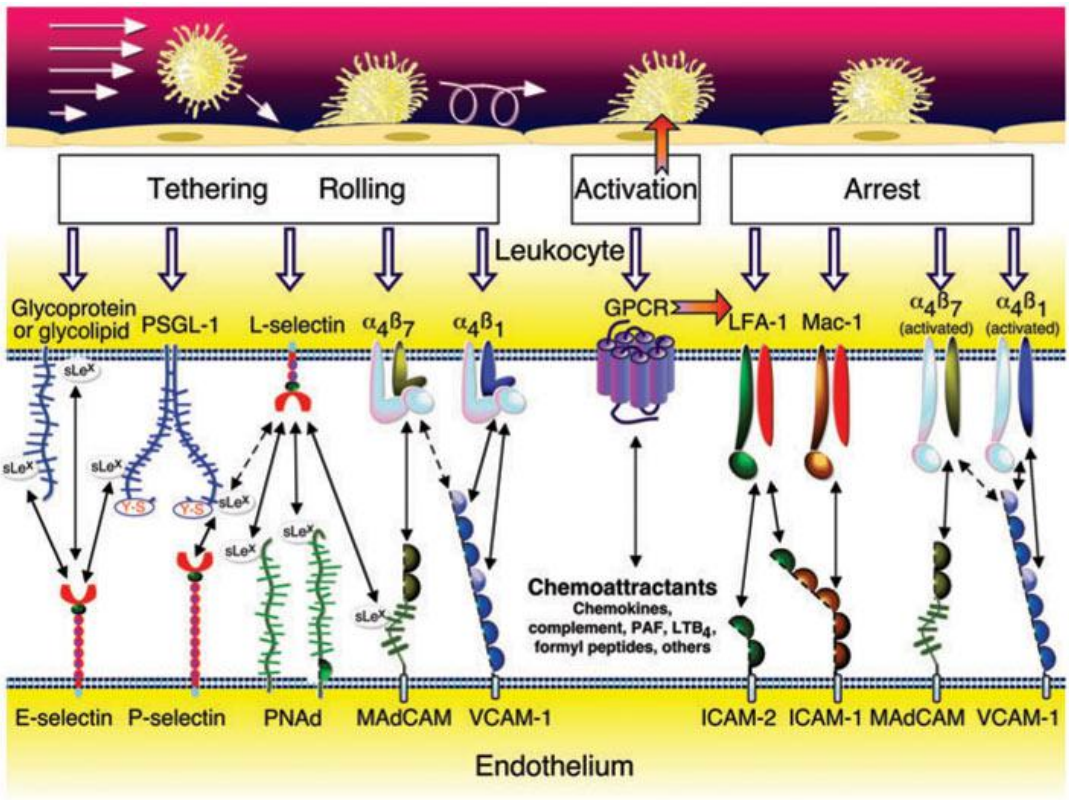


Figure 1.4.1a The surface expression of adhesion molecules by ECs and leukocytes. During the early stage of cell-cell interactions, the selectin family is responsible for the initial tethering and rolling of leukocytes via the binding of P-selectin to PSGL-1. Once E-selectin is expressed by ECs, it binds to glycoprotein or glycolipid of the leukocytes to further promote the rolling events. The CAM family (eg VCAM-1 and ICAM-1) binds to integrins on the rolling leukocytes and causes firm adhesion to the endothelium prior to transmigration towards the inflammatory site.

(Adapted from Luster et al [57])

1.5 The selectin family of adhesion molecules

The selecin family has three members, P-selectin (CD62P), E-selectin (CD62E) and L-selectin (CD62L) (Figure 1.5a). P-selectin is expressed by ECs and platelets, E-selectin is only expressed by ECs and L-selectin is expressed by leukocytes [58]. Although both P- and E-selectin are EC-expressed adhesion molecules, they are induced and upregulated in different manners. P-selectin is a transmembrane protein which consists of a very large lumenal domain and short cytoplasmic tail [59]. The lumenal domain of P-selectin directs itself to be incorporated into Weibel-Palade bodies (WPBs) in the cytoplasm of ECs or α-granules of platelets [58, 59]. P-selectin is pre-made and stored in ECs and platelets, and can thus be translocated and expressed on the cell surface rapidly upon stimulation [60]. By contract, E-selectin expression is regulated at the gene level, where transcriptional control of the *SELE* gene can be induced and enhanced by cytokines and stimuli, such as TNFα, IL-1, IL-6 and lipopolysaccharide (LPS) [61-64]. Of note, such inducible expression of E-selectin requires at least 2 to 4 hours of stimulation of ECs [64, 65]. Based on these characteristics, and despite both P-selectin and E-selectin participating in the early leukocyte-EC interactions during inflammation, P-selectin has been identified as the dominant adhesive molecule involved in the early phase of allergic inflammation [44].

L-selectin is also involved in the recruitment of circulating leukocytes during

allergic inflammation, where it is shed on the leukocytes via proteolytic cleavage by enzymes, such as A disintegrin and metallopeptidase (ADAM)-17 [66, 67]. This shedding enables the leukocytes to come in closer proximity to the vasculature and thus provide easier access to additional adhesive processes [68, 69].

All three selectins bind to sialyl-Lewis X-like carbohydrate ligands, such as P-selectin glycoprotein ligand (PSGL)-1, at high affinity [70]. PSGL-1 is expressed by leukocytes and ECs at a basal level, which allow the rapid cellular interactions during an inflammatory response [71]. Previous studies have shown that PSGL-1-deficient mice exhibit a significant delay of leukocyte rolling flux, which suggests that the binding of PSGL-1 to P-selectin is important for the recruitment of leukocytes during the early phase of allergic inflammation [72-74]. In fact, PSGL-1 is not the sole ligand for the selectin family, as L-selectin-expressed neutrophils have been shown to bind to ECs that are derived from PSGL-1-deficient mice, which demonstrates that such binding of L-selectin can also occur in a PSGL-1 independent manner [71]. However, the identity of this ligand is currently unknown.

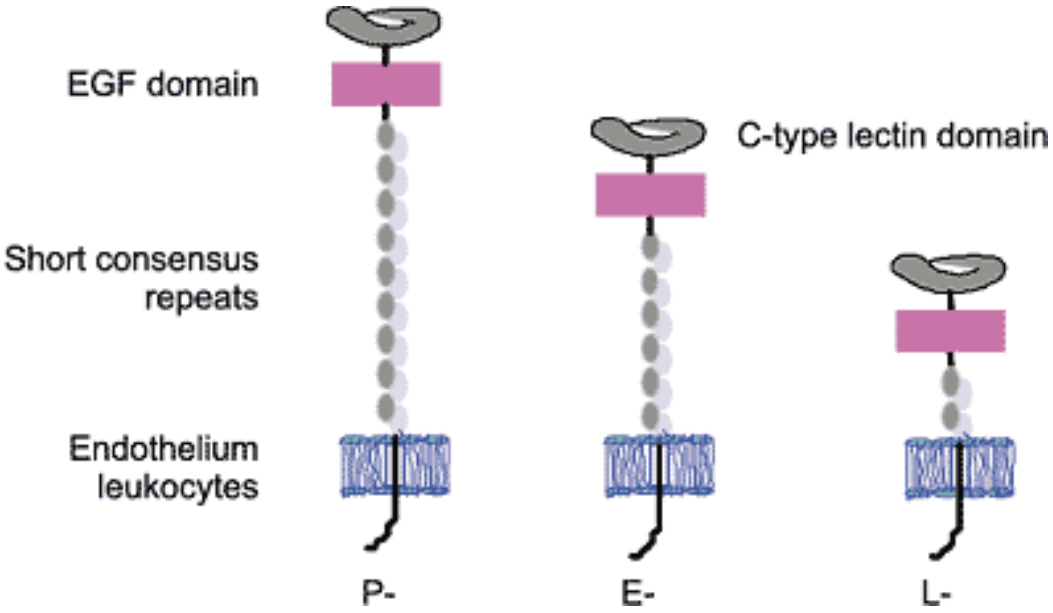


Figure 1.5a Schematic of the selectin family of adhesion molecules. All three members of the selectin family have the conserved C-type lectin domain and epidermal growth factor (EGF) domain, but differ in their short consensus repeats. P- depicts the structure of P-selectin, which is the largest selectin with a mass of 140kDa. E- and L- depict the structure of E-selectin with a mass of 100kDa and L-selectin with a mass of 58kDa, respectively.

(Adapted from Tilton et al [75])

1.5.1 Weibel-Palade bodies

Weibel-Palade bodies (WPBs) were identified by transmission electron microscopy by Ewald Weibel and George Palade in 1964. They are described as a rod-shape structures with a diameter of 0.1-0.3 μm and length of 1-5 μm and surrounded by a lipid bilayer [76]. Specific proteins and peptides have been identified as WPB cargo, which include P-selectin, von Willebrand factor (vWF), tissue-type plasminogen activator (tPA), IL-8, eotaxin-3, angiopoietin (Ang)-2, endothelin-1, endothelin-converting enzyme, calcitonin gene-related peptide and osteoprotegerin [77-79]. In resting ECs, WPBs are linked to actin cytoskeleton and microtubules in the cytoplasm and their movement are directed by the motor protein kinesin [76] (Figure 1.5.1a). A number of mediators can cause exocytosis of WPBs, including histamine and thrombin via an increase in intracellular calcium influx and phospholipase C, and epinephrine and vasopressin via an increase in intracellular cyclic adenosine monophosphate (cAMP) [80]. To exocytose WPBs, the microtubules and motor protein kinesin direct the cargo to the plasma membrane, with a complex of MyRIP and Rab27A anchoring to the actin filaments [81]. G protein-coupled receptors (GPCRs) are involved in the exocytosis of WPBs. For example, thrombin binds to protease-activated receptor (PAR)-1, which is one of the sub-classes of GPCRs, to induce the activation of a small protein, RalA [82]. The guanine exchange

factor RalGDS regulates the activation of RalA by the exchange of bound guanine diphosphate (GDP) to guanine triphosphate (GTP) [82]. The final step of WPB exocytosis is intracellular membrane fusion, which involves *N*-ethylmaleimide-sensitive factor (NSF), soluble NSF attached-protein (SNAP) and the SNAP receptor (ie. SNARE) [83]. The t-SNARE on the plasma membrane interacts with the v-SNARE on the vesicle to promote the fusion of WPBs by pulling the membranes together [83]. The contents of the WPBs can be released once the membranes are fused, and then NSF/SNAP are dissociated from the SNARE complex followed by recycling for other cargoes (Figure 1.5.1a) [83].

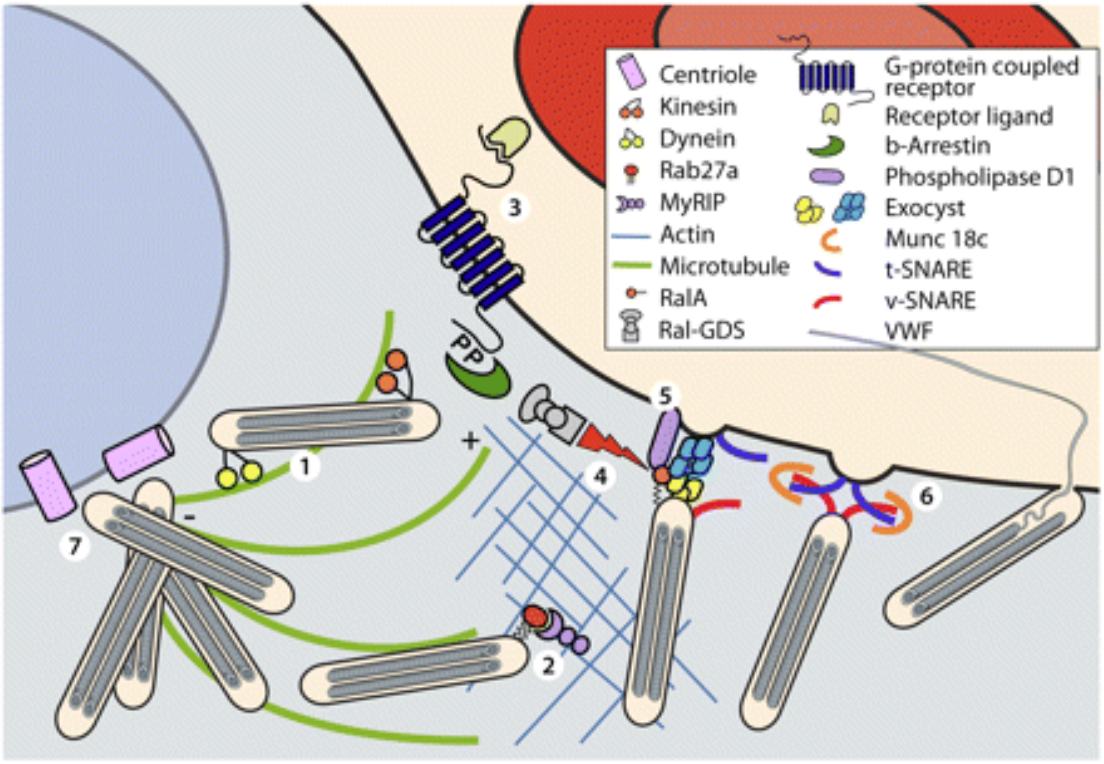


Figure 1.5.1a Exocytosis of WPBs. (1) WPBs rest in the cytoplasm via their attachment to microtubules and kinesin. (2) WPBs can also orientate themselves with actin filaments via the binding of Rab27/MyRIP complex. (3-5) Activated G protein-coupled receptors are involved in the exocytosis of WPBs via the dissociation of phospholipase D1 and exocyst, and the activation of RalA which is regulated by Ral-GDS. (6) t- and v-SNARE anchor WPBs to the plasma membrane for the fusion of WPBs and subsequent release their contents.

(Adapted from Valentijn et al [76])

In addition to the ‘conventional cascade’ of WPB exocytosis, two further mechanisms have been described to exist in ECs (Figure 1.5.1b). Firstly, the ‘Lingering-kiss’ occurs when WPBs containing multiple contents transiently fuse with the plasma membrane to form a small pore (~12 nm in diameter), which only allows the small contents (eg IL-8 and CD63) to be released, but not the large contents (eg P-selectin and vWF) [84]. Secondly, a mechanism termed ‘multigranular exocytosis’ has been described, wherein the different contents of WPBs coalesce to form a large intracellular secretory pod prior to the fusion with the plasma membrane [85]. In the secretory pod, the large peptide (ie. vWF) loses its normal tubular structure when compared to that of the ‘conventional cascade’ of WPBs [85]. As vWF and its precursor (Proregion) are widely expressed and represent more than 95% of the WPBs’ contents, most studies examine vWF as the indicator of WPB exocytosis [86]. The mechanism for P-selectin exocytosis has only been shown to utilize the ‘conventional cascade’ [79] and whether it is also transported by ‘multigranular exocytosis’ is yet to be examined.

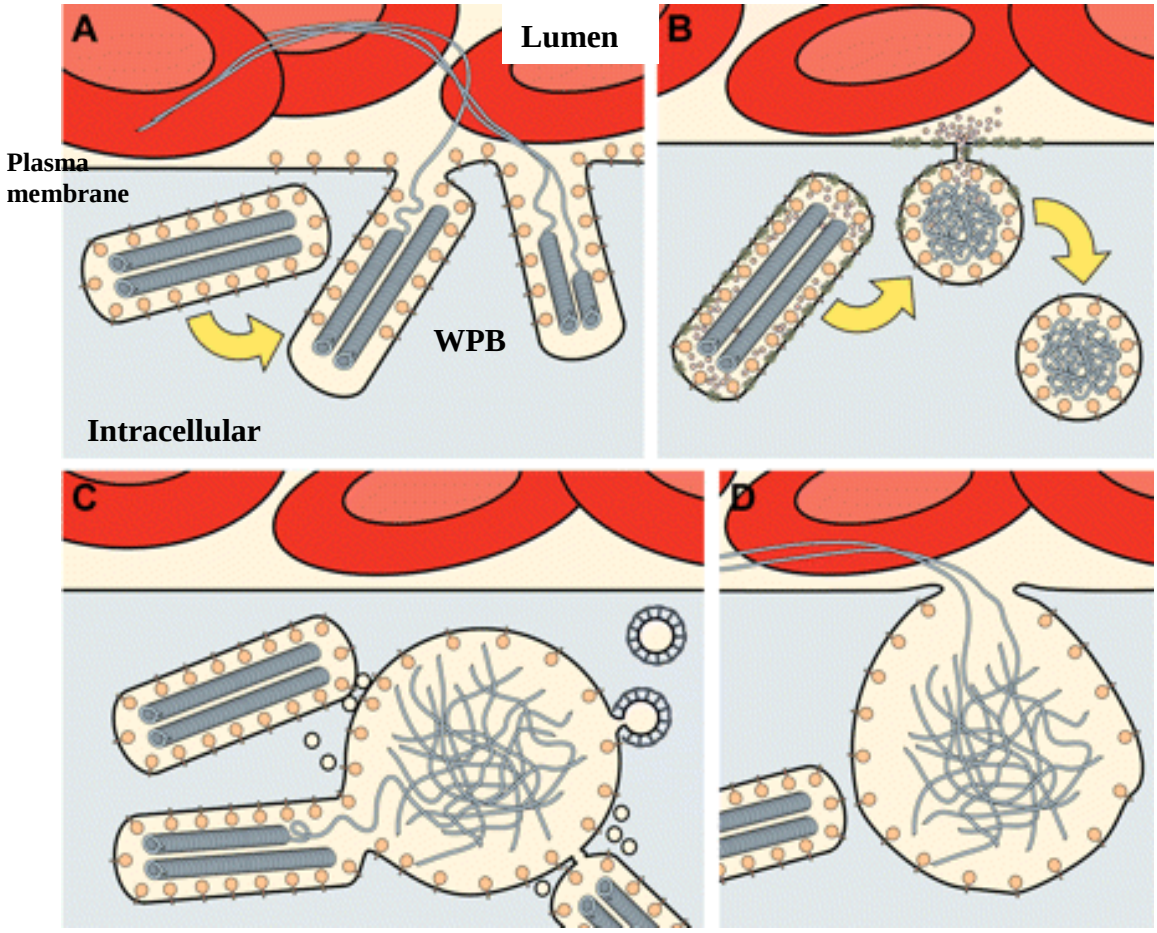


Figure 1.5.1b Different mechanisms of WPB exocytosis. (A) the 'conventional' mechanism dictates that a WPB is translocated and fused with the plasma membrane, with examples of P-selectin being expressed on the EC surface and vWF being released into the blood. (B) 'Lingering kiss' depicts that WPBs containing multiple contents can form a small pore with the plasma membrane which selectively release the smaller molecules of WPBs. (C & D) 'Multigranular exocytosis' involves the contents of WPBs being transiently loaded into a large pod followed by fusion with the plasma membrane and subsequently released.

(Modified and adapted from Valentijn et al [76])

1.5.2 P-selectin-expressed EC

As mentioned, P-selectin is pre-made and stored in WPBs of ECs, and is rapidly expressed on the surface of stimulated cells. The elongated structure of P-selectin can extend for up to 40 nm from the EC surface, which provides an advantage for capture and interaction with circulating leukocytes [75]. As stated above, the full mechanisms of P-selectin-derived WPB exocytosis by ECs is yet to be determined. Studies have shown that vWF-deficient mice had a reduction of P-selectin expression when challenged with histamine or TNFα, and resulted in an attenuation of neutrophil recruitment [76, 87]. This suggests that vWF is associated with the surface expression of P-selectin by ECs, or vWF and P-selectin are located in the same WPBs for exocytosis.

The surface expression of P-selectin is known to be switched ‘on’ and ‘off’ quickly by ECs during the early phase of allergic inflammation [88]. P-selectin is then recycled back to some WPBs for future exocytosis events. Interestingly, the recycled P-selectin is not found in the WPBs that contain Ang-2, suggesting that there is a specific mechanism for the mutually exclusive presence of P-selectin and Ang-2 in WPBs, and the reasons underpinning this are yet to be determined [89].

EC-expressed P-selectin can be induced in two phases with time. The early phase of P-selectin surface expression can be induced by histamine and thrombin

rapidly within minutes via the instant exocytosis of WPBs [44, 90]. By contrast, the chronic phase requires transcription of the P-selectin gene (*SELP*), which can be induced by inflammatory stimuli (eg. oncostatin M [91], IL-3 [92], IL-4 [93, 94], IL-13 [95] and substance P [96]), and such P-selectin surface expression by ECs can remain for days [92]. The promoter region of *SELP* varies significantly between humans and mice, where a NFκB binding region is present upstream of murine *SELP* but not located in the promoter of the human *SELP* [97, 98]. Therefore only in rodents can TNFα and IL-1 induce activation of the NFκB/IκBα complex via degradation of IκBα, subsequent release of NFκB and translocation to the nucleus for *SELP* regulation [99, 100]. Despite these advances, the full mechanisms underpinning rapid exocytosis of P-selectin to the surface of ECs for the recruitment of leukocytes are not fully understood.

The role of P-selectin in ECs is not limited to induction of the slow rolling events of leukocytes, it has also been shown to promote the activation of integrins during inflammation, which cause arrest and firm adhesion of leukocytes [101, 102]. The binding of leukocyte-expressed PSGL-1 to P-selectin on ECs induces Src kinase phosphorylation of Nef-associated factor (Naf)-1, which is then associated with phosphoinositide-3-OH kinase (PI3K) p85-p110δ heterodimer, leading to the activation of integrins on the leukocytes [60]. More importantly, P-selectin deficient

mice exhibit a significant attenuation of leukocyte adhesion during inflammation, which demonstrates the importance of P-selectin in the cell recruitment process [60].

1.5.3 Integrin and cellular adhesion molecule families

In humans, 24 different integrins have been identified, each of which consists of different subunits of α and β chains [103]. The structure of integrins includes the large extracellular domain, single transmembrane segments and short intracellular tails. In the presence of stimuli, integrins exhibit conformational changes as they become activated, which can induce binding to ligands [104]. Furthermore, the distinct levels of conformational changes of the integrins determine their moderate to high affinity to the ligands (Figure 1.5.3a) [105]. During the early phase of allergic inflammation, the binding of P-selectin on ECs to leukocyte-expressed PSGL-1 induces integrin signalling, where the leukocyte-expressed integrin $\alpha_L\beta_2$ (also known as LFA-1 or CD11a) becomes activated (ie. undergoes a conformational change) and then interacts with intracellular adhesion molecule (ICAM)-1 on ECs [104]. Subsequently, the rolling leukocytes become firmly adhered to the endothelium [104, 106]. In addition to $\alpha_L\beta_2$ and ICAM-1, we have shown that $\alpha_5\beta_1$ expressed by both ECs and leukocytes are activated in response to TNFα challenge, which causes adhesion of neutrophils via the mirror interaction with a ligand Ang-2 (ie. $\alpha_5\beta_1$:Ang-2:

$\alpha_5\beta_1$) (Appendix 1, p.238) [61].

Other members of the CAM family that are involved in leukocyte adhesion, include vascular cell adhesion molecule (VCAM)-1 and mucosal addressin cell adhesion molecule (MAdCAM)-1, which are the primary binding partners for leukocyte-expressed $\alpha_4\beta_1$ and $\alpha_4\beta_7$, respectively (reviewed in [57]).

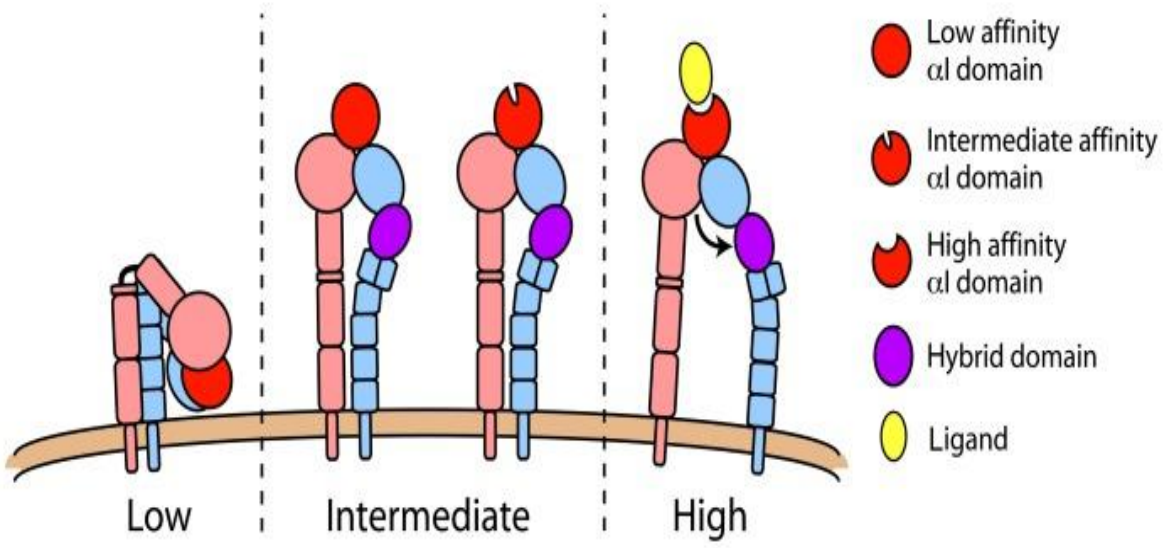


Figure 1.5.3a Conformational change of integrins upon antigen stimulation. Inactive integrin (left panel) exhibits a bent and inverted V shape structure at the extracellular domain which provides very low affinity to its ligand. Activation of the integrin causes an extension of the extracellular domain (middle panel), which significantly increases the ligand affinity. Different levels of conformational change at the αI domain result in a higher affinity to the ligand (right panel).

(Adapted from Lefort et al [105])

1.6 Sphingolipid metabolism

Bioactive sphingolipids, such as ceramide, sphingosine and sphingosine-1-phosphate (S1P), are involved in the sphingomyelin degradation pathway [reviewed in 107]. Their diverse roles in the regulation of cellular metabolism have been studied extensively over the last two decades. The balance of the levels of these sphingolipids in cells is called the 'sphingolipid rheostat', because each can determine cell fate (Figure 1.6a). For example, ceramide and sphingosine induce apoptosis [108], and S1P promotes cell survival and proliferation [109, 110]. Moreover, dysregulation of these sphingolipids has been implicated in many diseases, including cancer and allergic diseases [111, 112]. A number of excellent reviews have been published to discuss the roles of sphingolipids in disease and current opinion on the possible therapeutic approaches [113-115]. Herein, I will briefly describe the known roles of sphingosine, sphingosine kinase (SK) and S1P, and their contribution to allergic inflammation.

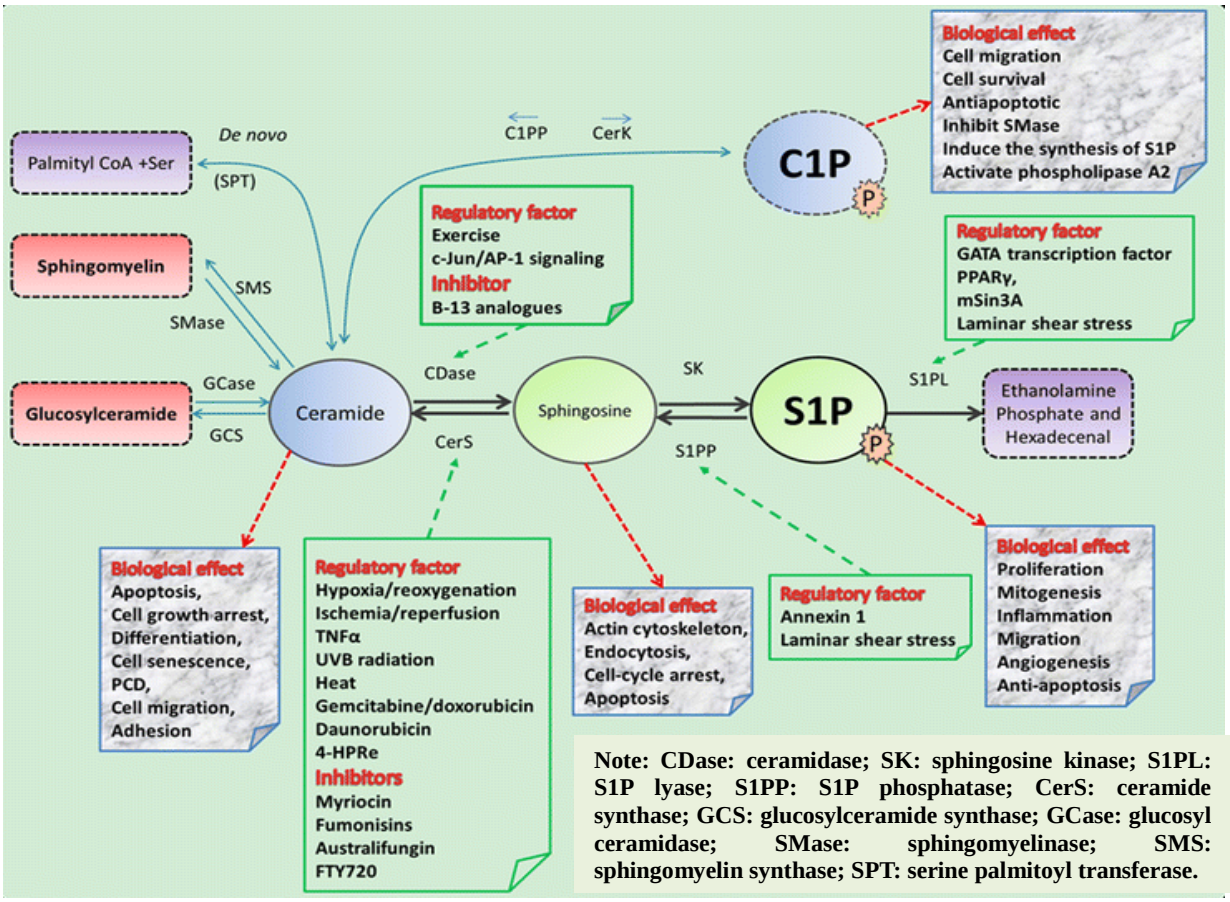


Figure 1.6a Regulation of the sphingomyelin metabolic pathway and the cellular effects. Sphingosine, SK and S1P are key mediators of the sphingomyelin degradation pathway. Ceramide is converted to sphingosine by CDase which can be further catalyzed to S1P by sphingosine kinase-1 and -2. These processes can be reversed by a different set of enzymes, with an exception of S1P lyase (S1PL) which degrades S1P irreversibly. The biological shift towards ceramide, sphingosine or S1P causes diverse biological effects. Ceramide and sphingsoine promote cell apoptosis, growth arrest and senescence, whereas S1P promotes cell proliferation, inflammation and angiogenesis.

(Modified and adapted from Liu et al [116])

1.6.1 Sphingosine

Sphingosine is a small cationic lipid with 18-carbon amino alcohol and an unsaturated hydrocarbon chain, which is generated from the breakdown of ceramide, or dephosphorylation of S1P [116]. Sphingosine is associated with a number of adaptor molecules and protein kinases to induce apoptosis, which include the protein kinase H homologue [117], phosphohydrolase [118], phospholipase D [118], diacylglycerol kinase [119] and 14-3-3 [120]. For example, the protein 14-3-3 belongs to the family of phospho-serine binding proteins and has a dimeric structure, which binds to pro-apoptotic mediators (eg the protein BAD) to maintain its inactive state and suppresses cell apoptosis [121]. However, the binding of sphingosine to 14-3-3 favours the phosphorylation of 14-3-3 at its dimer interface by protein kinase-A and -C, leading to disruption of the dimeric structure. As a result, this abolishes the pro-survival signalling of 14-3-3 [120]. Upregulation of SK can attenuate the binding of sphingosine to 14-3-3 to induce cell death, as SK catalyzes sphingosine to S1P, which has pro-survival properties [120].

1.6.2 Sphingosine kinase (SK)

Two human SK genes, *SPHK1* and *SPHK2*, are transcribed and translated into SK-1 and SK-2, which have been cloned and characterized in mammalian cells [122, 123]. These two isoforms of SK are different in size but share a high degree of polypeptide sequence similarity (80% similarity), with 47% of the amino acid sequence identified to be the same at both N- and C-terminal regions of SK-1 and SK-2 [107] (Figure 1.6.2a). In addition, in humans there are three sub-isoforms of SK-1 (SK-1a, SK-1b and SK-1c) and at least two sub-isoforms of SK-2 (SK-2a and SK-2b) generated by differential splicing or alternate translational start sites, leading to differences in the N- terminal region [123, 124].

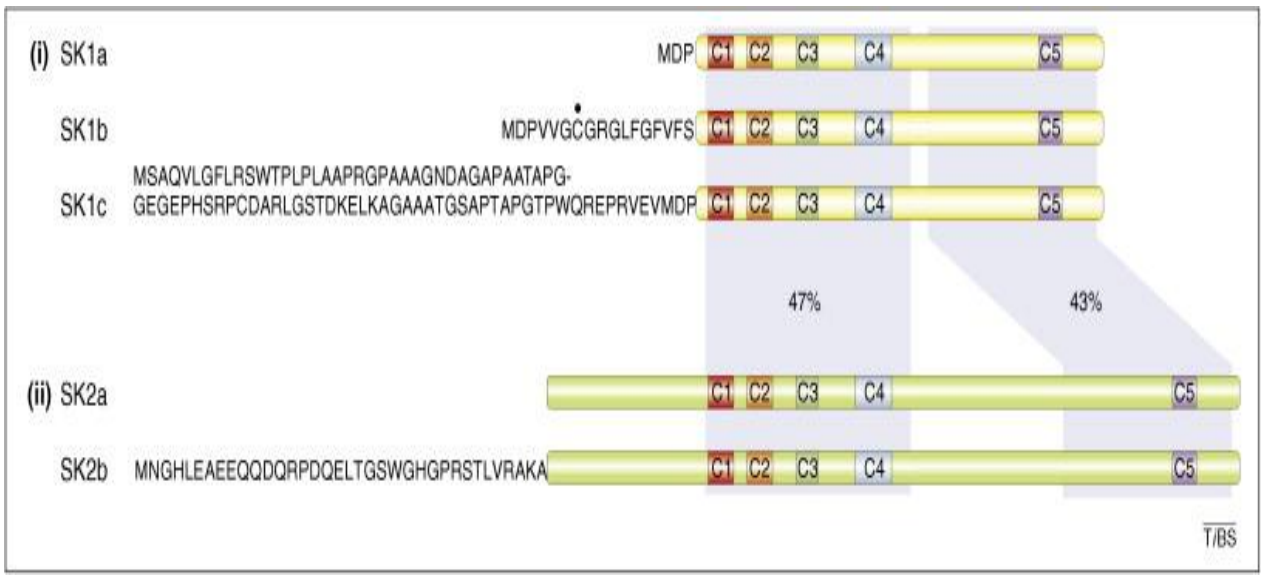


Figure 1.6.2a Different isoforms of human SK-1 and SK-2. Human SK-1 and SK-2 are generated from two separate genes but they share 47% and 43% amino acid sequence identity at the N- and C-terminal regions, respectively (the highlighted regions). C1 to C5 depict the five highly conserved regions within all sphingosine kinases in different species. Three sub-isoforms of SK-1 have been identified (SK-1a, -b and -c), which only differ at their N-termini. SK-1b and SK-1c have additional 14 and 86 amino acids when compared to SK-1a, respectively. SK-2b (also known as SK-2L) possesses an additional 36 amino acids when compared to SK-2a (also known as SK-2S).

(Adapted from Pitson [107])

Although both SK-1 and SK-2 can catalyse the phosphorylation of sphingosine to form S1P, their subcellular locations determine different outcomes of S1P downstream signalling effects [125] (Figure 1.6.2b). For example, SK-1 resides predominantly in the cytosol but can translocate to the inner plasma membrane following phosphorylation by extracellular signal regulated kinases 1 and 2 (ERK1/2) at serine (Ser) position 225 of SK-1 [109]. The enriched levels of sphingosine at the inner plasma membrane are the substrate for SK-1 and are catalyzed into S1P [109], which can then be retained inside the cell or transported out of the cell via ATP-binding cassette (ABC) transporters, such as ABCC1 [126]. This transportation allows S1P to act locally in an autocrine and/or directly in a paracrine manner [127]. Conversely, basal endogenous, as well as TNFα-induced, SK-1 activity can be deactivated via dephosphorylation of SK-1 at Ser225, via the protein phosphatase 2A (PP2A) [128, 129]. Furthermore, previous studies have shown that some cell types, such as endothelial cells, monocytes and macrophages, can secrete SK-1a from the cells via an unidentified channel or transporter, to allow for production of S1P outside of the cell [130-133]. SK-2 is not as well characterized than SK-1 but two specific phosphorylation sites at Ser351 and Thr578 have been proposed to mediate SK-2 activation [109]. The subcellular localization of SK-2 is more complex than that of SK-1 (Figure 1.6.2b). The predominant location of SK-2 is cell type specific. For

example, SK-2 is primarily located in the nucleus of COS-7 fibroblasts and in the cytoplasm of HEK293 cells [134]. When SK-2 is located in the nucleus and is associated with histone deacetylases (HDAC)-1 and -2 in repressor complexes, it can inhibit DNA synthesis to cause cell cycle arrest at G1/S phase [134, 135]. SK-2 can also be found at the endoplasmic reticulum (ER) and mitochondria, where it produces S1P [123, 136]. Notably, these S1P generated at the ER are likely to be dephosphorylated and degraded rapidly as they are in close proximity with ER-bound S1P lyase and S1P phosphatase. The dephosphorylation of S1P to sphingosine by S1P phosphatase can be further converted back to the ceramide by ER-localised ceramide synthase, which together can enhance the pro-apoptotic effects [137], whereas S1P lyase degrades S1P permanently [107].

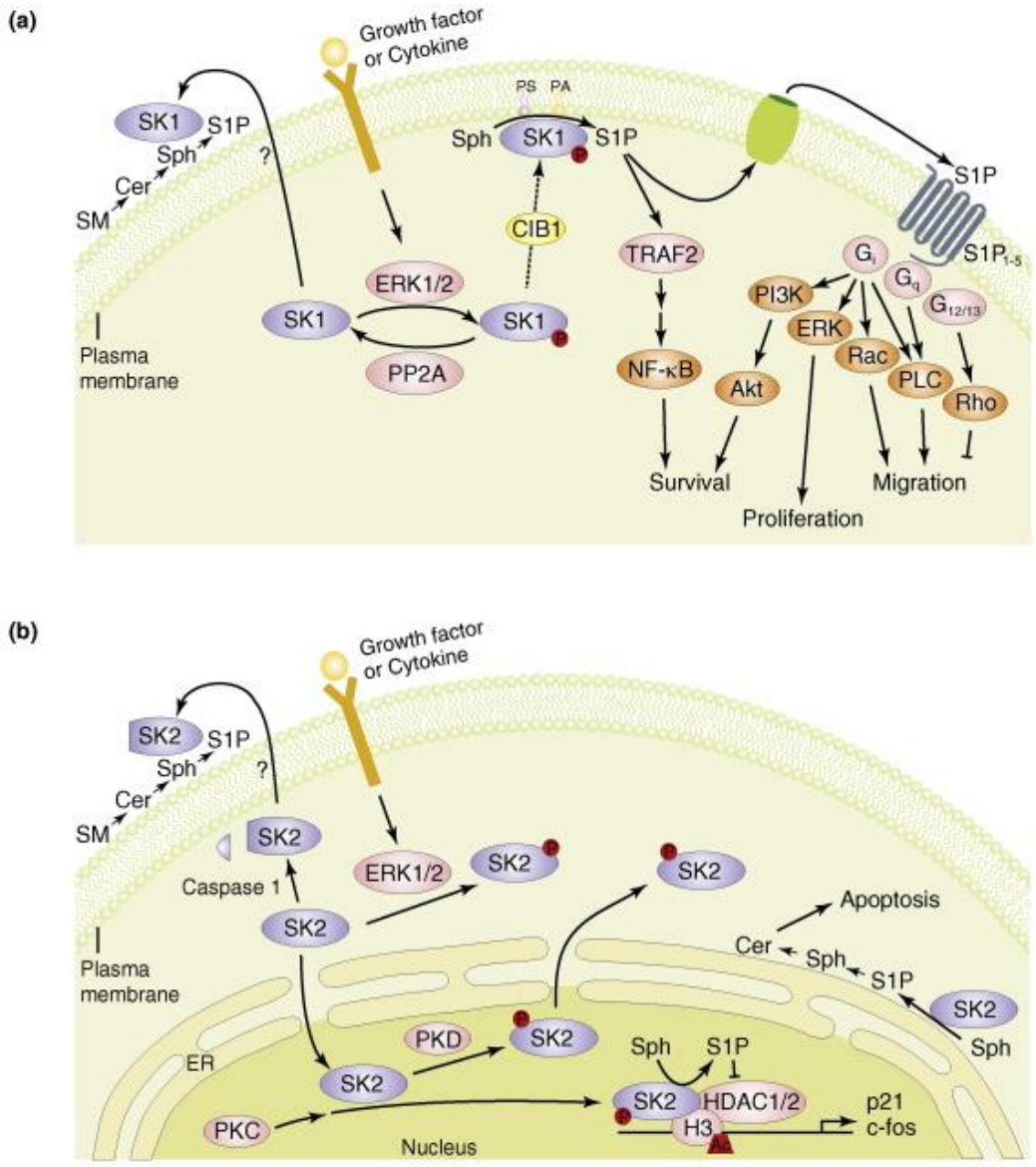


Figure 1.6.2b Subcellular locations of SK-1 and SK-2. (a) cytosolic SK-1 can be activated and deactivated by ERK1/2 and PP2A, respectively. The phosphorylated SK-1 is translocated to the inner plasma membrane to convert sphingosine to S1P. Intracellular S1P can be transported out of the cells and then act on its receptors $S1P_{1\text{-}5}$, or be retained inside the cells to interact with its target molecules, such as TRAF2. SK-1 can be secreted to the periphery by select cells and then produce S1P extracellularly. (b) SK-2 that resides in the nucleus can be activated and bind with

HDAC1/2 to regulate gene expression. SK-2 is also found in the cytoplasm, especially at the ER, where S1P production occurs. S1P produced at the ER is degraded or dephosphorylated rapidly to sphingosine and ceramide. Truncated SK-2 is also suggested to be transported out of the cells to produce extracellular S1P.

(Adapted from Pitson [107])

It has been shown that SK has intrinsic basal activity and can be further activated by a number of biological mediators, such as histamine [114], cross-linking of IgE receptor FcεRI [138], TNFα [62], vascular endothelial growth factor (VEGF), complement C5a [139], bradykinin [140] and IL-1β [141]. Hypoxia has also been shown to rapidly induce activation of SK-2 *in vivo* [142]. In response to these biological stimuli, the catalytic activity of SK-1 and SK-2 can increase by 2 to 6-fold [109, 143]. To further study the biological roles of SK-1 and SK-2, genetically modified mice with deletion of either *Sphk1* or *Sphk2* have been generated with no phenotypic abnormalities observed under normal conditions [144, 145]. In *Sphk1*$^{-/-}$ mice, SK-1 activity has been examined in most organs by the enzymatic activity assay and shown to be largely reduced when compared to that of wildtype (WT) mice [144]. Interestingly, the levels of S1P in those *Sphk1*$^{-/-}$ organs exhibit little to no reduction. In contrast, the serum and plasma of *Sphk1*$^{-/-}$ mice exhibit approximately a 50% reduction in S1P levels when compared to WT counterparts. This suggests that a compensatory effect by SK-2 exists [144]. In *Sphk2*$^{-/-}$ mice, while the catalytic activity of SK-1 is unaffected, the SK-2 activity is reduced by more than 90% in the organs by a similar enzymatic activity assay when compared to WT counterparts [146]. Surprisingly, *Sphk2*$^{-/-}$ mice have abnormally high levels of S1P in plasma (>150%) but only modestly elevated S1P level in lymphoid tissues when compared to WT mice

[146-148]. The mechanisms underpinning this phenomenon are still not fully understood. Importantly, deletion of both *Sphk1* and *Sphk2* is embryonic lethal in mice by day E13.5 due to the severe defects in vasculogenesis and neurogenesis [149]. Heterozygous-knockout mice with a loss of three out of four *Sphk* genes (ie $Sphk1^{-/-}Sphk2^{-/+}$ and $Sphk1^{-/+}Sphk2^{-/-}$) have been described. The female $Sphk1^{-/-}Sphk2^{-/+}$ mice show an early pregnancy loss due to breakage of blood vessels in the uterine, which suggests that a certain level of SK is required to maintain the vascular integrity [150]. Although generating $Sphk1^{-/-}Sphk2^{-/-}$ in the whole animal from birth is not feasible, recent technology allows $Sphk1^{-/-}Sphk2^{-/-}$ to be cell lineage restricted *in vivo*. For example, cross-breeding $Sphk1^{flox/flox}$ or $Sphk2^{flox/flox}$ with $LysM\text{-}Cre^{+}$ (lysozyme M promoter in myeloid cells) mice can generate the myeloid-specific $Sphk1^{-/-}Sphk2^{-/-}$ mice, with normal SK-1 and SK-2 activity in other non-myeloid cell types [151].

1.6.3 Sphingosine-1-phosphate (S1P)

As mentioned, S1P is the biological product of both SK-1 and SK-2, which can be secreted out of the cells or to be retained inside the cells. Notably, platelets release the highest levels of S1P with 0.4-1.1 μM identified in serum [152-155]. In the periphery, extracellular S1P can bind to the S1P receptors ($S1P_{1, 2, 3, 4, 5}$), which are the

G-coupled protein receptors associated with various Gα proteins (eg $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12/13}$) to activate downstream signalling pathways, including PI3K/Akt, Bcl2, ERK, phospholipase C and p53s [107, 156-159] (Figure 1.6.3a). The expression of S1P receptors is cell type specific, with an example that only $S1P_{1-3}$ being expressed by ECs [159]. The intracellular binding targets/receptors for S1P have also been investigated in the last decade. First, nuclear SK-2 is associated with histone H3 to produce S1P, which binds to histone deactylases (HDAC)-1 and -2 to regulate histone acetylation [135]. Second, SK-1-produced S1P binds to TNF receptor-associated factor 2 (TRAF2), which is a key component in NF-κB signalling, to regulate inflammation, anti-apoptotic and immune responses [160], and finally, S1P that is produced by mitochondrial SK-2 has high affinity and specificity to prohibitin (PHB)-2 which regulates mitochondrial assembly and function [161] (Figure 1.6.3b). Recently, we have shown that the transcriptional factor peroxisome proliferator-activated receptor (PPAR)-γ is the first transcriptional factor that acts as an intracellular binding partner for S1P (Parham et al, July 2014, manuscript currently under review with *FASEB J*). In addition to these intracellular binding partners for S1P, the transcription factor, interferon-regulatory factor 1 (IRF1), can form a complex with SK-1 and the apoptosis inhibitor cIAP2 to promote the release of CXCL10 and CCL5 for the recruitment of mononuclear cells during inflammation [162]

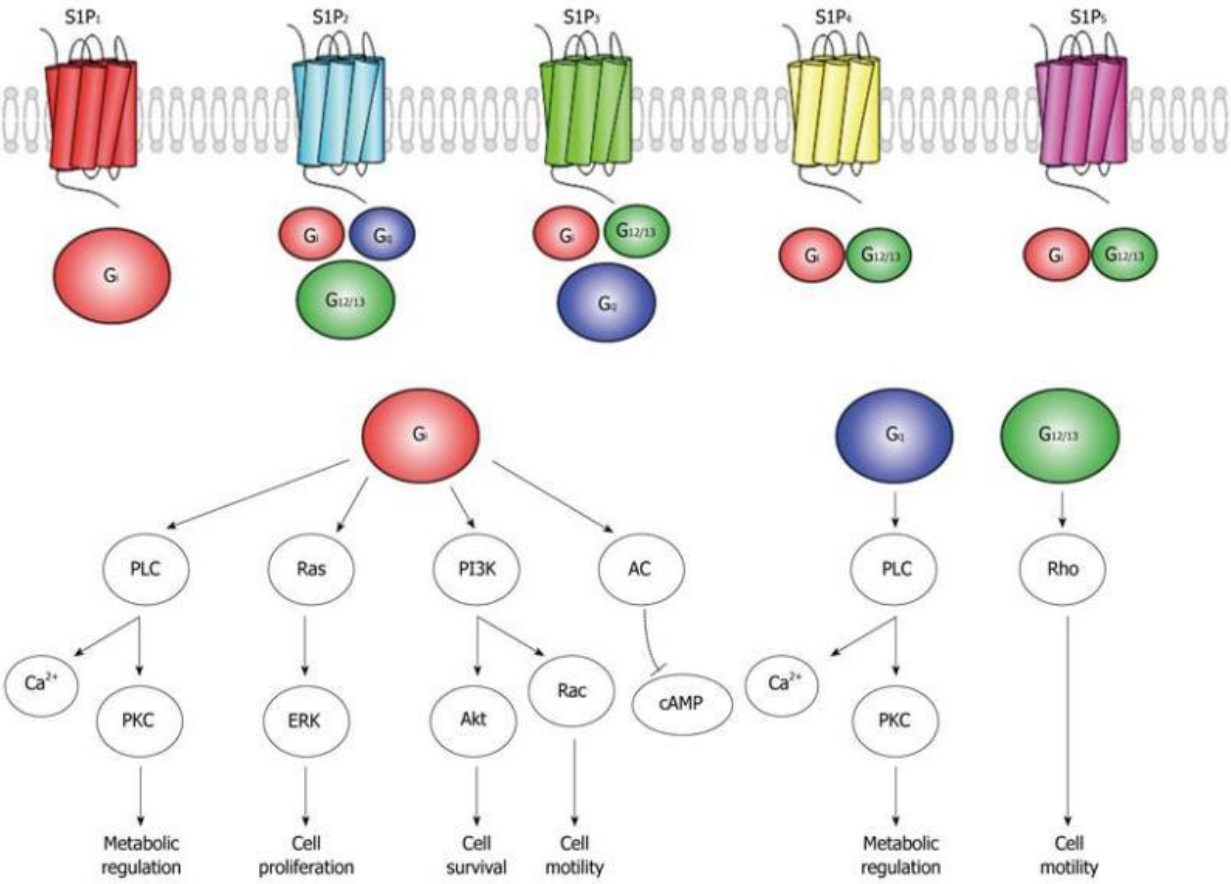


Figure 1.6.3a S1P cell surface receptors and their cellular signalling. $S1P_{1\text{-}5}$ depict the five G-protein-coupled receptors for S1P, each of which is associated with one or more cytosolic G proteins and secondary messengers that induce signalling pathways and subsequent cellular effects. G_i protein (red) is present in all S1P receptors; G_q protein (blue) is only associated with $S1P_{2\&3}$ and $G_{12/13}$ protein (green) is present in $S1P_{2\text{-}5}$.

(Adapted from Kawabori et al [163])

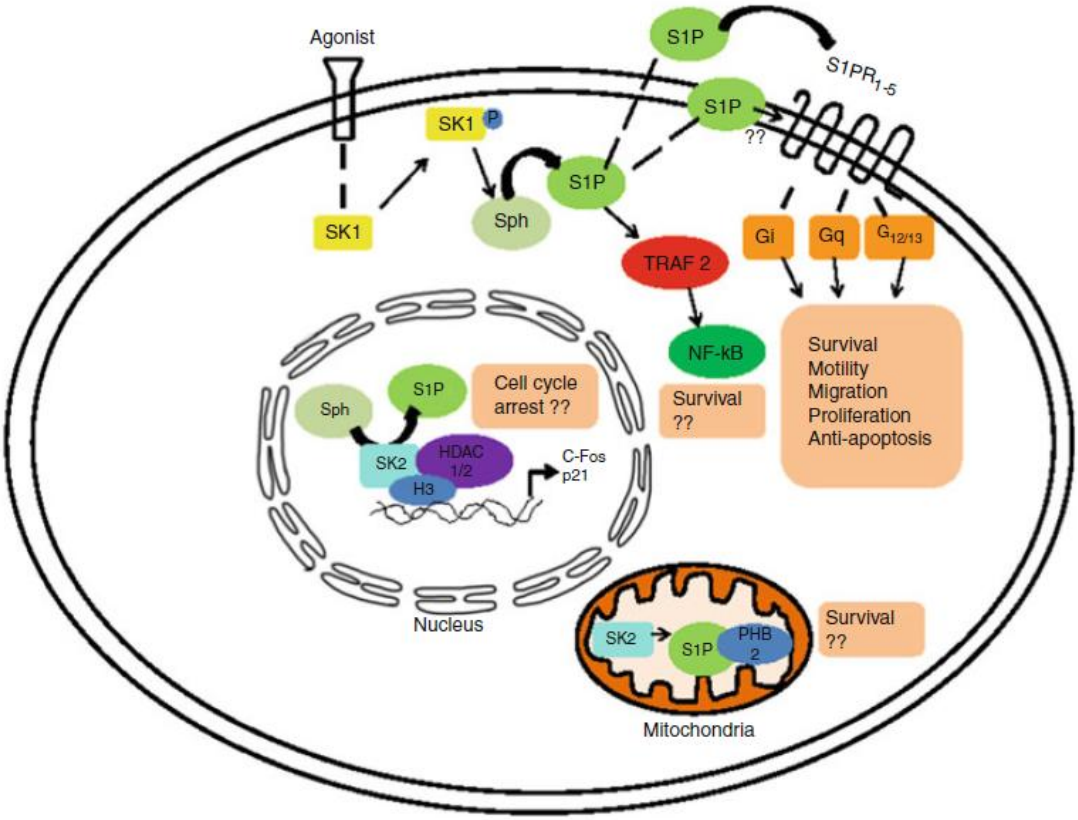


Figure 1.6.3b Intracellular targets for S1P. Two intracellular binding targets have been identified for SK-2-generated S1P, which are HDAC1/2 in the nucleus and PHB-2 in the mitochondria. The former can cause inhibition of DNA synthesis and arrest of cell cycle, whereas the latter can down-regulate mitochondrial functions and is suggested to reduce cell survival. Moreover, TRAF-2 has been identified as the cytosol binding target for SK-1-generated S1P, which is associated with the NFκB signalling for cell survival.

(Adapted from Selvam et al [164])

1.7 Role for SK/S1P in inflammatory cells

SK and S1P have been shown to regulate cellular functions of many cells, including mast cells [138], neutrophils [165], ECs [166], DCs [167] and T lymphocytes [168], all of which are important players during allergic inflammation (Figure 1.7a). The role of SK-1 and SK-2 remain controversial in mast cells, with Olivera et al showing that SK-1 primarily produces S1P, which regulates the severity of anaphylaxis, and SK-2 is involved in mast cell degranulation, cytokine and eicosanoid production *in vivo* [148]. However, Pushparaj et al showed that the mast cell-mediated anaphylactic reactions are SK-1, but not SK-2, dependent *in vitro* and *in vivo* [169]. These contradictory results may arise from using mast cells that are derived from different tissues, different species of origin and/or different culture conditions. For example, murine peritoneum-derived mast cells (PDMCs) have a denser granule content and express a higher level of mast cell protease-1, 2, 4, 5 and 9 than that of bone marrow-derived mast cells (BMMCs) [170]. Furthermore, Dillahunt et al have used PDMCs, BMMCs and liver-derived mast cells isolated from mice, as well as BMMCs and cord blood-derived mast cells isolated from humans to examine the roles of SK isoforms in mast cell functions [170]. They concluded that human mast cell functions are solely dependent on SK-1, whereas murine mast cell functions are more SK-2 dominant but also require SK-1 [170]. In addition to SK isoforms,

$S1P_1$ has been shown to induce migration of mast cells toward the allergens and $S1P_2$ can cause degranulation and inhibition of migration of mast cells [171].

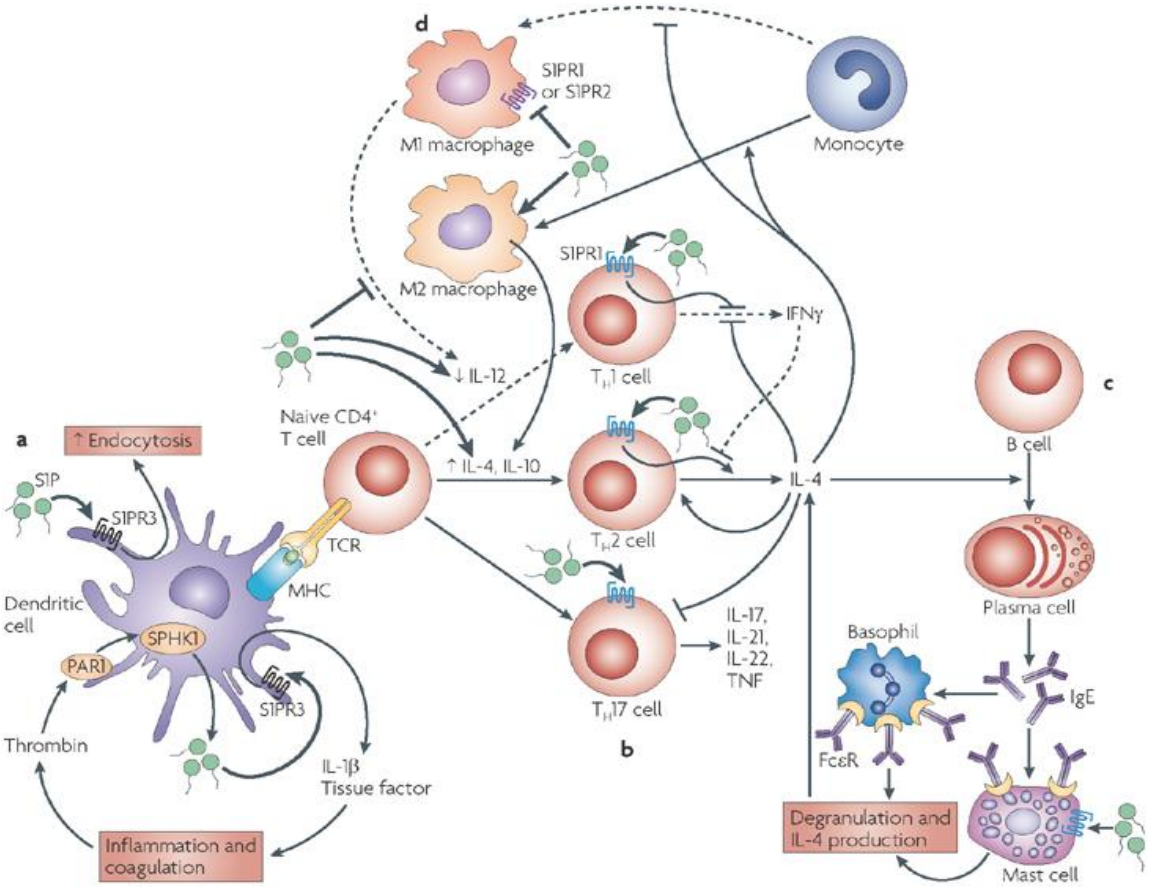


Figure 1.7a S1P and S1P receptors regulate the functions of inflammatory cells.

(a) In DCs, S1P mediates endocytosis and release of inflammatory cytokines via $S1P_3$. The DC-released cytokines and inflammatory mediators can promote SK-1 activity to produce S1P which further enhance the inflammatory functions of DCs. (b) In T lymphocytes, S1P promotes the production of Th2 and Th17 cytokines and inhibit the release of IFNγ by Th1 cells via $S1P_1$. (c) S1P promotes migration and degranulation of mast cells via $S1P_1$ and $S1P_2$, respectively. (d) S1P binds to $S1P_1$ and $S1P_2$ on macrophages to promote the polarization of macrophage and release of cytokines to enhance Th2 cellular functions.

(Adapted from Rivera et al [172])

Unlike mast cells, the roles of SK/S1P in neutrophils are less understood. Upon activation by platelet activating factor (PAF), TNFα and substance P, neutrophils exhibit an increase in SK activity and S1P production, which is associated with intracellular calcium flux for cell mobilization and migration [173]. Inhibition of SK abolishes the release of calcium from the internal store within neutrophils and thus, the complement C5a-mediated chemotaxis and degranulation of neutrophils are attenuated [174]. Furthermore, administration of S1P promotes the polymorphonuclear neutrophil Fcγ receptor-mediated intracellular calcium influx and reactive oxygen species production, which subsequently enhance the activation of neutrophils [175]. Binding and activation of $S1P_1$ signalling in neutrophils can further promote neutrophil infiltration to an inflammatory site resulting in tissue oedema and thermal hyperalgesia *in vivo* [165].

1.8 SK/S1P and EC adhesion molecule expression during allergic inflammation

The expression of adhesion molecules on ECs allows the inflammatory cells to be recruited from the circulation to a specific inflammatory site [reviewed in 54]. To this end, SK/S1P has been shown to be a key regulator of adhesion molecules expressed by ECs. For example, E-selectin [62], VCAM-1 [62] and ICAM-1 [176] are up-regulated on human umbilical vein ECs (HUVECs) in response to histamine or

TNFα. This process has shown to be $S1P_1$ dependent and via phosphorylation of the p38 mitogen-activated protein kinase (MAPK) pathway [177, 178].

In addition, integrins are expressed by ECs as an inactive state under basal conditions. We have shown that TNFα-induced SK-1 activity mediates the activation (but not the transcription or translation) of $\alpha_5\beta_1$ integrin, which is required for neutrophil adhesion under shear stress [61]. This TNFα-induced $\alpha_5\beta_1$ integrin activation is independent of cell surface S1P receptors. Furthermore, we have shown that the binding partner for EC $\alpha_5\beta_1$ integrin is $\alpha_5\beta_1$ integrin on neutrophils via a ligand angiopoietin-2 (Appendix 1, p.238) [61].

Taken together, the aforementioned studies demonstrate a role for SK/S1P in the expression of E-selectin, ICAM, VCAM and $\alpha_5\beta_1$ integrin. However, none of these adhesion molecules contribute to the rapid recruitment of leukocytes during allergic inflammation. Of note, P-selectin, which is pre-stored in WPBs, perfectly poised to be rapidly expressed on ECs and thus induce the initial tethering and rolling events observed during acute allergic inflammation [179]. Whether SK/S1P has a role in P-selectin surface expression has not been examined and thus, it forms Aim 1 of my study.

1.9 Inhibition of SK/S1P pathway as a therapeutic target for allergic inflammation

Increasing evidence shows that sphingolipids regulate the expression of adhesion molecules on the endothelium [61, 62, 176]. Therefore, inhibition of SK/S1P can be a biological target for controlling leukocyte recruitment to prevent and attenuate allergic inflammatory diseases. Furthermore, our review publication [114], discussed that inhibition of SK/S1P pathway can be a more effective therapeutic approach to tackle allergic inflammation when compared to the other treatments, such as anti-selectin therapy and immunosuppression (Appendix 3, p.240). Herein, aim 1 of this study is to examine whether SK/S1P regulates histamine-induced surface expression of P-selectin on ECs. Aim 2 focuses on testing and translating this work from the laboratory bench to clinical use. It incorporates *in vivo* experiments utilizing two different allergy models in mice thus allowing us to gain a better understanding of potentially new treatment options for allergic diseases. Investigation of a new and optimal SK/S1P inhibitor for human clinical trials is the ultimate aim of this study and thus a future direction.

Chapter 2: Aims and Hypotheses

The overall aim of this study is to examine whether SK/S1P has a role in the early phase of allergic inflammation and if they can be used as a therapeutic target to combat allergic inflammatory diseases.

There are two hypotheses and two main aims in this thesis, which form Chapter 3, 4, 5 and 6.

Hypothesis 1: SK/S1P mediates P-selectin surface expression and recruitment of neutrophils during the early phase of allergic inflammation.

Aim 1:

- 1.1 To examine whether SK/S1P regulate histamine-induced P-selectin surface expression by ECs
- 1.2 To examine whether S1P receptors and their downstream signalling pathways are involved in histamine-induced P-selectin surface expression by ECs
- 1.3 To examine whether inhibition of SK/S1P can attenuate histamine-induced P-selectin expression by ECs
- 1.4 To examine whether inhibition of SK/S1P can attenuate histamine-induced neutrophil recruitment *in vitro* and *in vivo*

- 1.5 To examine whether SK regulates exocytosis of P-selecin via intracellular calcium influx

Hypothesis 2: SK inhibitors (eg Fingolimod) can be used as a topical therapeutic agent to treat allergic inflammation via inhibition of SK activity

Aim 2:

- 2.1 To examine whether allergic inflammation can be induced by histamine in a dose dependent manner *in vivo*
- 2.2 To examine whether Fingolimod can attenuate allergic inflammation via inhibition of SK/S1P
- 2.3 To examine whether phosphorylation of Fingolimod is required to attenuate allergic inflammation
- 2.4 To examine whether Fingolimod can be used as a topical agent to prevent allergic inflammation
- 2.5 To examine whether Fingolimod can be applied topically following histamine or allergen challenge to reduce allergic inflammation
- 2.6 To examine whether topical application of Fingolimod can attenuate neutrophil recruitment and pro-inflammatory cytokines during allergic inflammation

- 2.7 To examine whether the novel SK inhibitor MP8 can be used as a topical agent to treat histamine-induced inflammation

Chapter 3: SK-1 regulates the surface expression of P-selectin during an early phase of allergic inflammation

During the initial/early phase of allergic inflammation, P-selectin is rapidly expressed onto the surface of ECs to mediate the tethering and rolling of leukocytes [44, 179]. The mechanism underpinning P-selectin surface expression by ECs is not fully elucidated and thus forms Aim 1 of this study.

Chapter 3 includes the published manuscript and troubleshooting addressing my first hypothesis: SK/S1P is a regulator of P-selectin surface expression on ECs to recruit neutrophils during the early phase of allergic inflammation.

3.1 Optimizing culture conditions for human umbilical vein endothelial cells to express P-selectin on the cell surface

In our laboratory, HUVECs are routinely isolated using collagenase and then cultured in 20% fetal calf serum (FCS) and endothelial growth factor supplements (GF) as per the previously published protocol [180]. However, this protocol of HUVEC culture does not retain all endothelial properties. For example, the ability of HUVECs to pre-form and pre-store P-selectin is lost within one passage [180]. Studies have described that retaining an ability to produce and express P-selectin on the HUVEC surface is one of the major challenges when working on this adhesion molecule [180]. Notably, retention of P-selectin expression is short-term and thus, cell manipulation to alter P-selectin expression by small interfering (si)-RNA transfection or viral infection, which require more than two passages in culture, are not feasible. Herein, I have found that HUVECs can express P-selectin in both primary and the first passage by culturing them in 20% human serum.

As shown in Figure 3.1a, untreated HUVECs in 20% human serum do not express P-selectin on the cell surface as determined by immunofluorescence antibody staining. However, in response to histamine challenge for 5 min, which mimics the time period during the early phase of allergic inflammation, surface expression of P-selectin was detected in the primary and first passage of HUVECs cultured in

human serum. To note, histamine-induced P-selectin surface expression on HUVECs was largely lost by the second passage when compared to that of primary or first passage HUVECs (Figure 3.1a). Thus, only primary or first passage of HUVECs that were cultured in 20% human serum without GF, were utilised in the subsequent experiments.

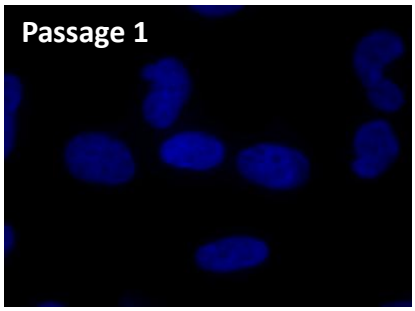
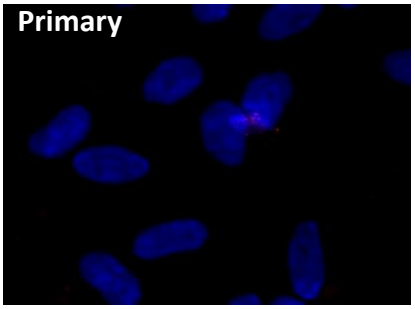
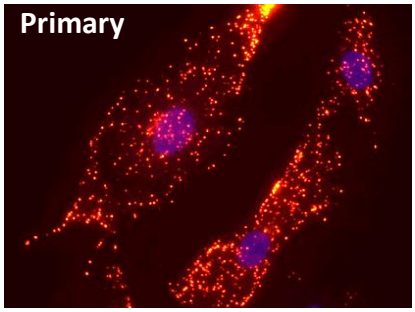
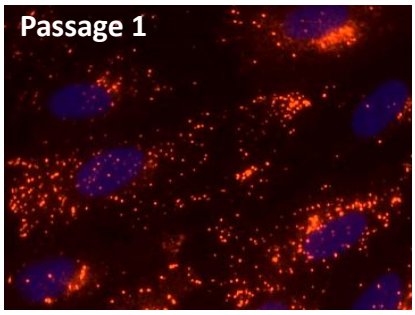
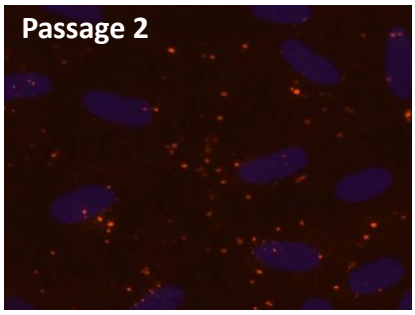
Treatment / Culture condition	Untreated	Histamine (25 µM, 5 min)
20% FCS + GF		
20% Human serum		  

Figure 3.1a Histamine-induced P-selectin surface expression by HUVECs that were cultured in 20% human serum. HUVECs were isolated and then cultured in either 20% FCS + GF or 20% human serum. Primary, first and second passages of HUVECs were treated without or with histamine (25 μM, 5 min) immediately following fixation, P-selectin antibody staining (red), permeabilization and then nuclear DAPI staining (blue). A representative image of immunofluorescence microscopy is shown (n=5), with magnification at X100.

3.1.1 Materials and methods (for Chapter 3.1)

Immunofluorescence microscopy

Collagenase-digested HUVECs (ie. primary passage HUVECs) were cultured on a fibronectin (Fn; 50 μg/mL; Sigma-Aldrich Pty. Ltd., Sydney, Australia) coated cover slip (15x15mm; ProSciTech, Queensland, Australia) in M199 medium (Sigma-Aldrich) containing either 20% FCS (JRH, Brooklyn, Vic, Australia) + GF (at 50 μg/mL; BD Biosciences, NSW, Australia) or in 20% human serum (Invitrogen, Carlsbad, CA, USA). Penicillin (100 U/mL) and streptomycin (100 μg/mL) (both from Invitrogen, Gibco BRL, Paisley, Scotland) were added to all cell culture. Fresh medium was changed after 48 h and experiments were performed when HUVECs reached 90% confluence.

In addition, HUVECs were replated at $1x10^5$ cells on the Fn-coated cover slip (ie first and second passages). Confluent HUVECs were treated without or with human recombinant histamine (25 μM, 5 min, Sigma-Aldrich) prior to fixation with 4% paraformaldehyde at room temperature (RT), for 15 min and blocking with 2% bovine serum albumin (BSA) in PBS (RT, 30 min). Antibody staining for P-selectin (AK-4 clone, 1 μg/mL, Bioscience, Franklin, NJ, USA) was performed overnight at 4°C followed by anti-mouse Alexa Fluor 594 (1:1000, Rockland Inc, South Australia, Australia) incubation at RT for 1 h. HUVECs were permeabilized with 0.1%

Triton-X100 in PBS (RT, 15 min) prior to DAPI staining (100 nM in PBS, RT, 3 min). Slides were mounted with Biomeda gel (ProSciTech) prior to visualization under an Olympus IX70 inverted microscope (Olympus, Tokyo, Japan) which was linked to a Bio-Rad Radiance 2100 confocal microscope (Bio-Rad Laboratories, Gladesville, Australia).

3.2 Rapid histamine-induced neutrophil recruitment is sphingosine kinase-1 dependent

Having optimized the culture conditions for HUVECs to be able to express P-selectin on the cell surface upon histamine challenge (Figure 3.1a), I then examined (1) whether or not SK/S1P regulated histamine-induced P-selectin surface expression by ECs, (2) which isoform of SK was involved in histamine-induced P-selectin surface expression, (3) the mechanism underpinning sphingolipid exocytosed WPBs that contain P-selectin to the EC surface, (4) which S1P receptors were at play if the S1P receptor signalling was required for this process of histamine-induced P-selectin expression, (5) whether histamine-induced P-selectin surface expression was required for the recruitment of neutrophils during the early phase of allergic inflammation, and (6) whether inhibition of SK/S1P could attenuate histamine-induced P-selectin surface expression and neutrophil recruitment.

Chapter 3.2 includes the published manuscript, Sun et al. 2012 *Am J Path*, which addressed my hypothesis 1: SK/S1P is a regulator of P-selectin surface expression to recruit neutrophils during the early phase of allergic inflammation. (Statement of Authorship, Chapter 3.2.1, p.136)

RAPID HISTAMINE-INDUCED NEUTROPHIL RECRUITMENT IS SPHINGOSINE KINASE-1 DEPENDENT

Wai Y. Sun*†§, Latasha D. Abeynaike‡, Samantha Escarbe*, Charles D. Smith¶, Stuart M. Pitson*§#, Michael J. Hickey‡ and Claudine S. Bonder*†§#

*Human Immunology, Centre for Cancer Biology, South Australia Pathology, Adelaide, South Australia; †School of Medicine, University of Adelaide, Adelaide, South Australia; §Co-operative Research Centre for Biomarker Translation, La Trobe University, Victoria, Australia; ‡Centre for Inflammatory Diseases, Department of Medicine, Monash Medical Centre, Monash University, Victoria, Australia; Medical University of South Carolina, South Carolina, USA and #School of Molecular and Biomedical Sciences, University of Adelaide, Adelaide, South Australia.

Address for correspondence: Dr. Claudine S. Bonder, Centre for Cancer Biology, South Australia Pathology, Frome Road, Adelaide, SA 5000, Australia. E-mail: claudine.bonder@health.sa.gov.au.

Content: 35 text pages, 5 figures, 2 supp. figure & 3 supp. videos

Running head: SK-1 regulates neutrophil rolling events

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ABSTRACT

Leukocyte recruitment to sites of inflammation is critical for the development of acute allergic responses. Rapid P-selectin upregulation by endothelial cells is a key promoter of leukocyte infiltration in response to mediators such as histamine. However, the mechanisms underpinning this process are still incompletely understood. Here, we examined the role of the sphingosine kinase/sphingosine-1-phosphate (SK/S1P) pathway and show that in human umbilical vein endothelial cells, histamine rapidly activated SK in an extracellular-signal regulated kinase (ERK) 1/2-dependent manner, concurrently with inducing P-selectin expression. Histamine activated both SK-1 and SK-2 isoforms but it is inhibition of SK-1, and not SK-2, which attenuated histamine-induced P-selectin upregulation and neutrophil rolling *in vitro*. S1P receptor antagonists failed to prevent histamine-induced P-selectin expression, and exogenous S1P did not increase P-selectin expression suggesting that S1P cell surface receptors were not involved in this process. Finally, the role of SK-1 and SK-2 in histamine-induced leukocyte rolling *in vivo* was assessed using pharmacologic and genetic means. Consistent with the *in vitro* findings, mice pretreated with either SKi or Fingolimod significantly attenuated histamine-induced leukocyte rolling in the cremaster muscle. Similarly, *Sphk1-/-* but not *Sphk2-/-* mice exhibited reduced histamine-induced leukocyte rolling. These studies demonstrate a key role for SK-1 in

histamine-induced rapid P-selectin upregulation and associated leukocyte rolling, and suggest that endothelial SK-1 is an important contributor to allergic inflammation.

INTRODUCTION

Inflammation is central to the development of acute allergic responses. The allergic inflammatory response is a multistep process involving increased vascular permeability, changes in expression of endothelial cell adhesion molecules and the triggering of cell-cell interactions between circulating leukocytes and the vascular endothelium. Several types of adhesion molecules are involved in leukocyte binding and transmigration and their expression is tightly regulated to produce the sequence of events that leads to leukocyte recruitment. In allergic inflammation these events are coordinated by inflammatory mediators including histamine. Histamine activates the local vasculature by binding to its G-protein coupled receptors, H_1 and H_2, on endothelial cells (ECs) causing a rapid exocytosis of preformed adhesion molecule P-selectin [181, 182]. Circulating neutrophils are immediately recruited by tethering and rolling along the vasculature via a P-selectin/P-selectin glycoprotein ligand-1 (PSGL-1) mechanism [181]. This ability of P-selectin to undergo a rapid increase in exposure on the endothelial surface plays a critical role in development of this initial phase of the allergic response. As such it is important that the molecular basis of this response be completely understood.

P-selectin is constitutively synthesized in endothelial cells [183], megakaryocytes/platelets [184] and resident peritoneal macrophages [185], where it is

packaged into Weibel-Palade body and alpha storage granules [184, 186]. Two distinct mechanisms regulate the inducible expression of P-selectin. In mice, mediators such as tumor necrosis factor (TNF), interleukin-1 and lipopolysaccharide (LPS) can induce transcription of P-selectin mRNA with subsequent protein synthesis and surface expression. However, this response is not seen in human endothelial cells due to the lack of binding sites for NF-κB and activating transcription factor (ATF)-2 in the human *Selp* gene promoter [97, 98, 187]. Alternatively in both species, P-selectin can be rapidly mobilized to the endothelial surface from Weibel-Palade bodies in response to mediators such as histamine, thrombin and other secretagogues [188]. This mechanism does not require new protein synthesis, instead being induced by rapidly-acting signaling molecules within endothelial cells. For mediators associated with allergic inflammation, such as histamine, the signaling molecules involved in this rapid response are not fully characterized, but one candidate is the sphingosine kinase pathway.

Sphingosine kinase (SK) is a highly conserved lipid kinase. Two isoforms (SK-1 and SK-2) have been identified, cloned and characterized [122, 123]. Both SK-1 and SK-2 catalyze the phosphorylation of sphingosine to form sphingosine-1-phosphate (S1P), but they exhibit different subcellular localization patterns, developmental expression, distribution in adult tissue and have been recognized to have both

overlapping and alternative biological functions [113]. S1P is a bioactive phospholipid and is an important signalling molecule which can be either retained inside or secreted out of the cell. Basal levels of S1P in cells are generally low but can increase rapidly when cells are exposed to various agonists through rapid and transient activation of SK activity as a result of phosphorylation on Ser225 by extracellular signal-regulated kinases (ERK) 1/2 [109]. Extracellular S1P acts on its G protein coupled receptors, $S1P_{1-5}$, in both autocrine and paracrine fashions with, for example, downstream signaling of phosphatidyl inositol 3-kinase (PI3K)/Akt and ERK1/2 [113]. Alternatively, endogenous S1P can associate with histone deacetylases (HDAC1 and HDAC2) [135], tumor necrosis factor receptor-associated factor 2 (TRAF2) [160], prohibitin [161] or via as yet unidentified targets. S1P has previously been shown to synergize with histamine during a 4 h exposure to promote gene and surface expression of E-selectin, ICAM-1, and VCAM-1 [177]. However the contribution of the SK pathway to rapid leukocyte recruitment typical of allergic responses has not been investigated.

Here, we identify SK-1 as a new potential target for controlling rapid recruitment of neutrophils following exposure to histamine. First, we demonstrate that both SK-1 and SK-2 are rapidly activated by histamine in human umbilical vein endothelial cells (HUVEC) and that this occurs in an ERK1/2-dependent manner. Second,

histamine-induced surface expression of P-selectin expression on HUVEC requires both ERK1/2 and SK-1 but does not involve SK-2 or the $S1P_{1-3}$ surface receptors. Finally, we demonstrate that histamine-induced SK-1, but not SK-2, activity mediates neutrophil recruitment *in vitro* and *in vivo*. Collectively, this study suggests that SK-1 may be a critical regulator controlling acute allergic responses.

MATERIALS AND METHODS

Reagents and antibodies

Antibodies against human P-selectin (AK-4) and isotype control were purchased from BD Biosciences (Franklin Lakes, NJ). Phosphorylated ERK1/2 and total ERK1/2 were purchased from Cell Signaling (Danvers, MA). Human SK-1 antibody was generated as previously described [109]. Secondary antibodies anti-rabbit-HRP (Pierce, Rockford, IL), anti-rabbit-Alexa 594, anti-mouse Alexa 488 and DAPI (Invitrogen, Carlsbad, CA) were used. Human recombinant histamine, histamine-1-receptor antagonist (Chlorpheniramine) and histamine-2-receptor antagonist (Cimetidine) were purchased from Sigma (St Louis, MO). Sphingosine kinase inhibitor (SKi) and S1P were purchased from Cayman Chemical Co. (Ann Arbor, MI). Inhibitors N,N-Dimethylsphingosine (DMS, Biomol, Plymouth Meeting, PA); $S1P_1$ receptor antagonist (W146, Cayman Chemical Co.); $S1P_2$ receptor inhibitor (JTE013, Cayman Chemical Co.); $S1P_3$ receptor antagonist (CAY10444, Cayman Chemical Co.); $S1P_{1\&3}$ receptor inhibitor (VPC23019, Avanti Polar Lipids Inc., Alabaster, AL); Fingolimod (FTY720; Sapphire Bioscience, Waterloo, NSW, Australia); MAPK pathway inhibitors (U0126, Cell Signaling; SB203580 and PD98059, Alexis Biochemicals (Plymouth Meeting, PA)) were purchased. SK-2 inhibitor (ABC294640) has been previously described [189].

Animals

Wildtype (WT), SK-1 knock-out (*Sphk1*$^{-/-}$) and SK-2 knock-out (*Sphk2*$^{-/-}$) mice were on a C57Bl/6 background [144, 145], housed under pathogen-free conditions at SA Pathology (South Australia, Australia) as well as Monash University (Victoria, Australia) and used between 6-12 weeks of age. All experimental procedures were approved by the Animal Ethics Committee of SA Pathology, the University of Adelaide and Monash University, and conform to the guidelines established by "Australian Code of Practice for the Care and Use of Animals for Scientific Purposes".

Cells and cell culture

The collection of human umbilical cords for use in this study was given ethical clearance from the Human Research Ethics Committee of the Children, Youth and Women's Health Service (CYWHS), North Adelaide, South Australia and informed written consent was obtained from all subjects in accordance with the Declaration of Helsinki. Human umbilical vein endothelial cells (HUVEC) were isolated as previously described [180]. HUVEC were grown in M199 medium (Sigma) containing 20% human serum (Invitrogen), 100 U/ml penicillin and 100µg/ml

streptomycin (Gibco BRL, Paisley, Scotland). Cells were cultured on 10% gelatin (Sigma) and used at passage 1.

Neutrophils and lymphocytes were enriched from the venepuncture of consenting healthy donors as previously described [190]. Briefly, dextran sedimentation preceded cells being were enriched by density-gradient centrifugation on Lymphoprep (Nycomed, Oslo, Norway) with the neutrophils pelleting at the base and the lymphocytes enriched at the interface. Following hypotonic lysis of erythrocytes, cells were resuspended in RPMI media containing 10 mM HEPES and 2.5% fetal bovine serum (FBS; Gibco BRL)) before use. Cytological examination of stained cytocentrifuged preparations by May-Grunwald Giemsa (Sigma) showed >95% of the cells were neutrophils or lymphocytes. Trypan blue staining confirmed over 98% of these cells were viable. The human Jurkat T cell line was cultured in complete RPMI1640 medium (Gibco BRL) with 10% FBS. To quantify the degree of Jurkat cell activation in response to histamine (25 μM, 30 min) or phorbol myristate acetate (PMA, 100 ng/ml 30 min), levels of L-selectin expression were measured using flow cytometry using 1 μg of mAb against L-selectin (Dreg56 mouse anti-human, gift E. Butcher) or a nonspecific isotype control (IgG_1, BD Biosciences) for 30 min on ice. Cells were then washed and incubated with Alexa488-conjugated anti-mouse Ig (1:1000 dilution, Invitrogen) for 30 min on ice. Stained cells were

resuspended in fluorescence-activated cell sorting (FACS) Fix (1% formaldehyde, 20 g/L glucose, 5 mM sodium azide in PBS) prior to analysis using a FACS Aria II (BD Biosciences) with FACS DIVA software (BD Biosciences). Further analysis was performed using FCS Express V3.0 (De Novo Software, Los Angeles, CA) against unstained cells gated at ≤1%.

SK activity assay

SK activity was determined as previously described [122]. For SK-1 activity, whole cell lysates were incubated with D-*erythro* sphingosine (Biomol) solubilised in either 0.05% or 0.1% Triton X-100 and [γ^{32}P]ATP (Perkin Elmer, Vic., Australia). For SK-2 activity, whole cell lysates were prepared in buffer containing 1M KCl and incubated with D-*erythro* sphingosine solubilised in BSA/PBS and [γ^{32}P]ATP. The radioactively labelled S1P was resolved by 2 thin-layer chromatography (TLC, Sigma) separations in the solvents containing butanol, ethanol, water and acetic acid (8:2:2:1). The radioactive spots were quantified by Phosphorimaging Typhoon 9410 (Fullerton, CA) and ImageQuant 5.2 program (GE Healthcare, Rydalmere, NSW, Aust.).

Western blotting

HUVEC were lysed in buffer containing 1% NP40 and sonicated. Cell lysates

were separated by 10% SDS-PAGE and transferred to Hybond-P membrane (Amersham Bioscience, NJ). Primary antibodies to pERK1/2 or total ERK1/2 probed the membrane overnight at 4℃ followed by secondary antibody incubation at room temperature (RT) for 1 h prior to visualization by ECL (GE Health Science, Piscataway, NJ) and a luminescent image analyser (LAS4000, Fujifilm, Stamford, CT).

MAPK, SK and S1P-receptor inhibition and S1P-receptor activation studies

SK inhibitor (SKi, 5 μM, 10 min), DMS (5 μM, 10 min), ERK1/2 pathway inhibitor (U0126, 10 μM, 30 min), p38 inhibitor (SB203580, 10 μM, 1 h), MEK inhibitor (PD98059, 25 μM, 30 min), S1P (1 μM, 10 min), Fingolimod (FTY720; 100 nM, 30 min), JTE013 (1 μM, 30 min), W146 (10 μM, 30 min), CAY10444 (10 μM, 30 min) or VPC23019 (10 μM, 30 min) were administered prior to histamine stimulation (25 μM, 5 min) in the activation and inhibition studies. All reagents were proven functionally effective in paralleled studies.

Immunofluorescence microscopy

HUVEC were replated at $5x10^4$ cells/well in fibronectin coated (50 μg/ml) LabTek chamber slides (Nunc, NY). Confluent cells were treated with SKi, DMS, S1P,

JTE013, VPC23019, W146, CAY10444, Fingolimod, U0126, SB203580, PD98059, Chlorpheniramine or Cimetidine without or with histamine stimulation (25 μM, 5 min). Cells were fixed with 4% paraformaldehyde at RT for 15 min prior to blocking with 2% BSA/PBS at RT for 30 min. P-selectin antibody (1 μg/ml) was added to cells overnight at 4℃ followed by anti-rabbit Alexa 594 antibodies (1:1000) incubation at RT for 1 h. Cells were then permeabilized with 0.1% Triton-X 100/PBS at RT for 10 min followed by DAPI staining (1:2000) at RT for 3 min. Slides were visualized by an Olympus IX70 inverted microscope linked to a BioRad Radiance 2100 confocal microscope (BioRad, Gladesville, NSW, Aust.). Five images were collected per sample and the fluorescence intensity was analysed using Analysis LifeSciences software (Olympus).

Parallel plate flow chamber assay

Confluent HUVEC cultured on Corning petri dishes (Sigma) were treated with isotype control antibody (10 μg/ml, 30 min), P-selectin blocking antibody (10 μg/ml, 30 min), SKi (5 μM, 10 min), DMS (5 μM, 10 min), Fingolimod (100 nM, 30 min), ABC294640 (10 μM, 10 min), U0126 (10 μM, 30 min), PD98059 (25 μM, 30 min) or SB203580 (10 μM, 1 h) prior to perfusion of histamine (25 μM, 2.5 min) followed by blood, neutrophils or lymphocytes. Using published methods, histamine (25 μM) was

prepared in Hank balanced salts solution (HBSS, Sigma) and perfused across the substratum by a syringe pump (NE-1000, New Era Pump System, Inc, Wartagh, NY) at a constant rate of 2 dynes/cm^2 for 2.5 min [114]. Peripheral blood was obtained by venepuncture from healthy donors after informed consent into heparinized syringes prior to 1:10 dilution with HBSS prior to perfusion for 5 min followed by HBSS wash. Alternatively, blood in acid-citrate-dextrose (ACD) was used to isolate neutrophils or lymphocytes prior to perfusion at 1x10^6 cells/ml for 5 min followed by HBSS wash. Unlabelled leukocyte, neutrophil or lymphocyte interactions were visualized by phase-contrast microscopy using 10X/0.3 NA objectives on an inverted microscope. Five random areas per dish were recorded using a digital camera (Olympus IX70 and SIS F-view, Olympus) for analyzing with AnalySIS Life Sciences software (Olympus). The number of rolling cells was analysed using a video microscopy system.

Intravital microscopy and *in vivo* experimental procedure

Intravital microscopy of the cremaster muscle was performed as previously described [191]. Two postcapillary venules (25-40 μm in diameter) were examined for each experiment. Images were visualized using a video camera and recorded on video-tape for subsequent analysis. Leukocyte rolling was assessed via playback analysis as previously described [191]. In experiments examining the effect of SK

inhibition, WT mice were injected subcutaneously with vehicle alone or SKi (50 mg/kg in DMSO/PBS) for 15 min prior or injected intraperitoneally with Fingolimod (0.5 mg/kg in PBS) for 60 min prior to intravital microscopy. A basal reading of leukocyte rolling flux was taken before histamine superfusion (100 μM in superfusion buffer) commenced. Additional recordings of leukocyte rolling were subsequently made at 5, 10, 20, and 30 min after commencing histamine superfusion. In a separate series of experiments, WT, *Sphk1*$^{-/-}$ and *Sphk2*$^{-/-}$ underwent the same model of histamine challenge.

Statistical analysis

Data is shown as mean ± SEM and statistically analysed by Student's t-test, 1- or 2-way ANOVA for multiple comparisons. $P<0.05$ was considered significant.

RESULTS

Histamine rapidly induces P-selectin expression and SK activity in HUVEC

Upon activation by histamine the vascular endothelium rapidly expresses preformed P-selectin at the cell surface for an immediate inflammatory response of leukocyte recruitment from the circulation and rolling along the vasculature [192]. Herein we used immunofluorescence microscopy to demonstrate that exposure of HUVEC to histamine for 5 min rapidly induces the surface expression of P-selectin (Fig. 1A). Histamine-induced P-selectin surface expression is not associated with increased mRNA levels (not shown) and occurs via the H_1 receptor, as pre-treatment of HUVEC with the H_1 receptor antagonist chlorpheniramine but not the H_2 receptor antagonist cimetidine inhibited these events (Fig. 1B) [193].

Huwiler et al previously demonstrated that prolonged exposure to histamine (> 2 h) increases sphingosine kinase (SK)-1 expression and activity in a human arterial EC line [140], and we recently demonstrated that TNFα-induced SK activity in HUVEC occurs in a biphasic manner with peaks observed at both 10 min and 4-6 h post treatment [61]. Based on these observations, we hypothesized that histamine activates SK within minutes of exposure. Indeed, this appears to be the case. As shown in Fig. 1C, a time course treatment of 25 μM histamine on HUVEC demonstrated an increase in SK activity at 2.5 min, peaking at 10 min and subsiding at 30 min. As TNF is also

known to increase SK activity in HUVEC within minutes [61] we investigated whether TNF could also exocytose P-selectin to the cell surface. Figure 1A suggests that the commonality observed between histamine and TNF to rapidly activate SK in HUVEC does not extend to P-selectin exocytosis on these cells.

To investigate whether the SK-1 or SK-2 isoform was preferentially activated by histamine, we executed experiments wherein the addition of 0.1% Triton X-100 or 1M KCl in the enzymatic assay can be used to distinguish between SK-1 and SK-2 activity, respectively [123]. As shown in Fig. 1D-E, HUVEC exposed to histamine for 5 min increased both SK-1 and SK-2 activity with the former being approximately 2-fold higher than the latter. Importantly, unstimulated HUVEC showed equivalent levels of basal SK-1 and SK-2 activity (data not shown). The specificity of these assays was confirmed in experiments using HUVEC pretreated with the SK-1 inhibitor (SKi) [194] and SK-2 inhibitor (ABC294640) [189], which demonstrated selective reductions in activity of the two SK isoforms (Fig. 1D-E).

Histamine-induced SK activity in HUVEC is ERK1/2 dependent

The catalytic activity of SK can be rapidly and transiently activated by a diverse range of growth factors, cytokines and other cell agonists [113] via phosphorylation on Ser225 by extracellular signal-regulated kinases (ERK)1/2 [109]. We next

investigated whether the signaling pathways by which histamine activates SKs in ECs also involve the phosphorylation of ERK1/2. As shown in Fig. 2A-B, 25 μM histamine treatment significantly increased the phosphorylation of ERK1/2 at 5 min, peaking at 10 min and subsiding at 20 min post exposure. Notably, the timing of ERK1/2 phosphorylation parallels that observed for histamine-induced SK activity (Fig. 1D). Fig. 2C shows that blocking the ERK1/2 pathway by administration of U0126 prevented histamine-induced SK activity in HUVEC. Inhibition of SK by SKi had no effect on histamine-induced ERK1/2 phosphorylation (Fig. 2D), consistent with ERK1/2 activation being upstream of SK activity. As expected, SK-1 protein levels did not alter during short exposure time of HUVEC to 25 μM histamine (Supplemental Fig. 1 at *http://ajp.amjpathol.org*).

Histamine-induced P-selectin surface expression is ERK1/2 and SK-1 dependent but S1P surface receptor independent

Using immunofluorescence microscopy we next examined a direct link between the MAPK pathway, SKs and P-selectin surface expression on histamine treated HUVEC. First, HUVEC pretreated with the ERK1/2 pathway inhibitor (U0126) prior to histamine administration exhibited a reduction in P-selectin surface expression similar to that observed in the absence of histamine (Fig. 3A). A similar reduction in

histamine-induced P-selectin expression was observed with administration of the MEK inhibitor (PD98059) but not the p38 inhibitor (SB203580) (Fig. 3A). Second, two separate SK inhibitors (Dimethylsphingosine (DMS, a competitive inhibitor for both SK-1 and SK-2) [122, 123] and SKi (an SK-1 inhibitor)) were utilized to examine the role of SK in histamine-induced P-selectin expression. As shown in Fig. 3A, a significant reduction in histamine-induced P-selectin expression was observed when HUVEC were pretreated with either DMS or SKi. These results suggest that histamine-induced P-selectin expression is SK dependent.

As $S1P_{1-2}$ receptors are known regulators of mast cell function during an allergic response [171] and $S1P_{1-3}$ have been identified on the surface of HUVEC [195], we used inhibitors for these 3 family members (W146 for $S1P_1$, JTE013 for $S1P_2$, CAY10444 for $S1P_3$ and VPC23019 for $S1P_{1\&3}$) to investigate whether S1P receptors are involved in histamine-induced P-selectin expression on ECs. As shown in Fig. 3B, histamine treated HUVEC exhibited a significant increase in P-selectin expression which was not affected by administration of inhibitors to $S1P_{1-3}$. Notably, blocking $S1P_1$, $S1P_3$ or $S1P_{1\&3}$ reduced histamine-induced P-selectin expression by approximately 30%, but expression was still significantly greater than untreated controls (Fig. 3B). To further evaluate whether the S1P receptors are involved, 1 μM exogenous S1P was added to HUVEC, a concentration suggested to only engage the

receptors for signaling events [196]. Figure 3B shows that S1P treatment of HUVEC did not induce P-selectin expression. Collectively, these data suggest that $S1P_{1-3}$ receptors play no major role in histamine-induced P-selectin expression by HUVEC. Also of interest, we investigated the effect of Fingolimod, a sphingosine-like fungal metabolite which has demonstrated direct inhibition of SK-1 [197-199]. Figure 3B shows that pretreatment of HUVEC with Fingolimod significantly reduced histamine-induced P-selectin expression.

Leukocyte rolling on histamine-treated HUVEC is SK-1 dependent

We next examined the role for the MAPK pathway, SK and P-selectin in histamine-induced recruitment of leukocytes *in vitro* by a parallel plate flow chamber assay. When human blood was perfused over untreated HUVEC at a physiological constant shear rate of 2 dynes/cm^2, very few leukocytes rolled along the endothelium (Fig. 4A). In contrast, HUVEC pre-perfused with 25 µM histamine for 2.5 min demonstrated a profound increase in the number of rolling leukocytes with approximately 100 cells per field of view (FOV). Adhesion of leukocytes was minimal to non-existent on both untreated and histamine treated cells (data not shown). Administration of a blocking antibody to P-selectin (AK-4) for 30 min prior to flow chamber assay significantly reduced the number of rolling leukocytes (Fig.

4A).

To investigate a role for ERK1/2 and SK-1 in this system, specific inhibitors were added prior to histamine perfusion. As shown in Fig. 4A and supplemental Video S1 (at *http://ajp.amjpathol.org*), a reduction in leukocyte rolling was observed when inhibitors to either the ERK pathway (U0126 and PD98059) or the SK pathway (DMS and SKi) were added. No reduction was observed with inhibition of the p38 pathway (SB203580) or with administration of the SK-2 inhibitor ABC294640 (Fig. 4A). Congruent with our P-selectin expression data, short term exposure of HUVEC to S1P failed to activate leukocyte rolling (data not shown). This supports the observations of histamine-induced P-selectin expression being $S1P_{1-3}$ receptor independent. Interestingly, pretreatment with Fingolimod also significantly suppressed histamine-induced leukocyte rolling (Fig. 4A), suggesting a potential utility for Fingolimod in the early phase of allergic inflammation.

As the leukocyte rolling studies to this point were performed with whole blood, we next asked whether these responses were also seen using isolated lymphocytes and neutrophils, with the latter previously demonstrating rolling capabilities on histamine-activated endothelial cells [200]. As shown in Fig. 4B, while very little if any lymphocytes exhibited rolling events, approximately 75 neutrophils rolled per FOV on histamine-treated HUVEC. As the lymphocytes isolated from peripheral

blood are likely naïve rather than memory or effector T cells, we used histamine to pre-activate Jurkat T cells and investigated their ability to interact with HUVEC. Supplemental Fig. 2A-B (at *http://ajp.amjpathol.org*) shows L-selectin shedding on histamine treated Jurkats thereby confirming an active state; however, this does not correlate with increased rolling on histamine treated HUVEC. Blocking P-selectin by antibody administration significantly attenuated the neutrophil rolling events (Fig. 4C). Similarly, HUVEC pretreated with the SK-1 inhibitor SKi demonstrated reduced neutrophil rolling (Fig. 4C). This was not observed with the SK-2 inhibitor ABC294640 (Fig. 4C). Collectively, these results suggest that histamine-induced neutrophil recruitment occurs via an SK-1 mediated P-selectin dependent process.

SK-1 mediates histamine-induced leukocyte rolling *in vivo*

We next performed *in vivo* experiments using intravital microscopy to assess the role for SKs in histamine-induced leukocyte rolling in cremasteric postcapillary venules. First, leukocyte rolling was assessed in wildtype mice pretreated with either SKi or vehicle. In vehicle-treated mice, histamine exposure rapidly increased leukocyte rolling flux from a basal level of ~50 cells/min to a peak of 168 ± 28 cells/min within 5 min, before rapidly returning to basal levels (Fig. 5A and supplemental Video S2 at *http://ajp.amjpathol.org*). These mice also exhibited a

transient reduction in rolling velocity from 89 ± 6 μM/sec to 41 ± 7 μM/sec which previous studies have shown is associated with increased sensitivity to chemoattractants [201, 202]. As anticipated from the *in vitro* studies, mice injected subcutaneously with SKi exhibited a significantly lower peak rolling flux of 89 ± 28 cells/min at the same time point (Fig. 5A and supplemental Video S2 at *http://ajp.amjpathol.org*), supporting the concept that histamine-induced leukocyte rolling *in vivo* is SK-1 dependent. Treatment of *Sphk1-/-* mice with SKi caused no further reduction in rolling, consistent with this agent being specific for SK-1 (data not shown). Administration of Fingolimod 60 min prior to histamine exposure also significantly attenuated neutrophil rolling *in vivo* (Fig. 5A and Supplemental Video S2 at *http://ajp.amjpathol.org*). Notably, the residual rolling neutrophils in the FYT720 treated mice did not exhibit a reduced rolling velocity (73 ± 11 μM/sec versus 67 ± 17μM/sec).

Second, to investigate the respective roles of SK-1 and SK-2 in histamine-induced leukocyte rolling *in vivo*, we utilized *Sphk1*$^{-/-}$ and *Sphk2*$^{-/-}$ mice. As shown in Table 1, other than the experiments performed in the *Sphk1*$^{-/-}$ mice being in post-capillary venules of a slightly reduced diameter, equivalent vascular and hemodynamic parameters as well as systemic leukocyte counts were observed by us and others [144, 145]. Furthermore, the equivalent baseline level of neutrophil rolling

in the WT, *Sphk1*$^{-/-}$ and *Sphk2*$^{-/-}$ mice is indicative of constitutive P-selectin expression in the cremasteric microvasculature of these strains [201, 203]. As shown in Fig. 5B and supplemental Video S3 (at *http://ajp.amjpathol.org*) WT mice exhibited a peak rolling flux of 142 ± 12 cells/min after 5 min of histamine superfusion. In *Sphk2*$^{-/-}$ mice, a slight but insignificant decrease in peak rolling flux (109 ± 12 cells/min) was observed. In contrast, *Sphk1*$^{-/-}$ mice demonstrated a profound reduction in histamine-induced rolling (63 ± 11 cells/min), supporting our *in vitro* data of SK-1 being the dominant SK isoform mediating histamine-induced neutrophil rolling.

DISCUSSION

Investigation of the cellular and soluble mediators that are involved in allergic inflammation not only helps in understanding the mechanisms of current treatments, but is also important for the identification of new targets. Herein, we demonstrate for the first time that SK-1 mediates the early phase of histamine-induced P-selectin mediated neutrophil recruitment. Evidence for this comes from experiments showing that (i) histamine increased ERK1/2 phosphorylation and SK activity in HUVEC, (ii) inhibition of either the ERK1/2 pathway or SK-1, but not SK-2, markedly attenuated histamine-induced P-selectin surface expression on ECs, (iii) addition of S1P or inhibition of $S1P_{1\text{-}3}$ receptors on histamine treated HUVEC did not alter P-selectin surface expression, (iv) histamine-induced neutrophil rolling on endothelium *in vitro* was P-selectin and SK-1 dependent, and (v) histamine-induced neutrophil influx *in vivo* was significantly reduced in wildtype mice pretreated with an SK-1 inhibitor as well as *Sphk1*$^{-/-}$ mice when compared to the WT and *Sphk2*$^{-/-}$ counterparts.

The importance of P-selectin in allergic inflammation has been well described with an *in vivo* study showing that P-selectin deficient mice exhibit a significant reduction in leukocyte rolling [192], histamine-induced P-selectin facilitating neutrophil adhesion via CD11/CD18 integrin activation [200] and the development of allergic inflammation [204]. The significance of P-selectin in mediating

leukocyte-endothelial cell interactions has been confirmed in patients with leukocyte adhesion deficiency (LAD II). These patients suffer from recurrent *Staphylococcal* infections and their neutrophils fail to roll and adhere adequately due to a lack of functional sialyl Lewis X expression, a fucose-containing glycoconjugate ligand for P-, E- and L-selectins [205]. Therefore, identifying the mechanisms underpinning the regulation of P-selectin surface expression may aid in the development of new pharmaceutical approaches to combat allergic inflammation. A role for S1P in histamine-induced gene regulation of E-selectin and ICAM-1 has been demonstrated by Shimamura et al [177]. It is contention that the SK/S1P pathway plays a critical role prior to gene regulation with the exocytosis of P-selectin occurring within minutes of exposure to histamine.

Our results suggest that HUVEC exposed to histamine rapidly activates SK-1 and SK-2. To delineate the contribution of SK-1 versus SK-2 in this system, we used both broad spectrum and specific SK inhibitors in our *in vitro* and *in vivo* experiments. DMS is an inhibitor of both SK-1 and SK-2 but affects other lipid and protein kinases, including protein kinase C (PKC) [113]. In contrast, SKi is a more specific inhibitor with a recent report suggesting it specifically targets SK-1 [194, 206], conversely the inhibitor ABC294640 specifically targets SK-2 [189]. Using these inhibitors our data suggest that only histamine-induced SK-1 activity is required for rapid surface

expression of P-selectin on ECs and neutrophil rolling events *in vitro*. Furthermore, extracellular S1P and the $S1P_{1-3}$ receptors appeared to play no major role in our study which differs from Matsushita et al who demonstrated that (i) exposure of the human aortic endothelial cell line (HAEC) to 1 μM S1P for 5 min caused release of von Willebrand Factor, another protein stored preformed in Weibel Palade bodies and (ii) 10 pM of S1P injected intravenously into mice increased soluble P-selectin within 1 h [207]. Our study raises an alternative possibility that intracellular second messengers modulated by S1P (eg HDAC1/2, TRAF2 or prohibitin) [135, 160, 161] may be involved. Clearly the difference observed between our study and that of Matsushita requires further investigation *in vitro* and *in vivo*, using multiple approaches, including, but not limited to, the family of SK and S1P receptor knockout mice. Our study demonstrated that pretreatment of HUVEC with Fingolimod caused a reduction in histamine-induced P-selectin expression and leukocyte rolling events. Fingolimod is an orally active immunomodulatory pro-drug which recently gained FDA approval to treat multiple sclerosis [208] based on its ability to inhibit lymphocyte egress from lymph nodes and thymus [209]. The mechanisms underpinning Fingolimod inhibition of histamine-induced P-selectin expression and leukocyte rolling flux are still unknown and likely due to the ability of Fingolimod to inhibit and degrade SK-1 *in vitro* [197, 198].

To provide additional definitive confirmation of the role of SK-1 in histamine-induced P-selectin expression in HUVEC, we attempted to use transient transfection with siRNA to knockdown SK-1 expression. However, these experiments were not technically feasible. A major limitation to working with P-selectin in primary HUVEC is that after two or more passages, HUVEC lose their ability express preformed P-selectin [210]. Given that siRNA experiments involve additional passages, this precluded our ability to combine siRNA treatment with assessment of histamine-induced P-selectin mobilization in HUVEC. However, our examination of *in vivo* responses in mice specifically lacking either SK-1 or SK-2 provided strong evidence supporting our hypothesis that SK-1 was critical to histamine-induced P-selectin upregulation.

Herein, our *in vivo* studies show that either pharmacological or genetic manipulation of SK-1 attenuates histamine-induced neutrophil rolling flux, which are critical for acute allergic inflammation. More specifically, we observed in WT mice that both SKi and Fingolimod significantly attenuated histamine-induced neutrophil rolling flux. Congruent with SK-1 mediating this process, *Sphk1*$^{-/-}$ mice exhibited significant resistance to histamine-induced neutrophil rolling flux, in contrast, *Sphk2*$^{-/-}$ mice did not. These data differ from that of Michaud et al, who reported equivalent neutrophil numbers in the lavage fluid of both WT and *Sphk1*$^{-/-}$ mice in an

inflammatory model of peritonitis using 4 h thioglycollate challenge [211]. The divergence in these data may be attributable to the difference in the time courses of the responses investigated (ie 5-10 min versus 4 h) and the nature of the inflammatory stimuli (ie histamine versus thioglycollate). Herein we also show that untreated WT, *Sphk1*$^{-/-}$ and *Sphk2*$^{-/-}$ mice exhibited similar levels of baseline neutrophil rolling flux. Constitutive P-selectin expression in the lung, skin, intestine, mesentery and cremaster muscle has been previously shown using the non-invasive dual radiolabelling antibody binding assay and as such is not the result of intravital microscopy intervention [201, 203]. Collectively, these data indicate that constitutive P-selectin expression in the cremaster muscle is SK-independent but that histamine-induced exocytosis of P-selectin expression is SK-dependent.

The physiological relevance of the differences in SK-1 and SK-2 activity levels, with respect to allergy, may be widespread [172] and are yet to be fully elucidated. Experimentally, Pushparaj et al showed both *in vitro* and *in vivo* that silencing SK-1 inhibited several mast cell effector functions triggered by FcεRI engagement, whereas silencing SK-2 had no effect [169]. However, there is still controversy over the different roles of SK-1 and SK-2 in mast cell responses with a study using *Sphk* deficient mice suggesting that SK-2, and not SK-1, was more important for degranulation and cytokine, or eicosanoid production by mast cells [148]. In addition,

Zemann et al showed that bone marrow-derived neutrophils from both *Sphk1*$^{-/-}$ and *Sphk2*$^{-/-}$ mice had normal functions of increasing intracellular Ca^{2+} and migration towards fMLP and C5a, when compared to WT mice [212]. Together, these studies suggest that the effects of SK isoforms may be cell-type specific.

The prevalence of all types of allergies continues to rise across all age, gender and racial groups with the Allergy and Asthma Foundation of America rating allergy as the 3rd most common chronic disease among children. An understanding of the cellular and soluble mediators that are involved in allergic inflammation not only helps in elucidating the mechanisms of current treatments, but is also important for the identification of new targets. Successful outcomes in future studies may establish SK as a therapeutic target to control histamine-induced allergic responses. More specifically, by targeting the early allergic response of neutrophil recruitment, we may be able to impact on the initiation of chronic diseases triggered by allergens. Our understanding of this complex relationship might also reveal new opportunities for other diseases in which histamine is suggested to play a role, such as multiple sclerosis, rheumatoid arthritis and psoriatic arthritis, for which traditional anti-histamines are generally regarded as ineffective.

Acknowledgements: We thank Michaelia Cockshell for preparing the endothelial cells, the staff and consenting donors at Women's and Children's Hospital and Burnside Memorial Hospital for collection of the umbilical cords.

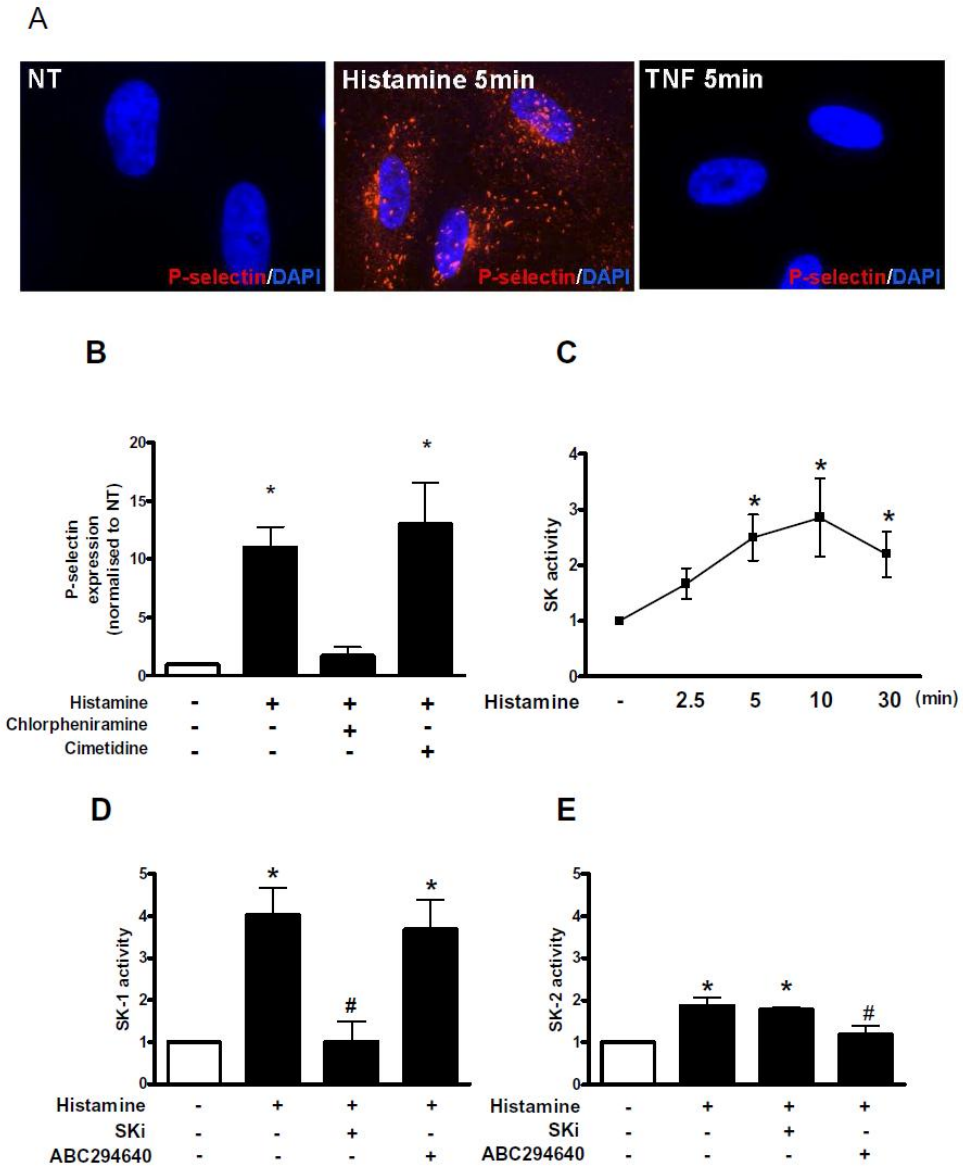


Figure 1. Histamine rapidly promotes P-selectin expression via the H_1 receptor and activates SK-1 and SK-2. In (A), immunofluorescence microscopy of HUVEC treated for 5 min without or with 25 μM histamine or 5 ng/ml TNF prior to P-selectin staining (red), permeablisation and DAPI staining (blue). A representative image of n=3 is shown. Original magnification is ×100 for all panels. In (B), pooled data of histamine treated HUVEC without and with H_1 antagonist (Chlorpheniramine) or H_2

antagonist (Cimetidine). Results show the mean fluorescence intensity mean ± sem of n=3, *, $p<0.05$ versus untreated (-). In (C), HUVEC stimulated without and with 25 μM histamine for 2.5, 5, 10 and 30 min prior to lysis for SK enzymatic assay. In (D-E), HUVEC were pre-incubated with either SK-1 inhibitor (SKi, 5 μM) or SK-2 inhibitor (ABC294640, 10 μM) 10 min prior to histamine stimulation for 5 min. Cells were lysed immediately for SK-1 or SK-2 enzymatic assay. Results show the mean ± sem of n=3-6, *, $p<0.05$ versus (-); ‡, $p<0.05$ versus histamine.

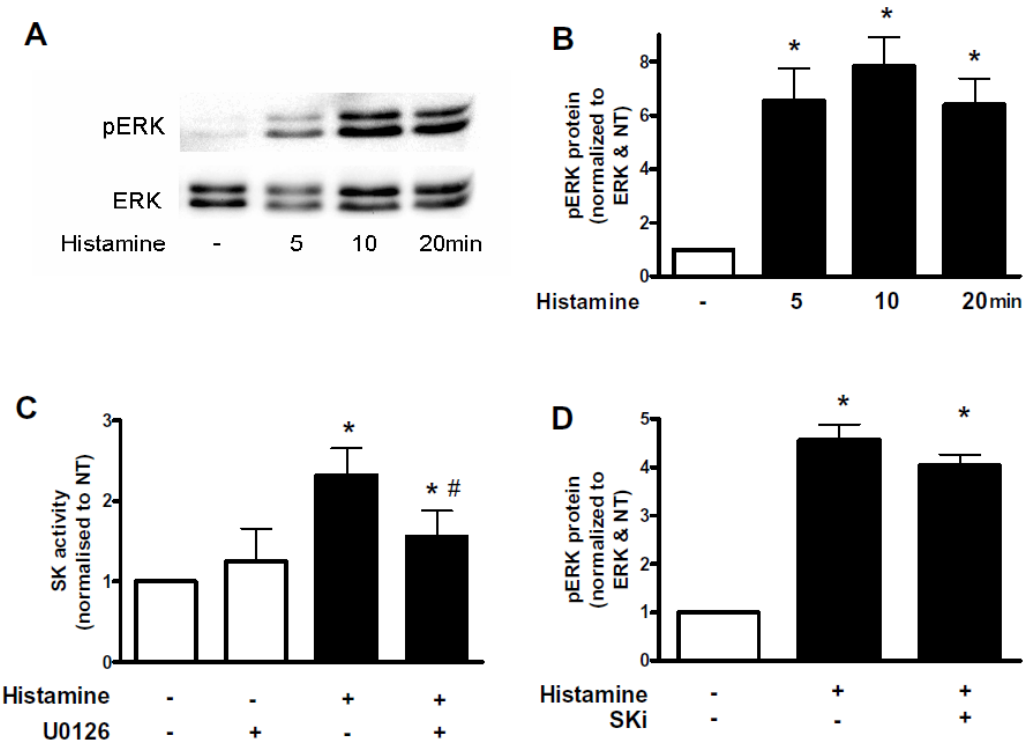


Figure 2. Histamine increases phosphorylation of ERK1/2 which activates SK. In (A), HUVEC were treated without and with histamine (25 μM for 5, 10 and 20 min) prior to lysis and Western blotting for phosphorylated ERK1/2 (pERK) and total ERK1/2 (ERK). A representative image of n=4 is shown. In (B), pooled data are shown as the mean ± sem of n=4; *, $p<0.05$ versus untreated (-). In (C), HUVEC were pre-treated with ERK1/2 pathway inhibitor (U0126, 10 μM, 30 min) prior to histamine stimulation (25 μM, 5min) and lysis for SK enzymatic assay. Data shown are the mean ± sem of n=5-7; *, $p<0.05$ versus untreated (-); ‡, $p<0.05$ versus histamine. In (D), HUVEC were pre-treated with SKi (5 μM, 10 min) prior to

histamine stimulation (25 μM, 5min) and examined for phosphorylated and total ERK1/2 by Western blotting. Data shown are the mean ± sem of n=5; *, $p<0.05$ versus untreated (-).

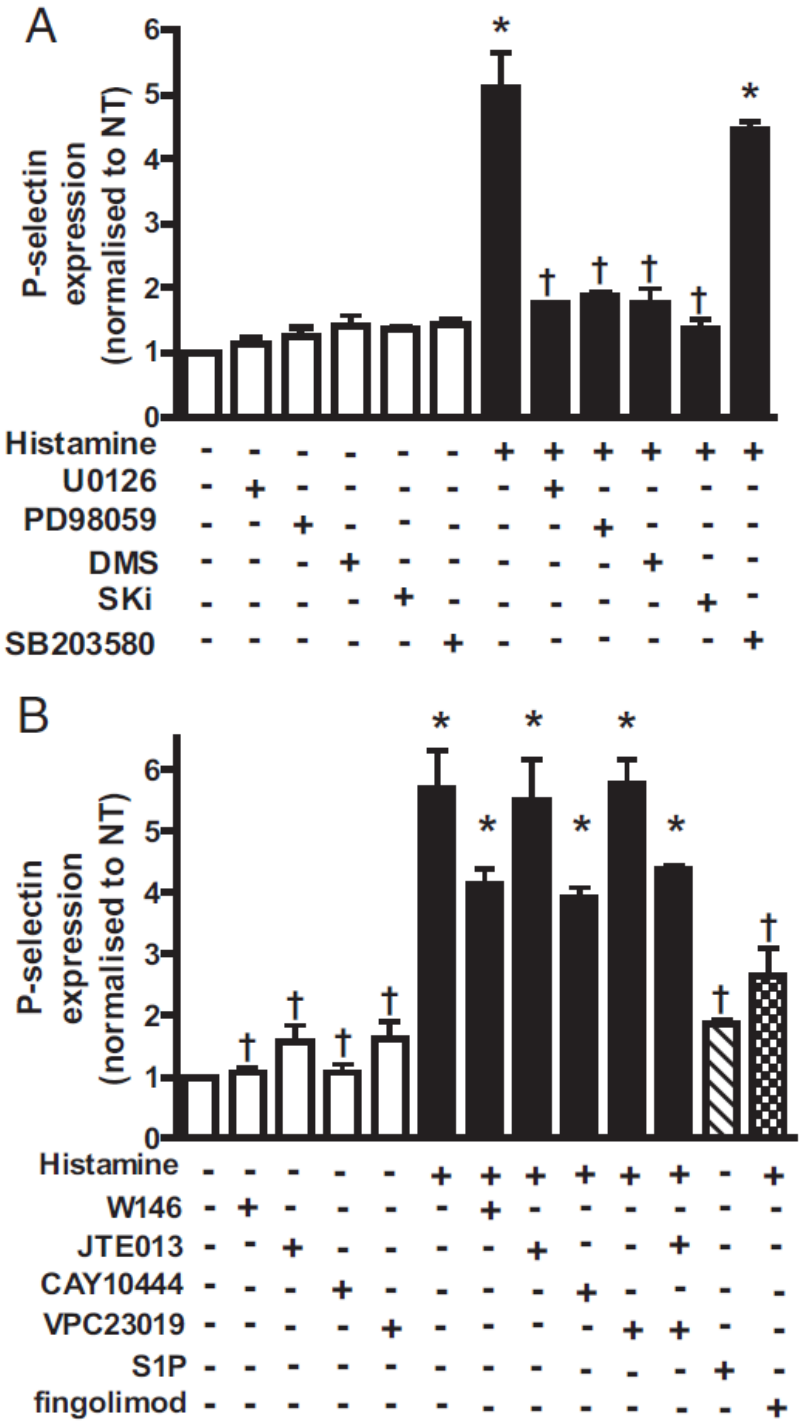


Figure 3. Inhibition of ERK1/2 pathway or SK attenuates histamine-induced P-selectin surface expression in an $S1P_{1-3}$ receptor independent manner. In (A), HUVEC were pre-incubated with U0126 (10 μM, 30 min), PD98059 (25 μM, 30 min), DMS (5 μM, 10 min), SKi (5 μM, 10 min), SB203580 (10 μM, 1 h) without or with

histamine treatment (25 μM, 5 min) and examined for P-selectin surface expression by immunofluorescence microscopy. In (B), HUVEC were treated with $S1P_1$ inhibitor (W146, 10 μM, 30 min), $S1P_2$ inhibitor (JTE013, 1 μM, 30 min), $S1P_3$ inhibitor (CAY10444, 10 μM, 30 min), $S1P_{1\&3}$ inhibitor (VPC23019, 10 μM, 30 min) or Fingolimod (100 nM, 30 min) prior to histamine (25 μM, 5 min) exposure. Similarly, exogenous S1P (1 μM, 30 min) was added to HUVEC. Cells were fixed and assessed for P-selectin expression by immunofluorescence microscopy. Results of the quantified mean fluorescence intensity are the mean ± sem of n=3-4; *, $p<0.05$ compared to corresponding (-) and ‡, $p<0.05$ versus histamine.

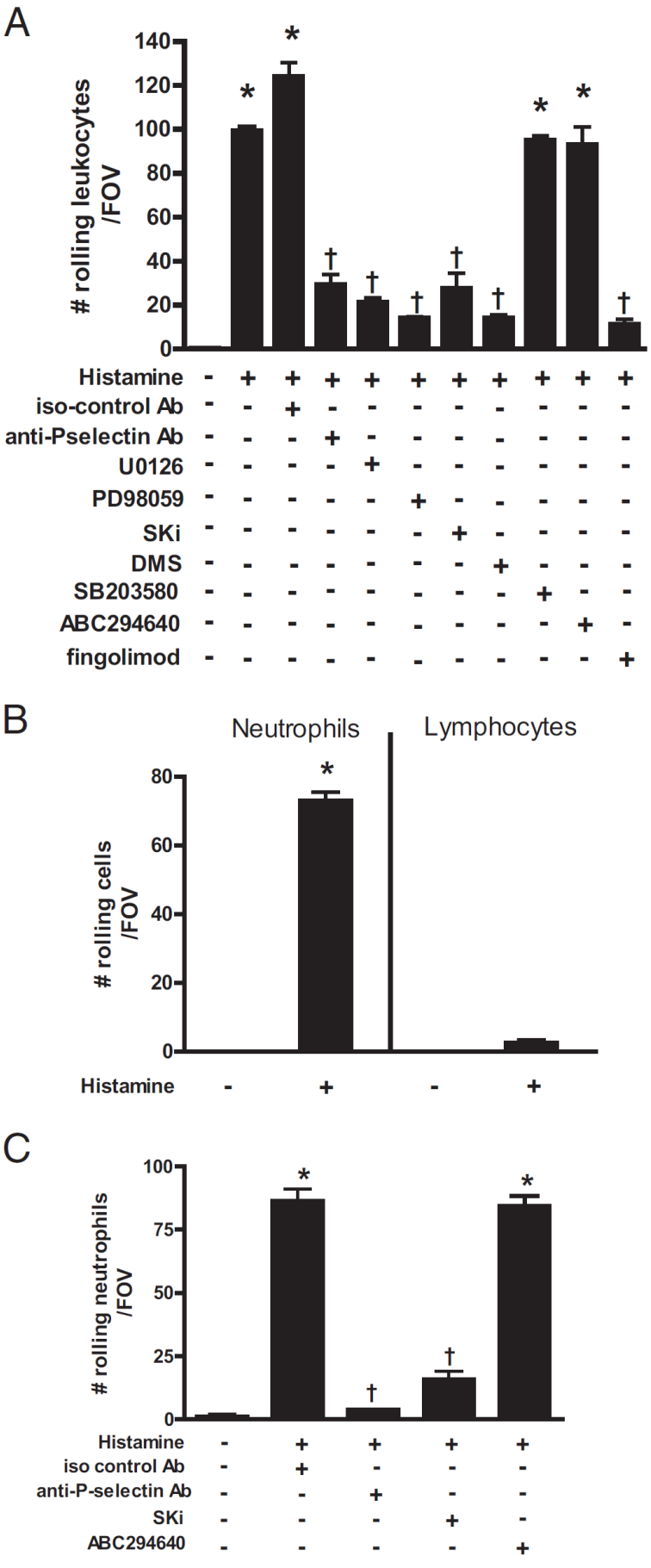

A
rolling leukocytes /FOV
140
120
100
80
60
40
20
0
Histamine
iso-control Ab
anti-Pselectin Ab
U0126
PD98059
SKi
DMS
SB203580
ABC294640
fingolimod
B
Neutrophils
Lymphocytes
rolling cells /FOV
80
60
40
20
0
Histamine
C
rolling neutrophils /FOV
100
75
50
25
0
Histamine
iso control Ab
anti-P-selectin Ab
SKi
ABC294640

Figure 4. Histamine-induced leukocyte and neutrophil rolling *in vitro* is ERK1/2 and SK-1 dependent but independent of $S1P_{1-3}$ surface receptors. In (A), HUVEC were pre-incubated without or with an isotype control antibody (10 μg, 30 min), P-selectin blocking antibody (10 μg, 30 min), ERK1/2 inhibitor (U0126, 10 μM, 30 min), MEK inhibitor (PD98059, 25 μM, 30 min), SK inhibitors (SKi; 5 μM, 10 min or DMS; 5 μM, 10 min), p38 inhibitor (SB203580, 10 μM, 1 h), SK-2 inhibitor (ABC294640, 10 μM, 10 min) or Fingolimod (100 nM, 30 min) prior to perfusion of histamine (25 μM, 2.5 min) and then human whole blood (5 min). Data are expressed as the mean ± sem rolling cells per field of view (FOV) with 4-5 FOV captured for n=3-4; *, $p<0.05$ versus untreated (-) and ‡, $p<0.05$ versus histamine. In (B), HUVEC were perfused without or with histamine (25 μM, 2.5 min) and freshly isolated human neutrophils or lymphocytes at $1x10^6$ cells per ml. Data are expressed as the rolling flux mean ± sem per field of view (FOV) with 4-5 FOV captured for n=3; *, $p<0.05$ versus untreated (-). (C) HUVEC were pre-treated without or with a control antibody (10 μg, 30 min), P-selectin blocking antibody (10 μg, 30 min), SK-1 inhibitor (SKi, 5 μM, 10 min) or SK-2 inhibitor (ABC294640, 10 μM, 10 min) prior to perfusion of histamine (25 μM, 2.5 min) and freshly isolated human neutrophils at $1x10^6$ cells per ml. Data are expressed as the rolling flux mean ± sem per FOV with 4-5 FOV captured for n=3-5; *, $p<0.05$ versus untreated (-) and ‡, $p<0.05$ versus histamine.

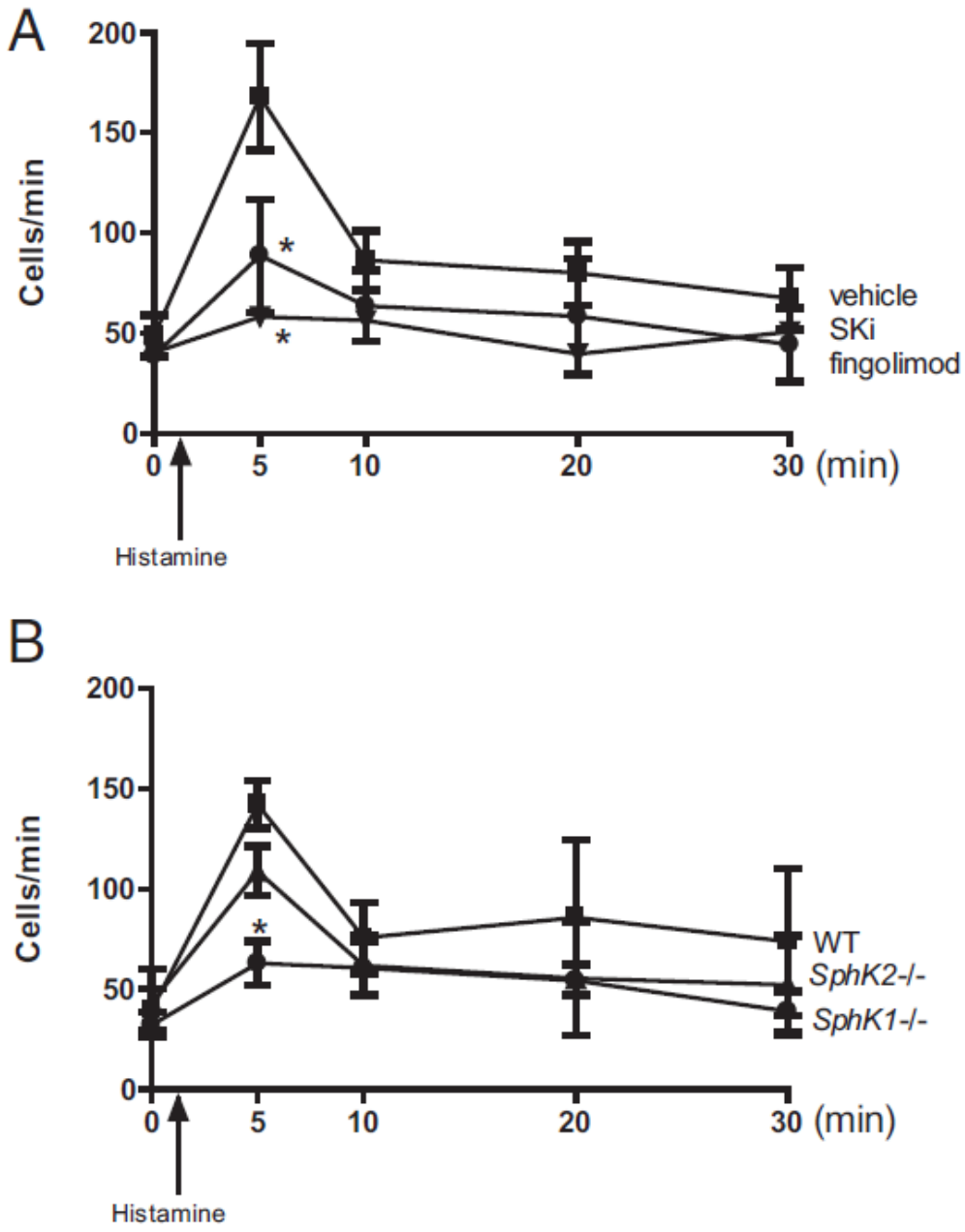


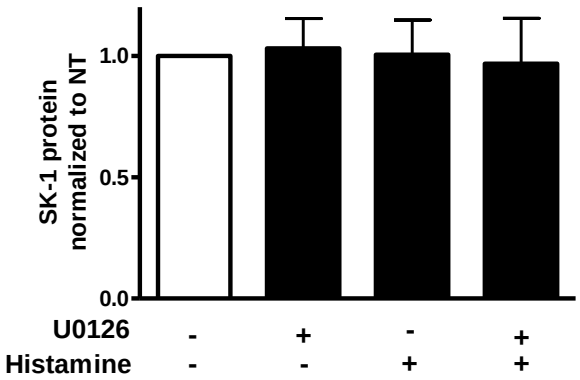
Figure 5. Histamine-induced neutrophil rolling flux in response to SK inhibitors and *Sphk* knockout mice. In (A), WT mice were injected with vehicle DMSO/PBS (squares), SKi (50 mg/kg, triangles) subcutaneously 15 min or Fingolimod (0.5 mg/kg, circles) intraperitoneally 60 min prior to histamine challenge (100 μM, superfused topically over the cremaster muscle) and examined via intravital microscopy.

Similarly, in (B), WT (squares), *Sphk1*$^{-/-}$ (circles) and *Sphk2*$^{-/-}$ (triangles) mice were superfused with histamine. Leukocyte rolling flux in the postcapillary venules of the mouse cremaster muscle was assessed at 5, 10, 20 and 30 min post-histamine challenge. Data are expressed as the mean ± sem from n=5-7 mice per group; *, $p<0.05$ versus WT.

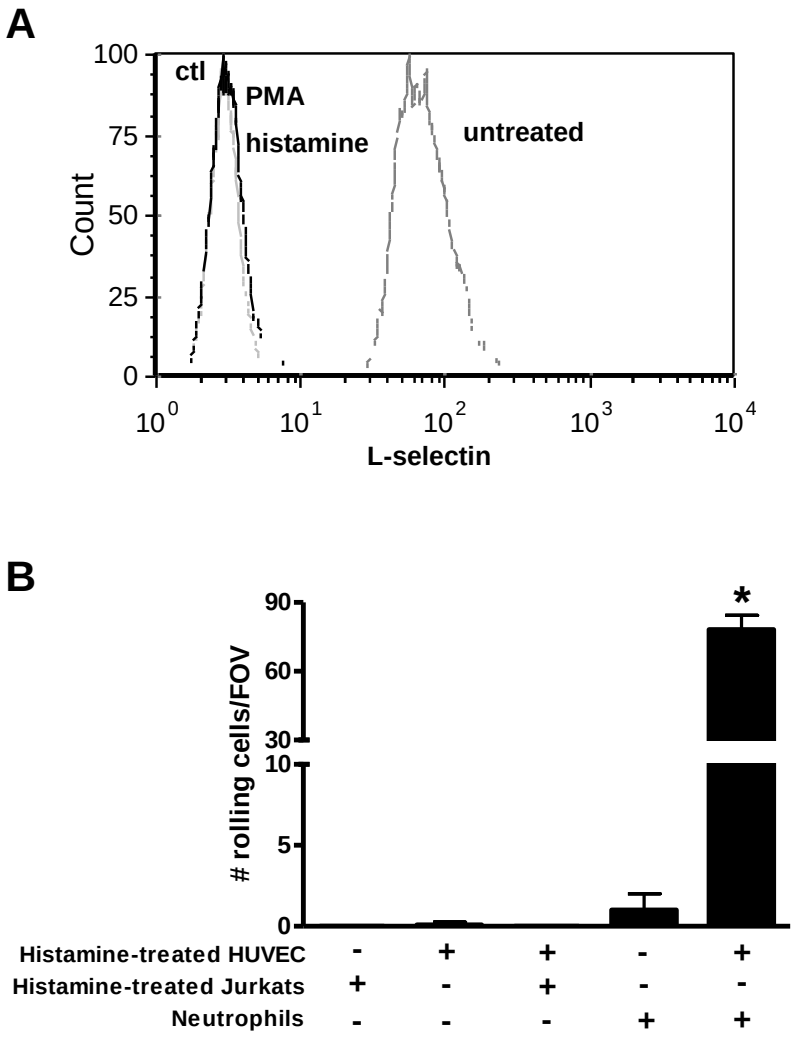
Table 1: Hemodynamic state of untreated animals

	WT	***Sphk1*$^{-/-}$**	***Sphk2*$^{-/-}$**
Vascular diameter (µm)	32.3 ± 1.0	28.4 ± 1.7*	29.9 ± 1.9
Mean RBC velocity (mm/s)	2.1 ± 0.6	1.1 ± 0.2	2.3 ± 0.4
Shear rate (s^{-1})	463 ± 126	318 ± 39	616 ± 83
Leukocyte counts			
Lymphocytes	65 ± 4	66 ± 2	63 ± 2
Neutrophils	28 ± 5	24 ± 4	25 ± 2
Monocytes	7 ± 3	10 ± 1	12 ± 1

*, $p<0.05$ relative to WT value (n=5-16).



Supp. Fig. 1. Phosphorylated ERK1/2 does not increase SK-1 protein levels. HUVEC were pre-incubated with ERK1/2 pathway inhibitor (U0126, 20 μM, 30 min) prior to histamine treatment (25 μM, 5 min), cell lysis and Western blotting for SK-1 protein. Quantified results are the mean ± sem of n=5.



Supp. Fig. 2. Interactions between T cells and HUVEC following histamine activation. In (A), untreated Jurkats (solid dark grey line) exhibit rapid shedding of L-selectin within 5 min of exposure to 25 μM histamine (solid black line) as determined by flow cytometry. These levels are similar to that expressed by phorbol myristate acetate (PMA, 100 ng/ml 30 min) treated Jurkats (solid light grey line) and untreated Jurkat T cells labelled with an isotype control antibody (dotted light grey line). Data are representative of 3 independent experiments. In (B), HUVEC were

perfused without or with histamine (25 μM, 2.5 min) prior to perfusion of Jurkat cells (treated without or with histamine (25 μM, 5 min)) or freshly isolated neutrophils at $1x10^6$ cells per ml. Data are expressed as the rolling flux mean ± sem per field of view (FOV) with 4-5 FOV captured for n=3; *, $p<0.05$ versus untreated (-).

Online Video S1. Parallel plate flow chamber video of HUVEC pre-treated without or with a control antibody (10 µg, 30 min), P-selectin blocking antibody (10 µg, 30 min) or SK-1 inhibitor (SKi, 5 µM, 10 min) prior to perfusion of histamine (25 µM, 2.5 min) and freshly isolated human blood (5 min). Playback speed is 1x.

Online Video S2. Intravital videos from untreated controls as well as vehicle control, SKi or Fingolimod treated mice prior to histamine superfusion (100 µM, 5 min). Videos are 7 sec images as shown in Fig. 5A.

Online Video S3. Intravital videos from WT, *Sphk1*$^{-/-}$ and *Sphk2*$^{-/-}$ following histamine superfusion (100 µM, 5 min). Videos are 7 sec images as shown in Fig. 5B.

Chapter 4: SK-1 regulates histamine-induced WPB exocytosis via intracellular calcium influx

Exocytosis of WPBs is a multi-step process, which includes formation of a granule, loading of a protein/molecule into the granule, priming the granule from the cytoplasm to the membrane, fusion of the granule with the plasma membrane and then recycling the granule [213] (See Figure 1.5.1a, p.50). A variety of WPB contents have been identified (Table 4a) and contribute to a multitude of biological functions, including inflammation and vasoconstriction. Table 4b details the known activators and their associated intracellular messengers identified to promote the exocytosis of WPBs. Clearly, cells releasing different contents of WPBs can lead to distinct vascular functions and as such it is important to understand the mechanisms underpinning WPBs exocytosis. Rondaij et al. performed a pull down assay for GTP-bound Ral in ECs to show that activation of Ral protein regulates thrombin and epinephrine-induced exocytosis of WPBs that contain vWF [214]. To this end, the present study (Chapter 3.2) has shown that histamine-induced P-selectin surface expression by ECs is SK-1 dependent. However, the mechanism underpinning the exocytosis of P-selectin containing WPBs from the cytoplasm to the cell surface is not completely understood, and the role for SK-1 in this process has not been examined.

This Chapter addresses the Aim that whether SK-1 regulates WPB exocytosis via intracellular influx in ECs.

Table 4a Contents of WPBs and vascular functions.

Function	Content	Reference
Inflammation	P-selectin	[215]
	IL-8	[216]
	Eotaxin	[217]
Thrombosis	vWF	[218]
	Factor XIIIa	[219]
Vasoconstriction	Endothelin	[220]
	Endothelin converting enzyme	[221]
Vasodilation	Calcitonin gene related peptide	[220]
Fibrinolysis	Tissue plasminogen activator (tPA)	[222]

Table 4b Activators and intracellular mediators of WPBs.

Class	Activator	Intracellular mediator	Reference
Polypeptides	Thrombin	Ca^{2+}	[223]
	VEGF	Ca^{2+}	[224]
	Complement	Ca^{2+}	[225]
Lipids	S1P	Ca^{2+}	[207]
	Ceramide	Ca^{2+}	[226]
Inflammatory mediators	Histamine	Ca^{2+}	[227]
	Leukotrienes	Ca^{2+}	[228]
	Epinephrine	cAMP	[229]
	Serotonin	cAMP	[230]

4.1 Gene manipulation of HUVECs for the surface expression of P-selectin

To examine whether SK-1-mediated P-selectin exocytosis is via activation of a co-factor, such as RalA, I attempted to perform manipulation of *RalA* in HUVECs using (1) viral infection, (2) lipid-based plasmid transfection and (3) electroporation followed by histamine stimulation for P-selectin exocytosis to HUVEC surface. However, P-selectin protein expression is lost or below the levels of detection when HUVECs have been passaged greater than two times. This limitation restricts experimental opportunities, where for example overexpression of Ral in HUVECs using the above experimental protocols would require more than two passages of the HUVECs. Furthermore, electroporation of primary or first passage HUVECs caused significant cell death and histamine-induced P-selectin surface expression was not detected in the remaining viable HUVECs (data not shown). Taking another approach, the levels of endogenous Ral in HUVECs were also determined and tested for SK-1 regulation of the GTP-bound Ral for histamine-induced P-selectin surface expression. However, immuno-blots failed to detect any endogenous Ral suggesting that these levels are below that of detection (data not shown).

4.2 Localization of P-selectin in WPBs

As both P-selectin, Ang-2 and vWF are known WPB contents, I examined whether they are co-localized in the same or separate WPBs. Immunofluorescence microscopy showed that vWF and P-selectin are largely co-localized (Figure 4.2d&e), which suggests that the exocytosis of P-selectin and vWF may occur at the same time by the same stimuli. By contrast, these same experiments showed that Ang-2 and P-selectin are not co-localized in HUVECs (Figure 4.2f&g), and agrees with the literature that their subcellular distribution is mutually exclusive [89].

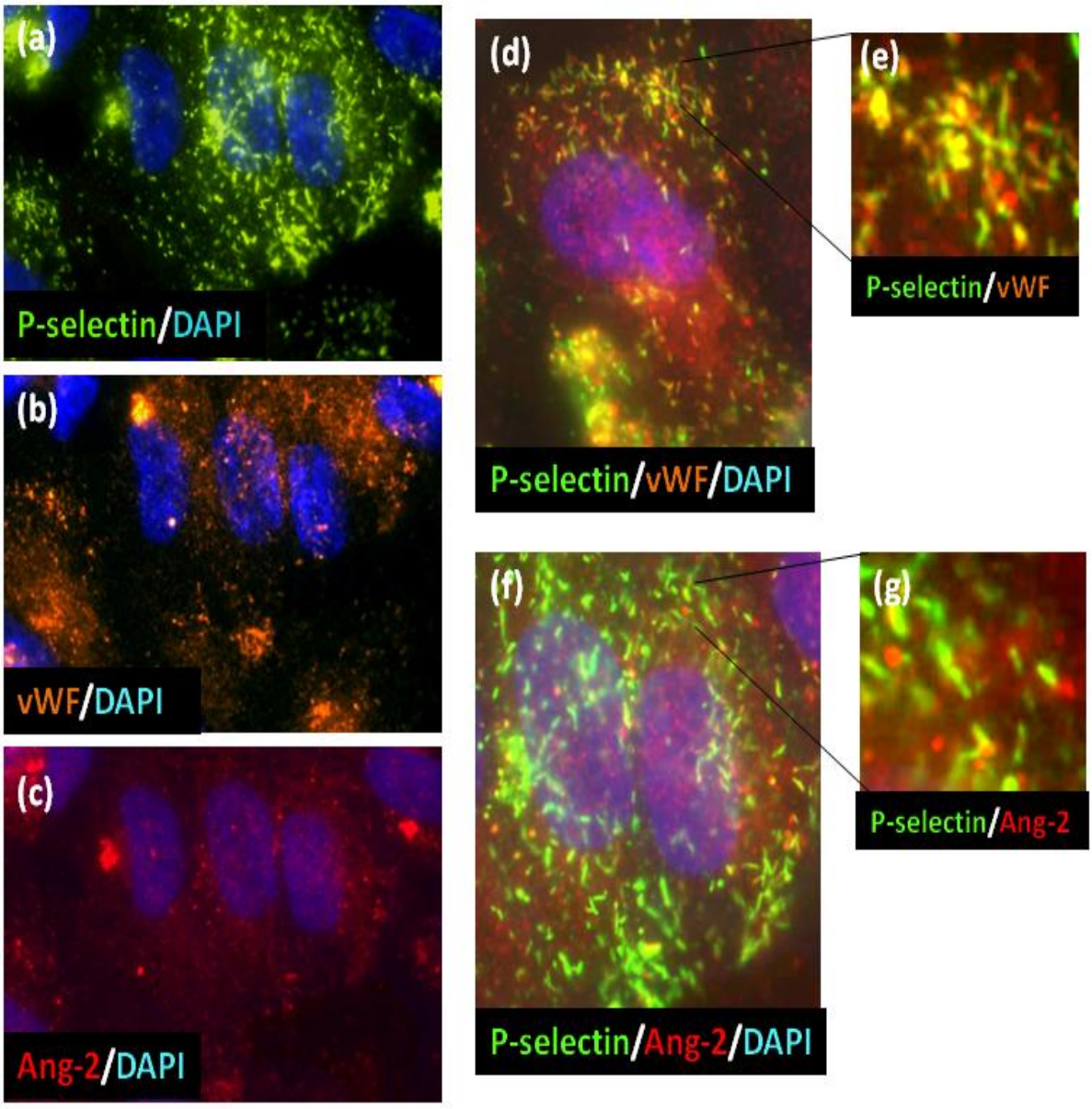


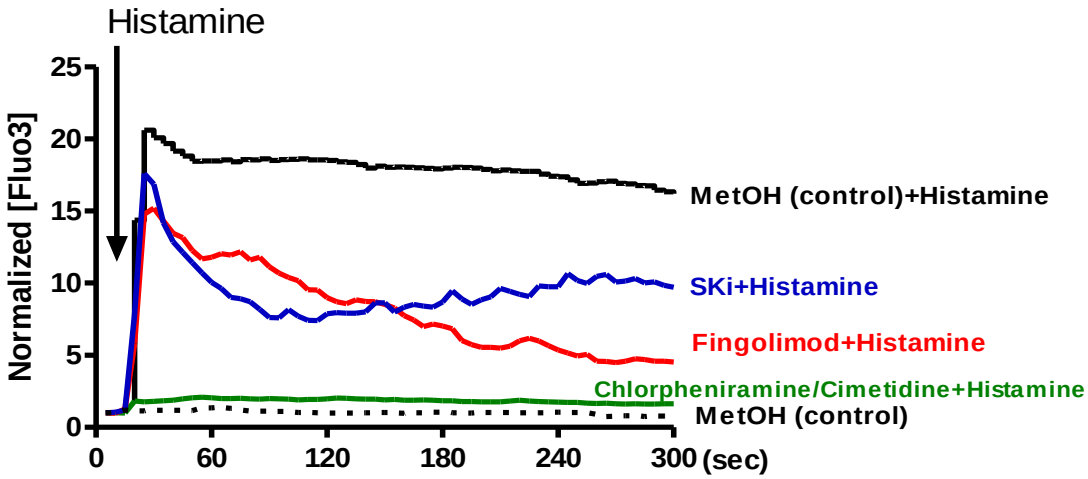
Figure 4.2 Co-localization of WPB contents. Immunofluoresence for (a) P-selectin (green), (b) vWF (orange) and (c) Ang-2 (red) were performed in permeabilized HUVECs followed by DAPI staining. Multi-parameter immunofluorescence imaging was captured prior to analysis. (d & e) Merged and enlarged image for P-selectin, vWF and DAPI, and the overlapped staining for P-selectin and vWF is shown in

yellow. (f & g) Merged and enlarged image for P-selectin, Ang-2 and DAPI. A representative image of immunofluorescence microscopy is shown (n=3), with magnification at X100.

4.3 SK-1 regulates histamine-induced intracellular calcium influx

Although it is not feasible to directly examine whether SK regulates the activation of RalA for P-selectin surface expression by HUVECs, histamine-induced exocytosis of WPBs is known to be associated with intracellular calcium influx ($[Ca^{2+}]i$) [227]. Thus, I examined whether inhibition of SK can attenuate histamine-induced $[Ca^{2+}]i$. The data (Figure 4.3i & ii and Video 4.3a) showed that histamine induced $[Ca^{2+}]i$ rapidly, within minutes, and was maintained for 5 min post-histamine stimulation. By contrast, pre-treatment with anti-histamines (Chlorpheniramine antagonizes H_1 and Cimetidine antagonizes H_2) abrogated histamine-induced $[Ca^{2+}]i$ in HUVECs. Importantly, HUVECs pre-treated with either SKi or Fingolimod also exhibited a reduction in histamine-induced $[Ca^{2+}]i$, but unlike those pre-treated with anti-histamines, the initial increase in histamine-induced $[Ca^{2+}]i$ was observed (Figure 4.3(i)). Take together, these results suggest that SK is involved in exocytosis of WPBs via $[Ca^{2+}]i$.

(i)


Histamine
Normalized [Fluo3]
25
20
15
10
5
0
0
60
120
180
240
300 (sec)
MetOH (control)+Histamine
SKi+Histamine
Fingolimod+Histamine
Chlorpheniramine/Cimetidine+Histamine
MetOH (control)

(ii)

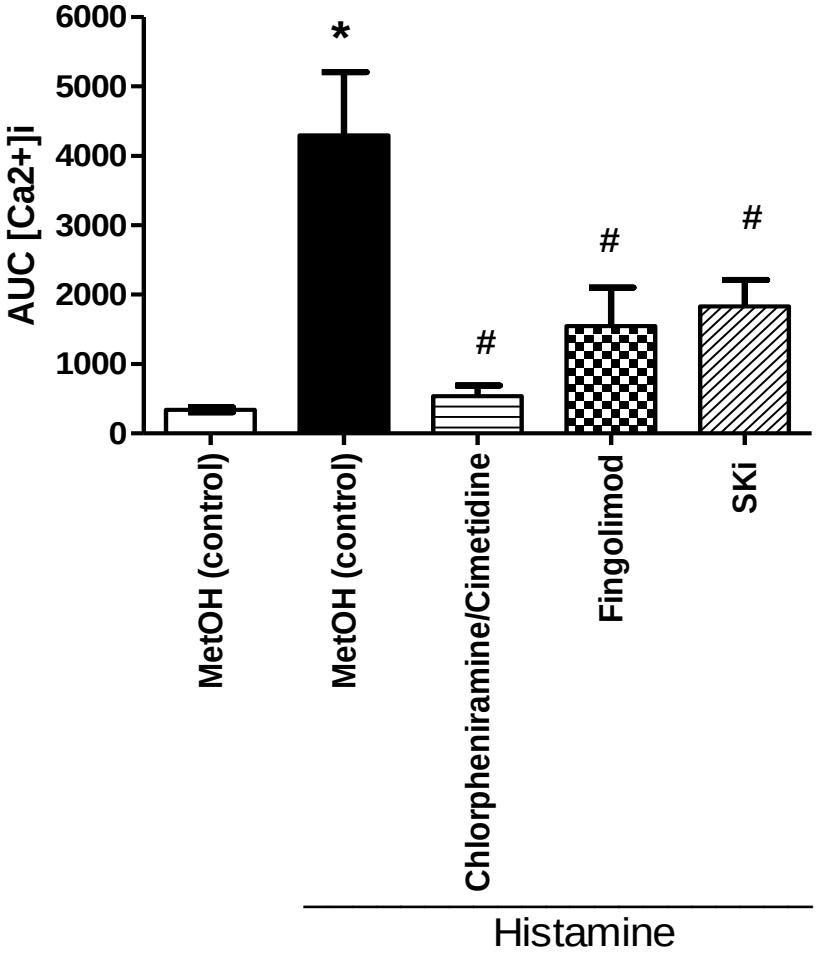

AUC [Ca2+]i
6000
5000
4000
3000
2000
1000
0
*
#
#
#
MetOH (control)
MetOH (control)
Chlorpheniramine/Cimetidine
Fingolimod
SKi
Histamine

Figure 4.3 Inhibition of SK attenuates histamine-induced intracellular calcium influx. Fluo3-labelled HUVECs were treated with vehicle control (Methanol (MetOH)), Fingolimod (100 nM), SKi (5 μM) or Chlorpheniramine/Cimetidine (both at 100 nM) for 1 h prior to confocal microscopy. Histamine (25 μM) was added to HUVECs at 10 sec of the time-lapse imaging video and the change of Fluo3 intensity was recorded over time for 5 min. (i) A representative figure is shown from 4 individual experiments with each treatment performed in duplicates. Fifty cells per video were gated for analysis using ImageJ. (ii) Area under the curve (AUC) was measured from 4 individual experiments, mean ± SEM, *, $p<0.05$ vs MetOH (control), $^{\#}$, $p<0.05$ vs MetOH (control) + Histamine, One-way ANOVA.

Video 4.3a. Intracellular calcium influx videos from vehicle control (MetOH), Chlorpheniramine/Cimetidine (both at 100 nM), Fingolimod (100 nM), SKi (5 μM) pre-treated HUVECs prior to histamine (25 μM) stimulation, Videos are 5 sec images.

4.4 Materials and methods

Immunofluorescence microscopy for vWF, P-selectin and Ang-2 antibody staining:

HUVECs were replated at $1x10^5$ cells on a Fn-coated (50 μg/mL, Sigma-Aldrich) cover slip (15x15 mm, ProSciTech) in M199 medium (Sigma-Aldrich) containing 20% human serum (Invitrogen), penicillin (100 U/mL, Invitrogen) and streptomycin (100 μg/mL, Invitrogen). Confluent HUVECs were fixed with 4% paraformaldehyde at RT for 15 min prior to blocking with 2% BSA in PBS (RT, 30 min). Cells were then permeabilized with 0.1% Triton-X100 in PBS (RT, 10 min) before primary antibody staining for vWF (H-300 clone, 1 μg/mL, Santa Cruz Biotechnology), P-selectin (AK-4 clone, 1 μg/mL, Bioscience) and Ang-2 (N-18 clone, 1 μg/mL, Santa Cruz Biotechnology) overnight at 4°C, followed by anti-rabbit Alexa Fluor 594 (1:1000, Rockland Inc), anti-mouse Alexa Fluor 488 (1:1000, Rockland Inc) and anti-goat Alexa Fluor 647 (1:1000, Rockland Inc) incubation at RT for 1 hour. Nuclear DAPI staining was performed (100 nM in PBS, RT, 3 min) and then slides were mounted with Biomeda gel (ProSciTechi) prior to visualization using an Olympus IX70 inverted microscope (Olympus) which was linked to a Bio-Rad Radiance 2100 confocal microscope (Bio-Rad Laboratories).

Measurement of intracellular calcium influx:

HUVECs were replaced at 5 x 10^4 cells/well in an Angiogenesis slide (Ibidi, Hallam, Victoria, Australia) overnight prior to incubation with the intracellular calcium binding dyes, Fluo3-AM (7 μM, 37°C, 30 min; Molecular Probes, Mulgrave, Victoria, Australia) in Tyrode's buffer (119 mM NaCl, 5 mM KCl, 25 mM HEPES buffer, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 6% glucose). Fluo3-labelled HUVECs were then untreated or treated with vehicle control (MetOH), Fingolimod (100 nM; Sapphire Bioscience), SKi (5 μM; Cayman Chemical Co) or Chlorpheniramine and Cimetidine (anti-histamines, both at 100 nM; Sigma-Aldrich) for 1 h. The intensity of Fluo3 was recorded by confocal microscopy (Carl Zeiss LSM 700, Carnegie, Australia) at every 5 sec for 5 min. Histamine (25 μM; Sigma) was added to HUVECs at 10 sec of the imaging videos. Time-lapse images were analysed using ImageJ (version 1.46r, Wayne Rasband, USA). Each treatment was done in duplicate and 50 cells per video were gated for analysis.

4.5 Discussion

With the current technology, gene manipulation of HUVEC for the surface expression of P-selectin is not yet feasible. Although I cannot directly examine which small molecule(s) and co-factor(s) are associated with histamine-induced P-selectin surface expression via SK-1, this chapter has shown that P-selectin is, at least in part, located in WPBs with vWF. This supports literature that vWF-deficient mice exhibit a reduction of histamine- and TNFα-induced P-selectin expression causing attenuation of neutrophil recruitment [76, 87].

Literature has also demonstrated that histamine-induced exocytosis of WPBs is associated with $[Ca^{2+}]i$ [227]. Herein, my result has shown that histamine-induced $[Ca^{2+}]i$ is SK dependent, where administration of different SK inhibitors can largely abrogate histamine-induced $[Ca^{2+}]i$. Notably, this read-out system measured the total amount of $[Ca^{2+}]i$ (ie. the release of intracellular calcium stores and the uptake of extracellular calcium upon stimulation). To examine which calcium channel(s) is/are regulated by SK, future experiments can include culturing HUVECs and performing the same assay in the absence of extracellular calcium in the medium [231]. Of note, other cell types, such as RBL-2H3 mast cell line [232], *SphK1-/-* or *SphK2-/- ex vivo* mast cells [148] and retinal amacrine cells [233], have been shown that SK is required for both calcium release from internal stores and influx across the plasma membrane.

This is likely due to inhibition of SK reducing the production of S1P, which is an activator for mobilization of calcium from the intracellular stores and as such can then lead to the influx of extracellular calcium (ie. in calcium-induced calcium influx manner) [234].

. Chapter 5: Epicutaneous Fingolimod treatment for allergic inflammation

Current treatments for allergic inflammation, such as dermatitis, include topical application of steroids. Epicutaneous treatment is commonly used and preferred as the medication acts locally on the affected areas/tissues and thus, reduces the systemic adverse effects. However, topical application of steroids can still lead to many undesirable side effects, such as skin atrophy (ie thinning of the skin), increased risk of infection and causing pigmentation of the skin [5]. Furthermore, topical application of steroids can be suboptimal or ineffective in patients and therefore alternative treatments for allergic inflammation are needed [5]. Of note, there is no topical formulation of anti-histamines (ie cream/lotion/ointment) available in Australia to treat allergic inflammation [5]. In a few countries, such as United Kingdom and New Zealand, anti-histamine cream and ointment are approved to be used to relieve insect bites and stings but these products are often found ineffective and not recommended [235]. Herein, the main aim of this Chapter is to examine whether SK inhibitors can be an effective topical medication to prevent and treat allergic inflammation. To address this, two *in vivo* models of ear inflammation were utilised, histamine-induced

allergic inflammation (Figure 5.1a) and passive cutaneous anaphylaxis (PCA) (Figure 5.1b).

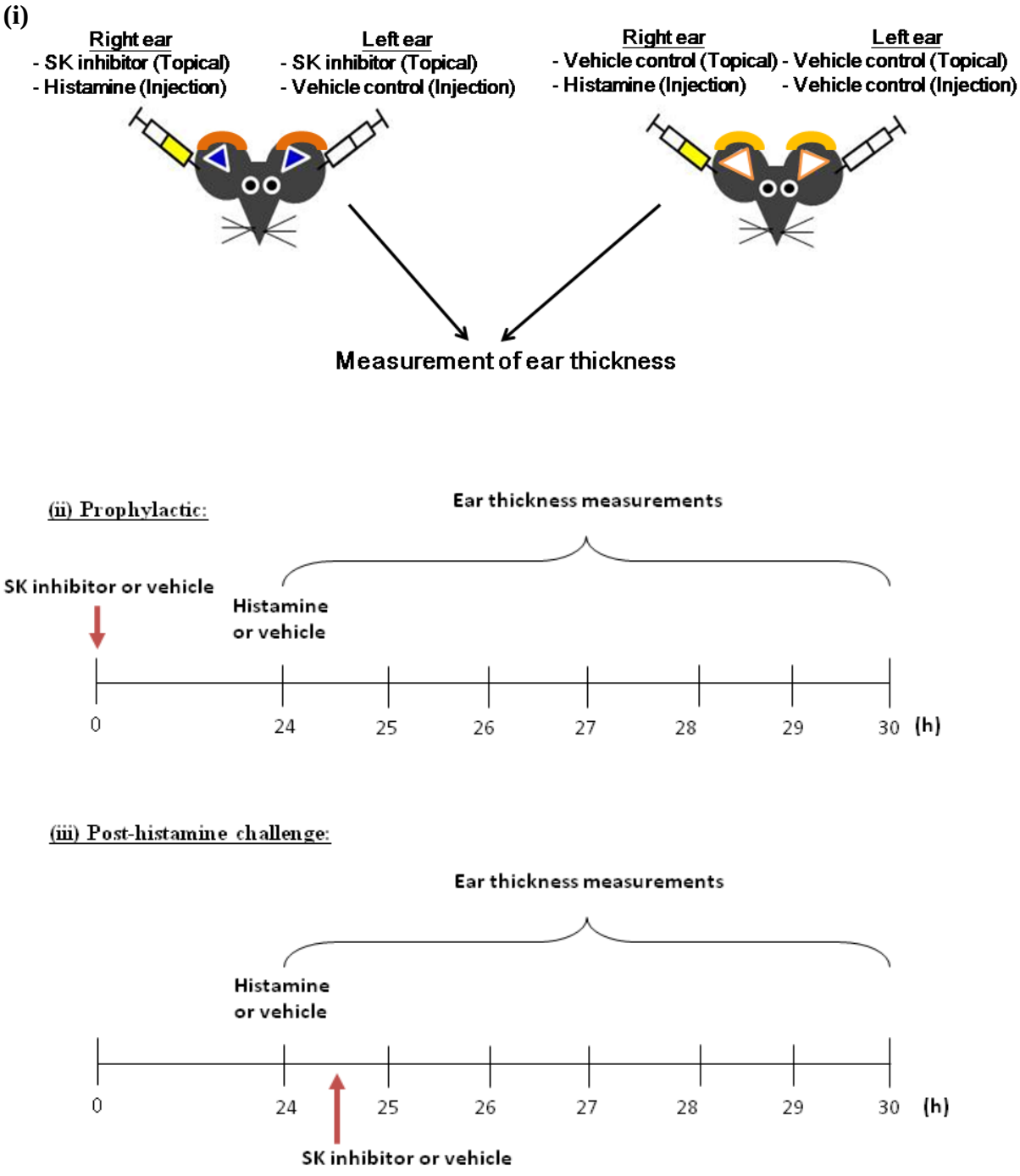


Figure 5.1a Histamine-induced inflammation of the ears. Schematic of histamine-induced inflammation of the ears is shown in (i). Prophylactic treatment of SK inhibitor is shown in (ii). A SK inhibitor was applied topically to both ears 24 h

prior to histamine or vehicle control (HMEM-Pipes) intradermal injection to the right and left ears, respectively. Vehicle control (Ethanol:Propylene glycol:water (EPH) ratio 2:2:1) was also applied topically to a separate group of mice as a negative control. Ear thickness was measured at intervals over 6 h post-histamine injection. Post-histamine challenge treatment with SK inhibitor is shown in (iii). Histamine or vehicle control (HMEM-Pipes) was intradermally injected to the right and left ears, respectively. At 30 min post-histamine challenge, a SK inhibitor was applied topically to the both ears and vehicle control (EPH) was applied to the both ears of the separate group of mice. Ear thickness was measured over time.

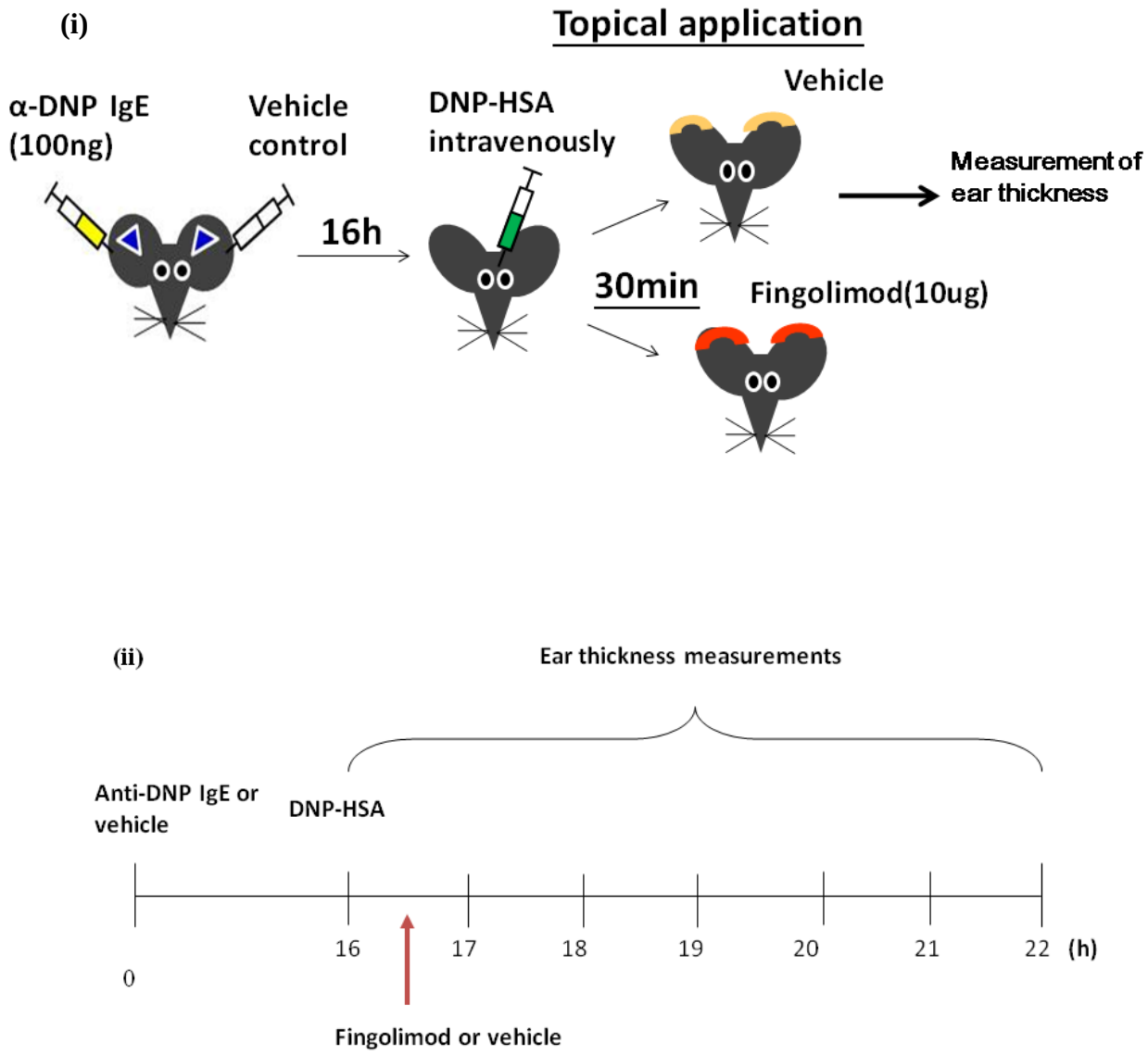


Figure 5.1b Passive cutaneous anaphylaxis. Schematic and the timeline of passive cutaneous anaphylaxis (PCA) are shown in (i) and (ii), respectively. Anti-2,4-Dinitrophenyl (DNP)-IgE and vehicle control (HMEM-Pipes) was injected intradermally to the right and left ears, respectively. After 16 h of DNP injection, DNP-conjugated Human Serum Albumin (HSA) or saline was injected retro-orbitally

(IV) to the mice. Topical Fingolimod (10 μg) was applied to both ears of mice at 30 min post-DNP-HSA injection. Vehicle control (EPH) was applied to both ears of the separate group of mice. Ear thickness was measured at intervals over 6 h post-DNP-HSA injection.

5.1 Optimizing the concentration of histamine in an *in vivo* model of histamine-induced inflammation of the ear

In the previous *in vivo* model of histamine-induced inflammation, we superfused the mouse cremaster muscle with 100 μM of histamine (MW=111) to induce neutrophil rolling flux in the post-capillary venules (Chapter 3.2 and Sun et al [236], Fig 5). Using this concentration of histamine as a guide to develop an ear inflammation model, I first administered 0.5 μg of histamine in 40 μL solvent intradermally to the ears of the mice. Ear thickness measurements were periodically taken to indicate the severity of allergic inflammation. However, I observed that 0.5 μg of histamine did not induce inflammatory reactions (eg. ear swelling) and thus, the dose range finding study of histamine with 0.2, 2, 8, 10 and 20 mg per mouse was performed to determine the optimal dose for subsequent experiments. Of note, histamine concentrations of 10 mg and 20 mg caused excessive irritation and tissue damage, and as such dictated that the highest and most optimal concentration of histamine causing inflammation could be 8 mg per mouse (Figure 5.1c).

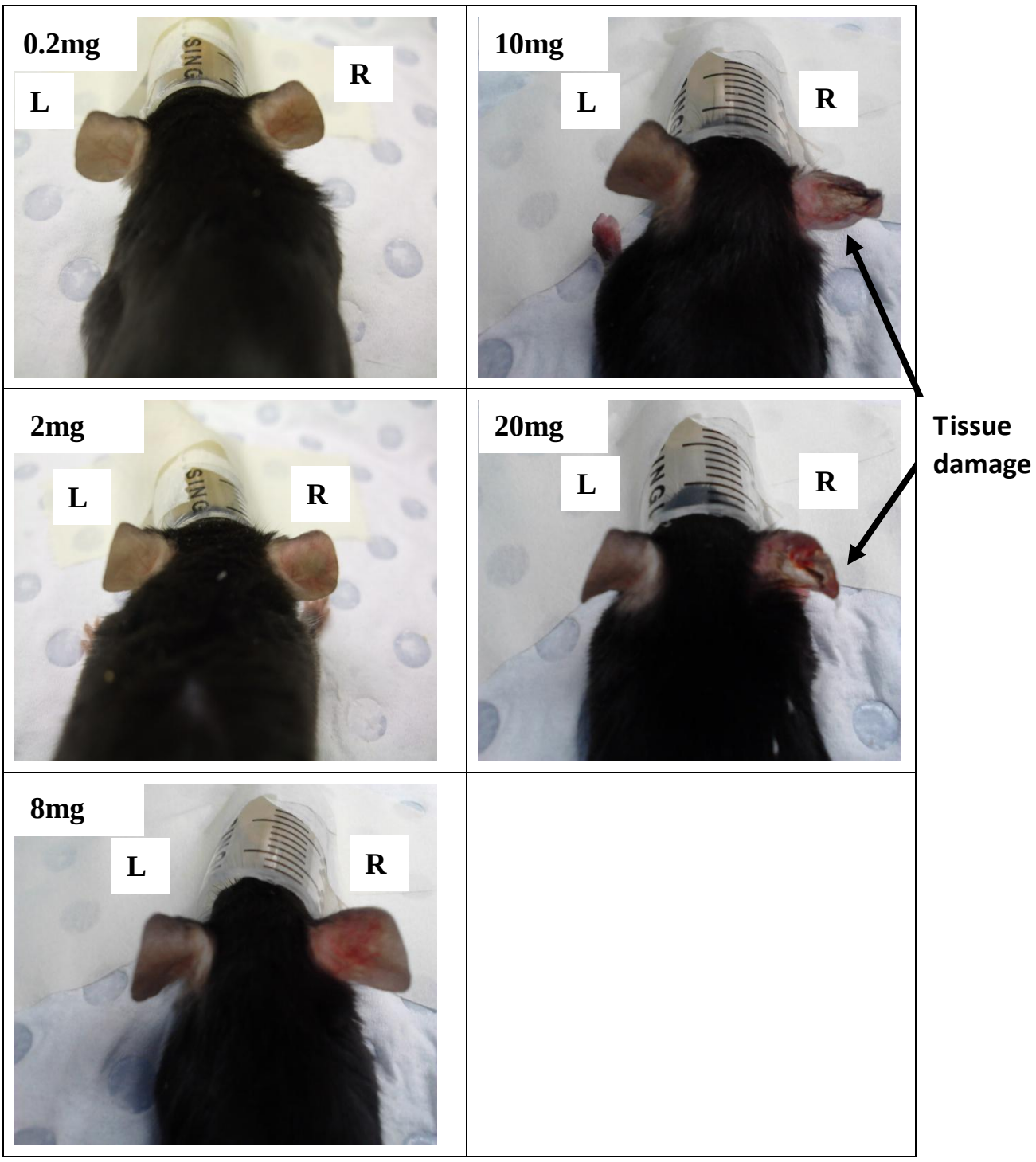


Figure 5.1c Histamine-induced inflammation of the ear. Histamine (0.2, 2, 8, 10 and 20 mg) and vehicle control were injected intradermally to the right ear (R) and left ear (L), respectively. A representative figure of 4 mice/group at 6 h post-histamine.

5.1.1 Materials and method (for Chapter 5.1)

Optimization of histamine concentration:

C57Bl/6 mice were anaesthetized with isoflurane prior to histamine (Sigma-Aldrich; at 0.2, 2, 8, 10 and 20 mg in a final volume of 20 μL in HMEM-Pipes) injection intradermally into the right ear. The same volume of vehicle control (ie HMEM-Pipes) was injected intradermally to the left ear. The change of ear thickness was measured using a dial thickness gauge (model G-1A, Ozaki Manufacturing Company Tokyo, Japan) at 0, 15, 30, 60, 120, 180, 240 and 360 min post-histamine injection.

5.2 Epicutaneous application of Fingolimod attenuates neutrophil recruitment during allergic inflammation

Chapter 5.2 examines the second hypothesis: SK can be a therapeutic target to treat allergic inflammation. It is presented in a manuscript format and will be submitted to *Journal of Clinical Investigation*. (Statement of authorship, Chapter 5.2.1, p.209)

Epicutaneous Application of Fingolimod Attenuates Neutrophil Recruitment During Allergic Inflammation

Wai Y. Sun[1,2,3], Stuart M. Pitson[1,2,3,4]

Michele A. Grimbaldeston[1,2,4] and Claudine S. Bonder[1,2,3,4]

[1]Centre for Cancer Biology, SA Pathology and University of South Australia, Adelaide, South Australia; [2]School of Medicine, University of Adelaide, Adelaide, South Australia; [3]Co-operative Research Centre for Biomarker Translation, La Trobe University, Victoria, Australia; and [4]School of Molecular and Biomedical Sciences, University of Adelaide, Adelaide, South Australia.

Address for correspondence:

A/Prof. Claudine S. Bonder, Centre for Cancer Biology, SA Pathology and the University of South Australia, Frome Road, Adelaide, SA 5000, Australia. E-mail: claudine.bonder@health.sa.gov.au.

ABSTRACT

Neutrophil flux at an inflammatory site is an important immune response during allergic inflammation. The Sphingosine kinase/sphingosine-1-phosphate (SK/S1P) pathway has been described to regulate certain inflammatory cell functions, including the recruitment of neutrophils during the early phase of allergic inflammation. However, the efficacy of SK inhibitors administrated via different routes has not been fully examined to treat allergic inflammation. This study examined the effects of topical application of Fingolimod in two animal models of skin inflammation, one induced by intradermal administration of histamine and the other induced by immunoglobulin (Ig)-E in a setting of passive cutaneous anaphylaxis (PCA). Prophylactic treatment of Fingolimod via topical application prevents histamine-induced allergic immune responses in mice. Strikingly, a single topical application of Fingolimod also attenuates skin inflammation induced by histamine or IgE-mediated PCA reactions. Fingolimod attenuates SK-1, but not SK-2 activity, to cause a reduction in pro-inflammatory chemoattractants and neutrophil flux at the inflammatory site. More importantly, topical application of Fingolimod results in a sustained therapeutic effect compared to similar administration of anti-histamines. Overall, this study demonstrates a new indication for Fingolimod to be used epicutaneously to treat allergic inflammation.

INTRODUCTION

Allergic inflammation is an IgE-mediated “immediate” type I hypersensitivity reaction of the immune system but in its chronic forms, such as asthma and atopic eczema, also involves late IgE responses and some T cell-mediated type IV responses [7]. The clinical phenotype is divided into respiratory allergies (eg allergic rhino-conjunctivitis and asthma), cutaneous allergies and systemic anaphylaxis [237]. Systemic anaphylaxis is a rapid onset, severe, life threatening allergic reaction to allergens found in food, medications or insect venoms [238]. Such allergic inflammation-associated diseases cause significant impact on co-morbidities and finance, as more than US$300 billion health care costs are incurred worldwide every year [239]. Clearly, a better therapeutic approach is required. The current understanding of allergic inflammation is that it is a multistep and progressive disease wherein the early phase includes activation of mast cells and basophils, which release pro-inflammatory mediators (eg histamine) [7]. These inflammatory mediators activate the local vasculature for increased surface expression of adhesion molecules and vasodilation which promote the recruitment of eosinophils and neutrophils via slow rolling, tethering, adhesion and transmigration [181]. Eosinophils have long been recognized to be associated with allergic inflammation [240]. By contrast, neutrophils have been largely ignored despite evidence that (i) neutrophils are often

the first cell to arrive [reviewed in 241], (ii) a correlation exists between neutrophil number and severity of allergic diseases [242-245] and (iii), neutrophils themselves are a major source of histamine and often accumulate at the affected site to achieve an overwhelming quantity compared to other histamine-producing cells (eg mast cells) [244].

Sphingosine kinase (SK) is a highly conserved lipid enzyme in the sphingomyelin pathway which catalyses the phosphorylation of sphingosine to form sphingosine-1-phosphate (S1P) [110]. Two isoforms of SK (SK-1 and SK-2) have been identified, cloned and characterized [122]. SK-1 and SK-2 exhibit different subcellular localization patterns, developmental expression and distribution in adult tissue [110, 122]. They have also been recognized to have both overlapping and alternative biological functions [109, 246, 247]. Mice lacking either SK gene (*Sphk1* or *Sphk2*) are viable, fertile and phenotypically normal [144, 145] but deletion of both genes causes embryonic lethality due to severe defects in angiogenesis and neurogenesis [149], suggesting a redundancy between these genes during development. Increasing evidence suggests that maintaining S1P homeostasis is important for preventing unwanted immune responses [147, 177, 178] and for maintaining appropriate vascular barrier integrity [248]. While the basal level of intracellular S1P is generally low, a number of biological stimuli (eg histamine and

TNFα) can rapidly increase the production of S1P via transient activation of extracellular signal-regulated kinases 1 and 2 (ERK-1/2) and SK activity [109]. Extracellular S1P binds to a family of G-protein coupled receptors ($S1P_{1-5}$) which induce downstream signaling, such as phosphatidyl inositol 3 kinase (PI3K)/Akt and ERK-1/2 [249]. $S1P_{1-3}$ are widely expressed and detected on endothelial cells and smooth muscle cells. $S1P_{4\&5}$ are more restricted in their expression to lymphocyte containing tissues and oligodendrocytes of the central nervous system, respectively [250, 251]. With SK activity and S1P concentrations increased in allergic inflammation [172, 236], a fundamental role for these enzymes is beginning to emerge. Notably, whether SK-1 and SK-2 differ in their functional roles during allergic inflammation is yet to be fully elucidated. This is highlighted in studies focusing on mast cells where discrepancies have emerged due to the distinct mast cell populations derived from origins and species [170]. For example, *in vitro* and *in vivo* silencing of SK-1 inhibited several human mast cell effector functions triggered by IgE crosslinking, whereas silencing SK-2 had no effect [169]. In contrast, a knockout mouse study suggested that SK-2, but not SK-1, was important for mast cell degranulation and cytokine production [148].

Fingolimod (also known as FTY720 or Gilenya®) is an orally active immunomodulatory prodrug that gained approval for the treatment of relapsing and

remitting multiple sclerosis by US Food and Drug Administration (FDA) and Australian Therapeutic Goods Administration (TGA). Fingolimod is predominantly phosphorylated by SK-2 [145] into its active form (FTY720-P) and acts primarily as an antagonist of $S1P_{1,3,4,5}$ where its action on $S1P_1$ of lymphocytes prevents their egress from lymph nodes and the thymus (ie lymphopenia) [209, 252]. More recent studies have revealed that non-phosphorylated Fingolimod can also inhibit SK-1 activity via degradation and thereby reduce S1P production [197, 198].

The preventative management of allergic inflammation is allergen avoidance; however this is extremely difficult and practically impossible due to the ubiquitous nature of some allergens. The only treatment which alters this hypersensitivity is immunotherapy which is invasive, costly and not without risks [253]. Patients with chronic symptoms often require long term treatment with anti-histamines and corticosteroids [254]. These treatments can be suboptimal in symptomatic control and can have long term side-effects on growth and metabolism, especially in children [254]. Clearly, a better understanding of the mechanisms underpinning allergic inflammation is required if new treatment options are to be developed.

We recently demonstrated that inhibition of histamine-induced SK-1 by Fingolimod attenuated neutrophil rolling along human umbilical vein endothelial cells (HUVEC) under shear stress and that i.p. injection of Fingolimod attenuated

histamine-induced neutrophil recruitment to the cremaster muscle [236]. This supports the study by Price and colleagues wherein Fingolimod attenuated airway hyperresponsiveness and inflammation in a mast cell-dependent mouse model of allergic asthma [255]. Herein, we demonstrate that a single topical dose of Fingolimod can effectively prevent and treat allergic inflammation using two animal models of allergic inflammation (histamine-induced inflammation and IgE-mediated passive cutaneous anaphylaxis (PCA)). Briefly, we show that epicutaneous application of Fingolimod attenuated neutrophil rolling and adhesion as well as pro-inflammatory cytokine levels. Importantly, this treatment regime resulted in attenuated inflammation which was restricted to the local area, remained active for up to 24 h post challenge and did not cause lymphopenia. Taken together, this study identifies a new indication for Fingolimod as a topical treatment option for allergic inflammation.

MATERIALS AND METHODS

Animals

Wildtype (WT), SK-1 knock-out (*Sphk1*-/-) and SK-2 knock-out (*Sphk2*-/-) female and male mice on a C57Bl/6 background [144, 145], housed under pathogen-free conditions at SA Pathology (Adelaide, SA, Australia) were used between 8-12 weeks of age. All experimental procedures were approved by the Animal Ethics Committees of SA Pathology and The University of Adelaide, and conform to the guidelines established by the "Australian Code of Practice for the Care and Use of Animals for Scientific Purposes".

Histamine-induced inflammation of the ear

Mice were anaesthetized prior to intradermal injections with histamine (Sigma, Saint Louis, MO, USA) at 0.2, 2 or 8 mg in 20 μL of HMEM-Pipes (Sigma) to the right ear and vehicle controls (ie 20 μL of HMEM-Pipes) to the left ear. Change in ear thickness from baseline was measured at 15, 30, 60, 120, 180, 240 and 360 min post-injection with a dial thickness gauge (model G-1A, Ozaki Manufacturing Company, Tokyo, Japan).

For the systemic prophylactic treatment, mice were anaesthetized prior to retro-orbital (intravenous) administration of Fingolimod at 0.5 mg/kg (Sapphire

Bioscience, Waterloo, NSW, Australia) in 100 μL of PBS/mouse or vehicle controls (ie 100 μL of PBS). Twenty four hours post treatment, 8 mg histamine and vehicle controls in 20 μL of HMEM-Pipes were injected intradermally to the right and left ears, respectively. Ear thickness was measured as detailed above.

For epicutaneous prophylactic treatment, the anaesthetized mice received a single topical application of SKi (Cayman Chemical Co, Ann Arbor, MI, USA) or Fingolimod, both at 10 μg in 40 μL of surfactant as per previously described [256] (100% ethanol, propylene glycol and water (EPH) at 2:1:1 vol/vol/vol) to both ears, and separate control groups received vehicle controls (ie EPH). Twenty four hours post-treatment, histamine challenge and measurement of ear thickness were performed as described above.

To investigate the effect of epicutaneous application of Fingolimod in allergic inflammation, the ears of mice were intradermally injected with 8 mg histamine in 20 μL of HMEM-Pipes. Thirty minutes post-histamine treatment, as detailed above a single topical application of Fingolimod (10 μg) to both ears, or vehicle (EPH) to both ears of a separate control mice was applied and ear thickness was measured over time.

IgE-mediated passive cutaneous anaphylaxis

For epicutaneous treatment in the IgE-mediated PCA model of allergic

inflammation, anaesthetized mice were injected intradermally as previously described [256], with anti-2,4-dinitrophenol (DNP) IgE mAb (clone SPE-7) at 5 μg/mL in 20 μL of HMEM-Pipes to the right ear and vehicle control (HMEM-Pipes) to the left ear. Sixteen hours after IgE sensitization, mice were injected intravenously (retro-orbital) with 2 mg/mL DNP-Human serum albumin (HSA) (Sigma) in 100 μL of 0.9% saline. Epicutaneous application of Fingolimod at 10 μg in 40 μL of EPH was performed at 30 min post-DNP-HSA injection. Change in ear thickness was measured over time as described above.

SK activity assay

SK activity was determined as previously described [257]. For SK-1 activity, whole ear lysates were digested with Liberase (Roche, Castle Hill, NSW, Australia) at 37°C for 2 h prior to separation of tissue debris with a 40 micron cell strainer (BD Biosciences, North Ryde, NSW, Australia). Lysates were incubated with D-*erythro* sphingosine (Cayman Chemical Co) solubilised in 0.05% Triton X-100 and [γ^{32}P]ATP (Perkin Elmer, Vic., Australia). For SK-2 activity, ear lysates were prepared in buffer containing 1M KCl and incubated with D-*erythro* sphingosine solubilised in BSA/PBS and [γ^{32}P]ATP. The radioactively labelled S1P was resolved by 2 thin-layer chromatography (Sigma) separations in the solvents containing butanol, ethanol,

water and acetic acid (8:2:2:1). The radioactive spots were quantified by Phosphorimaging Typhoon 9410 (Fullerton, CA, USA) and ImageQuant 5.2 program (GE Healthcare, Rydalmere, NSW, Australia).

Flow cytometric analysis

Whole ears were harvested, digested with Liberase at 37°C for 2 h and then filtered with a 40 micron cell strainer (BD Biosciences) to remove debris. Following FcR blocking, cells were incubated with anti-Gr1-PE and anti-F4/80-FITC antibodies (for granulocytes and macrophages) or IgG isotype controls (all at 2 μg/mL from eBioscience, San Diego, CA, USA) on ice for 30 min prior to analysis using an Accuri flow cytometer (BD Biosciences) and FCS Express 4 Flow Cytometry: Research Edition software (De Novo Software, Los Angeles, CA, USA).

Whole blood was collected from anaesthetized mice followed by red blood cell lysis using Ammonium-Chloride-Potassium (ACK) lysis buffer. Cells were incubated with CD3-FITC or IgG isotype control antibodies (2 μg/mL, eBioscience) on ice for 30 min prior to analysis using an Accuri flow cytometer and FCS Express 4 Flow Cytometry: Research Edition software.

The inflammatory cytokine assay utilised a cytometric bead array kit as per manufacturer's protocol (BD Biosciences). Briefly, whole ears were harvested,

digested and filtered to remove debris (as detailed above) prior to incubation with the microbeads that conjugated to cytokines (IL-6, IL-10, MCP-1, IFN-γ, TNF, IL-12p70 and KC). Sample analysis was performed using an Accuri flow cytometer with a selectable laser module (BD Biosciences) and FCAP Array Software (version 3.0, BD Biosciences).

Histology

Ear pinna were fixed in 10% (v/v) buffered formalin at 4°C overnight and then embedded in paraffin. Four-micron cross-sections were prepared for hematoxylin and eosin (H&E) staining. Analysis was performed using an Olympus CX41 microscope (Hachioji-shi, Tokyo, Japan), and images were captured using NanoZoomer Digital Slide Scanner (NDP-Hamamatsu, Hamamatsu, Japan).

Intravital microscopy and *in vivo* experimental procedure

For intravital microscopy experiments, WT mice were anaesthetized by intraperitoneal (ip) injection of ketamine (10% v/v) and xylazine (5% v/v) in 0.9% saline (10 μL/g). As described above, histamine was injected intradermally into the ear and epicutaneous application of Fingolimod was performed. A separate group of histamine-treated mice received epicutaneous application of 0.5% anti-histamines

(chlorpheniramine and cimetidine; 0.2 mg in 40 μL EPH; Sigma) at 30 min post-histamine challenge. To visualize blood vessels and granulocytes by 2-photon microscopy, mice were retro-orbitally (iv) injected with FITC-Dextran (150kDa, 1 mg in 100 μL of PBS, Sigma) and Rhodamine 6G (1 mg in 100 μL of PBS, Sigma).

Intravital microscopy (LSM710-NSO, Carl Zeiss Australia, Carnegie, NSW, Australia) of the ear was performed with a plain 20x objective lens and 10x eyepiece. The green (for FITC) and red (for Rhodamine 6G) lasers with external detectors (External Cascadable External Non Descanned Detector with GaAsP) were used. Live images were recorded every 2 sec for up to 4 min using Zen 2011 (version 7.0.4.0, Carl Zeiss Australia). Two post-capillary venules per animal ear were analysed for leukocyte trafficking by playback analysis. Leukocyte rolling was defined as slow rolling along the blood vasculature while adherent leukocytes were defined as those remaining stationary for at least 10 sec.

Statistical analysis

Data are expressed as mean ± SEM from at least 3 independent experiments with 2 to 3 mice/treatment group/experiment, and statistically analysed by Student's t-test or 2-way ANOVA for multiple comparisons. $p<0.05$ was considered significant.

RESULTS

Histamine-induced inflammation of the ear is dose dependent

Using physiologically relevant concentrations of histamine [258, 259], we injected histamine at 0.2, 2 and 8 mg intradermally into the ear pinna of mice and determined the change of ear thickness over the following 6 h. As shown in Figure 1, 0.2 mg of histamine caused a mild inflammatory response with modest increases in ear thickness. This peaked within 30 min and subsided significantly within 4 h post-injection. Histamine injection of 2 or 8 mg further increased the ear swelling and although ear thickness remained significantly higher than controls over the 6 h tested, it also peaked at 30 min and subsided over time. The highest dose of histamine tested (8 mg) induced the greatest effect in terms of ear swelling, redness and mild irritation. Notably, this dose of histamine did not cause the significant irritability or tissue damage observed when ≥10 mg of histamine was injected (data not shown). Based on these findings all subsequent experiments utilised 8 mg histamine.

Prophylactic treatment of Fingolimod attenuates inflammation and is SK-1 dependent

Increasing evidence suggests that SK and S1P have a role in the development of allergic inflammation, as they regulate the expression of adhesion molecules on

vascular endothelium and activate inflammatory cells [61, 148, 236]. Based on these previous findings, we hypothesized that the FDA/TGA approved prodrug, Fingolimod, may be used to treat allergic inflammation [198, 252]. Using the aforementioned model of histamine-induced inflammation in the ear, we firstly performed systemic administration of Fingolimod or vehicle controls via intravenous (IV) injection for 24 h prior to histamine challenge. Figure 2A shows that histamine-induced ear thickness is significantly attenuated in mice pre-treated with Fingolimod.

We next investigated whether Fingolimod can be applied topically to restrict a more localised allergic response. As shown in Figure 2B, mice that were pre-treated with Fingolimod topically to the ear 24 h prior to histamine challenge exhibited a significant attenuation in ear swelling. Interestingly, topical application of Fingolimod appeared to attenuate histamine-induced ear swelling to a similar level as that reduced by IV administration of the drug (Figure 2A). The known biological effect of Fingolimod via oral administration is to induce lymphopenia in MS patients [145]. Herein, we determined whether epicutaneous application of Fingolimod would also cause lymphopenia by comparing the levels of the circulating CD3+ lymphocytes in the untreated and Fingolimod-treated mice via topical application or IV injection. As shown in Supplemental Figure 1, epicutaneous application of Fingolimod did not cause lymphopenia in the mice but IV administration did, which agrees with the

literature that systemic administration of Fingolimod can cause lymphopenia [145]. Together, these results suggest that Fingolimod can be used topically to control local allergic inflammation without suppressing the immune system and thus supports allergic inflammation as a new indication for the use of Fingolimod.

To confirm the role of SK in histamine-induced allergic inflammation, we executed two separate but complementary experiments. First, a second SK inhibitor, SKi, was applied topically to the ears prior to histamine challenge. As shown in Figure 3A, like Fingolimod, epicutaneous application of SKi significantly attenuated histamine-induced ear swelling over time when compared to the untreated control group. To confirm that Fingolimod and SKi reduced SK activity in the aforementioned histamine-induced inflammation of the ear and to determine which SK isoform was attenuated, we executed SK-1 and SK-2 activity assays on the whole ear lysates. As shown in Figure 3B, both Fingolimod and SKi decreased histamine-induced SK-1 activity by ~50%. Notably, histamine did not increase SK-2 activity in mouse ears, nor did Fingolimod or SKi reduce SK-2 activity. Second, *Sphk1*$^{-/-}$ and *Sphk2*$^{-/-}$ mice were investigated for their response to intradermal injection of 8 mg histamine as well as epicutaneous pre-treatment with Fingolimod (or vehicle control). Figure 3C shows that *Sphk1*$^{-/-}$ mice were unable to respond to histamine for increased ear swelling. In contrast, *Sphk2*$^{-/-}$ exhibited a similar ear swelling to that

observed in WT mice (Figure 3A) and responded to topical pre-treatment of Fingolimod resulting in attenuated histamine-induced reactions. Together, these results suggest that histamine-induced inflammation is, at least in part, SK-1 dependent, and the effect of Fingolimod-attenuated inflammation is SK-2 independent.

Epicutaneous application of Fingolimod attenuates histamine-induced neutrophil recruitment and inflammatory chemoattractants

We and others have previously shown that neutrophils are rapidly recruited in response to stimuli (histamine, thrombin and TNFα) *in vitro* and *in vivo* [181, 236, 260]. To determine whether prophylactic epicutaneous application of Fingolimod regulates the recruitment of neutrophils via SK, H&E staining was performed on the fixed ears that were harvested at 6 and 24 h post-histamine challenge. As shown in Figure 4A, intradermal histamine challenge caused oedema and vasodilation of the ear at 6 h followed by significant cellular infiltration at 24 h in the histamine alone groups. Strikingly, prophylactic epicutaneous application of Fingolimod largely attenuated the histamine-induced vasodilation and cellular infiltration at 6 and 24 h.

To determine which cell type(s) were recruited during histamine-induced reactions at 24 h, flow cytometric analysis was performed on single cell suspensions

from ear digests to detect Gr-1^{+} and F4/80^{+} cells. Notably, using these markers, three populations can be detected within the 'granulocyte' gate as determined by forward scatter and side scatter profile. These include Gr-1^{hi}/F4/80^{lo}, Gr-1^{lo}/F4/80^{hi} and Gr-1^{hi}/F4/80^{hi} cells. As shown in Figure 4B, Gr-1^{hi}/F4/80^{lo} granulocytes within the ears of control mice constitute ~15%. When treated with histamine ~70% of the gated cells were Gr-1^{hi}/F4/80^{lo}. This suggests that within 24 h of exposure to histamine, neutrophils were recruited. Importantly, prophylactic epicutaneous treatment of Fingolimod attenuated the histamine-induced Gr-1^{hi}/F4/80^{lo} population, indicating that neutrophil infiltration was abrogated.

As murine KC is a potent chemoattractant for neutrophils [261] and histamine is a known stimulus of KC production [262], we next examined whether KC levels were modified in response to Fingolimod application. As shown in Figure 5, 24 h after histamine injection levels of KC were significantly elevated within the ears of the mice. Moreover, prophylactic epicutaneous treatment with Fingolimod significantly attenuated histamine-induced production of KC. We also investigated other known inflammatory markers of allergic inflammation, TNFα, MCP-1, IL-6, IL-12p70, IFNγ and IL-10. Also shown in Figure 5, histamine significantly increased the concentration of TNFα, MCP-1 and IL-6 in the ears. These were also reduced in mice treated with Fingolimod. No significant increase in IL-12p70, IFNγ and IL-10 was

observed in response to histamine (data not shown).

Epicutaneous application of Fingolimod post histamine challenge also reduces inflammation

With data suggesting that epicutaneous application of Fingolimod prior to histamine exposure can attenuate inflammation in the ear, we next examined whether Fingolimod can be used as a monotherapy for two established *in vivo* models of skin inflammation with one driven by histamine and another one associated with IgE-mediated PCA reactions. Figure 6A shows that histamine-induced ear swelling can be rapidly resolved when Fingolimod was applied epicutaneously 30 min post-histamine injection. To support our hypothesis that Fingolimod can be used to treat established allergic inflammation, we performed the second *in vivo* model, PCA, where DNP-HSA causes aggregation of adjacent IgE-bound FcεRIs to activate mast cells via cross-linking of the FcεRIs. The activated mast cells are able to degranulate and release inflammatory mediators such as histamine. As shown in Figure 6B and in agreement with Yip et al [256], ear swelling was observed within 15 min post-DNP-HSA injection in IgE-sensitized ear pinnae. This peaked at 30 min and then gradually subsided over 6 h. Importantly, epicutaneous application of Fingolimod to the ears 30 min post-anti-DNP-IgE injection significantly reduced ear swelling within

90 min of Fingolimod application, (Figure 6B). Taken together, these results show that a single topical dose of Fingolimod can attenuate ear swelling associated with IgE-mediated PCA.

Epicutaneous application of Fingolimod attenuates histamine-induced neutrophil recruitment

We have previously shown that Fingolimod attenuates leukocyte rolling and adhesion in histamine-induced activation of the cremaster muscle [236]. To determine whether epicutaneous application of Fingolimod also reduces histamine-induced leukocyte rolling and adhesion in the ear, we utilized intravital microscopy where IV injection of Rhodamine 6G was used to visualize all circulating leukocytes [263]. As shown in Figure 7A and Supplemental video S1, intradermal injection of histamine into the ear pinnae significantly increased leukocyte rolling in the post-capillary venules within one hour and was sustained at 24 h post histamine challenge. At 30 min post-histamine challenge, epicutaneous application of Fingolimod significantly attenuated leukocyte rolling events within 1 h of histamine exposure and was sustained for 24 h. By contrast, epicutaneous application of 0.5% Chlorpheniramine and 0.5% Cimetidine (H1 and H2 antagonists, respectively) failed to demonstrate any reduction in leukocyte rolling events. A similar result was also observed for leukocyte

adhesion with epicutaneous application of Fingolimod, but not anti-histamines, significantly attenuating the number of histamine-induced adherent leukocytes in the ear (Figure 7B and Supplemental video S1).

DISCUSSION

The prevalence of allergic diseases continues to rise across all age, gender and racial groups with the Australasian Society for Clinical Immunology and Allergy reporting ~7.7 million of Australians, which is a 70% increase in the number of Australians, with allergies by 2050 [264]. Current treatments for acute and chronic allergic inflammation include anti-histamines, epinephrine and corticosteroids [265], which with long term use can cause sedation, increased anti-cholinergic effects (ie dry mouth and blurred vision), and suppression of growth, bone metabolism and adrenal function [254]. Clearly, a better understanding of the mechanisms underpinning allergic inflammation is required if new therapeutic options are developed. The FDA and TGA approved oral drug, Fingolimod, has the known mode of action to antagonise $S1P_1$ and then downregulate its signalling pathway once it is phosphorylated (ie become phosphorylated Fingolimod or FTY720-P) [266]. A previous study also showed that Fingolimod without being phosphorylated can inhibit and degrade SK-1 [198]. Herein, we examined whether topical application of Fingolimod can inhibit SK-1 to attenuate skin inflammation in two animal models, one induced by histamine and another induced by IgE-mediated PCA reactions. Firstly, we observed that topical prophylactic application of Fingolimod attenuated histamine-mediated inflammation and IgE-mediated PCA reactions in the ear of mice.

Similar levels of reduction in histamine-induced ear swelling were also observed when mice were pre-treated with Fingolimod via IV injection. Secondly, we revealed that histamine induced SK-1, but not SK-2 activity within the mouse ears. Wildtype and *Sphk2*$^{-/-}$, but not *Sphk1*$^{-/-}$ mice responded to histamine for increased ear swelling which could be attenuated with epicutaneous application of Fingolimod. Fingolimod also demonstrated an ability to reduce ear swelling when the allergic response was already established in both models of histamine challenge as well as anti-DNP IgE challenge. These observations provide the first opportunity for Fingolimod as a therapeutic option for established skin inflammation. Upon investigation of the underlying mechanisms of Fingolimod, the epicutaneous application of Fingolimod attenuated histamine-induced neutrophil rolling and adhesion at the site of inflammation and reduced the inflammatory mediators KC, TNFα, IL-6 and MCP-1. Importantly, histamine-induced neutrophil influx was not attenuated in response to epicutaneous application of anti-histamines, thus supporting a previous study that the therapeutic effect of topical anti-histamines is short-acting and suboptimal [267].

An important role for neutrophils in allergic inflammation has been well described for food allergies [268], dermatitis [269] and anaphylaxis [270]. During the early phase of allergic inflammation, high concentrations of histamine (~1 to 100 g/L) are locally released to rapidly activate the vasculature for recruitment and activation

of neutrophils [271]. Interestingly, histamine is also important in the late phase of allergic inflammation as it supports the production of pro-inflammatory cytokine production from infiltrating inflammatory cells and endothelial cells [272]. Mast cells and basophils have long been recognized as the main producers of histamine during an allergic response. However, there is a growing body of evidence that neutrophils can also produce significant amounts of histamine during allergic inflammation [273, 274]. In fact, neutrophils isolated from allergic patients release five-times more histamine upon allergen challenge when compared to healthy individual counterparts [273]. These activated neutrophils are able to release cytokines and chemokines, including IL-8 (KC in mice), CCL3, chemokine (C-X-C motif) ligand (CXCL)-1, CXCL9 and CXCL10, to further promote the recruitment of neutrophils, monocytes, dendritic cells (DCs) and T lymphocytes [23, 48].

Increasing evidence suggests that the sphingolipid pathway is involved in allergic inflammation with SK and S1P regulating a number of cellular functions of mast cells [148], lymphocytes [145] and neutrophils [175], all of which are important for the development and enhancement of allergic inflammation. As Fingolimod interferes with the SK/S1P pathway, we and others have examined it’s suitability as a treatment for allergic inflammation. Our previous study showed that inhibition of SK-1 by Fingolimod attenuated histamine-induced P-selectin surface expression on

ECs, which was required for neutrophil rolling and recruitment during the early phase of allergic inflammation *in vitro* and *in vivo* [236]. In the comparison of the current therapeutic dose for MS in human (0.5 mg daily), Muller et al [275] defined Fingolimod at 0.1 mg/kg (~0.002 mg per mouse) as low dose and 2 mg/kg (~0.04 mg per mouse) as high dose in mice. Systemic administration of 0.002 mg Fingolimod followed by ventilation challenge with tidal volume and oxygen exhibited enhanced endothelial barrier function and reduced lung permeability. By contrast, systemic administration of 0.04 mg Fingolimod increased pulmonary vascular permeability and caused endothelial apoptosis [275]. Based on their observations, we selected to perform the dose of 0.01 mg Fingolimod, which is at the middle dose range of that described by Muller et al [275]. We observed that application of Fingolimod at 0.01 mg could significantly attenuate the ear swelling driven by histamine or IgE when compared to that of the vehicle control groups. Taken together, we propose that Fingolimod attenuates the recruitment of neutrophils via its down-regulation of P-selectin expression by the vascular endothelial cells as well as reduced vasodilation.

Notably, Fingolimod causes lymphopenia and thus acts as an immuno-suppressant which can suppress the systemic immune response when administered orally or intravenously [276]. The adverse effects of Fingolimod include transient bradycardia, increased risk of infection, macular edema and embryonic

development [277, 278]. There are a few studies have examined whether Fingolimod can be applied topically to retain the local therapeutic effect and minimize the systemic adverse effects. First, Reines et al [279] showed that topical prophylactic application of Fingolimod to mice at 10 μg/dose/day over a period of nine days inhibited dendritic cell (DC) migration in a mouse model of allergic contact dermatitis. However, reduction of the total peripheral lymphocytes was observed when compared to the control counterparts. Second, Kleinjan et al [280] showed that repeated intranasal administration of Fingolimod at 6 μg/dose/day for nine days over three weeks attenuated OVA-induced recruitment of eosinophils and DCs in the nasal mucosa of allergic rhinitis mice. Importantly, no systemic effect of lymphopenia was observed in their study. This suggests that different doses and different sites of topical application contribute to various levels of systemic absorption. Together, these studies demonstrated that Fingolimod can be applied topically as a prophylactic treatment to attenuate allergic inflammation. However, the therapeutic effect of a single topical dose of Fingolimod in allergic inflammation had not been previously examined, nor had the effect of Fingolimod applied post-allergen challenge been investigated. Herein, we have shown that a single topical dose of Fingolimod at 10 μg can be an effective prophylactic agent to reduce the histamine-induced ear swelling (Figure 2B). The percentage of the peripheral CD3 lymphocytes and the peripheral Gr-1^{hi}/F4/80^{lo}

granulocytes did not differ, which suggests that the single topical dose of Fingolimod is not absorbed systemically to cause lymphopenia and inhibit the mobilization of neutrophils from bone marrow, respectively (Supplemental Figure 2). Strikingly, the topical application of Fingolimod at 30 min following histamine challenge significantly reduced inflammation of the ear (Figure 6A). To further examine the effect of topical treatment of Fingolimod in allergic inflammation, the second mouse model of PCA was performed, which involves a cell mediated response with IgE and mast cells being the key players. The results showed that topical application of Fingolimod at 30 min after IgE-mediated PCA reactions significantly reduced the allergic inflammation of the ear (Figure 6B). Notably, histology and antibody staining revealed that topical treatment of Fingolimod largely attenuated neutrophil influx at the inflammatory site.

To determine whether the mechanism of Fingolimod-attenuated inflammation is via inhibition of SK/S1P pathway, we performed two different but complementary experiments. First, topical application of a SK inhibitor, SKi, also attenuated the histamine-induced ear swelling to the levels similar to that of Fingolimod treatment (Figure 3A). Second, genetically mice deficient in either *Sphk1* or *Sphk2* were used to compare with WT mice in the model of histamine-induced inflammation of the ear (Figure 3C&D). WT and *Sphk2*$^{-/-}$ mice had increased ear swelling in response to

histamine challenge, and more importantly, topical treatment of Fingolimod significantly reduced this inflammatory response (Figure 3D). This suggests that the mechanism of Fingolimod is unlikely SK-2 dependent. Strikingly, *Sphk1*$^{-/-}$ mice exhibited a large reduction of histamine-induced ear swelling when compared to *Sphk2*$^{-/-}$ and WT mice (Figure 3C&D). The residual ear swelling that was not completely abolished with Fingolimod treatment may be due to the constitutive basal expression of P-selectin found in skin [201].

Previous studies have shown that *Sphk1*$^{-/-}$ or *Sphk2*$^{-/-}$ mice demonstrate normal neutrophil functions in response to lipopolysaccharides challenge [212]. *Sphk1*$^{-/-}$ and *Sphk2*$^{-/-}$ mice also exhibit a similar basal level of neutrophil rolling flux in the cremaster muscle when compared to WT [236]. Thus, this suggests that Fingolimod is likely to regulate how neutrophils are recruited by ECs to inflammatory sites, and not via the inhibition of neutrophil activation and survival. This is supported by our previous studies wherein inhibition of SK-1 abrogated histamine-induced P-selectin and TNFα-induced and $\alpha_5\beta_1$ integrin expression by ECs [61]. Notably, these adhesion molecules are required for neutrophils to roll on and adhere to the endothelium [61, 236]. In addition, human pulmonary artery ECs and lung microvascular ECs exhibit a decrease in thrombin and neutrophil-activated permeability *in vitro* following treatment with SK inhibitors (SKI-II and DMS) [281], which can contribute to the

attenuation of neutrophil influx at the inflammatory sites. Herein, I also observed that the population of neutrophils (Gr-1^{hi}/F4/80^{lo}) was reduced following topical application of Fingolimod in the histamine-treated ears (Figure 4B) but did not differ in the peripheral blood (Supplemental Figure 2). These results suggest that Fingolimod does not affect the survival and mobilization of neutrophils. Besides, epicutaneous application of Fingolimod may inhibit the function of mast cells, as Dillahunt et al have shown that SK-1 is also involved in murine mast cell functions, including degranulation, calcium mobilization, and cytokine and leukotriene production, although all of these are primarily SK-2 dependent [170]. Using adoptive transfer of bone marrow-cultured mast cells (BMCMCs) from *Sphk1*$^{-/-}$ or *Sphk2*$^{-/-}$ mice into mast cell-deficient mice may address whether or not topical application of Fingolimod affects SK-1 and SK-2 in mast cells. However, this is beyond the scope of this study.

There is precedent for topical treatment with small molecules to minimize allergic inflammation. Topical prophylactic application of dasatinib and LCB 03-0110 (inhibitors for tyrosine kinases) [282], siRNA against CD86 (inhibitor for DC migration) [283] and PAP-1 (voltage-gated potassium channel inhibitor for CD8 T cell infiltration) [284] have been shown to attenuate allergic contact dermatitis in rodents, without causing the adverse effect of skin atrophy when compared to the

conventional steroid treatment. Prophylactic topical treatment with anti-histamines antagonizing both H_1 and H_2 receptors enhance barrier permeability but the effect is short-lived for only up to 2 h post-allergen challenge in mice [267]. Price et al has shown that repeated intranasal treatment with a specific SK-1 inhibitor (SK1-I) can suppress mast cell activation and proinflammatory cytokines via an NF-κB-dependent pathway in the murine model of ovalbumin (OVA)-induced asthma [255]. Our *in vivo* experiments using intravital microscopy further confirmed that a single dose of Fingolimod can be applied topically post-histamine challenge to attenuate neutrophil recruitment, with the effect still remaining at 24 h post-histamine (Figure 7).

Collectively, this study has shown that the mechanism of Fingolimod controlling allergic inflammation is via the recruitment of neutrophils by endothelial cells which is, at least in part, SK-1 dependent. This study has revealed a new indication for Fingolimod to be used topically as an effective pharmacological agent to treat allergic inflammation with its fast acting and relatively long-lasting effect.

ACKNOWLEDGEMENTS

We thank Zelig Eshar (Weizmann Institute of Science, Israel) for providing IgE anti-DNP mAb-producing mouse SPE-7 hybridoma cells, Dave Yip and Tim Hercus for IgE anti-DNP mAb purification.

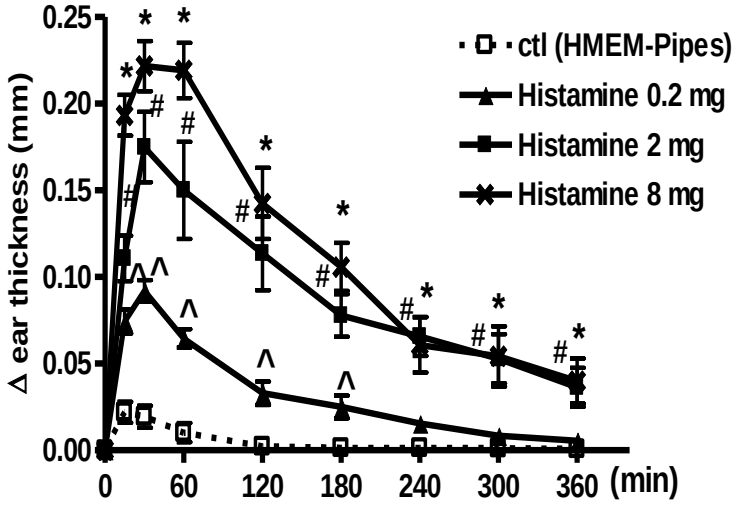


Figure 1. Histamine-induced inflammation of the ear is dose dependent. Mice were injected intradermally with HMEM-Pipes (control) to the left ear and various concentrations of histamine to the right ear. Ear thickness was measured at 0, 15, 30, 60, 120, 180, 240 and 360 min post-histamine injection. Mean ± SEM, n=6-10, in 3-5 individual experiments, *, $^{\#}$, ^, $p<0.05$ vs ctl, 2-way ANOVA.

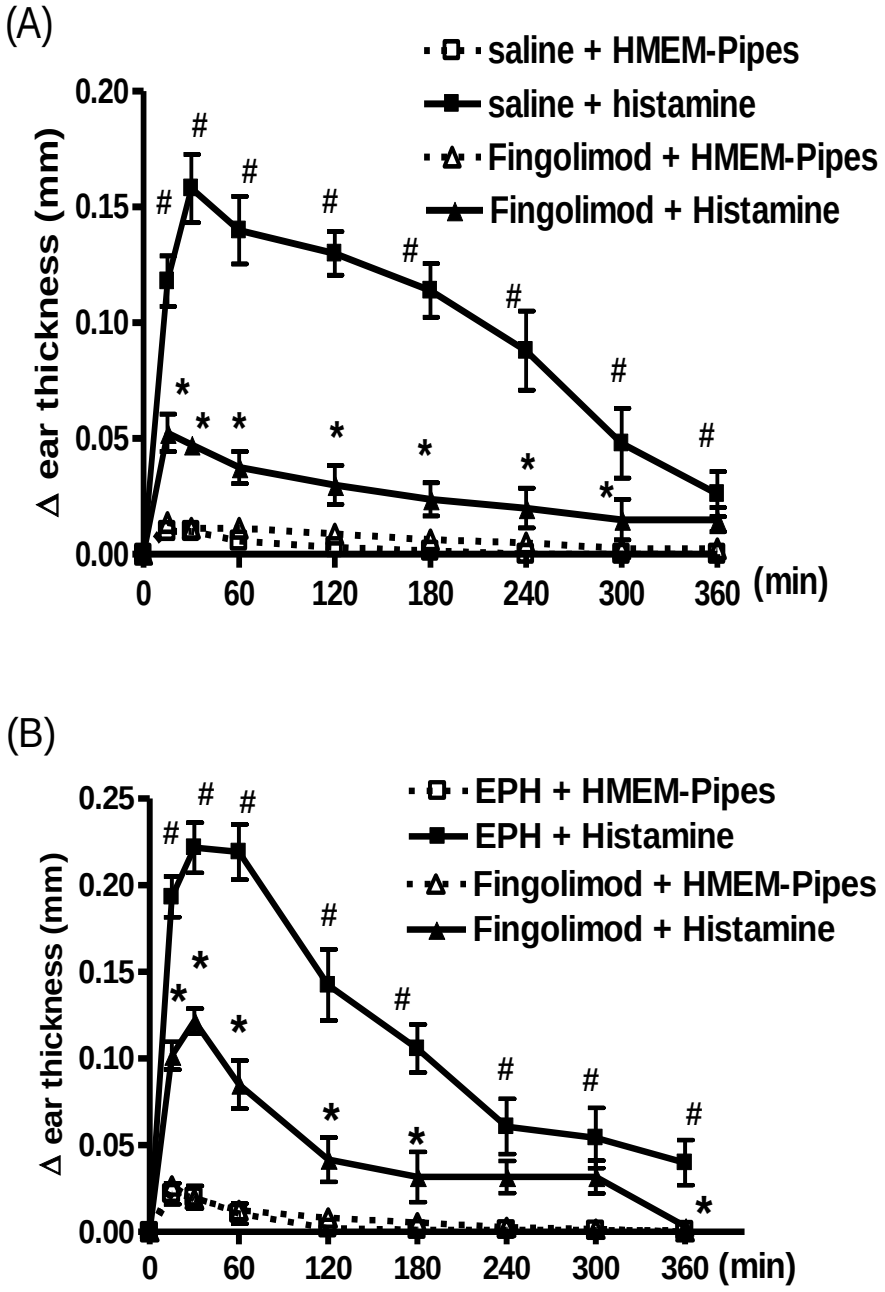


Figure 2. Systemic and topical prophylactic treatment of Fingolimod attenuates histamine-induced ear swelling. (A) Fingolimod (10 μg) or saline (control) was administered systemically via retro-orbital injection prior to histamine (8 mg) and HMEM-Pipes (control) injection intradermally to the right and left ears, respectively. Ear thickness was measured over time. Mean ± SEM, n=7-8, in 4 individual

experiments, #, $p<0.05$ vs saline+HMEM-Pipes, *, $p<0.05$ vs saline+histamine, 2-way ANOVA. (B) Fingolimod (10 μg) or EPH (control) was applied topically to the ears 24 h prior to histamine (8 mg) and HMEM-Pipes (control) injection intradermally to the right and left ears, respectively. Ear thickness was measured over time. Mean ± SEM, n=9, in 3 individual experiments, #, $p<0.05$ vs EPH+HMEM-Pipes, *, $p<0.05$ vs EPH+histamine, 2-way ANOVA.

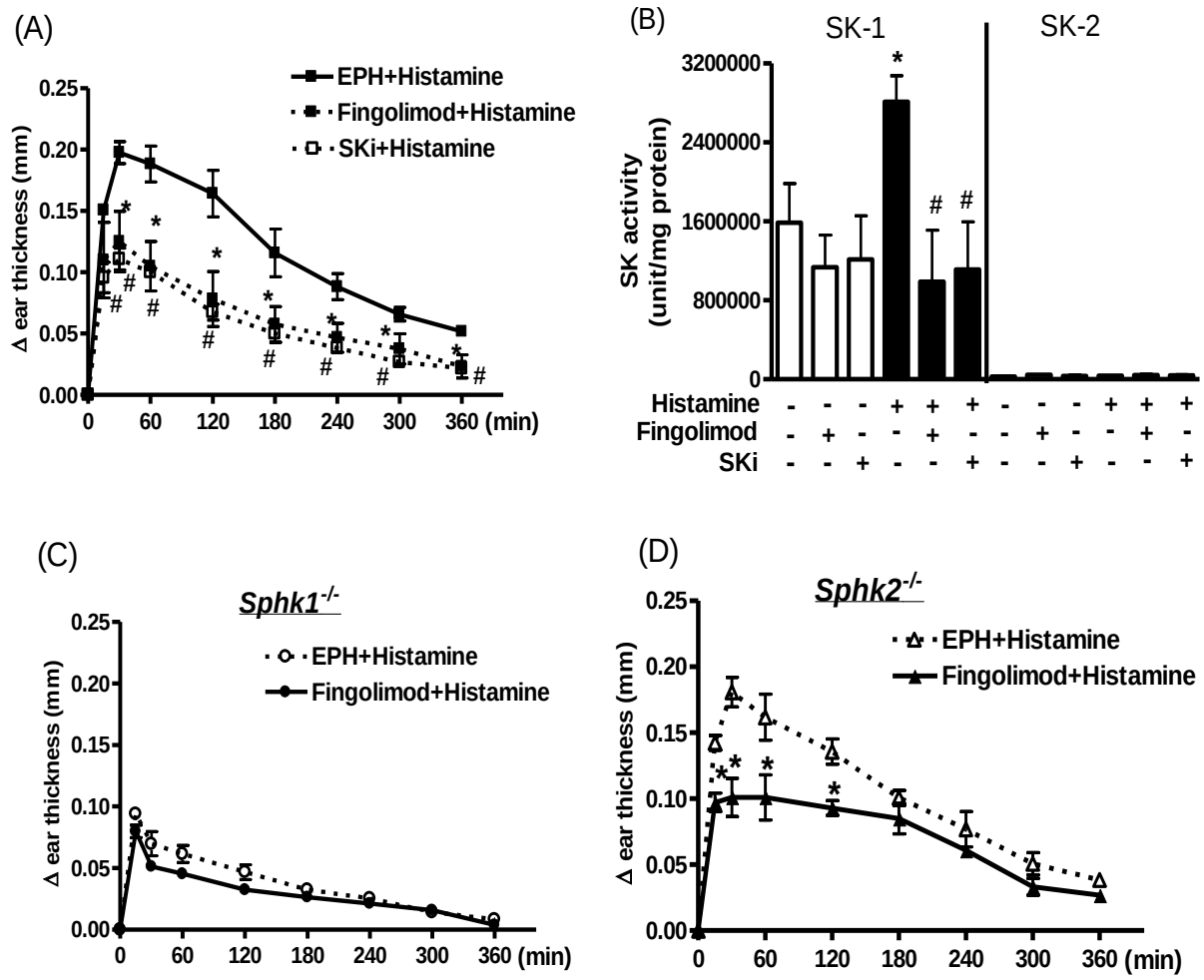
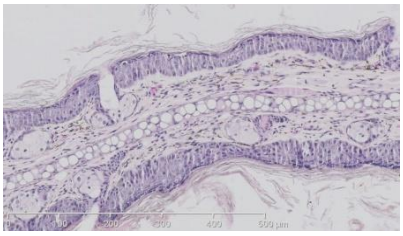
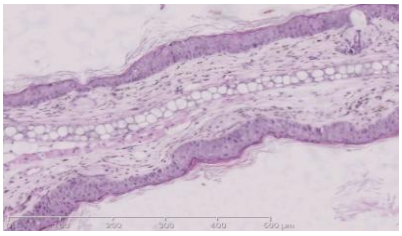
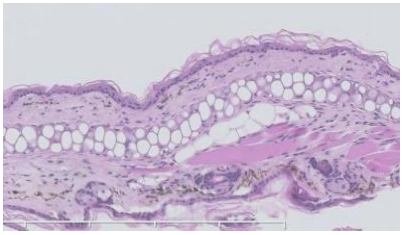
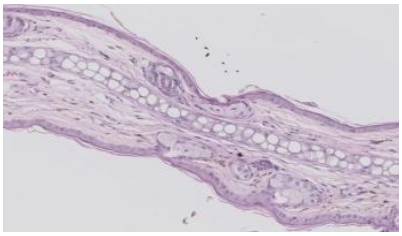
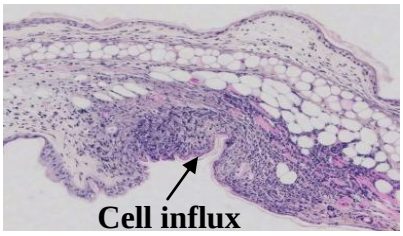
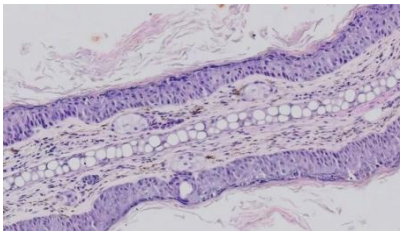
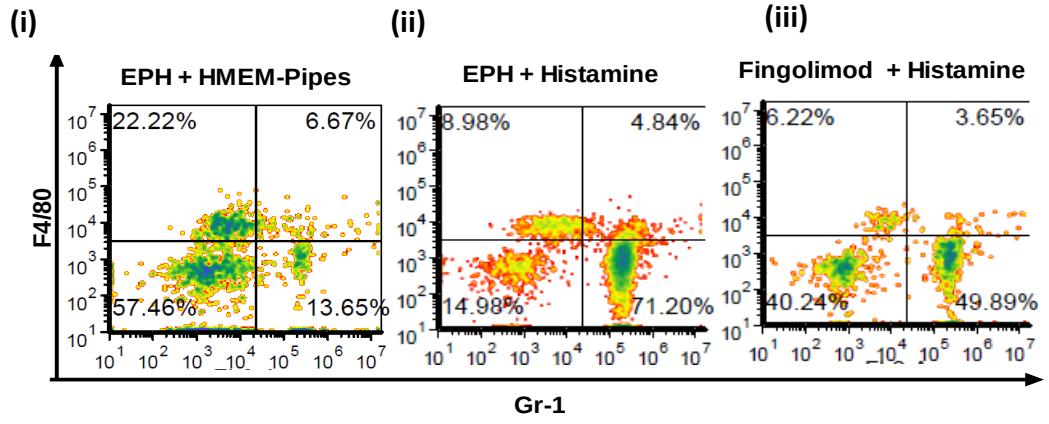


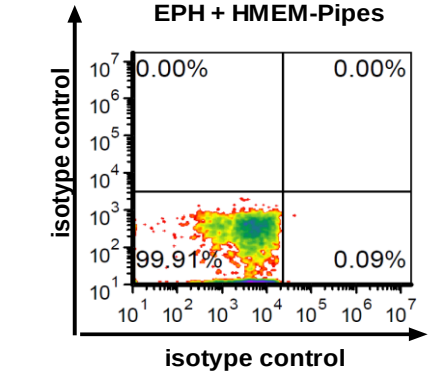
Figure 3. Attenuation of histamine-induced ear swelling is SK-1 dependent. (A) Fingolimod (10 μg), SKi (10 μg) or EPH (control) was applied topically to the ears 24 h prior to histamine (8 mg) injection intradermally and measurement of ear thickness over time. Mean ± SEM, n=7, in 3 individual experiments, *, #, $p<0.05$ vs EPH+histamine, 2-way ANOVA. (B) The whole ear lysates were collected and prepared from (A) at 24 h post-histamine. SK-1 and SK-2 activity assays were performed. Mean ± SEM, n=7, in 3 individual experiments, *, $p<0.05$ vs

EPH+HMEM-Pipes, #, $p<0.05$ vs EPH+Histamine, T-test. (C & D) Topical pre-treatment of Fingolimod and histamine-induced ear swelling were performed as detailed for (A) on *Sphk1-/-* and *Sphk2-/-* mice. Mean ± SEM, n=8, in 3 individual experiments, *, $p<0.05$ vs EPH+histamine, 2-way ANOVA.

(A)	EPH (Control)	Fingolimod
Histamine 0 min		
Histamine 6 h		
Histamine 24 h		

(B)





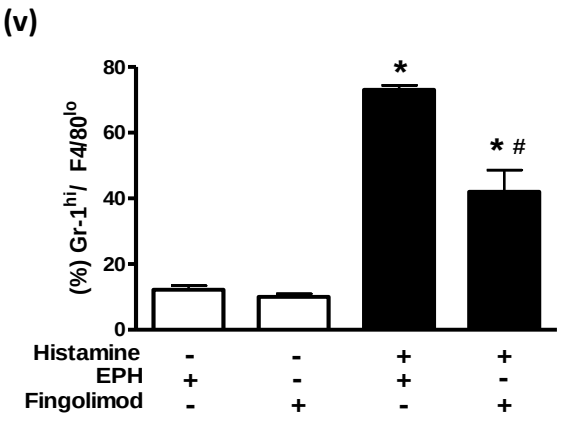


Figure 4. Topical treatment of Fingolimod attenuates oedema and cellular infiltration to the ears. (A) Fingolimod (10 μg) or EPH (control) was applied topically to the ears prior to histamine (8 mg) injection intradermally. The ears were harvested at 0, 6 and 24 h post-histamine injection followed by fixation and H&E staining. Cross sections of the fixed ear were shown for the change of ear thickness and cell influx without or with Fingolimod treatment over time. Results are representative of 3 experiments at 100X magnification. (B) Fingolimod or EPH (control) topically pre-treated ears were harvested followed by digestion of the whole ear lysates at 24 h post-histamine (8 mg) challenge. Immuno-staining for Gr-1 and F4/80 was performed for (i) EPH and untreated, (ii) EPH and histamine, and (iii) Fingolimod and histamine treated ear lysates. Isotype controls and the quantified results were shown in (iv) and (v), respectively. Mean ± SEM, n=9, in 3 individual experiments, *, $p<0.05$ vs HMEM-Pipes, #, $p<0.05$ vs EPH+Histamine, T-test.

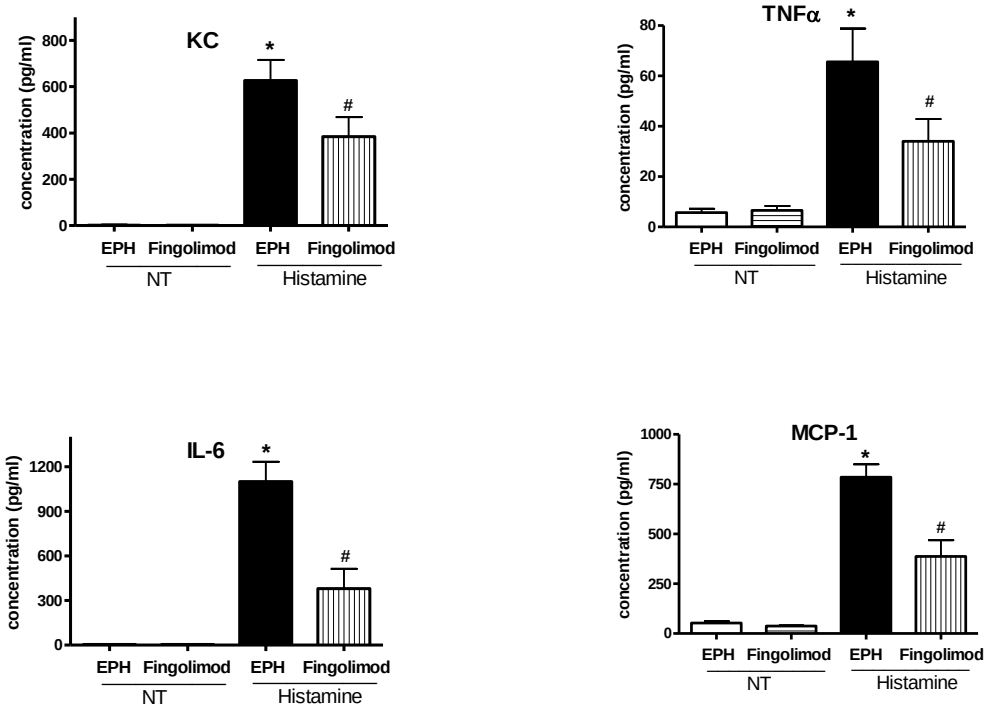


Figure 5. Topical treatment of Fingolimod attenuates the pro-inflammatory cytokines. Fingolimod or EPH (control) was applied topically to the ears for 24 h prior to histamine injection intradermally. The ears were harvested at 24 h post-histamine injection followed by Liberase digestion and cytokine detection using the CBA micro-bead array kit for FACS analysis. Quantified results are normalized to BSA (total protein) in each lysate for KC, TNFα, MCP-1 and IL-6. Mean ± SEM, n=7-9, in 3 individual experiments, *, $p<0.05$ vs EPH/NT, #, $p<0.05$ vs EPH/Histamine, t-test.

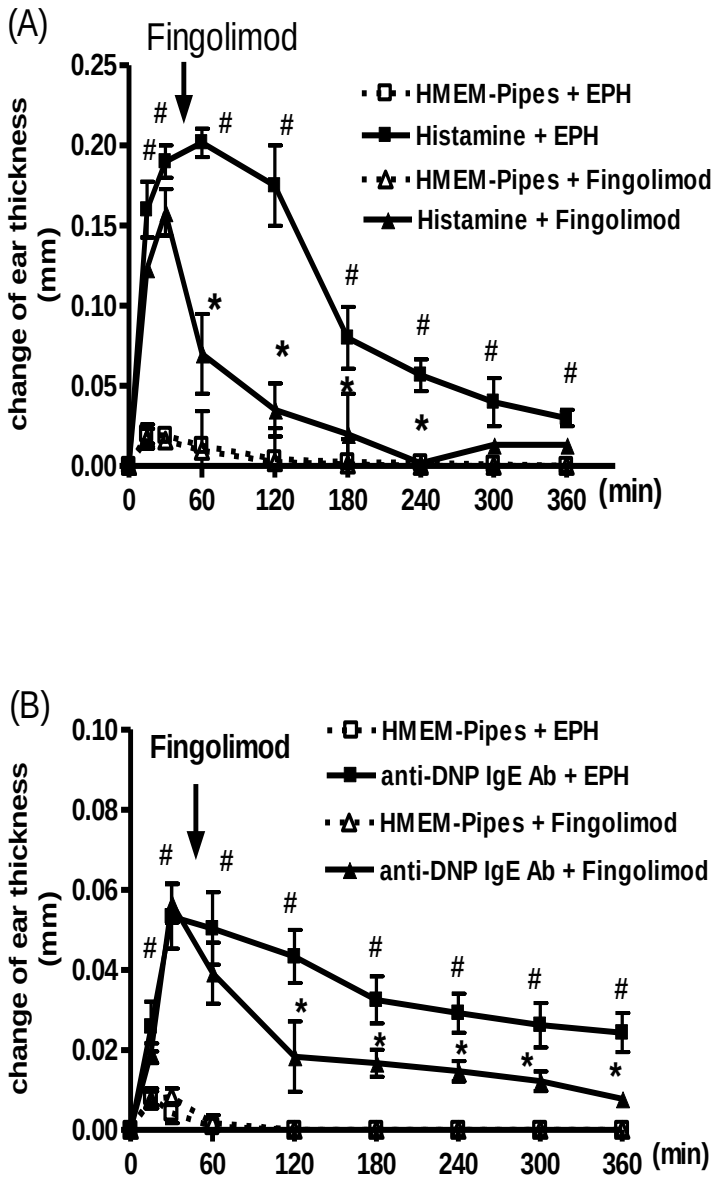


Figure 6. Topical treatment of Fingolimod attenuates ear swelling induced by histamine or PCA. (A) Fingolimod (10 μg) or EPH (control) was applied topically to both ears at 30 min post-histamine (8 mg) and HMEM-Pipes (control) intradermal injections. Ear thickness was then measured over time. Mean ± SEM, n=9, in 3 individual experiments, $^{\#}$, $p<0.05$ vs HMEM-Pipes+EPH *, $p<0.05$ vs histamine+EPH, 2-way ANOVA. (B) Fingolimod (10 μg) or EPH (control) was applied topically to the

ears at 30 min post-DNP-HSA or HMEM-Pipes (control) intradermal injections. Ear thickness was measured over time. Mean ± SEM, n=9, in 3 individual experiments, $^{\#}$, $p<0.05$ vs HMEM-Pipes+EPH, *, $p<0.05$ vs anti-DNP IgE Ab+EPH, 2-way ANOVA.

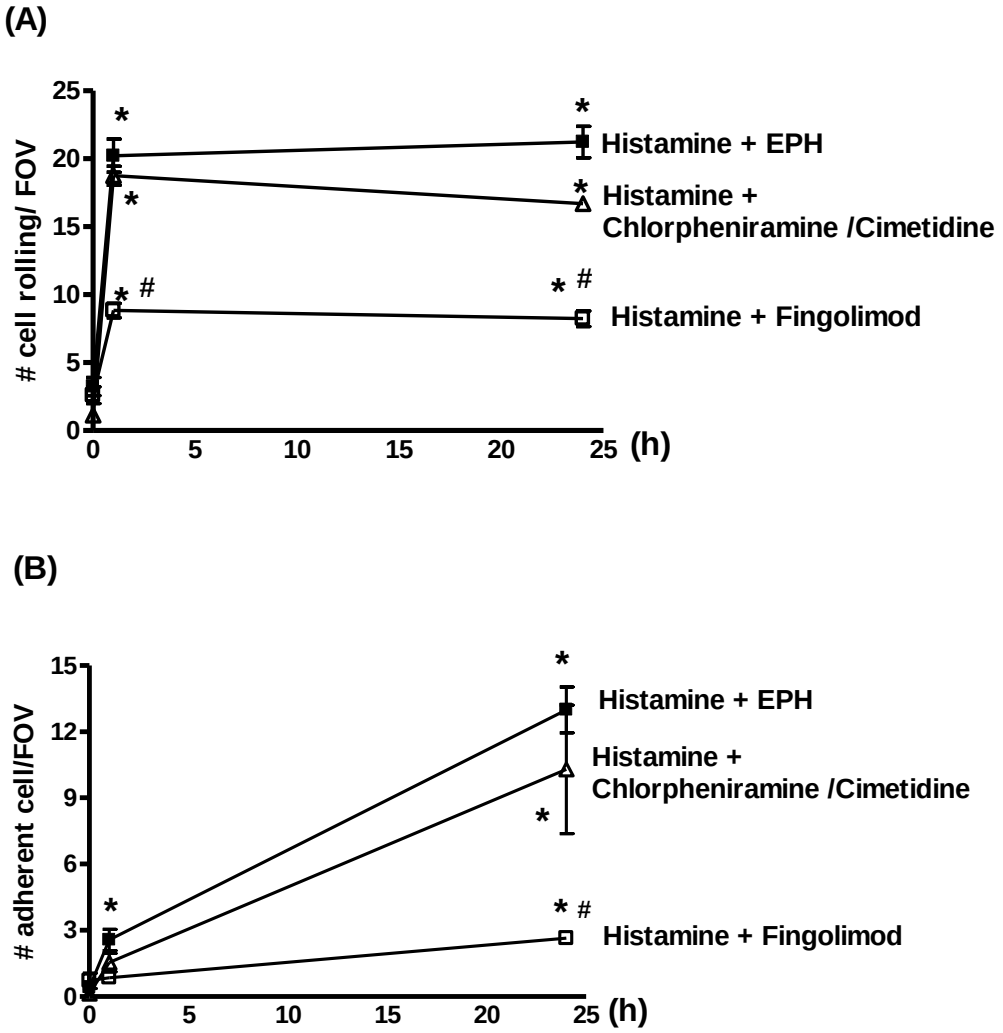
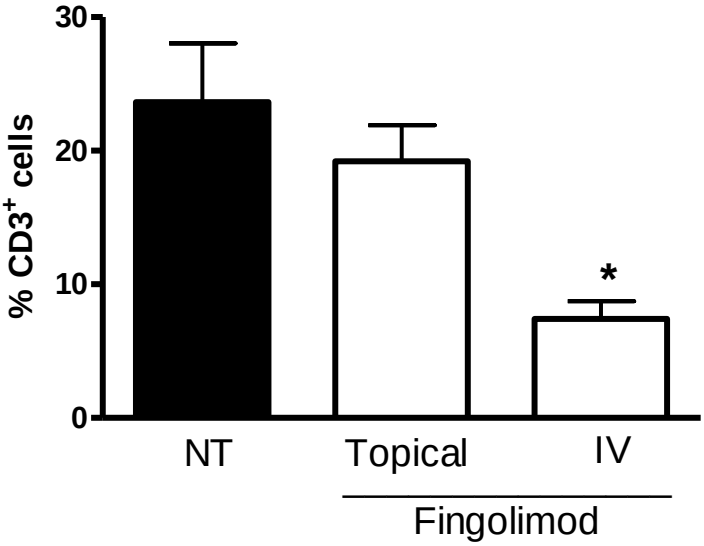
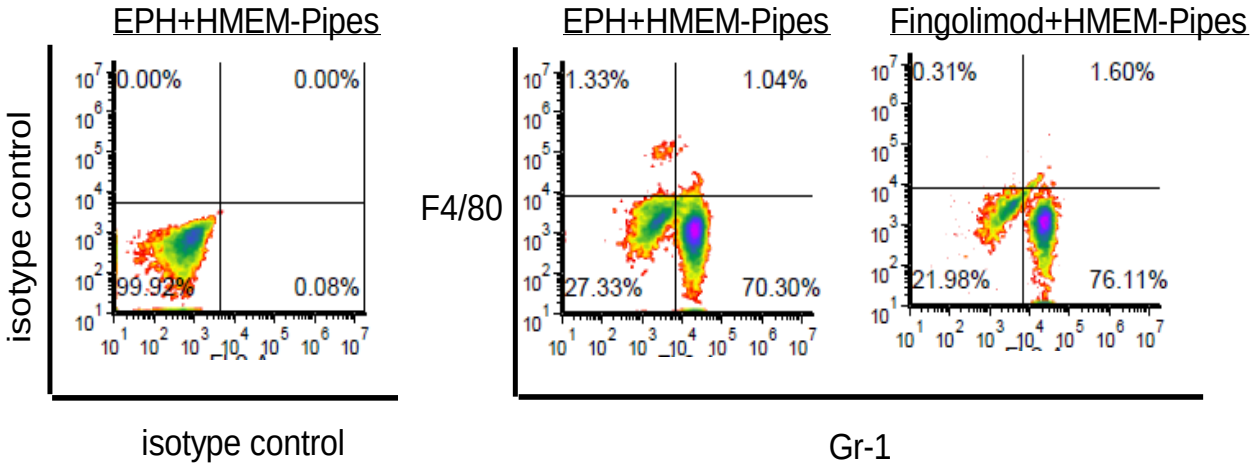


Figure 7. Topical treatment of Fingolimod attenuates histamine-induced cell rolling and adhesion. Mice were intradermally injected with histamine for 30 min prior to topical treatment of Fingolimod or Chlorpheniramine & Cimetidine. Rolling and adherent leukocytes were observed in post-capillary venules of the ears by 2-photon microscopy. Mean ± SEM, n=6-8, in 3 individual experiments, *, $p<0.05$ vs corresponding 0 h, $^{\#}$, $p<0.05$ vs Histamine+EPH at corresponding time points, 2-way ANOVA.

Supplemental video S1. Intravital videos from untreated and histamine treated (8 mg, 30 min) mice following anti-histamines (Chlorpheniramine & Cimetidine) or Fingolimod topical treatment to the ear. Videos are 2 sec images.



Supplemental Figure 1. Topical treatment of Fingolimod does not induce lymphopenia. Fingolimod was delivered via either topically to the ears or IV injection (retro-orbital) for 24 h prior to blood taken, red blood cell lysis and immuno-staining for CD3, Mean ± SEM, n=9, in 3 individual experiments, t-test.



Supplemental Figure 2. Topical treatment of Fingolimod does not reduce the mobilization of neutrophils. Fingolimod or vehicle control (EPH) was applied topically 24 h following HMEM-Pipes intradermal injection to the ears of mice. Blood was taken prior to red blood cell lysis and immuno-staining for Gr-1 and F4/80. A representative figure of the granulocyte gated cells from n=6 in 3 individual experiments.

Chapter 6: Investigation of a novel SK inhibitor for the treatment of allergic inflammation

SK/S1P are key players in the development of allergic inflammation, which suggests that they can be the therapeutic target to treat allergic diseases. Inhibition of SK can be done by using specific and commercially available SK inhibitors, gene manipulation via RNAi technology or genetic modification *in vivo* [144, 145, 285]. Notably, different types of SK inhibitors exhibit distinct levels of inhibition and off-target effects. Table 6a summarizes the inhibitory properties of the common SK inhibitors that are currently available for scientific research use and/or clinical use. Herein, this Chapter examines a novel SK inhibitor, which has a different mode of action when compared to the current SK inhibitors, in the setting of allergic skin inflammation *in vivo*.

Table 6a Inhibitory properties of SK inhibitors *in vitro*

	K_i		Type of inhibition	Off-target inhibitory effect	Reference
	SK-1	**SK-2**			
Sphingosine analog inhibitors					
D,L-*threo*-Dihydrosphingosine (DHS)	6 μM	*	Competitive	PKC, PI3K	[286]
Dimethylsphingosine (DMS)	5 μM	12 μM	Non-competitive	CK, MAPK, PKC, PI3K	[287, 288]
FTY720 (known as Fingolimod or Gilenya®)	2 μM		Competitive	Decrease dihydroceramides, ceramides, sphingosine, S1P, #	[289, 290]
SK1-I (known as BML-258)	10 μM		Competitive		[285]

Non-lipid small molecule inhibitors					
SKi-II (or known as SKi)	17μM**	50 μM	Mix	Nuclear factor erythroid 2-related factor 2, dihydroceramide desaturase	[15, 194, 291]
ABC294640		9.8μM	Competitive		[189]

Note: CK: Ceramide kinase; PI3K: phosphatidylinositol 3-kinase; PKC: Protein kinase C; MAPK: mitogen-activated protein kinase

*, shown as a substrate and to be metabolized by SK-2 [292]

#, FTY720-phosphate is a competitive inhibitor of nucleotide phosphodiesterase/pyrophosphatase (NPP) and lysophospholipase D that enhance metastasis and angiogenesis [293]

**, K_i=17 μM as competitive and K_i=48 μM as non-competitive inhibitor for SK-1 [294]; K_i=50 μM as competitive inhibitor for SK-2.

6.1 Inhibition of SK-1 by a novel SK inhibitor MP8 in ECs

Recently, Prof Stuart Pitson (Centre for Cancer Biology, SA, Australia) generated a new SK inhibitor, namely MP8, which has K_i value of 24 μM for SK-1 and 7 μM for SK-2 (Pitman et al July 2014, manuscript currently under review with *Nature Chemical Biology*). Briefly, MP8 is an ATP-competitive inhibitor of SK-1 and SK-2 and has been shown to be highly specific for SK inhibition *in vitro* and *in vivo*. It was screened against a panel of 140 kinases *in vitro* using the Dundee International Centre for Kinase Profiling and no substantial inhibition was observed at MP8 concentrations effective against SK-1 and SK-2. MP8 demonstrated inhibition of SK *in vivo*, as determined by a reduction of plasma S1P, and was well tolerated, with no changes in body weight, lymphocyte count, blood hemoglobin, platelet numbers and bone marrow hematopoietic lineages. The inhibitory effect of MP8 on SK has not been examined in ECs. Herein, I performed SK-1 and SK-2 activity assays to examine the effects of MP8 on endogenous SK-1 and SK-2 in HUVECs and compared it to that of other known SK inhibitors (Fingolimod and SKi). As shown in Figure 6.1a, all three SK inhibitors (Fingolimod at 100 nM, SKi at 5 μM and MP8 at 5 μM) significantly reduced SK-1 activity in HUVECs, with the largest reduction observed in MP8-treated HUVECs. Previously, Fingolimod at 10 μM has been shown to induce degradation of SK-1 in MCF-7 breast cancer cells [198]. Herein, a lower

concentration of Fingolimod (100 nM) was administered to HUVECs and also showed significant inhibition of SK-1 activity when compared to the vehicle control. Although these SK inhibitors also reduced SK-2 activity, the endogenous levels of SK-2 were low in HUVECs and therefore the result was statistically insignificant.

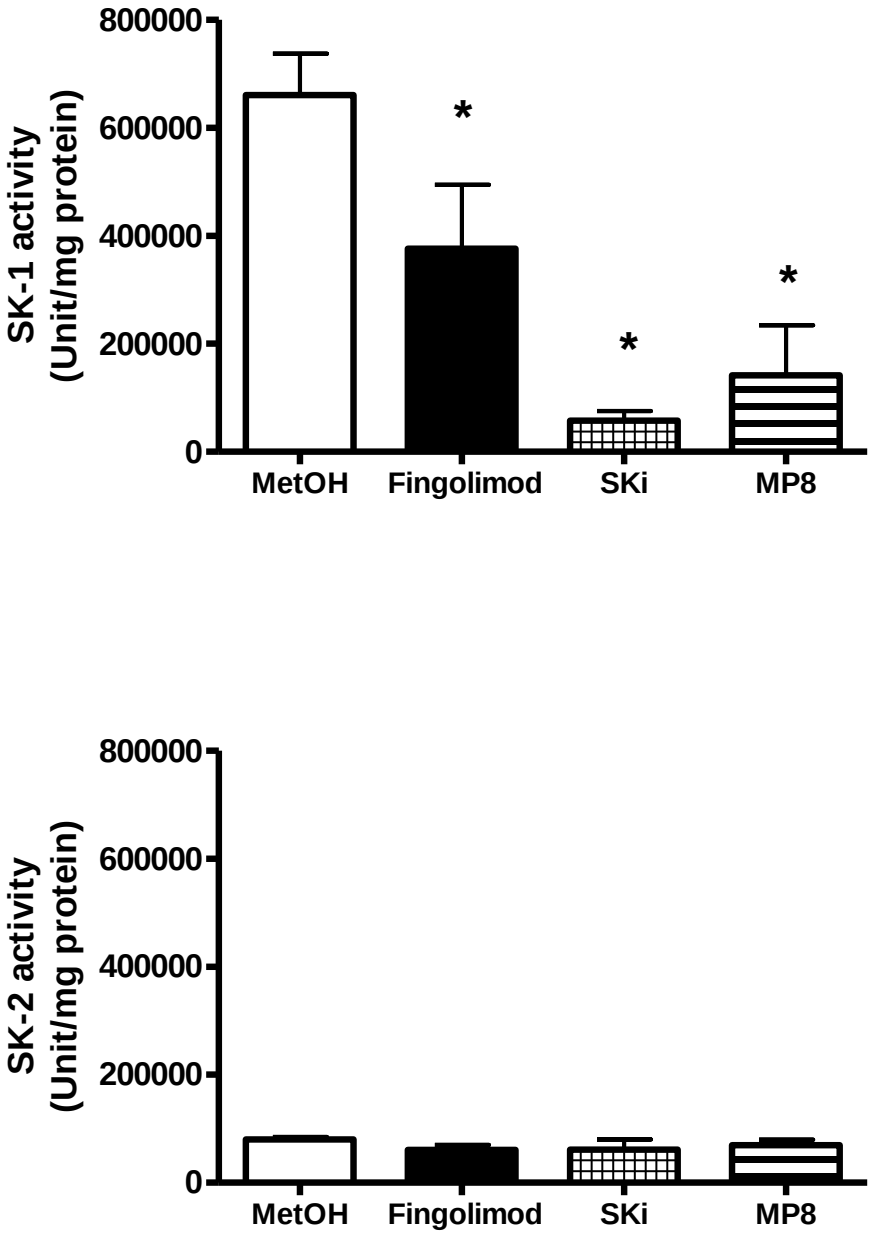


Figure 6.1a The inhibitory effect of SK inhibitors in HUVECs. MetOH (control), Fingolimod (100 nM), SKi (5 μM) or MP8 (5 μM) treated HUVEC lysates were prepared for the enzymatic activity assay. SK activity was normalized to corresponding protein concentrations. Mean±SD, n=3, *, $p<0.05$ vs MetOH, 1-way ANOVA.

6.2 Epicutaneous MP8 treatment in histamine-induced inflammation of the ear

In Chapter 5, we have shown that inhibition of SK by epicutaneous application of Fingolimod can attenuate allergic inflammation that is induced by histamine or IgE. It was also of interest to examine whether the novel SK inhibitor, MP8, could be used as a prophylactic topical agent to attenuate allergic inflammation. MP8 is structurally distinct from SKi and Fingolimod, which is only soluble in organic solvents, such as DMSO, at a minimum concentration of 30 mM. In order to administer 10 μg of MP8 topically, the final volume of MP8 required re-constitution in at least 150 μL. This volume was not feasible for topical application as a single dose to a mouse ear. Therefore, the total dose of MP8 (10 μg in 150 μL) was divided into 3 doses to administer at 1 h intervals for 24 h prior to histamine challenge.

Figure 6.2a shows that histamine-induced ear swelling, which peaked at 30 min and then subsided gradually over 6 h. Prophylactic treatment with MP8 reduced histamine-induced ear swelling, which suggests that this SK inhibitor can be applied topically to attenuate allergic inflammation. Notably, increased frequency of application/dosing was required which often translates to poor compliance in the population at large, and hence sub-optimal therapeutic outcomes. Thus, Fingolimod, which is a small molecule (MW=343) with high lipophilic solubility, was the main focus in this thesis for the treatment of allergic inflammation.

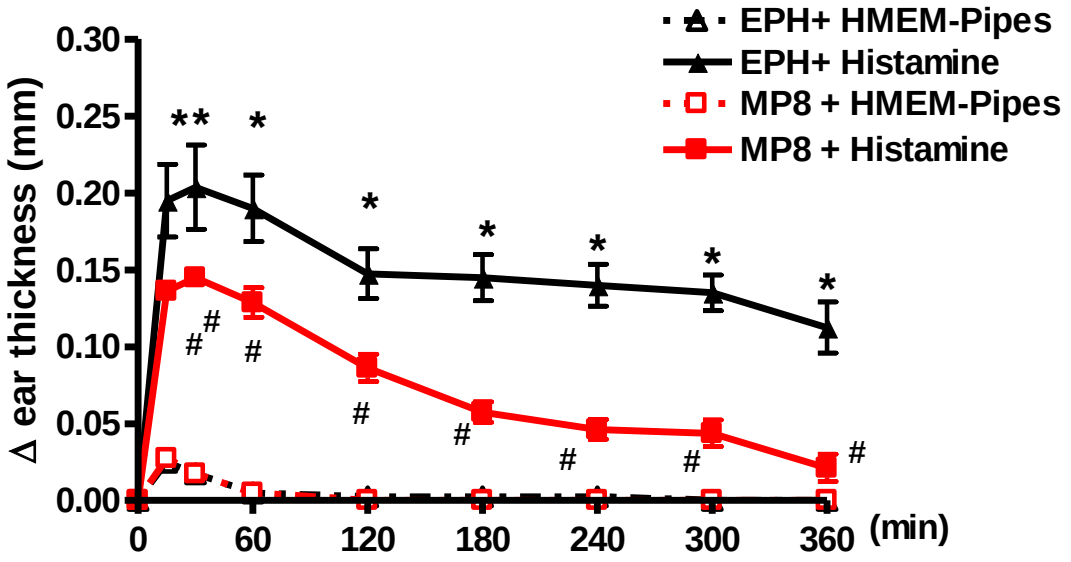


Figure 6.2a Topical MP8 treatment in histamine-induced inflammation of the ear.

MP8 was applied topically to the ears prior to histamine challenge. Ear thickness was measured at intervals over a period of 6 hours post-histamine challenge as a read out system to indicate allergic inflammation. Mean ± SD, n=3, *, $p<0.05$ vs EPH+HMEM-Pipes, #, $p<0.05$ vs EPH + histamine, 2-way ANOVA.

6.3 Materials and methods

SK activity assay

HUVECs were treated with vehicle control (MetOH), Fingolimod (100 nM), SKi (5 μM) or MP8 (5 μM) for 1 h prior to cell lysis in extraction buffer as previously described [122]. HUVEC lysates were sonicated for 5 min followed by measurement of protein concentration using the Bradford method as per manufacturer's protocol (BioRad). The enzymatic assays were performed as per previously described [257], and briefly for SK-1 activity, whole cell lysates were incubated with D-*erythro* sphingosine (Cayman Chemical Co) solubilised in 0.05% Triton X-100 and [γ^{32}P]ATP (Perkin Elmer). For SK-2 activity, whole cell lysates were prepared in buffer containing 1M KCl and incubated with *D-erythro* sphingosine solubilised in BSA/PBS and [γ^{32}P]ATP. The radioactively labelled S1P was resolved by 2 thin-layer chromatography (Sigma) separations in solvents containing butanol, ethanol, water and acetic acid (8:2:2:1). The radioactive spots were quantified by Phosphorimaging Typhoon 9410 (Fullerton) and ImageQuant 7.0 program (GE Healthcare).

Epicutaneous application of MP8 in histamine-induced inflammation of the ear

MP8 (gift, Prof. S. Pitson, Centre for Cancer Biology, Adelaide, SA, Australia) was re-constituted in a mixed solvent (Ethanol: Propylene glycol: Water (EPH) in a ratio of 1:4:1) followed by heating at 60°C, 15 min or until fully dissolved.

MP8 was topically applied to the ears of the anaesthetized C57Bl/6 mice, 3 times at 1 h intervals (total dose was 10 μg in a final volume of 150 μL EPH). The same volume of vehicle control was applied to a second group of C57Bl/6 mice. At 24 h post-MP8 treatment, anaesthetized mice were intradermally injected with histamine (8 mg in 20 μL of HMEM-Pipes) and vehicle control (20 μL of HMEM-Pipes) to the right and left ears, respectively. Ear thickness was measured at intervals over 6 h post-histamine injection.

6.4 Discussion

To investigate an effective therapeutic agent that can target the SK/S1P axis for the treatment of allergic inflammation, the specificity and potency of the agent are important to consider. In Chapter 5, we have shown that Fingolimod can be used as a topical agent to treat allergic skin inflammation. However, the potency and adverse effects of Fingolimod via topical application are not fully understood, as the previous studies and human clinical trials were conducted via oral and IV administration [294-296]. In comparison with other SK inhibitors (including Fingolimod), our new SK inhibitor, MP8, has a better specificity as it is a direct ATP-competitive inhibitor which does not affect other kinases *in vitro*. (Pitman et al 2014, submitted). Unlike Fingolimod, which causes lymphopenia via oral/IV administration [297], MP8 does not change the lymphocyte counts nor blood cell profile *in vivo* (Pitman et al 2014, submitted). This renders MP8 a relatively better agent without causing any obvious off-target effects when administered systemically.

This Chapter has further examined MP8 and shown that it is capable of attenuating SK activity to similar levels of other SK inhibitors (SKi and Fingolimod) in ECs. Topical application of MP8 could also effectively decrease histamine-induced ear inflammation in mice, suggesting that the recruitment of inflammatory cells that would cause skin inflammation was attenuated. However, the solubility of MP8 was poor due to its molecular structure (less lipophilic than Fingolimod) and therefore

multiple administrations were required in our *in vivo* experiments. Nevertheless, the present study has revealed an opportunity for MP8 as a potential agent to treat skin inflammation.

Chapter 7: Discussion and conclusion

The overall aim of this study was to investigate a new therapeutic approach to combat allergic inflammation via attenuation of neutrophil recruitment. Chapter 3 revealed that SK-1, but not SK-2, is a key regulator of histamine-induced P-selectin surface expression by ECs, which is likely via intracellular calcium influx as shown in Chapter 4, and as such rapid P-selectin surface expression is required for the recruitment of neutrophils during the early phase of allergic inflammation (Figure 7a). Based on these findings, I then hypothesized that SK/S1P may be a therapeutic target to attenuate the immune responses in the early phase of allergic inflammation. There are few scientific approaches to inhibit SK *in vitro* and *in vivo*, such as SK inhibitors, RNAi against SK and S1P receptors, and genetically modified mice [reviewed in 113]. Chapter 5 selectively examined the application of SK inhibitors (Fingolimod and SKi) using two *in vivo* models for skin inflammation in the ear; the first induced by histamine and the second an IgE-mediated PCA of the ear. Results showed that Fingolimod can be applied topically to prevent and treat allergic inflammation. More importantly, topical application of Fingolimod does not cause lymphopenia or inhibition of neutrophil mobilization from the bone marrow. The therapeutic effect of Fingolimod in inflammation via topical application is significantly longer lasting than the same application of anti-histamines. Finally, Chapter 6 has summarized the

pharmacological properties and the off-target effects of the common SK inhibitors. By understanding these current SK inhibitors, a novel SK inhibitor MP8 has been developed and I examined it in a setting of histamine-induced ear inflammation model.

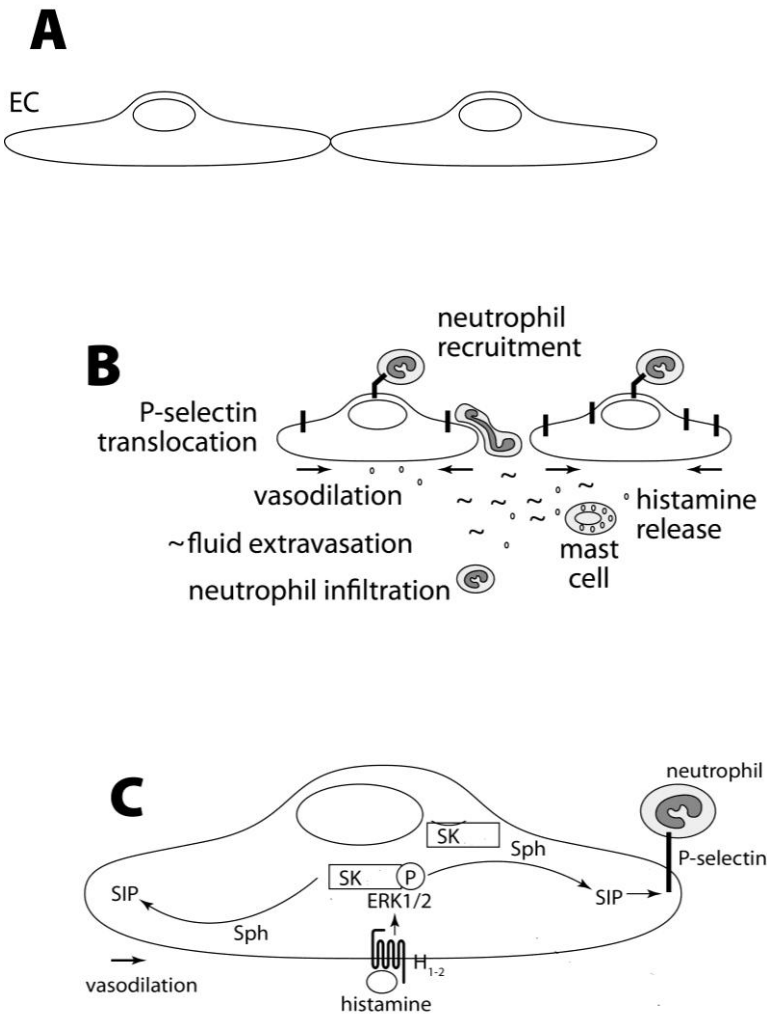


Figure 7a Schematic of the mechanism of histamine-induced P-selectin surface expression by ECs. (A) The resting ECs form a monolayer inside the blood vessels with tight cell junctions. (B) During allergic inflammation, mast cells release histamine to activate ECs, which lead to an upregulation of P-selectin surface expression and vasodilation causing recruitment of circulating neutrophils to the inflammatory site(s). (C) In response to histamine, ECs rapidly increase endogenous SK-1 activity via the activation of ERK1/2 pathway to produce S1P. As a result, the

pre-stored P-selectin in WPBs is translocated to EC surface within minutes of histamine exposure to induce neutrophil tethering and rolling events.

Our experiments utilized histamine, one of the potent inflammatory mediators, to induce P-selectin expression for neutrophil recruitment during inflammation *in vitro* and *in vivo*. Erent et al have shown that the rate of histamine-induced WPB exocytosis by ECs occurs in a dose-dependent manner, where a higher concentration of histamine (100 μM) can promote the intracellular calcium flux 5-times faster than a lower concentration of histamine (0.3 μM) [298]. Therefore, high concentrations of histamine enhance the intracellular calcium-evoked WPB exocytosis [298]. As allergic inflammation develops, mast cells are activated by, for example, binding of a specific allergen and cross-linking of the Fc region of mast cells via FcεRI [17]. The activated mast cells can rapidly degranulate to release cytokines and inflammatory mediators, including histamine, which is required to induce the recruitment of neutrophils [20]. Circulating neutrophils are one of the first responders to migrate toward an inflammatory site [41]. Once recruited, the activated neutrophils can also produce and release a range of chemokines, including IL-8, CCL3, CXCL-1, CXCL9 and CXCL10, to further enhance the recruitment of neutrophils, monocytes, DCs and T lymphocytes [23, 48]. Of note, neutrophils have been shown to be the dominant producer of histamine as allergic inflammation develops [273]. Thus, the total levels of histamine in local tissues are likely to be significantly elevated with time as contributed by both mast cells and neutrophils, which implies that the exocytosis of

WPBs containing P-selectin may be further enhanced. Herein, Chapter 4 has shown that inhibition of SK-1 can attenuate the total amount of histamine-induced intracellular calcium influx and reduce P-selectin-derived WPB exocytosis. Previous studies have demonstrated that administration of S1P can induce the release of calcium in HEK293 cells [239] and can open calcium entry channels in human neutrophils [299]. To this end, whether SK-1 is associated with (i) calcium release from internal stores and/or (ii) activation of the calcium channels on the plasma membrane for external calcium influx into ECs, is yet to be elucidated.

Notably, inhibition of SK-1 reduces the production of S1P and previous studies have shown that ceramide and S1P are the biological activators for the exocytosis of WPBs by ECs [207, 226, 300]. Although a compensatory effect of S1P production by SK-2 is suggested, SK-1 is still a dominant producer for S1P *in vitro*. One of the explanations is that SK-1 is located predominantly in the cytoplasm and at the plasma membrane, whereas SK-2 is mainly found in the nucleus and at the ER membrane, which is in close proximity with the ER-located S1P lyase and S1P phosphatase [137]. Therefore, the S1P produced by SK-2 can be quickly degraded or dephosphorylated. As mentioned in Chapter 3.3, IL-8 is also one of the biological contents of WPBs that is released by ECs to chemoattract neutrophils during inflammation [216]. Thus, this study suggests that inhibition of SK-1 may have the dual effects on the attenuation of

P-selectin and IL-8 exocytosis, which both contents of WPBs are associated with the recruitment of neutrophils.

Different SK inhibitors have their distinct modes of action. For example, DMS, SKi and Fingolimod are direct inhibitors of SK catalytic activity [122, 194, 199], whereas Resveratrol and Glabridin are indirect inhibitors of SK by targeting the upstream signalling pathways of sphingolipids to prevent SK activation [301, 302]. Notably, SKi and Fingolimod are sphingosine analogue inhibitors, which competitively occupy the substrate binding site of SK [303]. Furthermore, they have been shown to be orally bioavailable *in vivo*, with a half-life of eight hours for SKi in rodents [303] and a half-life of six to nine days for Fingolimod in humans [209]. In terms of off-target effects, SKi does not exhibit any significant inhibition of PI3 kinase, ERK1/2 and PKC signalling pathways at 60 μM *in vitro* [194], although it has recently been shown to inhibit dihydroceramide desaturase (at 10 μM), which is involved in the *de novo* synthesis of ceramide [15], and activate nuclear factor erythroid 2-related factor 2 (at 1 μM), which mediates cellular senescence *in vitro* [291]. Fingolimod (at 2.5 μM) can competitively inhibit ceramide synthases for the production of ceramide, with the IC_{50} of 6.4 μM *in vitro* [289]. However, Lahiri et al demonstrated that Fingolimod (at 25μM) but not its phosphorylated form is an uncompetitive inhibitor for ceramide synthesis [304]. Moreover, there are a few

adverse effects of Fingolimod (following oral administration) in humans, which have been reported and include bradycardia, heart block (initiation of treatment), macular oedema and increased risk of infection [305]. Of 418-431 MS patients who were involved in the phase III clinical trials of Fingolimod, five and two melanoma cases were reported in the groups taking Fingolimod and placebo, respectively [306].

In addition to the mechanism of SK inhibition by Fingolimod, the phosphorylated Fingolimod acts as an $S1P_1$ agonist to induce downstream signalling and internalization of the receptors [307, 308]. The $S1P_1$ receptors can be internalized rapidly within 30 min rendering the cells unable to respond to the extracellular S1P [309]. Thus, this mechanism of action of phosphorylated Fingolimod is known as functional antagonist. Unlike $S1P_1$ receptor, the phosphorylated Fingolimod-bound $S1P_4$ and $S1P_3$ do not result in the internalization of the receptors and only exhibit partial internalization, respectively [310, 311]. .

This study not only examined on the application of Fingolimod as an epicutaneous treatment for allergic inflammation (Chapter 5) but also examined and compared with the novel SK inhibitor, MP8, which was generated by Prof. S. Pitson within the Centre for Cancer Biology (Chapter 6). Unlike SKi and Fingolimod, MP8 targets the ATP-binding site but not the substrate-binding site of SK (Figure 7b). The ATP-binding site of SK has been shown to be specific and different from that of

protein kinases, such as PKB and PKC [122, 312]. Therefore, inhibition of SK by MP8 should increase the specificity and reduce the off-target effects (Pitman et al June 2014, submitted). The SK-1 and SK-2 enzymatic assay confirmed that SKi, Fingolimod and MP8 reduced the endogenous SK activity significantly in HUVECs (Chapter 6). High-performance liquid chromatography (HPLC) was also conducted to examine the total levels of intracellular S1P in response to these SK inhibitors. Of note, I utilized HUVECs which are not a cell line but a collection of freshly isolated primary human cells that represent the population at large. However, one of the disadvantages when working with primary human cells is the limitation of cell expansion potential. As per the established protocol of HPLC within our department, intracellular level of S1P can be detected in cell lysates made from approximately twenty million cells. Notably, the maximal expansion of HUVECs from a single donor is unable to reach a total of twenty million cells in culture. Thus, we are unable to detect the endogenous level of S1P by HPLC with the maximal HUVEC counts per donor.

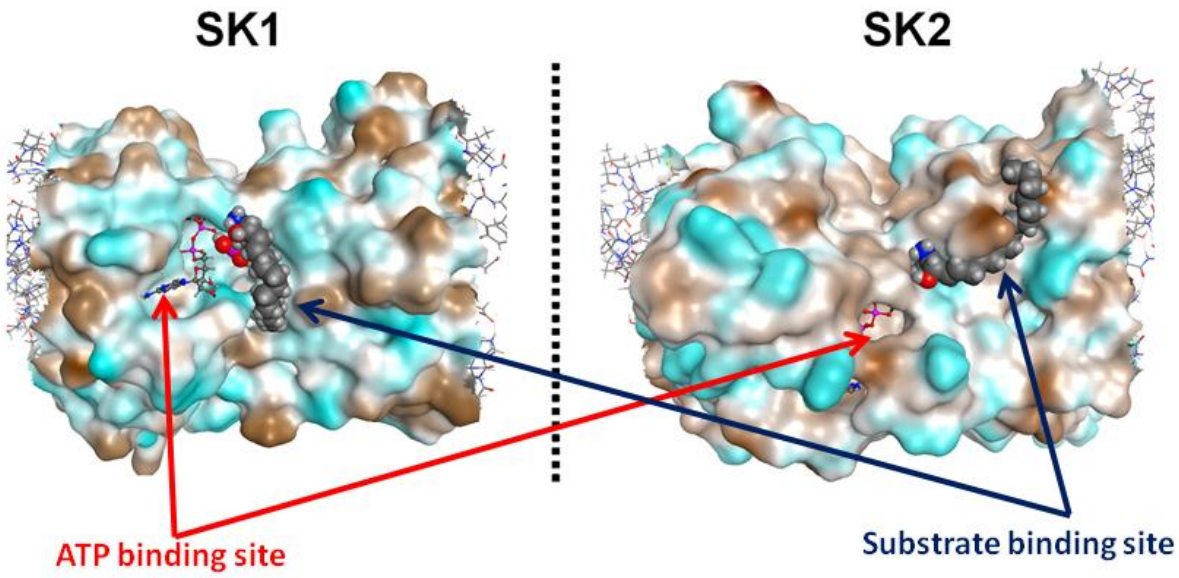


Fig 7b. Schematic of SK-1 and SK-2. SK-1 and SK-2 are shown in blue (hydrophobic residues) and brown (hydrophilic residues). Black arrows indicate the substrate-binding sites of SK which is targeted by SKi and Fingolimod. Red arrows indicate the ATP-binding sites of SK which is targeted by MP8.

(Adapted and modified from Gao et al [313])

This study has focused on the SK and S1P axis in ECs during allergic inflammation. However, SKs are ubiquitously expressed by many cell types, such as mast cells, T lymphocytes, vascular smooth muscle cells (VSMC) and neutrophils [reviewed in 314]. It is expected that administration of a drug that targets SK/S1P signalling pathway *in vivo*, such as Fingolimod, also exhibits the inhibitory effects on the aforementioned cells. Herein, my work (Chapter 5) has shown that topical application of Fingolimod to the ears of mice attenuated the levels of histamine-induced inflammatory chemoattractants and neutrophil influx. I have also shown that topical application does not cause lymphopenia with circulating CD3+ lymphocytes remaining at normal levels, nor inhibition of neutrophil mobilization from bone marrow. In addition to our previous findings that histamine-induced P-selectin surface expression by ECs is SK-1 dependent and is required for neutrophil rolling on the endothelium (Chapter 3.3 and [236]), Fingolimod has also been shown to reduce $S1P_1$ and $S1P_3$ expression by VSMCs, which results in attenuation of VSMC proliferation and migration, respectively [315]. Recently, Rahman et al showed that S1P enhances IL-8 gene expression and secretion by airway smooth muscle cells to promote neutrophil chemotaxis [316]. Their findings imply that inhibition of the SK/S1P axis can attenuate SMC-released IL-8 and recruitment of

neutrophils. Taken together, these studies demonstrated that SK/S1P signalling pathway is important to regulate the vascular tone during allergic inflammation.

The role of sphingolipids in neutrophil recruitment begins to be more understood. Zemann et al have shown that bone-marrow-derived neutrophils from *Sphk1*$^{-/-}$ and *Sphk2*$^{-/-}$ mice have normal increase of intracellular calcium influx for neutrophil activation and chemotaxis [212]. This demonstrated that SK is not required for neutrophil activation. However, S1P is shown to be involved in Fcγ receptor-mediated neutrophil activation via the mechanisms of an increase in intracellular calcium levels and reactive oxygen species [175]. The activated neutrophils can promote EC permeability to favour their transmigration via the vasculature. Importantly, such levels of vascular permeability are attenuated when either ECs or neutrophils are pre-treated with an SK inhibitor (eg DMS and SKi) [281]. Furthermore, S1P lyase-deficient mice exhibit an elevated level of S1P in both periphery and tissues, which promotes the mobilization of neutrophils into the circulation. However, these circulating neutrophils are unable to infiltrate into the inflammatory tissues [317]. Therefore, the homeostatic feedback loop fails to regulate the release of neutrophils in the periphery and as a result, neutrophilia is observed in the S1P lyase-deficient mice [317]. Overall, these previous studies have clearly indicated the importance of the right balance of sphingolipids in neutrophils.

We have shown a new indication for Fingolimod in allergic inflammation via epicutaneous application as evident by the two *in vivo* models of skin inflammation that is driven by histamine or IgE (Chapter 5). These findings are likely to be translated into human clinical use as the SK isoforms are highly conserved between mouse and human with more than 80% amino acid sequence to be identical between these species [318]. The area of application is also an important factor to be considered as this study performed the topical application of Fingolimod to the ears of mice where it is well vascularized. To mimic the allergic disease in human, such as dermatitis, future experiments will include topical application of Fingolimod to different sites of the mice (eg dorsal skin).

Chapter 8: Significance and future directions

Overall, this study has shown for the first time that SK-1 regulates histamine-induced P-selectin surface expression by ECs during the early phase of allergic inflammation. More importantly, inhibition of SK-1 by topical application of Fingolimod can attenuate skin inflammation that is induced by histamine or IgE-medicated PCA reactions *in vivo*. The significance of these findings can be translated into clinical use with epicutaneous application of Fingolimod being more effective for the prevention and treatment of allergic inflammation when compared to topical application of anti-histamines. Currently in Australia, there is no topical formulation of anti-histamines available (ie. cream, lotion or ointment), except for the intranasal spray. Oral anti-histamine tablets are one of the treatment choices for allergic inflammation, including mild dermatitis and skin rash [5]. However, anti-cholinergic effects, such as dry mouth, cough and blurred vision, are the common adverse effects of anti-histamines, and such adverse effects are even more likely to occur in the first generation of anti-histamine (or known as the sedative anti-histamines). Thus, patients who have a medical history of closed-angle glaucoma, increased intraocular pressure, bladder neck obstruction or hyperthyroidism should be carefully considered prior to treatment [5]. Herein, this study has examined the

therapeutic effect of Fingolimod in allergic inflammation and suggested that topical application of Fingolimod may be a new treatment option for allergy.

Future directions of this study include (i) examining the pharmacokinetics of Fingolimod via topical application, such as the absorption rate and half-life after a single application, and (ii) human clinical trials. In fact, we have gained the approval for human ethics to conduct a human pre-clinical study using topical application of Fingolimod in a skin-prick test with histamine. Briefly, a histamine skin-prick test, routinely conducted in the clinic, will be performed on the forearms of the healthy participants. After 10 and 20 min, Fingolimod will be applied topically to the forearms at the sites of histamine administration. The allergic inflammation (ie. wheal and flare reaction) will be measured at intervals over time to indicate the therapeutic effects of Fingolimod. This pre-clinical study, if successful, will ultimately lead to the phase I-III clinical trials with a large sample size and dosing experiments. Furthermore, focus should also be on investigating a suitable solvent to improve the solubility of the next generation SK inhibitors, such as MP8, so that they become more feasible for the topical application to treat allergic inflammation.

Appendix 1:

Tumor necrosis factor-induced neutrophil adhesion occurs via sphingosine kinase-1-dependent activation of endothelial $\alpha_5\beta_1$ integrin.

Sun WY, Pitson SM, Bonder CS.

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Vascular Biology, Atherosclerosis and Endothelium Biology

Tumor Necrosis Factor-Induced Neutrophil Adhesion Occurs Via Sphingosine Kinase-1-Dependent Activation of Endothelial $\alpha_5\beta_1$ Integrin

Wai Y. Sun,* Stuart M. Pitson,*† and Claudine S. Bonder*‡

From the Department of Human Immunology, Centre for Cancer Biology, South Australia Pathology, School of Molecular and Biomedical Science,† and School of Medicine,‡ University of Adelaide, Adelaide, South Australia, Australia*

Leukocyte recruitment plays a major role in the immune response to infectious pathogens, as well as during inflammatory and autoimmune disorders. The process of leukocyte extravasation from the blood requires a complex cascade of adhesive events between the leukocytes and the endothelium, including initial leukocyte rolling, adhesion, and finally transendothelial migration. Current research in this area aims to identify the key leukocyte subsets that initiate a given disease and to identify the trafficking molecule(s) that will most specifically inhibit those cells. Herein we demonstrate that tumor necrosis factor (TNF)α activates the integrin $\alpha_5\beta_1$ without altering total expression levels of β_1 integrin on human umbilical vein endothelial cells. Moreover, our studies suggest that TNFα-induced β_1 activation is dependent on sphingosine kinase-1, but independent of the sphingosine-1-phosphate family of G protein-coupled receptors. We also show, using a parallel plate flow chamber assay, that neutrophil adhesion to TNFα-activated endothelium can be attenuated by blocking $\alpha_5\beta_1$ or its ligand angiopoietin-2. These observations add new complexities that broaden the accepted concept of cellular trafficking with neutrophil adhesion to TNFα activated endothelial cells being sphingosine kinase-1, $\alpha_5\beta_1$, and angiopoietin-2 dependent. Moreover, this work supports the notion that sphingosine kinase-1 may be the single target required for an effective broad spectrum approach to combat inflammation and immune disorders. ***(Am J Pathol 2010, 177:436–446; DOI: 10.2353/ajpath.2010.091016)***

To fulfill their surveillance function, leukocytes continuously patrol the human body, shuttling back and forth between the blood stream, the lymphatic fluid, secondary lymphoid organs, and peripheral tissues.[1] Leukocyte recruitment to sites of inflammation is critical for the development and maintenance of the immune response. During injury and pathogen invasion, inflammatory cytokines, such as tumor necrosis factor (TNF)α, are released to recruit leukocytes. However, excessive and remaining cytokines at these sites often result in prolonged inflammation, tissue damage, and disease. When leukocytes leave the blood stream, they undergo a sequential adhesion cascade to overcome both the high shear forces within the blood vessel and the tight seal of endothelial cells that line these vessels. The classical paradigm for leukocyte recruitment states that the selectin-family (ie, P-selectin, E-selectin, and L-selectin) uses transient interactions with carbohydrates to initiate tethering and rolling (reviewed in 2). Leukocyte arrest during rolling is rapidly triggered by chemoattractants (eg, chemokines) and is mediated by the binding of leukocyte integrins to immunoglobulin superfamily members, such as vascular and cellular adhesion molecule (VCAM)−1 and intercellular adhesion molecule (ICAM)−1, expressed by endothelial cells (ECs). This stabilization of the rolling leukocytes to the endothelium enables their emigration from the microvasculature. Undoubtedly, the diversity in selectivity and extent of leukocyte recruitment are regulated by the intrinsic complexity of pro-adhesive signaling networks expressed by the vasculature.

The family of integrins are significant contributors to leukocyte adhesion, with their qualitative and quantitative variations of expression and activation states. In the past

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Address reprint requests to Dr. Claudine S. Bonder, Ph.D., Human Immunology, Centre for Cancer Biology, SA Pathology, Frome Rd, Adelaide, SA 5000, Australia. E-mail: claudine.bonder@health.sa.gov.au.

decade, new insights have been gained in understanding the combination and activation of the 18α and 8β integrin subunit family members, which associate in pairs to form at least 24 $\alpha\beta$ receptors (reviewed in 3, 4). Moreover, modulation of integrin ligand affinity is now widely recognized as a crucial step in agonist-induced leukocyte arrest under flow.[5] Indeed, specific integrin blocking molecules are effective therapeutic strategies in multiple sclerosis and psoriasis as they modulate leukocyte trafficking.[6,7] However, their inability to provide absolute protection suggests that the precise mechanisms underpinning cellular recruitment remain incompletely understood.[8]

TNFα is one of the most pleiotropic cytokines involved in systemic inflammation and has been implicated in a multitude of pathologies including autoimmune disease, insulin resistance, and cancer (reviewed in 9). A major site for TNFα action is the vascular endothelium where it binds to membrane receptors and instigates a cascade of intracellular signaling events for EC production of cytokines and induction of adhesion molecule expression. TNFα also stimulates the activation of sphingomyelinase and sphingosine kinase (SK)−1, yielding sphingosine-1-phosphate (S1P) (reviewed in 10). Although most cells can synthesize S1P, large amounts are present in platelets,[11] and recent reports have identified erythrocytes as well as vascular endothelium as major contributors of S1P in circulation.[12–14] S1P can act extracellularly through the G protein coupled S1P receptors ($S1P_{1\text{-}5}$). Mature ECs express S1P receptors $S1P_{1\text{-}3}$ and these ligand/receptor interactions promote EC survival, migration, proliferation, adherens junction assembly, increased revascularization, and wound healing both *in vitro* and *in vivo* (reviewed in 15). However, S1P can also act intracellularly, possibly through histone deacetylases[16] or other as yet unknown binding partners, where the ablation of receptor signaling through both chemical or genetic mechanisms does not abrogate S1P effects on cell proliferation, Ca^{2+} mobilization, EC survival, nor the differentiation of endothelial progenitor cells.[10,17] SK-1 has two functional states, an intrinsic or basal state and an agonist-induced activated state, which requires its phosphorylation and is responsible for its oncogenic properties.[18] More recently we observed that SK-1 activates $\alpha_v\beta_3$ integrin to mediate EC survival signaling pathway via formation of a heterotrimeric complex between SK-1, $\alpha_v\beta_3$ and CD31.[19] In ECs, TNFα-induced up-regulation of E-selectin, VCAM-1 and ICAM-1 expression is an SK-1-dependent process.[20,21] Together, it is tempting to speculate that SK-1 may also contribute to integrin-mediated leukocyte adhesion under shear stress, and as such, act as a master switch for adhesion molecules and thus become a single target for therapeutic intervention.

In this study, we have examined the role of SK-1 in direct leukocyte adhesion under physiologically relevant shear flow. We show that although TNFα stimulation of human umbilical vein endothelial cells (HUVECs) does not alter total β_1 protein levels it significantly increases the formation of $\alpha_5\beta_1$ (very late antigen−5) and promotes angiopoietin (Ang)−2 expression at the EC surface. Furthermore, we hypothesize that an active conformation of β_1 integrin exists on these cells as identified by a unique anti-β_1 integrin monoclonal antibody, QE.2E5. This antibody was originally developed by Faull et al[22] following immunization of mice with lipopolysaccharide-activated HUVECs and binds to a site remote from that of the ligand, as well as other function-modifying β_1 antibodies. Importantly, QE.2E5 is itself without effect on the adhesive, proliferative, and tube-forming capabilities of both untreated and phorbol-12-myristate-13-acetate-treated HUVECs.[23] This study now proposes a novel mechanism of neutrophil adhesion under shear flow involving TNFα-induced $\alpha_5\beta_1$ activation and Ang-2 expression, which is SK-1-dependent.

Materials and Methods

Reagents and Antibodies

Murine monoclonal antibodies directed against β_1 (QE.2E5, 61.2C4, and 58.7H2), VCAM-1 (51.10C9) and E-selectin (68.5H11) were used along with isotype control (23.1F11; gifts, Prof Jennifer Gamble, Centenary Institute, NSW, Australia); α_5 (P1D6), α_2 (P1E6), PECAM-1 (M-20), and Ang-2 (N-18) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); $\alpha_5\beta_1$ (BMC5 and MAB1999), $\alpha_v\beta_3$ (LM609), and actin were from Chemicon International Inc (Temecula, CA); VCAM (BBIG-V1) and ICAM (BBIG-I1) were purchased from R&D Systems, Inc. (MN); $\alpha_5\beta_1$ (BMC5), ERK (137F5), and P-ERK (197G2) were purchased from Cell Signaling Technologies (Danvers, MA). Secondary anti-goat Alex-555, anti-mouse Alexa-488 and -594 antibodies were purchased from Invitrogen (Carlsbad, CA), and anti-mouse-, goat-, and rabbit-HRP were purchased from Pierce (Rockford, IL). Human recombinant TNFα was purchased from R&D Systems. Small interfering (si)RNA against human Ang-2 was purchased from Invitrogen (Mount Waverley, Victoria, Australia).

Cells and Cell Culture

HUVECs were grown in M199 medium containing 20% fetal calf serum (JRH, Brooklyn, Victoria, Australia) and endothelial growth factor supplement (BD BioSciences, North Ryde, NSW, Australia) as previously described.[24] Cells were used at passage two or less.

Flow Cytometry

Expression of β_1, α_5, $\alpha_5\beta_1$, VCAM-1, and E-selectin were determined on HUVECs with one-color flow cytometry using 1 μg of antibodies described above added to the cells at 4°C for 30 minutes. The secondary antibody was anti-mouse Alexa 488 at 1:1000 dilution. The median fluorescence intensity was determined using a Beckman Coulter XL-MCL (Gladesville, NSW, Australia).

SK Activity Assay

SK activity was determined as previously described.[25] D-Erythro sphingosine (Biomol, Plymouth Meeting, PA) solubilized by 0.05% Triton X-100 and [γ^{32}P]ATP (Perkin Elmer, Victoria, Australia) were used as substrates to incubate with the whole cell lysates. The radioactively labeled phospholipid was resolved by two thin-layer chromatography (Sigma, St. Louis, MO) separations in the solvents containing butanol, ethanol, water, and acetic acid (8:2:2:1 ml). The radioactive spots were quantified by Phosphorimaging Typhoon 9410 (Fullerton, CA) and ImageQuant 5.2 program (GE Health care, Rydalmere, NSW, Australia).

Inhibition Studies Using Human SK-1 Adenovirus Transduction

Generation of wild-type human SK-1 wild-type (SK-1) and mutants possessing an aspartate at Glycine82 (SK-1-DN) have been made as previously described.[26] For infection with adenoviral constructs, HUVECs were exposed to one plaque forming unit/cell for 2 hours in M119 medium with 2% fetal calf serum and a further 72 hours with medium containing 20% fetal calf serum. Cells were infected with a dose of virus previously determined to lead to a five- to tenfold increase in SK-1 activity and the same dose of control EV adenovirus was used.[17]

Immunofluorescence Microscopy

Untreated or adenovirus infected EV, SK-1, and SK-1-DN HUVECs were replated at 5×10^4 cells/well in LabTek chamber slides (Nunc, NY) with fibronectin coated at 50 μg/ml. Confluent cells were fixed with 4% paraformaldehyde at room temperature for 15 minutes before blocking with 2% bovine serum albumin/PBS at room temperature for 30 minutes. Activated β_1 (QE.2E5), $\alpha_5\beta_1$ (BMC5), Ang-2, or control antibodies (1 μg) were added to cells at 4°C overnight followed by anti-mouse Alexa 488 or anti-goat Alexa 594 antibodies (1:1000) incubation at room temperature for 1 hour. The slides were mounted with anti-fading agents (Biomeda Corp, CA) before visualization by fluorescence microscopy (Olympus IX2-UCB, Olympus, Mt Waverley, Victoria, Australia). Five images, with four cells per image, were collected per sample and analyzed using Analysis LifeSciences software (Olympus).

S1P Receptor Activation and Inhibition Studies

S1P receptor activation and inhibition studies used S1P (1 μmol/L, Cayman Chemical Co., Ann Arbor, MI), JTE-013 (1 μmol/L, Cayman Chemical Co.), and VPC23019 (10 μmol/L, Avanti Polar Lipids Inc., Alabaster, AL), which were administered either alone (for S1P) or 30 minutes before TNFα stimulation of cells (5 ng/ml, 5 hours). All reagents were proven functionally effective in paralleled human umbilical vein EC studies.[17]

Parallel Plate Flow Chamber Assay

Confluent HUVECs were cultured on Corning petri dishes (Sigma) and treated with or without 5 ng/ml TNFα for 5 hours. Control, blocking antibodies (10 μg/ml), or SK inhibitor SKi (1 μmol/L, Cayman Chemical Co.) were added for 30 minutes before cell perfusion. Using published methods, heparinized whole blood was diluted 1:10 in Hank's balanced salt solution (Sigma)[27] or used to isolate neutrophils[28] and cells (diluted blood or 1×10^6 isolated neutrophils) were perfused across the substratum by a syringe pump (NE-1000, New Era Pump System, Inc, Wartagh, NY) at a constant rate of 2 dynes/cm^2 for 5 minutes followed by Hank's balanced salt solution wash. Interactions of unlabeled cells were visualized using $\times$10/0.3 NA objectives and phase-contrast microscopy on an inverted microscope and images were recorded using a digital camera (Olympus IX70 and SIS F-view, Olympus). Five random areas per dish were recorded for analysis using AnalySIS Life Sciences software (Olympus). Adherent cells were defined as those remaining stationary for at least 10 seconds. Dishes were stained using the using May-Grunwald Giemsa (Sigma) according to the product protocol to identify leukocyte subsets.

siRNA Transfection

Adapted from methods previously described,[29] Ang-2 siRNA (50 nmol/L) (HSS100480: 5′-AAUACUUCAGCACAGUCUCUGAAGC-3′;HSS100482:5′-UUCUCCUGAAGGGUUACCAAAUCCC-3′) were transfected into HUVECs using HiPerfect (Qiagen, Doncaster, Victoria, Aust.) in EGMII medium (Clonetics/Lonza, Basel, Switzerland) when cells were at 60% confluency. After 24 hours, culture media were changed to the aforementioned tissue culture media until cells were harvested at 48 hours post-transfection.

Immunoprecipitation and Immunoblotting

HUVECs were harvested and lysed in lysis buffer containing 1% NP40 and sonicated for immunoprecipitation. Following the manufacturers instructions, cell lysates were incubated with $\alpha_5\beta_1$ (MAB199) or Ang-2 (N-18) antibody and protein A magnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany) on ice for 30 minutes before selection by magnetic columns (Miltenyi Biotech). Whole lysates or immunoprecipitates were separated by 8% SDS-polyacrylamide gel electrophoresis and transferred to Hybond-P (Amersham Biosciences, NJ). Primary antibodies to phosphorylated ERK (197G2), total ERK (137F5), Ang-2 (N-18), or β_1 (58.7H2) and secondary antibodies were used to probe the membranes before visualization by enhanced chemiluminescence (GE Health Science, Piscataway, NJ) and a luminescent image analyzer (LAS4000, Fujiflim; Stamford, CT).

Statistical Analysis

Data are shown as mean $\pm$ SEM and statistically analyzed by Student's *t*-test, 1- or 2-way analysis of variance

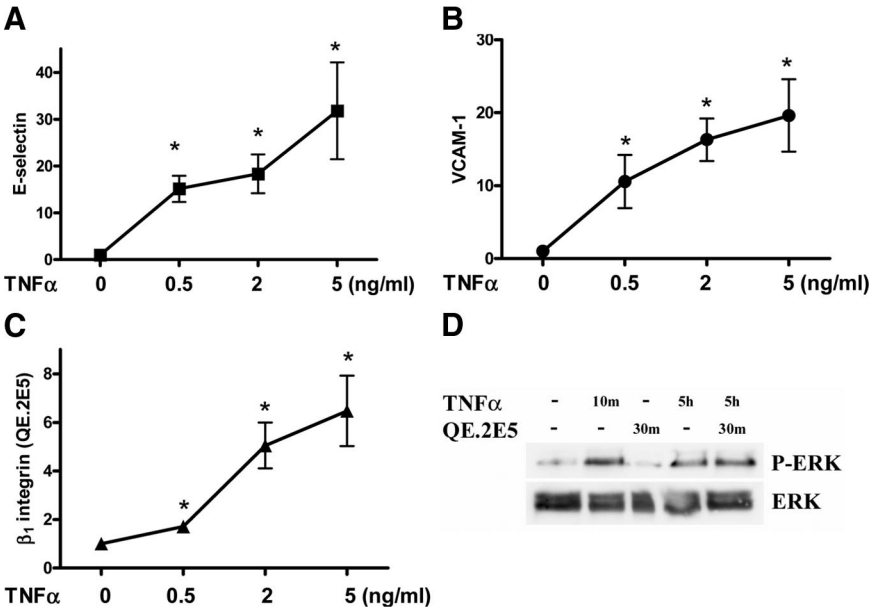

Figure 1. TNFα induced E-selectin, VCAM-1, and β_1 integrin expression on ECs. HUVECs were treated with TNFα (0, 0.5, 2, or 5 ng/ml, four hours) and examined for E-selectin (**A**), VCAM-1 (**B**), and activated β_1 integrin (**C**) using the QE.2E5 Ab using flow cytometric analysis. Results are normalized to untreated control and expressed as the mean ± SEM of seven experiments; $^*P < 0.05$ versus untreated controls (0). In **D**, HUVECs treated without and with TNFα for ten minutes or five hours without or with QE.2E5 (30 minutes at 4°C) before immunoblotting equal lysate amounts for P-ERK and total ERK. Results are representative of three experiments.

for multiple comparisons. $P < 0.05$ was considered as significant.

Results

TNFα Activates β_1 Integrin on ECs

With a similar profile to that observed for TNFα-induced E-selectin and VCAM-1 expression, Figure 1, A–C shows a dose-dependent increase in β_1 integrin on HUVECs. As these flow cytometric data were obtained using the β_1 integrin QE.2E5 antibody, we propose that this increase in β_1 integrin may in fact represent an increase in β_1 activation. The concept of QE.2E5 binding to activated β_1, without causing activation, stems from it originally being developed against activated HUVECs[22] and previous observations of it being without effect on the adhesive, proliferative, and tube-forming capabilities of both untreated and phorbol-12-myristate-13-acetate-treated HUVECs.[23] To confirm that QE.2E5 does not activate HUVECs, we examined phosphorylation of ERK, an integrin-activated downstream signaling molecule. As shown in Figure 1D, addition of QE.2E5 to HUVEC for 30 minutes at 4°C did not phosphorylate ERK. By contrast, ERK is phosphorylated by TNFα at both 10 minutes and 5 hours (Figure 1D).

TNFα-Induced Activation of β_1 Integrin Is Sphingosine Kinase-1-Dependent

To study the underlying mechanism of TNFα-induced β_1 integrin activation we investigated a role for SK-1. First, TNFα-induced activation of SK-1 in ECs was confirmed using an enzymatic assay. As shown in Figure 2, TNFα activates SK-1 in a biphasic pattern with significant increases observed at 10 minutes and 4 to 6 hours after TNFα treatment. We next examined a role for SK-1 in β_1 integrin activation using an adenoviral delivery system to either overexpress SK-1 or knock down SK-1 catalytic activity with the dominant negative SK-1 mutant (SK-1-DN).[26,30] Data shown as supplemental Figure S1 (at *http://ajp.amjpathol.org*) demonstrate that both SK-1 and SK-1-DN infected ECs exhibit a significant increase in SK-1 protein when compared with empty vector (EV) controls but that only SK-1 infected ECs express catalytically active SK-1 above control levels (see supplemental Figure S1, A and B, at *http://ajp.amjpathol.org*). Moreover, ECs overexpressing SK-1 exhibited a further increase in SK activity following TNFα stimulation for 4 hours. This was not observed in the SK-1-DN cells (see supplemental Figure S1B at *http://ajp.amjpathol.org*).

Flow cytometric analysis (using the QE.2E5 antibody) suggests that levels of activated β_1 integrin in untreated ECs do not differ between EV controls and SK-1 or SK-1-DN overexpressing cells (Figure 3B). Following TNFα activation, levels of active β_1 in EV-infected ECs increased approximately sevenfold. This increase was fur-

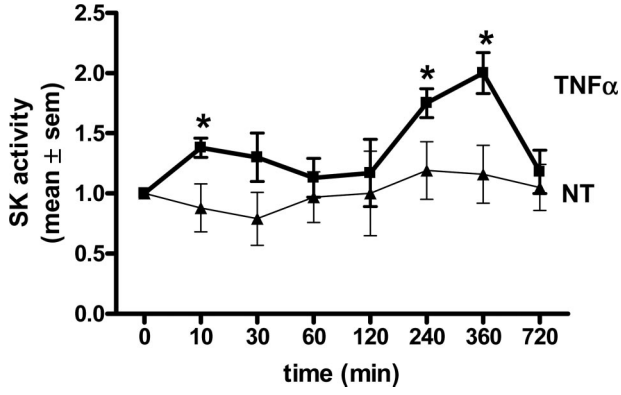

Figure 2. SK activity in ECs stimulated with TNFα. Equal lysate amounts from HUVECs treated without (triangle, NT) and with TNFα (square) (5 ng/ml: 10, 30, 60, 120, 240, 360, and 720 minutes) were assessed for SK activity by enzymatic assay. Results are the mean ± SEM of $n = 4$. $^*P < 0.05$ versus NT controls.

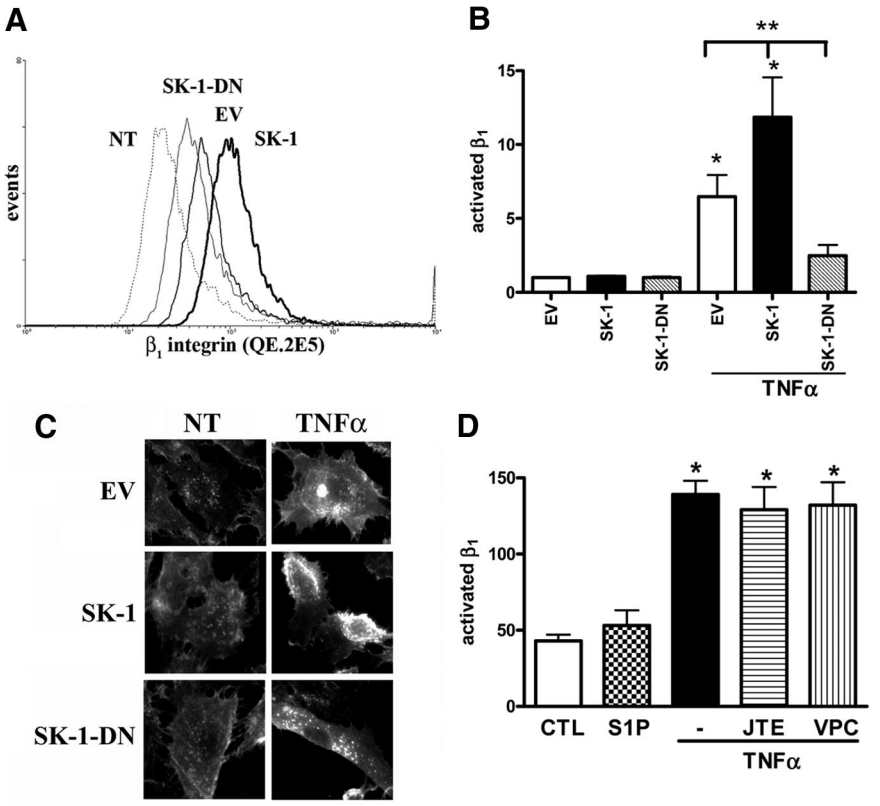

Figure 3. TNFα induces β_1 integrin activation in ECs overexpressing SK-1. HUVECs overexpressing SK-1, SK-1 dominant negative mutant (SK-1-DN), or EV control were stimulated without and with TNFα (5 ng/ml, 4 hours) and assessed for β_1 activation. In **A**, trace files show a representative experiment with untreated (NT) controls and TNFα treated EV, SK-1, and SK-1-DN. In **B**, flow cytometric analysis of QE.2E5 stained pooled experiments as the mean ± SEM, $n = 7$. $^*P < 0.05$ versus untreated control; $^{**}P < 0.05$ versus TNFα treated EV. In **C**, immunofluorescence and confocal microscopy using the QE.2E5 Ab in NT and TNFα treated EV, SK-1, and SK-1-DN cells. In **D**, HUVECs were cultured without or with JTE-013 (1 μmol/L) or VPC23019 (10 μmol/L) 30 minutes before S1P (1 μmol/L, 4 hours) or TNFα (5 ng/ml, 4 hours) stimulation. β_1 activation on ECs was assessed using immunofluorescence and mean fluorescence intensity quantified. Results are the mean ± SEM of four cells/view, five views per sample, and three experiments. $^*P < 0.05$ versus untreated control.

ther augmented in the ECs overexpressing SK-1 and not observed in the SK-1-DN ECs, which exhibited significantly less active β_1 integrin than both EV and SK-1 ECs (Figure 3, A and B). These results were supported by fluorescence microscopy, wherein increased active β_1 integrin was observed on the cell surface and edges of TNFα-treated EV control cells. This effect was augmented in TNFα-treated ECs overexpressing SK-1 and absent in the TNFα-treated SK-1-DN cells (Figure 3C).

We next investigated whether the family of S1P receptors were involved in activation of β_1 integrin, using two methods. First, ECs cultured with 1 μmol/L S1P, a concentration known to result in receptor-mediated signaling events,[20] exhibited no change in β_1 activation (Figure 3D). Second, chemical inhibition of the three S1P receptors identified on ECs (ie, $S1P_1$, $S1P_2$, and $S1P_3$)[31] using JTE-013 and VPC23019 previously shown to inhibit S1P-induced SK-1 activation in HUVECs[17] failed to inhibit TNFα-induced activation of β_1 integrin (Figure 3D).

Together, these results suggest that TNFα activates β_1 integrin at the cell surface, that SK-1 is integral to this process, and that this occurs independently of $S1P_{1\text{-}3}$.

Activation of β_1 Integrin by TNFα Does Not Correlate with Increased β_1 Integrin Protein

We next investigated whether TNFα-induced activation of β_1 integrin coincided with an increase in β_1 protein levels at the cell surface. Using flow cytometry and the 58.7H2 β_1 antibody, which does not specifically identify an activated conformation, we observed that the surface expression levels of β_1 integrin in EV, SK-1, and SK-1-DN were similar without and with TNFα treatment (Figure 4A). These observations were confirmed with a second antibody that recognizes a ligand binding site of β_1 integrin (61.2C4; Figure 4B). In addition to surface expressed β_1 integrin levels, we investigated total β_1 protein levels via immunoblotting. With a representative immunoblot shown in supplemental Figure S2 (at *http://ajp.amjpathol.org*) and pooled experimental results shown in Figure 4C; EV, SK-1, and SK-1-DN expressing ECs exhibit similar levels of β_1 integrin protein both without and with TNFα treatment. Together, these results suggest that neither TNFα treatment nor SK-1 overexpression altered total β_1 integrin protein levels either within or on the surface of ECs.

TNFα Activates $\alpha_5\beta_1$ Co-Association in ECs

To identify the α integrin subunit partnering β_1 following TNFα activation we performed immunoprecipitation assays using TNFα-treated EC lysates and the activated β_1 integrin antibody QE.2E5. As shown in Figure 5A, immunoblots identified a close association between β_1 and α_5 in untreated as well as TNFα-treated ECs. Notably, from five experiments we observed an average of 18 ± 5% increase in α_5 association with β_1 integrin following TNFα stimulation. Despite previous studies reporting α_2 subunit associating with β_1 in TNFα-treated ECs, we were unable to detect this association following activated β_1 (QE.2E5) antibody pull-down (Figure 5A). Flow cytometric analysis confirmed a significant increase in surface expression

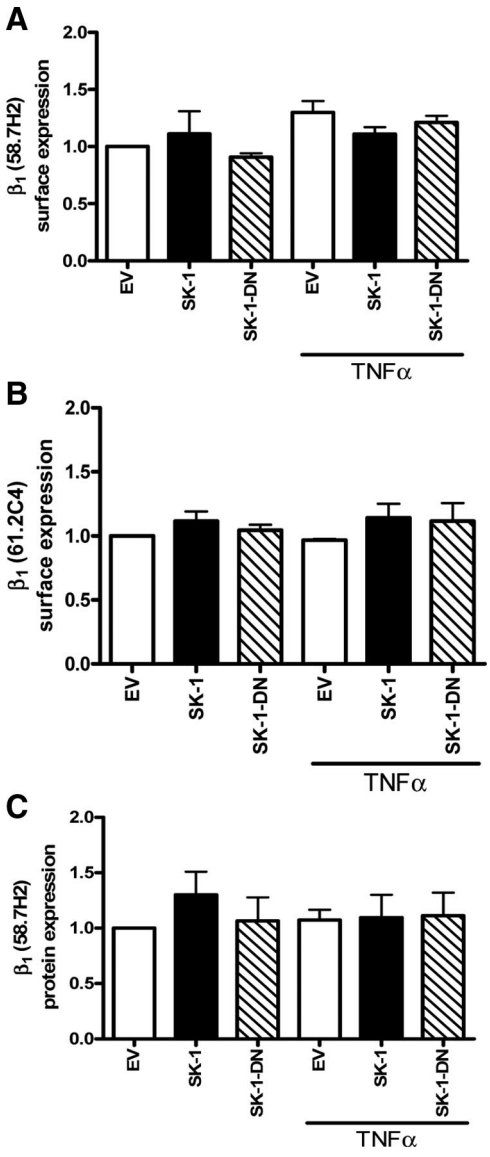

Figure 4. β_1 integrin surface expression and protein levels in ECs in response to TNFα treatment. HUVECs overexpressing SK-1, SK-1 dominant negative mutant (SK-1-DN), or EV control, were stimulated without and with TNFα (5 ng/ml, four hours) and assessed for β_1 expression by flow cytometric analysis using 58.7H2 and 61.2C4 Abs (**A** and **B**) and immunoblotting using the 58.7H2 Ab (**C**). Results are expressed as the mean ± SEM of three experiments (**A** and **B**) or four experiments (**C**).

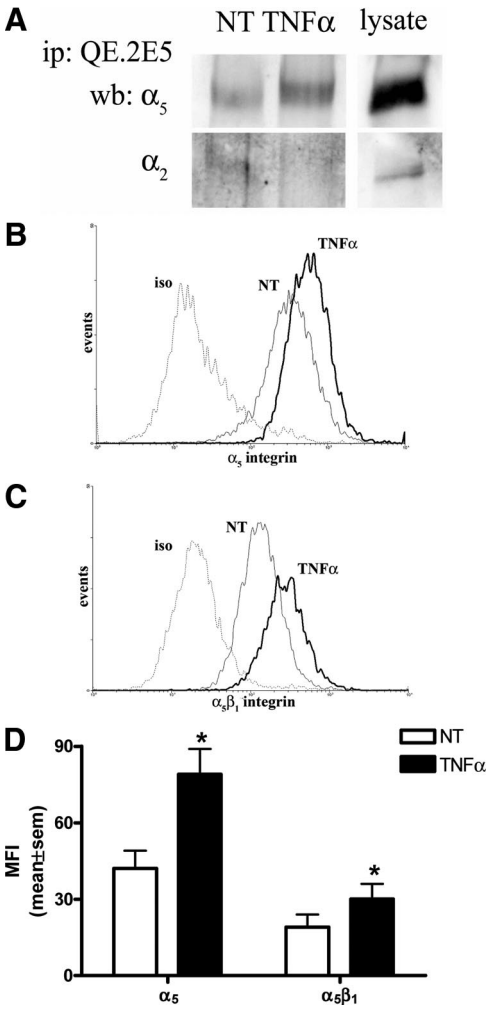

Figure 5. TNFα activated β_1 integrin is co-associated with α_5. HUVECs were treated without and with TNFα (5 ng/ml, four hours) before immunoprecipitation of equal lysate amounts with QE.2E5 to pull down activated β_1 integrin (**A**). Precipitates were examined for β_1 co-association with α_5 and α_2 integrin via immunoblotting. Gel lanes from the same experiment have been cut and re-orientated to better illustrate the whole HUVEC lysate positive controls (lysate). Results are representative of five experiments. In **B** and **C**, HUVECs were treated without (NT) and with TNFα (5 ng/ml, four hours) before flow cytometric analyses for α_5 and $\alpha_5\beta_1$ integrin surface expression, respectively, and are a representative of three experiments relative to isotype control (iso). In **D,** similar experiments were quantified for mean fluorescence intensity (MFI) of α_5 and $\alpha_5\beta_1$ surface expression. Results are mean ± SEM, $n = 3$; $^*P < 0.05$ versus NT.

of α_5, as well as $\alpha_5\beta_1$, on TNFα-treated HUVEC (Figure 5, B–D).

TNFα-Induced Activation of $\alpha_5\beta_1$ on HUVECs Mediates Leukocyte Adhesion

To investigate the function of TNFα-induced activation of β_1 integrin on ECs, we examined its role in leukocyte adhesion by a parallel plate flow chamber assay. As shown in Figure 6A (and supplemental Video S1, at *http://ajp.amjpathol.org*), when human blood was perfused over untreated HUVECs at a rate of 2 dynes/cm^2, negligible leukocyte adhesion was observed. By contrast, when stimulated with TNFα, a significant increase in leukocyte adhesion occurred. When TNFα stimulated ECs were exposed to a β_1 blocking antibody (61.2C4) 30 minutes before flow chamber assay, the number of adherent cells was significantly reduced. A similar profile of reduced leukocyte adhesion was also observed with an α_5 blocking antibody. No change was observed with either an isotype control or a blocking antibody to α_2 (confirmed to inhibit ECs attachment to collagen) (Figure 6A). A role for SK in TNFα-induced leukocyte adhesion was confirmed in experiments wherein an SK inhibitor, SKi, was added 30 minutes before flow chamber assay (Figure 6A). These results suggest that TNFα-induced leukocyte adhesion to ECs is, at least in part, mediated by the integrin $\alpha_5\beta_1$ and the SK/S1P pathway. The role for α_5 and β_1 combined in leukocyte adhesion was next examined us-

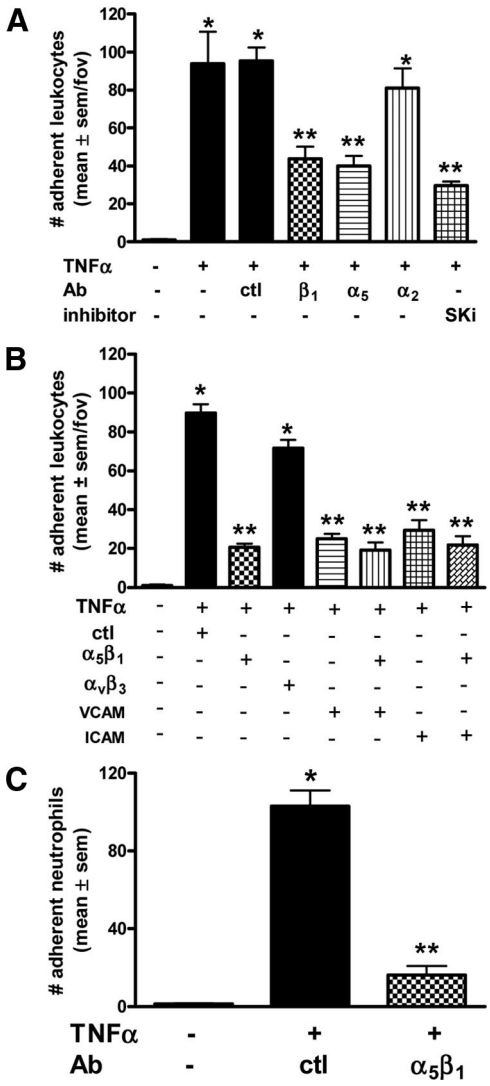

Figure 6. Leukocyte adhesion to TNFα-activated ECs under shear flow. Adhesion of leukocytes on HUVECs stimulated without and with TNFα (5 ng/ml, four to five hours) using the parallel plate flow chamber assay at a constant shear rate of 2 dynes/cm^2 and pre-incubation of ECs in **A** with control (ctl) or blocking Abs to β_1, α_5, α_2, or the SK inhibitor (SKi, 1 μmol/L), for 30 minutes prior. In **B**, blocking Abs to $\alpha_5\beta_1$, $\alpha_v\beta_3$, VCAM, and ICAM for 30 minutes prior. In **C**, isolated neutrophils are perfused (1×10^6/ml) as above and pre-incubation of ECs with control (ctl) or blocking Abs to $\alpha_5\beta_1$ for 30 minutes prior. Data are expressed as the mean ± SEM per field of view (fov) with four to five fov captured for three to four separate experiments. *$P < 0.05$ relative to untreated (−) controls; **$P < 0.05$ relative to TNFα stimulated adhesion.

ing the $\alpha_5\beta_1$ blocking antibody (BMC5). As shown in Figure 6B, administration of the $\alpha_5\beta_1$ antibody significantly reduced leukocyte adhesion to TNFα-treated HUVECs. This inhibition was not observed with the $\alpha_v\beta_3$ blocking antibody (LM609) (Figure 6B), an integrin also activated by SK-1.[19] As TNFα is also known to regulate VCAM-1 and ICAM-1 expression on HUVECs via SK-1,[20] we next investigated their contribution to $\alpha_5\beta_1$-mediated leukocyte adhesion. As shown in Figure 6B, antibodies to $\alpha_5\beta_1$, VCAM-1 and ICAM-1, alone, or together, demonstrated a similar level of inhibition.

May-Grunwald Giemsa staining of the dishes at completion of the aforementioned experiments identified neutrophils as the leukocyte subset targeted by $\alpha_5\beta_1$ for adhesion (data not shown). This was confirmed with isolated neutrophils in flow chamber assays. As shown in Figure 6C, when TNFα-stimulated ECs were exposed to the $\alpha_5\beta_1$ blocking antibody (BMC5) 30 minutes before flow chamber assay, the majority of neutrophils could no longer adhere.

Angiopoietin-2 Associates with $\alpha_5\beta_1$ to Mediate TNFα-Induced Leukocyte Adhesion

Previous studies have shown that Ang-2 production by ECs is significantly increased following TNFα stimulation.[32] More importantly for this study, Ang-2 is a known ligand for $\alpha_5\beta_1$ on ECs.[33] Increased Ang-2 expression by ECs following TNFα activation was first confirmed by fluorescence microscopy on HUVECs overexpressing SK-1, SK-1-DN, or EV control. As shown in supplemental Figure S3A (at *http://ajp.amjpathol.org*), untreated ECs exhibit an Ang-2 expression that is low and diffuse over the cell surface. This expression increases in a similar pattern following TNFα activation. Figure 7A shows quantitation of the immunofluorescence data and suggests that TNFα promotes Ang-2 surface presentation by approximately fourfold. When ECs overexpress SK-1, TNFα-mediated Ang-2 expression is further increased. This effect is not observed in the SK-1-DN ECs where TNFα-induced Ang-2 expression is significantly abrogated (Figure 7A).

As Ang-2 is a known ligand for $\alpha_5\beta_1$ on ECs[33] we next investigated their endogenous association in ECs without and with TNFα activation. Immunoprecipitations targeting Ang-2 identified its close association with β_1 integrin (Figure 7B) with repeated experiments suggesting an increase of approximately 10% following TNFα activation. Reciprocal immunoprecipitations targeting $\alpha_5\beta_1$ and immunoblotting against Ang-2 confirmed an increase in $\alpha_5\beta_1$:Ang-2 complex formation in ECs following TNFα activation. From repeated experiments, an average of 21 ± 12% increase in Ang-2 association with $\alpha_5\beta_1$ integrin occurred following TNFα activation.

We next investigated whether Ang-2 is involved in leukocyte adhesion under flow using Ang-2 specific siRNAs. Immunoblots first confirmed a significant reduction in Ang-2 protein levels when compared with ECs transfected with a negative control siRNA (see supplemental Figure S3B at *http://ajp.amjpathol.org*). Importantly, when ECs are depleted of Ang-2 by siRNA the number of adherent leukocytes under flow is significantly reduced when compared with the control siRNA-transfected ECs (Figure 7C and supplemental Video S2 at *http://ajp.amjpathol.org*). These results were confirmed using a second Ang-2 siRNA and Giemsa May-Grunwald staining of the dishes supported previous data of neutrophils being targeted by this process. Finally, Figure 7D shows immunofluorescence staining following flow chamber assay for $\alpha_5\beta_1$ (green) and Ang-2 (red), which indicate similar expression patterning on TNFα-treated HUVECs. The close

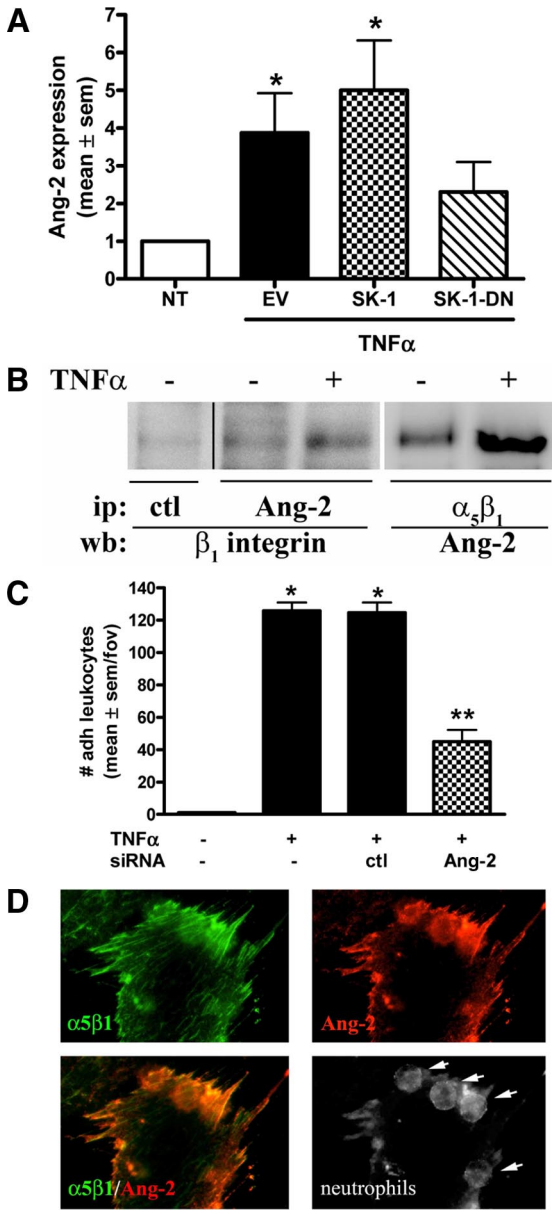

Figure 7. Ang-2 expression in TNFα-treated ECs and leukocyte adhesion under shear flow. In **A**, HUVECs overexpressing SK-1, SK-1 dominant negative mutant (SK-1-DN), or EV control were stimulated without (NT) and with TNFα (5 ng/ml, four hours) and quantified for Ang-2 surface expression by immunofluorescence. Results are the normalized mean ± SEM of approximately 100 cells from a total of five experiments; $^{*}P < 0.05$ versus untreated controls (NT). In **B**, equal lysate amounts from HUVECs treated without and with TNFα (5 ng/ml, four hours) were immunoprecipitated for Ang-2 or $\alpha_5\beta_1$. Precipitates were examined for $\alpha_5\beta_1$ and Ang-2 co-association via immunoblotting. Gel lanes from the same experiment have been cut and re-orientated to better illustrate the changes from isotype control Ab (ctl) ip. Results are representative of five experiments. In **C**, HUVECs containing siRNA to Ang-2 or ctl were stimulated without and with TNFα (5 ng/ml, four to five hours) and assessed for leukocyte adhesion by flow chamber assay at a constant shear rate of 2 dynes/cm^2. Data are expressed as the mean ± SEM per field of view (fov) with four to five fov captured for five separate experiments. $^{*}P < 0.05$ relative to untreated (−) controls; $^{**}P < 0.05$ relative to TNFα stimulated ctl siRNA cells. In **D**, $\alpha_5\beta_1$ (green) and Ang-2 (red) surface expression, as well as neutrophil localization (**arrows**) were viewed by immunofluorescence in HUVECs treated with TNFα (5 ng/ml, four hours) by confocal microscopy.

association of these two proteins is further supported by the merged image in Figure 7D ($\alpha_5\beta_1$/Ang-2) where $\alpha_5\beta_1$ and Ang-2 clearly overlay. Furthermore, a change in focal plane of this image identified the localization of neutrophils in close proximity to $\alpha_5\beta_1$ and Ang-2 at the cell junction (Figure 7D, neutrophils, arrows). Together these results support our hypothesis that TNFα-induced neutrophil adhesion to ECs under flow is mediated by a complex of active $\alpha_5\beta_1$ and Ang-2.

Discussion

Inappropriate activation or recruitment of leukocytes has been implicated in the pathogenesis of various inflammatory diseases (reviewed in 2). Herein we demonstrate that TNFα induces $\alpha_5\beta_1$ activation via an SK-1-dependent pathway in HUVECs. We show that SK-1 overexpression in ECs, to levels of activity equivalent to that seen after vascular endothelial growth factor or other growth factor stimulation, sensitizes the cells to TNFα and has a crucial role in $\alpha_5\beta_1$ integrin activation and neutrophil adhesion under shear flow. Evidence for this comes from firstly, the binding of the QE.2E5 antibody, previously shown to bind to activated β_1 integrin via a conformation sensitive epitope[22] when SK-1 is overexpressed. Notably, this TNFα-induced activation of β_1 does not coincide with increased protein levels but does correlate with increased expression of α_5 as well as formation of $\alpha_5\beta_1$, as demonstrated by immunoblotting and flow cytometric analysis of TNFα-treated ECs. TNFα-induced $\alpha_5\beta_1$ activation in ECs promotes neutrophil adhesion under physiologically relevant shear flow and this occurs in an SK-1-dependent, $S1P_{1\text{-}3}$-independent, manner. Finally, in response to TNFα activation, ECs exhibit an SK-1-dependent, increased surface expression of Ang-2, which is biologically important for neutrophil adhesion.

Herein, we propose that TNFα treated HUVEC exhibit increased activation of β_1 integrin. This concept is primarily derived from increased binding of the QE.2E5 monoclonal antibody, which was originally developed against lipopolysaccharide-activated HUVEC.[22] QE.2E5 is unique in that it binds to β_1 integrin at amino acid residues 426-587, a site 194 amino acids distal from the binding site of ligands and other function-modifying β_1 antibodies.[22] Our data support the notion that QE.2E5 identifies activated β_1 integrin on HUVECs with 1) TNFα-activated HUVECs expressing more $\alpha_5\beta_1$ complex at their cell surface, 2) no change in β_1 surface expression or protein using antibodies 58.7H2 and 61.2C4, and 3) increased binding of Ang-2 to $\alpha_5\beta_1$ on TNFα-treated HUVEC. With the assumption that β_1 integrin does not exist without an α partner at the cell surface, this implies that the TNFα-induced $\alpha_5\beta_1$ formation is a result of β_1 switching from another integrin heterodimer. The integrin from which this β_1 subunit is derived is yet to be determined with $\alpha_2\beta_1$ a likely candidate (Figure 5A). Interestingly, despite QE.2E5 previously shown to activate β_1 integrin function on the erythroleukemic cell line K562[22] and thymocytes[34] it remains without effect on the adhesive, proliferative, and tube-forming capabilities of both untreated and phorbol-12-myristate-13-acetate-treated HUVECs,[23] as well as ERK activation on TNFα-treated HUVECs (Figure 1). The physiological significance of such alternative conformational effects by QE.2E5 on

different cell types is uncertain. It is tempting to speculate that the lipopolysaccharide-activated HUVECs, first used to develop QE.2E5, underwent an inside-out signaling event, which altered the conformation of β_1 integrin at residues 426–587, and as such, identifies agonist induced β_1 activate conformation specific to HUVECs.

Despite our significant progress in the knowledge of the neutrophil adhesion cascade, there are still several caveats in our understanding. In particular, the relative importance of each adhesion receptors in response to specific stimuli for tissue- and vascular bed-specific inflammatory cell recruitment needs to be established. Some light may have been shed on this issue by previous studies, which demonstrated a role for $\alpha_5\beta_1$ in the migration of neutrophils,[35–37] as well as the adhesion of monocytes.[38] These studies clearly suggest that leukocyte interactions with the endothelium may rely on the assembly of complexes, which contain different adhesion molecules. Our observations that inhibiting β_1 abrogated, in part, neutrophil adhesion to TNFα-treated HUVEC under shear flow is in keeping with other studies demonstrating the contribution of TNFα-induced expression of VCAM-1 and ICAM-1 to leukocyte adhesion (reviewed in 39). Furthermore, when $\alpha_5\beta_1$ is blocked in addition to VCAM-1 and ICAM-1 a slight, but insignificant reduction in neutrophil adhesion was observed. The full relationship between $\alpha_5\beta_1$ and other TNFα-induced adhesion molecules is still to be fully elucidated, but our work suggests that an interrelationship between these molecules may exist. Given the wide distribution of integrins throughout the body, the need for stringent regulation of their interactions might be well anticipated. Indeed, studies to date suggest that a certain threshold level of integrin expression, as well as conversion to the active form, is required to engage ligands—a conversion that is cell type- and stimuli-specific. Such selectivity is exemplified by opioids that trigger $\alpha_5\beta_1$ integrin-mediated monocyte adhesion[38] and f-Met-Leu-Phe, but not leukotriene B4 activation of $\alpha_5\beta_1$ for neutrophil adhesion to fibronectin.[40] In further support, studies suggest that the activated form of $\alpha_5\beta_1$ is a transient property of activated ECs, and can therefore be used only within a confined time frame (reviewed in 3). Our data now extend this work by suggesting that TNFα activates the endothelium for $\alpha_5\beta_1$-dependent neutrophil adhesion under shear flow.

Ang-1 and Ang-2 are structurally related endothelial growth factors found on the extracellular surface of ECs with Tie-2 considered to be their principle cell surface receptor.[41] While Ang-1 mediates vessel maturation and maintains vessel integrity, Ang-2, in contrast, is classically considered as a functional antagonist of Ang-1 and binds to Tie-2 without inducing signal transduction.[42] Mice transgenically overexpressing *Ang-2* have an embryonic lethal phenotype that essentially phenocopies *Ang-1*-deficient and *Tie-2*-deficient phenotypes.[41–43] Interestingly, genetic ablation of Ang-2 is also lethal with postnatal mortality of newborn pups occurring by day 14. However, this lethality is entirely dependent on the genetic background of the mice with the 129/J and C57BL/6 strains being more and less susceptible, respectively.[44,45] Ang-2 acts by an autocrine mechanism and is stored in Weibel-Palade bodies from where it can be rapidly released on stimulation.[46] A role for Ang-2 in leukocyte recruitment is evidenced by Fiedler and colleagues who recently showed that *Angpt-2−/−* mice could not elicit an inflammatory response in thioglycollate-induced or *Staphylococcus aureus*-induced peritonitis, or in the dorsal skin fold chamber model.[45] Furthermore, intravital microscopy showed normal TNFα-induced leukocyte rolling but not adhesion in the vasculature of *Angpt-2−/−* mice. Cellular experiments also suggest that Ang-2 promotes cell adhesion by modulating TNFα-induced expression of the EC adhesion molecules ICAM and VCAM.[45] Finally, a close association between the angiopoietins and $\alpha_5\beta_1$ has previously been demonstrated in pull-down assays and is suggested to be Tie-2 independent.[47,48] Our study supports these observations with TNFα-induced neutrophil recruitment being both $\alpha_5\beta_1$- and Ang-2-dependent and that a relationship with VCAM-1 and ICAM-1, but not $\alpha_v\beta_3$, may exist.

SK-1 can exist in a basal, intrinsic state that we have shown inhibits EC permeability.[29] SK-1 also has an agonist-induced activated state, which occurs, at least in response to TNFα and phorbol ester, as a direct consequence of phosphorylation at serine 225 by ERK1/2.[30] The effects of this single phosphorylation are two-fold: it is required for agonist induced increases in the catalytic activity of SK-1, and it is necessary for translocation of this protein from the cytosol to the plasma membrane.[18] We have recently demonstrated a requirement of SK-1 phosphorylation at serine 225 for increased heterotrimeric complex formation between SK-1, $\alpha_v\beta_3$ and CD31 following factor deprivation where $\alpha_v\beta_3$ activation and subsequent EC survival signals include the Bcl-X and nuclear factor κB pathways.[19] Herein we demonstrate, for the first time, that SK-1 is integral for TNFα-induced $\alpha_5\beta_1$ activation, Ang-2 expression and neutrophil adhesion under shear flow. Whether phosphorylation of SK-1 is also involved in this process is still to be determined. Furthermore, we suggest that TNFα-induced activation of $\alpha_5\beta_1$ is S1P receptor independent as inhibition of $S1P_{1\text{-}3}$ function, as well as S1P administration (at a concentration specific for receptor engagement[49]) did not alter QE.2E5 antibody binding capabilities. The possibility that TNFα-induced adhesion molecule expression requires SK-1/S1P to act intracellularly supports the initial observations by Xia and co-workers.[50] Briefly, Xia et al[50] demonstrated that the effective concentrations of S1P to mediate adhesion molecule expression were in the micromolar range despite the K_d for $S1P_{1\text{-}3}$ on ECs being 20 to 60 nmol/L. Furthermore, neither pertussis toxin (a $G\alpha_i$ inhibitor) nor suramin (a nonspecific phospholipid receptor inhibitor) could effectively block S1P induction of adhesion molecules.[20] These observations are however in contrast to the suppressive effect of S1P on $\alpha_5\beta_1$-mediated monocyte adhesion to ECs.[51] Aoki and colleagues[51] recently demonstrated that S1P-induced activation of HUVECs inhibited U937 adhesion by shifting the localization of $\alpha_5\beta_1$ from the apical surface to the basal surface. This S1P-induced suppressive effect was $S1P_1$ and $S1P_3$ receptor-mediated, as demonstrated by inhibition studies, as well as specific inhibitors of $G\alpha_i$ protein, Src family proteins, phosphatidylinositol 3-kinase, and Rac1.[51] The

differences observed between this and our own observations may be explained by the use of a myeloid cell line and a static adhesion assay by Aoki et al[51] versus ours of whole blood or neutrophils and adhesion under conditions of physiologically relevant shear flow. The very recent identification of histone deacetylases as intracellular targets of S1P by Hait and colleagues[16] suggests a new paradigm of S1P signaling within the nucleus. Whether transcriptional regulation of EC adhesion molecules occurs via this mechanism is interesting to speculate and requires further investigation. Another aspect that requires further elucidation with respect to TNFα-induced, SK-1-dependent adhesion molecule expression is the timing of SK activity. We show here a biphasic response of SK activity in ECs following TNFα activation: the first occurring within 10 minutes and the second approximately 4 to 6 hours later. Whether TNFα-induced E-selectin, VCAM-1, ICAM-1, and now, $\alpha_5\beta_1$, expression are reliant on the first and/or second wave of SK activity is currently under investigation within our laboratory.

Neutrophils are the most abundant blood-borne leukocytes in healthy humans and they accumulate within hours at sites of acute inflammation. Moreover, they are essential for combating bacterial and fungal infections, but their activation also releases cytotoxic mediators, causing tissue damage. These studies support that the paradigm of neutrophil trafficking as a multistep cascade determined by a variety of adhesion receptors continues to serve as a useful model, but needs to be refined to accommodate Ang-2 and other non-selectin and cellular adhesion molecules. The findings presented here add new complexities that broaden the accepted concept of neutrophil trafficking, as we show that primary adhesive events of neutrophils, *in vitro*, are dependent on $\alpha_5\beta_1$ and Ang-2. The precise mechanisms underpinning $\alpha_5\beta_1$:Ang-2-mediated neutrophil adhesion require further investigation. We propose a sandwich type configuration between $\alpha_5\beta_1$ and Ang-2, where Ang-2 acts as a bridge to mediate $\alpha_5\beta_1$:$\alpha_5\beta_1$-dependent cellular events between neutrophils and TNFα-activated HUVECs. A similar system was recently observed with Th1 and Th2 lymphocytes, wherein hyaluronan mediates the CD44:CD44 dependent rolling and adhesion to the intestine of TNFα-treated mice.[27] Our observations now suggest that SK-1/S1P is integral to controlling all three families of adhesion molecules, namely, selectins, cellular adhesion molecules, and integrins. Taken together these results suggest that SK-1 may be the single target required for an effective broad spectrum therapeutic target to combat inflammatory and immune disorders.

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Appendix 2:

Rapid histamine-induced neutrophil recruitment is sphingosine kinase-1 dependent.

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Rapid Histamine-Induced Neutrophil Recruitment Is Sphingosine Kinase-1 Dependent

Wai Y. Sun,*†‡ Latasha D. Abeynaike,§ Samantha Escarbe,* Charles D. Smith,¶ Stuart M. Pitson,*‡‖ Michael J. Hickey,§ and Claudine S. Bonder*†‡‖

From the Division of Human Immunology, Centre for Cancer Biology, SA Pathology, Adelaide, Australia; the School of Medicine† and Molecular and Biomedical Sciences,‖ University of Adelaide, Adelaide, Australia; the Co-Operative Research Centre for Biomarker Translation,‡ La Trobe University, Bundoora, Australia; the Department of Medicine,§ Centre for Inflammatory Diseases, Monash Medical Centre, Monash University, Clayton, Australia; and the Medical University of South Carolina,¶ Charleston, South Carolina*

Leukocyte recruitment to sites of inflammation is critical for the development of acute allergic responses. Rapid P-selectin up-regulation by endothelial cells is a key promoter of leukocyte infiltration in response to mediators such as histamine. However, the mechanisms underpinning this process are still incompletely understood. We examined the role of the sphingosine kinase/sphingosine-1-phosphate (SK/S1P) pathway and showed that in human umbilical vein endothelial cells, histamine rapidly activates SK in an extracellular signal-regulated kinase (ERK) 1/2-dependent manner, concurrent with the induction of P-selectin expression. Histamine activated both SK-1 and SK-2 isoforms; inhibition of SK-1, but not SK-2, attenuated histamine-induced P-selectin up-regulation and neutrophil rolling *in vitro*. S1P receptor antagonists failed to prevent histamine-induced P-selectin expression, and exogenous S1P did not increase P-selectin expression, suggesting that S1P cell surface receptors are not involved in this process. Finally, the role of both SK-1 and SK-2 in histamine-induced leukocyte rolling *in vivo* was assessed using pharmacological and genetic methods. Consistent with the *in vitro* findings, mice pretreated with either sphingosine kinase inhibitor or fingolimod (FTY720) significantly attenuated histamine-induced leukocyte rolling in the cremaster muscle. Similarly, *Sphk1*$^{-/-}$ but not *Sphk2*$^{-/-}$ mice exhibited reduced histamine-induced leukocyte rolling. These findings demonstrate a key role for SK-1 in histamine-induced rapid P-selectin up-regulation and associated leukocyte rolling, and suggest that endothelial SK-1 is an important contributor to allergic inflammation. ***(Am J Pathol 2012, 180:1740–1750; DOI: 10.1016/j.ajpath.2011.12.024)***

Inflammation is central to the development of acute allergic responses. The allergic inflammatory response is a multistep process involving increased vascular permeability, changes in expression of endothelial cell adhesion molecules, and the triggering of cell-cell interactions between circulating leukocytes and the vascular endothelium. Several types of adhesion molecules are involved in leukocyte binding and transmigration, and their expression is tightly regulated to produce the sequence of events that leads to leukocyte recruitment. In allergic inflammation, these events are coordinated by inflammatory mediators, including histamine. Histamine activates the local vasculature by binding to its G-protein coupled receptors, H_1 and H_2, on endothelial cells and thus causing a rapid exocytosis of the preformed adhesion molecule P-selectin.[1,2] Circulating neutrophils are immediately recruited by tethering and rolling along the vasculature via a P-selectin/P-selectin glycoprotein ligand-1 (PSGL-1) mechanism.[1] The ability of P-selectin to undergo a rapid increase in exposure on the endothelial surface plays a critical role in development of this initial phase of the allergic response. It is therefore important

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Address reprint requests to Claudine S. Bonder, Ph.D., Centre for Cancer Biology, South Australia Pathology, Frome Road, Adelaide, SA 5000, Australia. E-mail: claudine.bonder@health.sa.gov.au.

that the molecular basis of this response be completely understood.

P-selectin is constitutively synthesized in endothelial cells,[3] megakaryocytes/platelets,[4] and resident peritoneal macrophages,[5] where it is packaged into Weibel-Palade body and α storage granules.[4,6] Two distinct mechanisms regulate the inducible expression of P-selectin. In mice, mediators such as tumor necrosis factor (TNF), interleukin-1, and lipopolysaccharide can induce transcription of P-selectin mRNA, with subsequent protein synthesis and surface expression. This response is not seen in human endothelial cells, however, because of the lack of binding sites for NF-κB and activating transcription factor-2 (ATF-2) in the human *SELP* gene promoter.[7–9] Alternatively, in both mice and humans, P-selectin can be rapidly mobilized to the endothelial surface from Weibel-Palade bodies in response to mediators such as histamine, thrombin, and other secretagogues.[10] This mechanism does not require new protein synthesis, instead being induced by rapidly acting signaling molecules within endothelial cells. For mediators associated with allergic inflammation, such as histamine, the signaling molecules involved in this rapid response are not fully characterized, but the sphingosine kinase pathway is one candidate.

Sphingosine kinase (SK) is a highly conserved lipid kinase. Two isoforms (SK-1 and SK-2) have been identified, cloned, and characterized.[11,12] Both SK-1 and SK-2 catalyze the phosphorylation of sphingosine to form sphingosine-1-phosphate (S1P), but they exhibit different subcellular localization patterns, developmental expression, and distribution in adult tissue and have been recognized to have both overlapping and alternative biological functions.[13] S1P is a bioactive phospholipid and is an important signaling molecule that can be either retained inside or secreted out of the cell. Basal levels of S1P in cells are generally low, but can increase rapidly when cells are exposed to various agonists through rapid and transient activation of SK activity as a result of phosphorylation on Ser225 by extracellular signal-regulated kinases 1 and 2 (ERK-1/2).[14] Extracellular S1P acts on its G-protein coupled receptors, $S1P_{1–5}$, in both autocrine and paracrine fashions with, for example, downstream signaling of phosphatidyl inositol 3-kinase (PI3K)/Akt and ERK-1/2.[13] Alternatively, endogenous S1P can associate with histone deacetylases (HDAC1 and HDAC2),[15] tumor necrosis factor receptor-associated factor 2 (TRAF2),[16] prohibitin,[17] or as yet unidentified targets. S1P has been shown to synergize with histamine during a 4-hour exposure to promote gene and surface expression of E-selectin, ICAM-1, and VCAM-1.[18] However, the contribution of the SK pathway to rapid leukocyte recruitment typical of allergic responses has not previously been investigated.

With the present study, we identify SK-1 as a new potential target for controlling rapid recruitment of neutrophils after exposure to histamine. First, we demonstrate that both SK-1 and SK-2 are rapidly activated by histamine in human umbilical vein endothelial cells (HUVECs) and that this occurs in an ERK-1/2-dependent manner. Second, we demonstrate that histamine-induced surface expression of P-selectin on HUVECs requires both ERK-1/2 and SK-1 but does not involve SK-2 or the $S1P_{1–3}$ surface receptors. Finally, we demonstrate that histamine-induced SK-1, but not SK-2, activity mediates neutrophil recruitment *in vitro* and *in vivo*. Collectively, the present findings suggest that SK-1 may be a critical regulator controlling acute allergic responses.

Materials and Methods

Reagents and Antibodies

Antibodies against human P-selectin (AK-4) and isotype control were purchased from BD Biosciences (Franklin Lakes, NJ). Phosphorylated ERK-1/2 and total ERK-1/2 were purchased from Cell Signaling Technology (Danvers, MA). Human SK-1 antibody was generated as described previously.[14] Secondary antibodies anti-rabbit-HRP (Pierce; Thermo Fisher Scientific, Rockford, IL), anti-rabbit Alexa Fluor 594, anti-mouse Alexa Fluor 488, and DAPI (Invitrogen, Carlsbad, CA) were used. Human recombinant histamine, histamine-1-receptor antagonist (chlorpheniramine), and histamine-2-receptor antagonist (cimetidine) were purchased from Sigma-Aldrich (St. Louis, MO). Sphingosine kinase inhibitor (SKi) and S1P were purchased from Cayman Chemical (Ann Arbor, MI). Other inhibitors were purchased as follows: *N*,*N*-dimethyl sphingosine (DMS; Biomol International-Enzo Life Sciences, Plymouth Meeting, PA); $S1P_1$ receptor antagonist (W146; Cayman Chemical); $S1P_2$ receptor inhibitor (JTE013; Cayman Chemical); $S1P_3$ receptor antagonist (CAY10444; Cayman Chemical); $S1P_{1\&3}$ receptor inhibitor (VPC23019; Avanti Polar Lipids, Alabaster, AL); fingolimod (FTY720; Sapphire Bioscience, Waterloo, Australia); and MAPK pathway inhibitors (U0126, Cell Signaling Technology; SB203580 and PD98059, Alexis Biochemicals-Enzo Life Sciences, Plymouth Meeting, PA). The SK-2 inhibitor ABC294640 was synthesized as described previously.[19]

Animals

Wild-type (WT), SK-1 knockout (*Sphk1*$^{-/-}$), and SK-2 knockout (*Sphk2*$^{-/-}$) mice on a C57Bl/6 background[20,21] were housed under pathogen-free conditions at SA Pathology and at Monash University and were used between 6 and 12 weeks of age. All experimental procedures were approved by the Animal Ethics Committee of South Australia Pathology, the University of Adelaide, and Monash University and conform to the guidelines established by the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Cells and Cell Culture

The collection of human umbilical cords for use in the present study was given ethical clearance from the Human Research Ethics Committee of the Children, Youth and Women's Health Service (CYWHS), North Adelaide; informed written consent was obtained from all subjects in accordance with the Declaration of Helsinki. Human

umbilical vein endothelial cells (HUVECs) were isolated as described previously.[22] HUVECs were grown in M199 medium (Sigma-Aldrich) containing 20% human serum (Invitrogen), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen, Gibco BRL, Paisley, Scotland). Cells were cultured on 10% gelatin (Sigma-Aldrich) and used at passage 1.

Neutrophils and lymphocytes were enriched from venipuncture samples of consenting healthy donors, as described previously.[23] Briefly, after dextran sedimentation the cells were enriched by density-gradient centrifugation on Lymphoprep medium (Nycomed, Oslo, Norway), with the neutrophils pelleting at the base and the lymphocytes enriched at the interface. After hypotonic lysis of erythrocytes, cells were resuspended in RPMI 1640 medium containing 10 mmol/L HEPES and 2.5% fetal bovine serum (Invitrogen, Gibco BRL) before use. Cytological examination of cytocentrifuged preparations with May-Grünwald Giemsa staining (Sigma-Aldrich) showed that >95% of the cells were neutrophils or lymphocytes. Trypan Blue staining confirmed that >98% of these cells were viable. The human Jurkat T-cell line was cultured in complete RPMI 1640 medium (Gibco BRL) with 10% fetal bovine serum. To quantify the degree of Jurkat cell activation in response to histamine (25 μmol/L, 30 minutes) or phorbol myristate acetate (100 ng/mL 30 minutes), levels of L-selectin expression were measured using flow cytometry with 1 μg of monoclonal antibody against L-selectin (Dreg56 mouse anti-human, a kind gift from E. Butcher) or a nonspecific isotype control (IgG_1; BD Biosciences) for 30 minutes on ice. Cells were then washed and incubated with Alexa Fluor 488-conjugated anti-mouse Ig (1:1000 dilution; Invitrogen) for 30 minutes on ice. Stained cells were resuspended in fluorescence-activated cell sorting Fix medium (1% formaldehyde, 20 g/L glucose, 5 mmol/L sodium azide in PBS) before analysis using a Beckman Coulter XL-MCL using CXP Cytometry List Mode Data Acquisition & Analysis Software version 2.2 (Gladesville, Australia). Further analysis was performed using FCS Express version 3.0 software (De Novo Software, Los Angeles, CA) against unstained cells gated at ≤1%.

SK Activity Assay

SK activity was determined as described previously.[11] For SK-1 activity, whole-cell lysates were incubated with D-erythro sphingosine (Biomol) solubilized in either 0.05% or 0.1% Triton X-100 and [γ^{32}P]ATP (PerkinElmer, Melbourne, Australia). For SK-2 activity, whole-cell lysates were prepared in buffer containing 1 mol/L KCl and incubated with D-erythro sphingosine solubilized in bovine serum albumin/PBS and [γ^{32}P]ATP. The radiolabeled S1P was resolved by two thin-layer chromatography (Sigma-Aldrich) separations in the solvents containing butanol, ethanol, water, and acetic acid (8:2:2:1). The radioactive spots were quantified using Phosphorimaging Typhoon 9410 (Beckman Coulter, Fullerton, CA) and ImageQuant software version 5.2 (GE Healthcare, Rydalmere, Australia).

Western Blotting

HUVECs were lysed in buffer containing 1% NP40 surfactant and then sonicated. Cell lysates were separated by 10% SDS-PAGE and transferred to Hybond-P membrane (Amersham; GE Healthcare, Piscataway, NJ). Primary antibodies to pERK-1/2 or total ERK-1/2 were used to probe the membrane overnight at 4°C, followed by secondary antibody incubation at room temperature (RT) for 1 hour before visualization by enzymatic chemiluminescence (GE Healthcare) and a luminescent image analyzer (LAS4000; Fujifilm, Stamford, CT).

MAPK, SK, and S1P-Receptor Inhibition and S1P-Receptor Activation Studies

In the activation and inhibition studies, SK inhibitor (SKi; 5 μmol/L, 10 minutes), DMS (5 μmol/L, 10 minutes), ERK-1/2 pathway inhibitor (U0126; 10 μmol/L, 30 minutes), p38 inhibitor (SB203580; 10 μmol/L, 1 hour), MEK inhibitor (PD98059; 25 μmol/L, 30 minutes), S1P (1 μmol/L, 10 minutes), fingolimod (FTY720, 100 nmol/L, 30 minutes), JTE013 (1 μmol/L, 30 minutes), W146 (10 μmol/L, 30 minutes), CAY10444 (10 μmol/L, 30 minutes), or VPC23019 (10 μmol/L, 30 minutes) were administered before histamine stimulation (25 μmol/L, 5 minutes). All reagents were proven functionally effective in paralleled studies.

Immunofluorescence Microscopy

HUVECs were replated at 5×10^4 cells/well in fibronectin-coated (50 μg/mL) Lab-Tek chamber slides (Nalge Nunc International, Rochester, NY). Confluent cells were treated with SKi, DMS, S1P, JTE013, VPC23019, W146, CAY10444, fingolimod, U0126, SB203580, PD98059, chlorpheniramine, or cimetidine without or with histamine stimulation (25 μmol/L, 5 minutes). Cells were fixed with 4% paraformaldehyde at RT for 15 minutes before blocking with 2% bovine serum albumin/PBS at RT for 30 minutes. P-selectin antibody (1 μg/mL) was added to cells overnight at 4°C, followed by anti-rabbit Alexa Fluor 594-conjugated antibody (1:1000) incubation at RT for 1 hour. Cells were then permeabilized with 0.1% Triton-X 100/PBS at RT for 10 minutes, followed by DAPI staining (1:2000) at RT for 3 minutes. Slides were visualized under an Olympus IX70 inverted microscope (Olympus, Tokyo, Japan) linked to a Bio-Rad Radiance 2100 confocal microscope (Bio-Rad Laboratories, Hercules, CA; Gladesville, Australia). Five images were acquired per sample. The fluorescence intensity was analyzed using Olympus AnalySIS Life Science imaging software version 3.0.

Parallel Plate Flow Chamber Assay

Confluent HUVECs cultured on Corning Petri dishes (Sigma-Aldrich) were treated with isotype control antibody (10 μg/mL, 30 minutes), P-selectin blocking antibody (10 μg/mL, 30 minutes), SKi (5 μmol/L, 10 minutes), DMS (5 μmol/L, 10 minutes), fingolimod (100 nmol/L, 30 minutes), ABC294640 (10 μmol/L, 10 minutes), U0126 (10 μmol/L,

30 minutes), PD98059 (25 μmol/L, 30 minutes), or SB203580 (10 μmol/L, 1 hour) before perfusion of histamine (25 μmol/L, 2.5 minutes) followed by blood, neutrophils, or lymphocytes. Using published methods, histamine (25 μmol/L) was prepared in Hank's balanced salt solution (HBSS; Sigma-Aldrich) and perfused across the substratum using a syringe pump (NE-1000; New Era Pump Systems, Farmingdale, NY) at a constant rate of 2 dynes/cm^2 for 2.5 minutes.[24] Peripheral blood was obtained by venipuncture from healthy donors after informed consent into heparinized syringes; samples were diluted 1:10 with HBSS and then perfused for 5 minutes, followed by an HBSS wash. Alternatively, blood in acid-citrate-dextrose was used to isolate neutrophils or lymphocytes before perfusion at 1×10^6 cells/mL for 5 minutes, followed by HBSS wash. Unlabeled leukocyte, neutrophil, or lymphocyte interactions were visualized under phase-contrast microscopy using 10×/0.3 NA objectives on an inverted microscope. Five random areas per dish were recorded using a digital camera (Olympus IX70 and SIS F-view) and analyzed using AnalySIS Life Science imaging software version 3.0 (Olympus). The number of rolling cells was analyzed using the aforementioned video microscopy system.

Intravital Microscopy and in Vivo *Experimental Procedure*

Intravital microscopy of the cremaster muscle was performed as described previously.[25] Briefly, microscopy (Axioplan 2 Imaging; Carl Zeiss Australia, Carnegie, Australia) with a 20× objective lens (20×/0.40 numerical aperture) and 10× eyepiece was used to observe the cremasteric microcirculation. A color video camera (Sony SSC-DC50AP; Carl Zeiss Australia) was used to project the images onto a calibrated monitor (Sony PVM-20N5E), and the images were recorded for playback analysis using a DVD recorder (Panasonic DMR-EH57; Retravision, Moorabbin, Australia). Two postcapillary venules (25 to 40 μm in diameter) were examined for each experiment. Leukocyte rolling was assessed via playback analysis, as described previously.[25] In experiments examining the effect of SK inhibition, WT mice were injected subcutaneously with vehicle alone or SKi (50 mg/kg in dimethyl sulfoxide/PBS) for 15 minutes or injected intraperitoneally with fingolimod (0.5 mg/kg in PBS) for 60 minutes before intravital microscopy. A basal reading of leukocyte rolling flux was taken before histamine superfusion (100 μmol/L in superfusion buffer) commenced. Additional recordings of leukocyte rolling were subsequently made at 5, 10, 20, and 30 minutes after histamine superfusion commenced. In a separate series of experiments, WT, *Sphk1*$^{-/-}$, and *Sphk2*$^{-/-}$ mice underwent the same model of histamine challenge.

Statistical Analysis

Data were statistically analyzed by Student's *t*-test or one-way or two-way analysis of variance for multiple comparisons and are expressed as means ± SEM. $P < 0.05$ was considered significant.

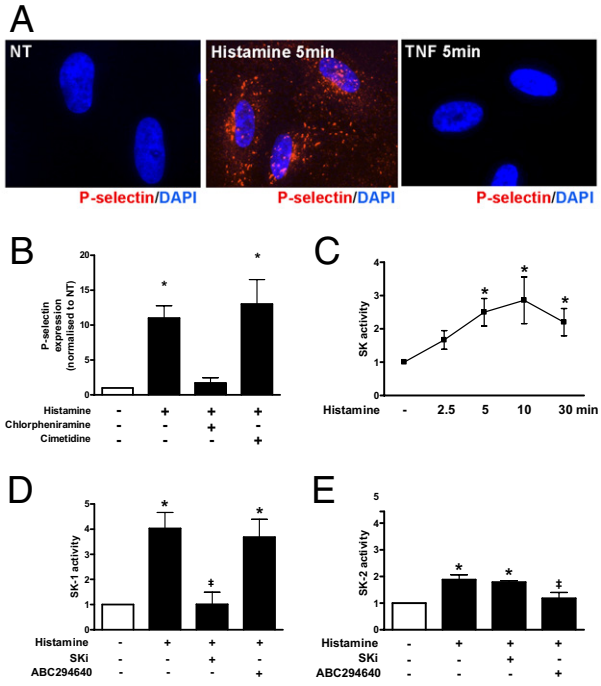


Figure 1. Histamine rapidly promotes P-selectin expression via the H_1 receptor and activates SK-1 and SK-2. **A:** Immunofluorescence microscopy of HUVECs treated for 5 minutes without or with 25 μmol/L histamine or 5 ng/mL TNF before P-selectin staining (red), permeabilization, and DAPI staining (blue). A representative image is shown ($n = 3$). NT, no treatment. Original magnification, ×100. **B:** Pooled fluorescence intensity data of histamine-treated HUVECs without and with H_1 antagonist (chlorpheniramine) or H_2 antagonist (cimetidine). Data are expressed as means ± SEM ($n = 3$). $^*P < 0.05$ versus untreated. **C:** HUVECs stimulated without and with 25 μmol/L histamine for 2.5, 5, 10, and 30 minutes before lysis for SK enzymatic assay. Data are expressed as means ± SEM ($n = 6$). **D** and **E:** HUVECs were preincubated with either SK-1 inhibitor (SKi; 5 μmol/L) or SK-2 inhibitor (ABC294640; 10 μmol/L) 10 minutes before histamine stimulation for 5 minutes. Cells were lysed immediately for SK-1 (**D**) or SK-2 (**E**) enzymatic assay. Data are expressed as means ± SEM ($n = 3$ to 6). $^*P < 0.05$ versus untreated; $^\dagger P < 0.05$ versus histamine.

Results

Histamine Rapidly Induces P-Selectin Expression and SK Activity in HUVECs

On activation by histamine, the vascular endothelium rapidly expresses preformed P-selectin at the cell surface for an immediate inflammatory response of leukocyte recruitment from the circulation and rolling along the vasculature.[26] In the present study, we used immunofluorescence microscopy to demonstrate that exposure of HUVECs to histamine for 5 minutes rapidly induces the surface expression of P-selectin (Figure 1A). Histamine-induced P-selectin surface expression is not associated with increased mRNA levels (data not shown) and occurs via the H_1 receptor, because pretreatment of HUVECs with the H_1 receptor antagonist chlorpheniramine but not the H_2 receptor antagonist cimetidine inhibited these events (Figure 1B).[27]

Huwiler et al[28] demonstrated that prolonged exposure to histamine (>2 hours) increases SK-1 expression and activity in a human arterial endothelial cell line, and we recently demonstrated that TNFα-induced SK activity in HUVECs occurs in a biphasic manner, with peaks observed both at 10 minutes and at 4 to 6 hours after treatment.[29] Based on these observations, we hypothesized that histamine activates SK within minutes of exposure. Indeed, this appears to be the case. A time-course treatment of 25 μmol/L histamine on HUVECs demonstrated an increase in SK activity at 2.5 minutes, peaking at 10 minutes and subsiding at 30 minutes (Figure 1C). Because TNF is also known to increase SK activity in HUVECs within minutes,[29] we investigated whether TNF could also exocytose P-selectin to the cell surface. The commonality observed between histamine and TNF in rapidly activating SK in HUVECs does not seem to extend to P-selectin exocytosis on these cells (Figure 1A).

To investigate whether the SK-1 or SK-2 isoform is preferentially activated by histamine, we executed experiments wherein the addition of 0.1% Triton X-100 or 1 mol/L KCl in the enzymatic assay is used to distinguish between SK-1 and SK-2 activity, respectively.[12] HUVECs exposed to histamine for 5 minutes exhibited increased activity of both SK-1 and SK-2, with SK-1 activity approximately twofold higher than that of SK-2 (Figure 1, D and E). Notably, unstimulated HUVECs exhibited equivalent levels of basal SK-1 and SK-2 activity (data not shown). The specificity of these assays was confirmed in experiments using HUVECs pretreated with SKi[30] and the SK-2 inhibitor ABC294640,[19,31] which demonstrated selective reductions in activity of the two SK isoforms (Figure 1, D and E).

Histamine-Induced SK Activity in HUVECs is ERK-1/2 dependent

The catalytic activity of SK can be rapidly and transiently activated by a diverse range of growth factors, cytokines, and other cell agonists[13] via phosphorylation on Ser225 by ERK-1/2.[14] We next investigated whether the signaling pathways by which histamine activates SKs in endothelial cells also involve the phosphorylation of ERK-1/2. The 25 μmol/L histamine treatment significantly increased the phosphorylation of ERK-1/2 at 5 minutes (Figure 2, A and B); phosphorylation peaked at 10 minutes and subsided at 20 minutes after exposure. Notably, the timing of ERK-1/2 phosphorylation parallels that observed for histamine-induced SK activity (Figure 1D). Blocking the ERK-1/2 pathway by administration of U0126 prevented histamine-induced SK activity in HUVECs (Figure 2C). Inhibition of SK by SKi had no effect on histamine-induced ERK-1/2 phosphorylation (Figure 2D), consistent with ERK-1/2 activation being upstream of SK activity. As expected, SK-1 protein levels did not alter during short-term exposure of HUVECs to 25 μmol/L histamine (see Supplemental Figure S1 at *http://ajp.amjpathol.org*).

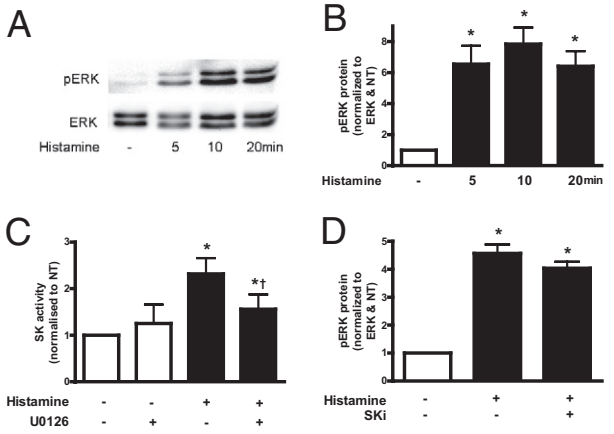


Figure 2. Histamine increases phosphorylation of ERK-1/2, which activates SK. **A:** HUVECs were treated without and with histamine (25 μmol/L for 5, 10, and 20 minutes) before lysis and Western blotting for phosphorylated ERK-1/2 (pERK) and total ERK-1/2 (ERK). Representative blots are shown ($n = 4$). **B:** Pooled data are expressed as means ± SEM ($n = 4$). $^*P < 0.05$ versus untreated. **C:** HUVECs were pretreated with ERK-1/2 pathway inhibitor (U0126; 10 μmol/L, 30 minutes) before histamine stimulation (25 μmol/L, 5 minutes) and lysis for SK enzymatic assay. Data are expressed as means ± SEM ($n = 5$ to 7). $^*P < 0.05$ versus untreated; $^\dagger P < 0.05$ versus histamine. **D:** HUVECs were pretreated with SKi (5 μmol/L, 10 minutes) before histamine stimulation (25 μmol/L, 5 minutes) and examined for phosphorylated and total ERK-1/2 by Western blotting. Data are expressed as means ± SEM ($n = 5$). $^*P < 0.05$ versus untreated.

Histamine-Induced P-selectin Surface Expression Is ERK-1/2 and SK-1 Dependent but Is S1P Surface Receptor Independent

Using immunofluorescence microscopy, we next examined a direct link between the MAPK pathway, SKs, and P-selectin surface expression on histamine-treated HUVECs. First, HUVECs treated with the ERK-1/2 pathway inhibitor U0126 before histamine administration exhibited a reduction in P-selectin surface expression similar to that observed in the absence of histamine (Figure 3A). A similar reduction in histamine-induced P-selectin expression was observed with administration of the MEK inhibitor PD98059 but not the p38 inhibitor SB203580 (Figure 3A). Second, two separate SK inhibitors [dimethyl sphingosine (DMS), a competitive inhibitor for both SK-1 and SK-2,[11,12] and SKi] were used to examine the role of SK in histamine-induced P-selectin expression. A significant reduction in histamine-induced P-selectin expression was observed when HUVECs were pretreated with either DMS or SKi (Figure 3A). These results suggest that histamine-induced P-selectin expression is SK dependent.

Given that $S1P_{1-2}$ receptors are known regulators of mast-cell function during an allergic response,[32] and that $S1P_{1-3}$ proteins have been identified on the surface of HUVECs,[33] we used inhibitors for these three family members (W146 for $S1P_1$, JTE013 for $S1P_2$, CAY10444 for $S1P_3$, and VPC23019 for $S1P_{1\&3}$) to investigate whether S1P receptors are involved in histamine-induced P-selectin expression on endothelial cells. Histamine-treated HUVECs exhibited a significant increase in P-selectin expression that was not affected by administration of inhibitors to $S1P_{1-3}$ (Figure 3B). Notably, blocking

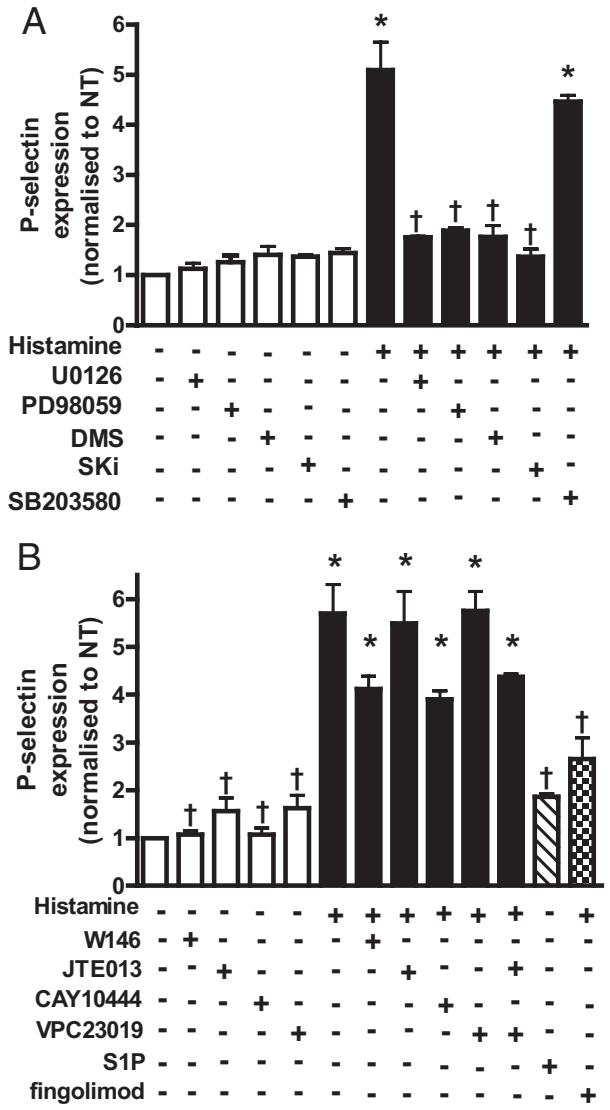


Figure 3. Inhibition of ERK-1/2 pathway or SK attenuates histamine-induced P-selectin surface expression in an $S1P_{1–3}$ receptor-independent manner. **A:** HUVECs were preincubated with U0126 (10 μmol/L, 30 minutes), PD98059 (25 μmol/L, 30 minutes), DMS (5 μmol/L, 10 minutes), SKi (5 μmol/L, 10 minutes), SB203580 (10 μmol/L, 1 hour) without or with histamine treatment (25 μmol/L, 5 minutes) and examined for P-selectin surface expression under immunofluorescence microscopy. **B:** HUVECs were treated with $S1P_1$ inhibitor (W146; 10 μmol/L, 30 minutes), $S1P_2$ inhibitor (JTE013; 1 μmol/L, 30 minutes), $S1P_3$ inhibitor (CAY10444; 10 μmol/L, 30 minutes), $S1P_{1\&3}$ inhibitor (VPC23019; 10 μmol/L, 30 minutes), or fingolimod (100 nmol/L, 30 minutes) before histamine exposure (25 μmol/L, 5 minutes). Similarly, exogenous S1P (1 μmol/L, 30 minutes) was added to HUVECs. Cells were fixed and assessed for P-selectin expression under immunofluorescence microscopy. Data are expressed as means ± SEM for quantified fluorescence intensity (n = 3 or 4). $^*P < 0.05$ versus corresponding untreated; $^\dagger P < 0.05$ versus histamine.

$S1P_1$, $S1P_3$, or $S1P_{1\&3}$ reduced histamine-induced P-selectin expression by approximately 30%, but expression was still significantly greater than that of untreated controls (Figure 3B). To further evaluate whether the S1P receptors are involved, 1 μmol/L exogenous S1P was added to HUVECs, a concentration thought to engage only the receptors for signaling events.[34] S1P treatment of HUVECs did not induce P-selectin expression (Figure 3B). Collectively, these findings suggest that $S1P_{1–3}$ receptors play no major role in histamine-induced P-selectin expression by HUVECs. Also of interest, we investigated the effect of fingolimod, a sphingosine-like fungal metabolite with demonstrated direct inhibition of SK-1.[35–37] Pretreatment of HUVECs with fingolimod significantly reduced histamine-induced P-selectin expression (Figure 3B).

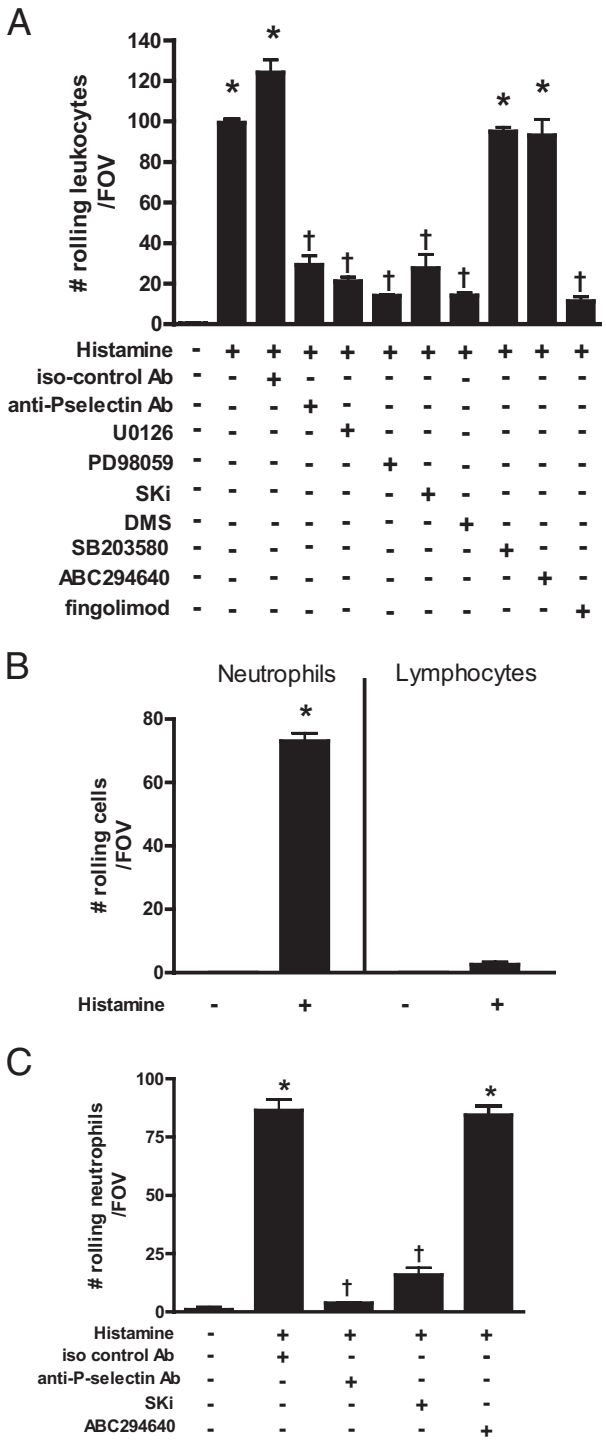
Leukocyte Rolling on Histamine-Treated HUVECs Is SK-1 Dependent

We next examined the role for the MAPK pathway, SK, and P-selectin in histamine-induced recruitment of leukocytes *in vitro* by a parallel plate flow chamber assay. When human blood was perfused over untreated HUVECs at a physiological constant shear rate of 2 dynes/cm^2, very few leukocytes rolled along the endothelium (Figure 4A). In contrast, HUVECs preperfused with 25 μmol/L histamine for 2.5 minutes demonstrated a profound increase in the number of rolling leukocytes, with approximately 100 cells per field of view (FOV). Adhesion of leukocytes was minimal to nonexistent on both untreated and histamine-treated cells (data not shown). Administration of a blocking antibody to P-selectin (AK-4) for 30 minutes before flow chamber assay significantly reduced the number of rolling leukocytes (Figure 4A).

For investigation of a role for ERK-1/2 and SK-1 in this system, specific inhibitors were added before histamine perfusion. A reduction in leukocyte rolling was observed when inhibitors to either the ERK pathway (U0126 and PD98059) or the SK pathway (DMS and SKi) were added (Figure 4A; see also Supplemental Video S1 at *http://ajp.amjpathol.org*). No reduction was observed with inhibition of the p38 pathway (SB203580) or with administration of the SK-2 inhibitor ABC294640 (Figure 4A). Consistent with our P-selectin expression data, short-term exposure of HUVECs to S1P failed to activate leukocyte rolling (data not shown). This supports the observations of histamine-induced P-selectin expression being $S1P_{1–3}$ receptor independent. Of note, pretreatment with fingolimod also significantly suppressed histamine-induced leukocyte rolling (Figure 4A), suggesting a potential utility for fingolimod in the early phase of allergic inflammation.

Because the leukocyte rolling studies to this point were performed with whole blood, we next asked whether these responses were also seen using isolated lymphocytes and neutrophils; for the latter, rolling capabilities on histamine-activated endothelial cells have been demonstrated.[38] Although very few, if any, lymphocytes exhibited rolling events, approximately 75 neutrophils rolled per FOV on histamine-treated HUVECs (Figure 4B). Because the lymphocytes isolated from peripheral blood are likely naïve rather than memory or effector T cells, we used histamine to preactivate Jurkat T cells and investigated their ability to interact with HUVECs. L-selectin shedding was observed on histamine-treated Jurkat cells (see Supplemental Figure S2, A and B, at *http://ajp.amjpathol.org*), thereby confirming an active state; however, this does not correlate with increased rolling on

histamine-treated HUVECs. Blocking P-selectin by antibody administration significantly attenuated the neutrophil rolling events (Figure 4C). Similarly, HUVECs pretreated with SKi demonstrated reduced neutrophil rolling (Figure 4C). This was not observed with the SK-2 inhibitor ABC294640 (Figure 4C). Collectively, these findings suggest that histamine-induced neutrophil recruitment occurs via an SK-1-mediated P-selectin dependent process.



SK-1 Mediates Histamine-Induced Leukocyte Rolling in Vivo

We next performed *in vivo* experiments using intravital microscopy to assess the role for SKs in histamine-induced leukocyte rolling in cremasteric postcapillary venules. First, leukocyte rolling was assessed in WT mice pretreated with either SKi or vehicle. In vehicle-treated mice, histamine exposure rapidly increased leukocyte rolling flux from a basal level of ~50 cells/minute to a peak of 168 ± 28 cells/minute within 5 minutes, before rapidly returning to basal levels (Figure 5A; see also Supplemental Video S2 at *http://ajp.amjpathol.org*). These mice also exhibited a transient reduction in rolling velocity from 89 ± 6 μmol/L per second to 41 ± 7 μmol/L per second, which previous studies have shown is associated with increased sensitivity to chemoattractants.[39,40] As expected from the *in vitro* studies, mice injected subcutaneously with SKi exhibited a significantly lower peak rolling flux (89 ± 28 cells/minute) at the same time point (Figure 5A; see also Supplemental Video S2 at *http://ajp.amjpathol.org*), supporting the concept that histamine-induced leukocyte rolling *in vivo* is SK-1 dependent. Treatment of *Sphk1*$^{-/-}$ mice with SKi caused no further reduction in rolling, consistent with this agent being specific for SK-1 (data not shown). Administration of fingolimod 60 minutes before histamine exposure also significantly attenuated neutrophil rolling *in vivo* (Figure 5A; see also Supplemental Video S2 at *http://ajp.amjpathol.org*). Notably, the residual rolling neutrophils in the fingolimod-treated mice did not exhibit a reduced rolling velocity (73 ± 11 μmol/L per second versus 67 ± 17 μmol/L per second).

Second, to investigate the respective roles of SK-1 and SK-2 in histamine-induced leukocyte rolling *in vivo*, we used *Sphk1*$^{-/-}$ and *Sphk2*$^{-/-}$ mice. Except that the experiments performed in the *Sphk1*$^{-/-}$ mice were in postcapillary venules of a slightly reduced diameter, equivalent vascular parameters, hemodynamic parameters, and systemic leukocyte counts were observed both in the present study (Table 1) and as reported by others.[20,21] Furthermore, the equivalent baseline level of neutrophil

Figure 4. Histamine-induced leukocyte and neutrophil rolling *in vitro* is ERK-1/2 and SK-1 dependent but independent of $S1P_{1-3}$ surface receptors. **A:** HUVECs were preincubated without or with an isotype control antibody (10 μg, 30 minutes), P-selectin blocking antibody (10 μg, 30 minutes), ERK-1/2 inhibitor (U0126; 10 μmol/L, 30 minutes), MEK inhibitor (PD98059; 25 μmol/L, 30 minutes), SK inhibitors (SKi or DMS; each at 5 μmol/L, 10 minutes), p38 inhibitor (SB203580; 10 μmol/L, 1 hour), SK-2 inhibitor (ABC294640; 10 μmol/L, 10 minutes), or fingolimod (100 nmol/L, 30 minutes) before perfusion of histamine (25 μmol/L, 2.5 minutes) and then human whole blood (5 minutes). Data are expressed as means ± SEM for rolling cells per FOV, with four to five FOV captured ($n = 3$ or 4). $^{*}P < 0.05$ versus untreated; $^{\dagger}P < 0.05$ versus histamine. **B:** HUVECs were perfused without or with histamine (25 μmol/L, 2.5 minutes) and freshly isolated human neutrophils or lymphocytes at 1×10^6 cells/mL. Data are expressed as means ± SEM for rolling flux per FOV, with four to five FOV captured ($n = 3$). $^{*}P < 0.05$ versus untreated. **C:** HUVECs were pretreated without or with a control antibody (10 μg, 30 minutes), P-selectin blocking antibody (10 μg, 30 minutes), SK-1 inhibitor (SKi; 5 μmol/L, 10 minutes), or SK-2 inhibitor (ABC294640; 10 μmol/L, 10 minutes) before perfusion of histamine (25 μmol/L, 2.5 minutes) and freshly isolated human neutrophils at 1×10^6 cells/mL. Data are expressed as means ± SEM for rolling flux per FOV, with four to five FOV captured ($n = 3$ to 5). $^{*}P < 0.05$ versus untreated; $^{\dagger}P < 0.05$ versus histamine.

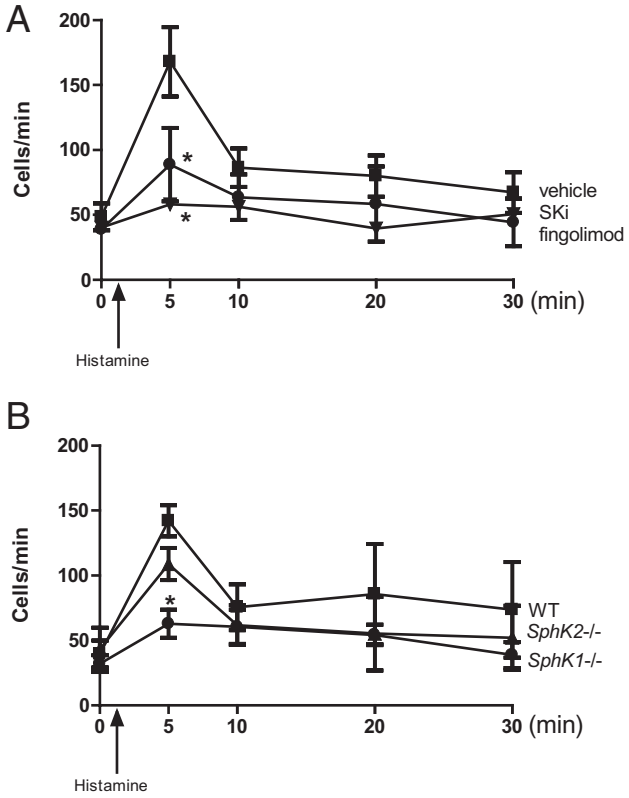


Figure 5. Histamine-induced neutrophil rolling flux in response to SK inhibitors and Sphk knockout in mice. **A:** WT mice were injected with vehicle dimethyl sulfoxide/PBS (squares), SKi (50 mg/kg, triangles) subcutaneously 15 minutes, or fingolimod (0.5 mg/kg, circles) i.p. 60 minutes before histamine challenge (100 μmol/L, superfused topically over the cremaster muscle) and were examined under intravital microscopy. **B:** Similarly, WT (**squares**), *Sphk1*$^{-/-}$ (**circles**), and *Sphk2*$^{-/-}$ (**triangles**) mice were superfused with histamine. Leukocyte rolling flux in the postcapillary venules of the mouse cremaster muscle was assessed at 5, 10, 20, and 30 minutes after histamine challenge. Data are expressed as means ± SEM (n = 5 to 7 mice per group). *$P < 0.05$ versus WT.

rolling in the WT, *Sphk1*$^{-/-}$, and *Sphk2*$^{-/-}$ mice is indicative of constitutive P-selectin expression in the cremasteric microvasculature of these strains.[40,41] The WT mice exhibited a peak rolling flux of 142 ± 12 cells/minute after 5 minutes of histamine superfusion (Figure 5B; see also Supplemental Video S3, available at *http://ajp.amjpathol.org*). In *Sphk2*$^{-/-}$ mice, a slight but nonsignificant decrease in peak rolling flux (109 ± 12 cells/minute) was observed. In contrast, *Sphk1*$^{-/-}$ mice demonstrated a profound reduction in histamine-induced rolling (63 ± 11 cells/minute), supporting our *in vitro* data of SK-1 being the dominant SK isoform mediating histamine-induced neutrophil rolling.

Discussion

Investigation of the cellular and soluble mediators involved in allergic inflammation not only contributes to understanding of the mechanisms of current treatments, but is also important for the identification of new targets. With the present study, we demonstrate for the first time that SK-1 mediates the early phase of histamine-induced P-selectin-mediated neutrophil recruitment. Evidence for this comes from experiments showing that i) histamine increased ERK-1/2 phosphorylation and SK activity in HUVECs; ii) inhibition of either the ERK-1/2 pathway or SK-1, but not SK-2, markedly attenuated histamine-induced P-selectin surface expression on endothelial cells; iii) addition of S1P or inhibition of $S1P_{1-3}$ receptors on histamine-treated HUVECs did not alter P-selectin surface expression; iv) histamine-induced neutrophil rolling on endothelium *in vitro* was P-selectin and SK-1 dependent; and v) histamine-induced neutrophil influx *in vivo* was significantly reduced in WT mice pretreated with an SK-1 inhibitor, as well as in *Sphk1*$^{-/-}$ mice, compared with the WT and *Sphk2*$^{-/-}$ counterparts.

The importance of P-selectin in allergic inflammation has been well described, with an *in vivo* study showing that P-selectin deficient mice exhibit a significant reduction in leukocyte rolling,[26] and other studies showing histamine-induced P-selectin facilitating neutrophil adhesion via CD11/CD18 integrin activation[38] and the development of allergic inflammation.[42] The significance of P-selectin in mediating leukocyte-endothelial cell interactions has been confirmed in patients with leukocyte adhesion deficiency (LAD II). These patients experience recurrent staphylococcal infections, and their neutrophils fail to roll and adhere adequately for lack of functional expression of sialyl Lewis X, a fucose-containing glycoconjugate ligand for P-, E-, and L-selectins.[43] Identifying the mechanisms underpinning the regulation of P-selectin surface expression may therefore aid in development of new pharmaceutical approaches to combat allergic inflammation. A role for S1P in histamine-induced gene regulation of E-selectin and ICAM-1 was demonstrated by Shimamura et al,[18] and it is our contention that the SK/S1P pathway in fact plays a critical role before gene regulation, with exocytosis of P-selectin occurring within minutes of exposure to histamine.

Table 1. Hemodynamic State of Untreated Animals

Variable	WT	*SphK1*$^{-/-}$	*SphK2*$^{-/-}$
Vascular diameter (μm)	32.3 ± 1.0	28.4 ± 1.7*	29.9 ± 1.9
Mean red blood cell velocity (mm/second)	2.1 ± 0.6	1.1 ± 0.2	2.3 ± 0.4
Shear rate (s^{-1})	463 ± 126	318 ± 39	616 ± 83
Leukocyte count (no.)			
Lymphocytes	65 ± 4	66 ± 2	63 ± 2
Neutrophils	28 ± 5	24 ± 4	25 ± 2
Monocytes	7 ± 3	10 ± 1	12 ± 1

*$P < 0.05$ versus WT (n = 5 to 16).

Our results suggest that HUVECs exposed to histamine rapidly activate SK-1 and SK-2. To delineate the contribution of SK-1 versus SK-2 in this system, we used both broad-spectrum and specific SK inhibitors in *in vitro* and *in vivo* experiments. DMS is an inhibitor of both SK-1 and SK-2, but it also affects other lipid and protein kinases, including protein kinase C (PKC).[44] In contrast, SKi is a more specific inhibitor. A recent report suggests that it specifically targets SK-1.[30,31] Conversely, the inhibitor ABC294640 specifically targets SK-2.[19] Our data from using these inhibitors suggest that only histamine-induced SK-1 activity is required for rapid surface expression of P-selectin on endothelial cells and neutrophil rolling events *in vitro*. Furthermore, extracellular S1P and the $S1P_{1-3}$ receptors appeared to play no major role in the present study, which differs from the findings of Matsushita et al,[45] who demonstrated that exposure of the human aortic endothelial cell line HAEC to 1 μmol/L S1P for 5 minutes caused release of von Willebrand factor, another protein stored preformed in Weibel-Palade bodies, and that 10 pmol/L of S1P injected intravenously into mice increased soluble P-selectin within 1 hour.

The present study raises an alternative possibility, that intracellular second messengers modulated by S1P (eg, HDAC1/2, TRAF2, or prohibitin[15–17]) may be involved. Clearly, the difference observed between the present findings and those of Matsushita et al[45] requires further investigation *in vitro* and *in vivo*, using multiple approaches (including, but not limited to, the family of SK and S1P receptor knockout mice). In the present study, pretreatment of HUVECs with fingolimod caused a reduction in histamine-induced P-selectin expression and leukocyte rolling events. Fingolimod is an orally active immunomodulatory prodrug that recently gained U.S. Food and Drug Administration approval for treatment of multiple sclerosis,[46] based on its ability to inhibit lymphocyte egress from lymph nodes and thymus.[47] The mechanisms underpinning fingolimod inhibition of histamine-induced P-selectin expression and leukocyte rolling flux are still unknown, but likely are due to the ability of fingolimod to inhibit and degrade SK-1 *in vitro*.[35–37]

To provide additional definitive confirmation of the role of SK-1 in histamine-induced P-selectin expression in HUVECs, we attempted to use transient transfection with siRNA to knock down SK-1 expression; however, these experiments proved to be not technically feasible. A major limitation to working with P-selectin in primary HUVECs is that, after two or more passages, HUVECs lose their ability express preformed P-selectin.[48] siRNA experiments necessarily involve additional passages, which precluded our ability to combine siRNA treatment with assessment of histamine-induced P-selectin mobilization in HUVECs. Nonetheless, our examination of *in vivo* responses in mice specifically lacking either SK-1 or SK-2 provided strong evidence supporting our hypothesis that SK-1 is critical to histamine-induced P-selectin up-regulation.

Our *in vivo* studies showed that either pharmacological or genetic manipulation of SK-1 attenuates histamine-induced neutrophil rolling flux, which is critical for acute allergic inflammation. More specifically, we observed in WT mice that both SKi and fingolimod significantly attenuated histamine-induced neutrophil rolling flux. Consistent with SK-1 mediation of this process, *Sphk1*$^{-/-}$ mice exhibited significant resistance to histamine-induced neutrophil rolling flux, but *Sphk2*$^{-/-}$ mice did not. These findings differ from those of Michaud et al,[49] who reported equivalent neutrophil numbers in the lavage fluid of both WT and *Sphk1*$^{-/-}$ mice in an inflammatory model of peritonitis using a 4-hour thioglycolate challenge.[49] The divergence in these data may be attributable to the difference in the time courses of the responses investigated (ie, 5 to 10 minutes versus 4 hours) and the nature of the inflammatory stimuli (ie, histamine versus thioglycolate). We also showed that untreated WT, *Sphk1*$^{-/-}$, and *Sphk2*$^{-/-}$ mice exhibited similar levels of baseline neutrophil rolling flux. Constitutive P-selectin expression in the lung, skin, intestine, mesentery, and cremaster muscle has been previously shown using the noninvasive dual radiolabeling antibody binding assay, so the finding is not the result of intravital microscopy intervention.[40,41] Collectively, these data indicate that constitutive P-selectin expression in the cremaster muscle is SK independent, but that histamine-induced exocytosis of P-selectin expression is SK dependent.

The physiological relevance of the differences in SK-1 and SK-2 activity levels with respect to allergy may be widespread,[50] and are yet to be fully elucidated. Experimentally, Pushparaj et al[51] showed both *in vitro* and *in vivo* that silencing SK-1 inhibited several mast-cell effector functions triggered by FcεRI engagement, whereas silencing SK-2 had no effect. However, there is still controversy concerning the different roles of SK-1 and SK-2 in mast-cell responses. Findings from a study using *Sphk*-deficient mice suggested that SK-2, and not SK-1, is more important for degranulation and cytokine or eicosanoid production by mast cells.[52] In addition, Zemann et al[53] showed that bone marrow-derived neutrophils from both *Sphk1*$^{-/-}$ and *Sphk2*$^{-/-}$ mice had normal functions of increasing intracellular Ca^{2+} and migration toward chemoattractants fMLP and C5a, compared with WT mice. Together, these studies suggest that the effects of SK isoforms may be cell-type specific.

The prevalence of all types of allergies continues to rise across all age, sex, and racial groups. The Allergy and Asthma Foundation of America rating allergy as the third most common chronic disease among children.[54] An understanding of the cellular and soluble mediators that are involved in allergic inflammation not only helps in elucidating the mechanisms of current treatments, but is also important for the identification of new therapeutic targets. Successful outcomes in future studies may establish SK as a therapeutic target to control histamine-induced allergic responses. More specifically, by targeting the early allergic response of neutrophil recruitment, we may be able to interfere in the initiation of chronic diseases triggered by allergens. Our understanding of this complex relationship might also reveal new opportunities for treatment of other diseases in which histamine is suggested to play a role (such as multiple sclerosis,

rheumatoid arthritis, and psoriatic arthritis) but for which traditional antihistamines are generally regarded as ineffective.

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Appendix 3:

Sphingolipids: a potential molecular approach to treat allergic inflammation.

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Review Article

Sphingolipids: A Potential Molecular Approach to Treat Allergic Inflammation

Wai Y. Sun[1,2,3] and Claudine S. Bonder[1,2,3,4]

[1] *Centre for Cancer Biology, SA Pathology, Frome Road, Adelaide, SA 5000, Australia*
[2] *School of Medicine, University of Adelaide, Adelaide, SA 5000, Australia*
[3] *Cooperative Research Centre for Biomarker Translation, La Trobe University, Bundoora, VIC 3086, Australia*
[4] *School of Molecular and Biomedical Sciences, University of Adelaide, Adelaide, SA 5000, Australia*

Correspondence should be addressed to Claudine S. Bonder, claudine.bonder@health.sa.gov.au

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Allergic inflammation is an immune response to foreign antigens, which begins within minutes of exposure to the allergen followed by a late phase leading to chronic inflammation. Prolonged allergic inflammation manifests in diseases such as urticaria and rhinoconjunctivitis, as well as chronic asthma and life-threatening anaphylaxis. The prevalence of allergic diseases is profound with 25% of the worldwide population affected and a rising trend across all ages, gender, and racial groups. The identification and avoidance of allergens can manage this disease, but this is not always possible with triggers being common foods, prevalent air-borne particles and only extremely low levels of allergen exposure required for sensitization. Patients who are sensitive to multiple allergens require prophylactic and symptomatic treatments. Current treatments are often suboptimal and associated with adverse effects, such as the interruption of cognition, sleep cycles, and endocrine homeostasis, all of which affect quality of life and are a financial burden to society. Clearly, a better therapeutic approach for allergic diseases is required. Herein, we review the current knowledge of allergic inflammation and discuss the role of sphingolipids as potential targets to regulate inflammatory development *in vivo* and in humans. We also discuss the benefits and risks of using sphingolipid inhibitors.

1. Introduction

Allergic inflammation can occur rapidly or delayed via the classical inflammatory immune reaction involving the production of specific IgE antibodies as well as the activation of inflammatory cells and the endothelium [1]. Many proinflammatory mediators and cytokines including histamine, leukotriene, and tumor necrosis factor α (TNFα) can activate the vascular endothelial cells (ECs) to cause proinflammatory microvasodilation and mediate leukocyte recruitment from the circulation to the sites of allergic inflammation [2, 3]. Excessive and prolonged leukocyte recruitment can result in extracellular matrix (ECM) remodelling and tissue damage [4]; thus controlling EC activation provides a strategy to minimize allergic inflammation. This review discusses the pathophysiology of vascular ECs during allergic inflammation, current treatments and new therapeutic approaches. We focus on the role of sphingolipids in the regulation of vasculature during the early phase of allergic inflammation, in particular, studies utilizing sphingolipid knockout animals which support their potential as new therapeutic targets.

2. Pathophysiology in Acute Allergic Inflammation

Histamine is a potent proinflammatory mediator primarily released by mast cells and basophils with up to 0.01–1 mol/m^3 found in the periphery during an allergic response [5, 6]. Histamine mediates dendritic cell maturation [7], T lymphocyte differentiation and migration [8–10], and

Table 1: Common antihistamines marketed in Australia.

Some common antihistamines			
First generation	Second generation		Third generation
Systemic	Systemic	Topical	Systemic/topical
Promethazine	Cetirizine	Azelastine	Levocetirizine
Pheniramine	Loratadine	Levocabastine	Desloratadine
Cyproheptadine	Terfenadine		Fexofenadine
Dexchlorpheniramine	Ketotifen		
Trimeprazine	Mizolastine		

endothelial cell proliferation [11] via a family of four G-protein-coupled receptors (H_{1-4}) [12]. Histamine receptors are differentially expressed with only H_1 and H_2 expressed by vascular ECs [13, 14] (Figure 1). Within minutes of histamine exposure and binding to H_1 and H_2, the G-protein subunit αq is recruited to decrease cAMP accumulation and subsequent EC contraction [15]. By contrast, the G protein β and γ subunits are activated to induce the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) [16]. Ligand interaction with the H_1 receptor causes vascular permeability, synthesis of prostacyclin and platelet activating factor, and release of von Willebrand Factor (vWF) and nitric oxide [17, 18]. H_2 receptor stimulation is linked to the Gαs subunit for the activation of adenylate cyclase and formation of cyclic adenosine monophosphate (cAMP), which induces intracellular calcium-mediated vasodilatation at a slower rate of onset than that of H_1 receptor [19, 20]. In addition, the H_2 receptor can negatively regulate the release of histamine by mast cells and basophils [21] and suppress the production of TNFα and IL-12 from inflammatory cells [10, 22, 23].

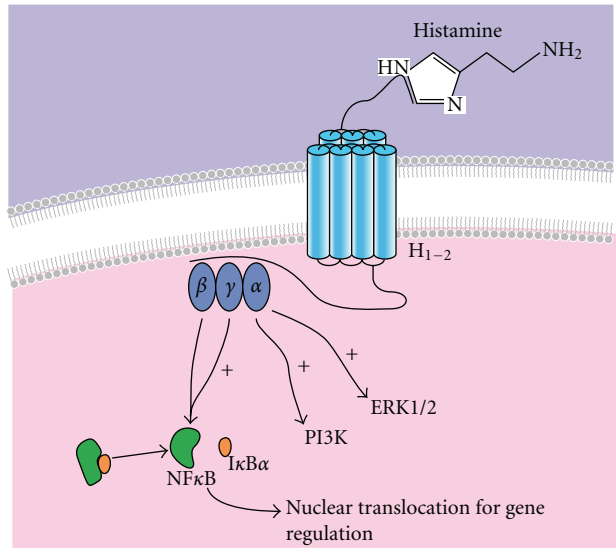


Figure 1: Histamine receptors on ECs. Two histamine receptors (H_1 and H_2) are found on ECs. Within minutes of histamine binding to its receptors, the G-protein subunits are activated to initiate intracellular signalling. The αq subunit of the G-protein contributes to reduced cAMP accumulation, induced ERK1/2, and induced inositol phospholipid (PI3K) signalling. The β and γ subunits contribute to the activation of NFκB and subsequent translocation into the nucleus where transcriptional processes are regulated causing cellular changes, such as vascular contraction and permeability, all of which are important for immune regulation and inflammation.

3. Antihistamines as the Current Mainstay Treatment for Allergic Inflammation

Antihistamines (e.g., diphenhydramine and chlorpheniramine) were first developed in the 1930s as an inverse agonist for the histamine receptors and have been commonly used to treat and prevent allergic symptoms ever since [24] (Table 1). Patients treated with H_1 antihistamines exhibit reduced production of histamine and leukotrienes as well as downregulation of adhesion molecule expression on the vasculature which in turn attenuates allergic symptoms by 40–50% [20, 25–28]. Long term treatment with H_1 antihistamines can retard the progression of respiratory disease by inactivating functions of macrophages and other Th2 cells thus preventing local tissue remodelling and damage [29, 30]. Second- and third-generation antihistamines (e.g., loratadine, fexofenadine, and cetirizine) (Table 1) were generated in the 1980s. These drugs also target the H_1 receptor but, in general, are less lipophilic and therefore exhibit reduced ability to penetrate the blood-brain barrier resulting in a less sedating effect than the first generation counterparts [28, 31]. Notably, 2–5 times higher dose of these second-generation antihistamines are required to control mild seasonal allergic symptoms when compared to the first-generation medications [32]. Using H_1 antihistamines at a high dose remains controversial as (i) animal studies have shown that mice treated with high doses of fexofenadine during the allergen challenge exhibited reduced lung inflammation, reduced Th2 responses, and reduced the secretion of IL-4, -5, and -13 [7, 29], (ii) a recent human clinical study demonstrated that high-doses of desloratadine only marginally improved allergic symptoms in patients without an increase in adverse effects when compared to the standard doses [33] and (iii) long-term high-dose use of antihistamines in patients with chronic urticaria retained adverse effects, such as rapid eye movement, sleep disturbance, and negative impacted on learning and performance [34]. Clearly, other effective clinical approaches are needed to combat allergic inflammation.

4. Antiselectin Therapy for Inflammatory Diseases

Another approach is to target the expression of adhesion molecules on ECs, such as selectins, which are known to initiate the early capturing and rolling of leukocytes from the circulation. Antagonism of the selectins is recognized to be a therapeutic approach to prevent and minimize inflammatory reactions. Evidence for this comes from P-selectin-deficient mice which, when challenged with the inflammatory irritant thioglycollate, exhibit attenuated leukocyte rolling in the blood vessels for up to 4 hours [35]. They also exhibit a significant reduction in leukocyte infiltration at the inflammatory hindlimb by ischemia on postoperative day 14 when compared to wildtype (WT) controls [36]. In humans, the recruitment of activated neutrophils to the local inflamed tissue is largely dependent on adhesion molecules as evidenced by patients with leukocyte adhesion deficiency (LAD II) whose neutrophils lack functional sialyl Lewis X expression (a fucose-containing glycoconjugate ligand for P-, E-, and L-selectin), exhibit reduced rolling and firm adhesion on the endothelium [37]. Together, these show that controlling expression of adhesion molecules can influence the early phase as well as the chronic phase of inflammatory reactions.

Selectin antagonists have been examined in preclinical studies, including cutaneous inflammation, allergy and ischemia-reperfusion injury [38, 39]. The first selectin antagonist CY1503 (Cylexin), an analogue of sialyl Lewis X which inhibits E-, P-, and L-selectins, has demonstrated a reduction in the degree of myocardial infarct size associated with a canine model of coronary artery ischemia and reperfusion, and reduced leukocyte accumulation at 4.5 hours after operation [40]. However, the effects of CY1503 remain controversial as a second similar study failed to consistently reduce myocardial injury and neutrophil accumulation at 48 hours post-operation [41]. Treatment with CY1503 also failed to attenuate the "no-reflow" phenomenon of leukocytes and could not limit the myocardial infarct size in the rabbit [42]. More recently, the oral P-selectin blocking agent, Pentosan Polysulfate Sodium (PPS), has been examined in a Phase I clinical study, wherein a single dose of PPS showed improvement of microvascular blood flow in patients with sickle cell disease [43]. However, no study to date has examined the efficacy of PPS in controlling leukocyte recruitment during allergic inflammation.

To date, four classes of selectin blocking agents have been developed: (i) carbohydrate based inhibitors targeting all P-, E-, and L-selectins [44], (ii) antihuman selectin antibodies [45], (iii) a recombinant truncated form of PSGL-1 immunoglobulin fusion protein [46], and (iv) small-molecule inhibitors of selectins [47]. Notably, most of the selectin blocking agents have failed in phase II/III clinical trials or the clinical studies were ceased due to their unfavorable pharmacokinetic properties and high cost [39]. Animal models also suggest that the timing and potency of selectin blockade are crucial to preventing the development of allergic inflammation with a greater than 90% reduction in leukocyte rolling required for firm adhesion events to be significantly attenuated [48, 49]. Given that the direct selectin blockade by the current compounds remains unsuccessful to regulate allergic inflammation, new therapeutic approaches which target the regulation and expression of adhesion molecules are warranted.

5. Sphingomyelin Pathway

The lipid enzyme, sphingosine kinase (SK), was originally identified for its role in the sphingomyelin degradation pathway but is increasingly being recognized as an important signalling molecule (Figure 2). There are excellent reviews focusing on the roles of SK/S1P in diseases, such as cancer [50], immunity [51], asthma [52], multiple sclerosis [53], rheumatoid arthritis [54], and pancreatic islet transplantation [55]. Herein, we discuss how SK can be used as a new therapeutic target to combat allergic inflammation, referencing animal models and human trials, together with the benefits and adverse effects of manipulating SK using inhibitors.

6. Sphingosine Kinase

Two isoforms of SK (i.e., SK-1 and SK-2) have been cloned and characterized in mammalian cells, which both catalyze the phosphorylation of sphingosine to form sphingosine-1-phosphate (S1P) [56, 57]. SK-1 has been shown to be the primary contributor to serum S1P levels with *SphK1*−/− mice exhibiting a ∼50% reduction in serum S1P when compared to wildtype (WT) mice [58] and the *SphK2*−/− mice serum S1P levels exhibiting no reduction. In fact, Zemann et al. showed an increase in serum S1P of *SphK2*−/− mice [59]. Notably, S1P was undetectable in plasma and lymph of the conditional double knockout mice [60].

The polypeptide sequences of SK-1 and SK-2 contain 80% similarity, which supports compensatory effects when one isoform of SK is knocked down [56, 57]. Interestingly, the localization of SK-1 and SK-2 differs with SK-1 being predominantly found in the cytoplasm and at the plasma membrane leading to prosurvival effects [61, 62], and SK-2 being predominantly found in the nucleus and at the endoplasmic reticulum (ER) promoting proapoptotic effects [63, 64] (Figure 3). Three splice isoforms of SK-1 have been identified (i.e., SK-1a, SK-1b, and SK-1c) that differ at their N-termini with additional 14 and 86 amino acids in SK-1b and SK-1c, respectively [65]. Two variants of SK-2 have also been identified (i.e., SK-2 and SK-2 long (SK2L)) arising from alternate start sites [57]. The specific physiological role for each SK variant is yet to be further elucidated.

SK has intrinsic activity and can be further activated by many biological stimuli, including histamine [66], cross-linking of immunoglobulin receptors [11], TNFα [67], vascular endothelial growth factor (VEGF), interleukins, complement C5a [68], and bradykinin [11]. Upon stimulation, the catalytic activity of SK-1 increases via the phosphorylation of extracellular signal regulated kinase (ERK)-1/2 at Ser225 which results in the translocation to the inner plasma membrane [69]. The binding of SK-1 to lipid phosphatidylserine can enhance SK-1 activity and plasma

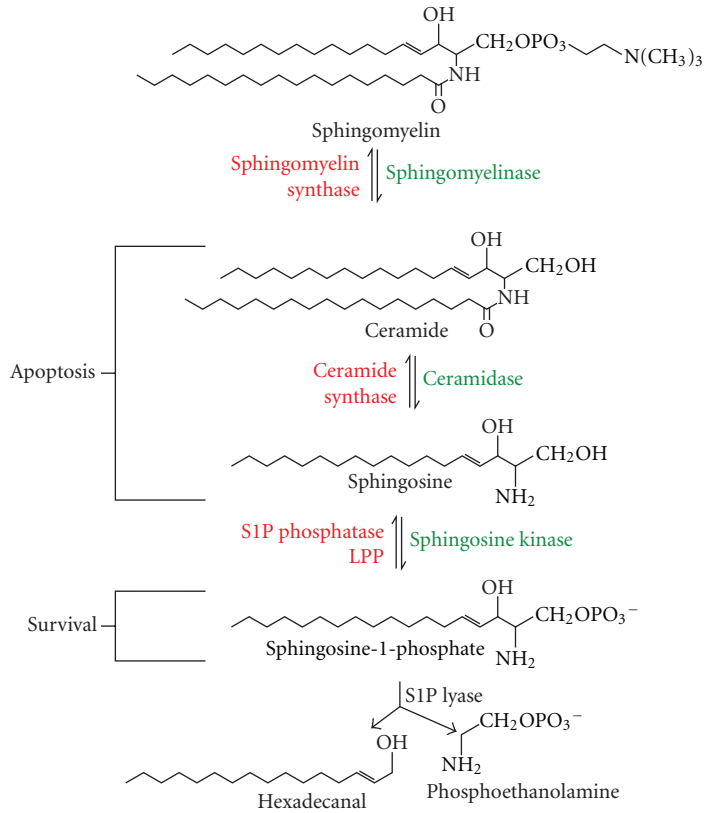


Figure 2: Sphingomyelin pathway. Sphingomylein is hydrolysed to ceramide, which is then metabolized to sphingosine and sphingosine-1 phosphate (S1P) by different kinases (green). This process is reversible via the activities of different synthases and phosphatases (red). The levels of the biological product, S1P, are regulated by S1P lyase which degrades it into hexadecanal and phosphoethanolamine. Although the structures of each sphingolipid are similar, they have divergent cellular functions with ceramide and sphingoine being pro-apoptotic, and S1P being prosurvival.

membrane translocation [70]. More recently, calcium- and integrin-binding protein (CIB)-1 protein has been identified to translocate SK-1 to the plasma membrane [71]. Conversely, dephosphorylation at Ser225 causes deactivation of basal and TNFα-induced SK-1, a process shown to be regulated by protein phosphatase 2A (PP2A) [72, 73]. In contrast, SK-2 does not possess the Ser225 phosphorylation site but its activation, also via the ERK pathway, is suggested to occur by phosphorylation at Ser351 and Thr578, which induces translocation from the nucleus to endoplasmic reticulum [57, 74].

7. Sphingosine-1-Phosphate

S1P is the biological product of SKs and is predominantly formed in the cytoplasm. S1P can be retained intracellularly or released by platelets, neutrophils, leukocytes, ECs, and mast cells via the transporters, ATP-binding cassette (ABC) transporter ABCC1, ABCA1 and ABCG1 [89–92]. S1P is bound to high-density lipoproteins (HDL) and plasma proteins, such as albumin, which stabilizes S1P in the circulation [93]. Platelets secrete the highest levels of S1P but ECs also upregulate their release of S1P in response to activation and shear stress [94]. The concentration of S1P ranges from 4×10^{-4} to 1.2×10^{-3} mol/m^3 in serum, 2×10^{-4} to 5×10^{-4} mol/m^3 in plasma, and 5×10^{-7} to 7.5×10^{-6} mol/m^3 in tissue [93, 95–97]. Interestingly, S1P can also be formed outside the cell as SK-1 has been shown to be secreted by human umbilical vein ECs (HUVEC) and macrophages [98, 99].

Increasing evidence supports intracellular targets for S1P signalling with S1P binding to histone deacetylases (HDAC)-1 and -2 to regulate histone acetylation [100], TNF receptor-associated factor 2 (TRAF2) to regulate inflammation, anti-apoptotic and immune responses via the NFκB pathway [101], and prohibitin 2 (PHB2) for regulation of mitochondrial assembly and function [102]. By contrast, extracellular S1P-mediated signalling has been well described with five S1P receptors ($S1P_{1,2,3,4,5}$) coupled with various Gα proteins (e.g., Gα_i, Gα_q, and G$\alpha_{12/13}$) which activate different downstream targets, such as PI3 K/Akt, Bcl2, adenylyl cyclase, ERK, phospholipase C, and p53 for cellular responses in both an autocrine and paracrine manner [103–107]. Briefly, $S1P_1$ is important to regulate the egress of lymphocytes into the blood stream [108], and $S1P_2$ is involved in mast cell degranulation and recovery from anaphylaxis *in vivo* [109, 110], $S1P_3$ is involved in vascular

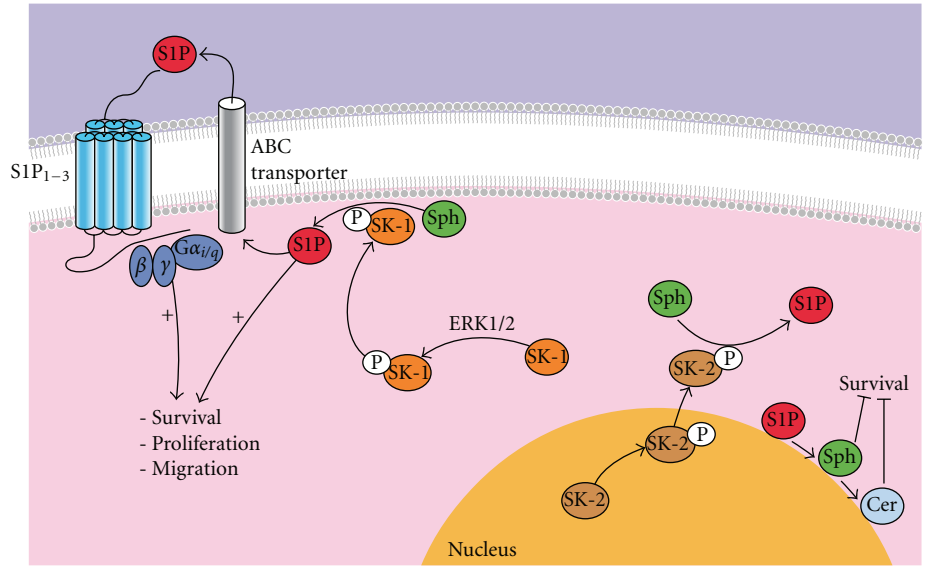


Figure 3: Intracellular SK-1 and SK-2 activity. The activation of SK-1 and SK-2 occurs via ERK1/2 phosphorylation in response to pro-inflammatory mediators, such as histamine and TNFα. Upon the activation, SK-1 is translocated from the cytoplasm to plasma membrane where it catalyses sphingosine to form S1P. S1P can then be transported outside the cell and then act back on its receptors to induce the activation of G-proteins for subsequent cellular changes, such as survival, proliferation, and migration. In contrast, SK-2 activity is associated primarily with the nuclear membrane, where it is phosphorylated prior to being translocated out of the nucleus. At the nuclear membrane and endoplasmic reticulum, S1P can be dephosphorylated to sphingosine and ceramide via the sphingolipid salvage pathway where many enzymes including sphingomyelinases, cerebrosidases, ceramides, and ceramide synthases are involved to induce apoptosis.

development in the embryo [111]. $S1P_4$ and $S1P_5$ are not well studied but have been shown to be expressed by dendritic cells and lymphocytes, respectively [112, 113].

8. Genetic Manipulation of SK/S1P *In Vivo*

To investigate the physiological roles of SK/S1P *in vivo* and whether their manipulation can regulate disease development, genetically modified mice with depletion of either SK-1 or SK-2 gene (*Sphk1* or *Sphk2*) have been generated and no phenotypical abnormalities have been identified under normal conditions [58, 77]. By contrast, the depletion of both *Sphk1* and *Sphk2* is embryonic lethal by day 13.5 due to the severe defects in vasculogenesis and neurogenesis involved in CNS development [114]. More recently, the *Sphk1* and *Sphk2* heterozygous-knockout mice (i.e., *Sphk1*−/−*Sphk2*+/−) have been generated [115]. Although *Sphk1*−/−*Sphk2*+/− mice have not been studied extensively, the female mice exhibit a significant breakage of blood vessels in the uterine causing early pregnancy loss, which suggests that a basal level of SK is required for blood vessel integrity or stability [115]. To investigate the inhibitory effects of both SKs, administration of specific SK inhibitors serves as an alternative approach to attain the double knockdown effects, for example, administration of ABC294640 (SK-2 specific inhibitor) to *SphK1*−/− mice and administration of CB5468139 (SK-1 specific inhibitor) to *SphK2*−/− mice. However, studies using this alternative approach are lacking, which are likely due to the complicated pharmacokinetics and pharmacodynamic of the SK inhibitory agents *in vivo*.

9. SK/S1P in Allergic Inflammation

SK and S1P are involved in multiple cellular functions, such as survival, differentiation, activation and migration (reviewed in [107]). Notably, these cellular properties are involved in many disease developments, including allergic inflammation. To better understand the role of SK/S1P in allergic inflammation, a number of studies have examined the specific roles of each SK isoform and S1P receptors via genetically modified mice. For example, both *Sphk1*−/− and *Sphk2*−/− mice have been shown to exhibit a reduction in ovalbumin (OVA)-induced IgE and IgG production via an inability to increase mast cell protease 1 in response to OVA, an enzyme required for IgE-induced anaphylaxis [116]. Our recent work has shown that *Sphk1*−/− mice but not *Sphk2*−/− mice exhibit an attenuated histamine-induced P-selectin expression and neutrophil recruitment [66]. This is in agreement with a study by Baker et al. who generated hTNF/*Sphk1*−/− mice (i.e., *Sphk1*−/− mice carrying the human modified copy of TNFα) and showed that only hTNF/*Sphk1*−/− mice but not hTNF/*Sphk1*+/+, hTNF/*Sphk1*−/+, or hTNF/*Sphk2*−/− mice exhibited a reduction in paw inflammation and bone deformity [117, 118]. Moreover, this was determined to be due to decreased articular COX2 protein and Th17 cell contribution to inflammation [117]. In terms of recovery from allergic inflammation, *Sphk1*−/− and $S1P_2$−/− mice were observed to have increased vasodilation, poor recovery from anaphylaxis and delayed clearance of histamine. This was not observed in the *Sphk2*−/− mice [109]. Administration of S1P to *Sphk1*−/− mice can rescue these

phenomena, which suggests that SK-1 activity aids in the recovery from anaphylaxis [109].

In humans, increasing evidence suggests that SK and S1P are involved in the pathophysiology of inflammatory diseases, such as asthma [119], chronic obstructive pulmonary disease (COPD) [120], microbial-induced sepsis [121], acute pancreatitis [122], and rheumatoid arthritis [123]. Studies have shown that the SK-1 protein and activity are upregulated markedly in peripheral immune cells including neutrophils, lymphocytes, and macrophages during the early phase of these diseases, which allow for their activation and release of the proinflammatory cytokines TNFα, IL-1β and IL-6 [121, 122]. Not surprisingly, high levels of S1P were detected in the synovial fluid of arthritic patients, which enhances COX-2 expression and prostaglandin E(2) production via the $S1P_1$ receptor [123]. Blockade of SK-1 in tissue samples extracted from these patients exhibited a decrease in proinflammatory cytokine expression [121], which suggests that the regulation of SK-1/S1P pathway is a potential therapy for inflammatory diseases.

10. Pharmacological Manipulation of SK/S1P

There are a number of SK and S1P receptor inhibitors that have been generated and studied in the last few decades (Table 2) (reviewed in [124, 125]). Blockade of SK-1 by inhibitors can attenuate prostate cancer [65], melanoma [126], inflammation in rheumatoid arthritis [123] and asthma [127] *in vivo*. Of all of the SK/S1P inhibitors, only a few have proceeded to clinical trials and been approved for human use based on their pharmacokinetics, target specificity, efficacy, adverse effects, and safety profile. The best example to date is FTY720 (Fingolimod), which was the first oral prodrug to be approved by the Food and Drug Administration (FDA) and Therapeutic Goods Administration(TGA) for the clinical treatment of multiple sclerosis (MS) [128]. The first described mechanism of FTY720 is predominantly phosphorylated by SK-2 to form FTY720-P, which is then able to bind to S1P receptors ($S1P_{1,3,4,5}$) [77, 129]. In MS, FTY720-P blocks S1P signalling largely by the internalization of the $S1P_1$ on lymphocytes causing lymphocyte egress from the lymphoid organs and lymphopenia in the periphery [108].

Interestingly, later studies have shown that FTY720 without phosphorylation can potently inhibit SK-1 by competing with sphingosine as a substrate for SKs and thereby preventing subsequent S1P formation [129–131]. Furthermore, the analogues of FTY720 (i.e., (S)- and (R)- FTY720-vinylphosphonate) bind to an allosteric site of SK-1 to induce proteasomal degradation in cells in a noncompetitive manner [132]. As FTY720 itself can inhibit SK-1, studies have also examined whether high concentrations (larger than the recent clinical dose of 0.5 mg once daily) and multiple dosing of FTY720 can be a potential therapy for cancer and renal transplantation [133, 134]. Unfortunately, results showed that FTY720 does not improve the prognosis for postrenal transplantation when compared to the current protocols [134, 135], likely due to the multiple inhibitory effects of FTY720 on S1P receptors, SK-1, autotoxin, protein phosphatase 2A, ceramide synthases, S1P lysase, protein kinase C and cytosolic phospholipase A [reviewed in [136]]. Clearly, new and specific SK/S1P inhibitors are required. To this end, Schnute et al. recently generated a specific and potent SK-1 inhibitor, PF-543, which inhibits SK-1 by competing with sphingosine and resulting in rapid reduction of S1P formation [79]. The inhibitory effect of SK-1 by PF-543 is over 1000-fold more potent than other SK inhibitors such as N,N-dimethylsphingosine (DMS) and SKI-II. However, the efficacy of PF-543 *in vivo* remains to be examined. In addition, Kharel et al. reported that their two new amidine-based SK-1 inhibitors (1a and 1b) can selectively inhibit SK-1 at high potency for rapid reduction in S1P levels without toxicity *in vitro* and *in vivo* [81].

Although SK-2 is less well studied than SK-1, a role for SK-2 (via the administration of the SK-2 inhibitor, ABC294640) has been described in tumor development [82, 137], Crohn's disease [138], hepatic ischemia-perfusion [139], and osteoarthritis [140]. However, this SK-2 inhibitor also binds to oestrogen receptor [141], which suggests that administration of this compound may result in additional off-target effects. Interestingly, a new selective SK-2 inhibitor, SLR080811, has been shown to inhibit SK-2 at a higher potency than ABC294640 *in vitro* and drive an SK-1-dependent increase in blood S1P in WT mice [83]. Whether this small molecule is suitable for the clinic still requires long-term efficacy and safety data development.

Notably, pharmacological manipulation of SK/S1P does not always lead to the same results as observed for genetic manipulation *in vivo*. As mentioned above, the hTNF/*Sphk2*−/− mice exhibited no significant difference in arthritic inflammation when compared to controls [118]. However, the hTNF mice treated with ABC294640 exhibited severe arthritic inflammation in the same study, which may suggest that high dose of the agent and acute inhibition of SK-2 contribute to this phenomenon [118]. Moreover, other animal models include that thioglycollate-induced peritonitis and collagen-induced arthritis (CIA) have shown that the recruitment of neutrophils and lymphocytes to sites of inflammation in *Sphk1*−/− mice did not differ from that of WT mice [142]. By contrast, Lai et al. have shown that knockdown of either SK-1 protein or gene in mice by DMS and small interfering (si)RNA, respectively, exhibit reduced CIA severity [123, 143]. These different observations may be due to the different time period of stimulus challenge, animal strains and models for susceptibility. Nevertheless, taken together these studies clearly indicate that SK and S1P are involved in the development of allergic inflammation.

11. Adverse Effects of SK Inhibition

The inhibition of SK/S1P pathway may be an effective therapeutic approach to control allergic diseases as shown by the *in vivo* studies discussed above. However, excessive or prolonged blockade of SK/S1P may lead to profound adverse effects as evidenced by $S1P_1$−/− and double knockout of *Sphk1*−/− *Sphk2*−/− animals being embryonic lethal [106, 114] as well as $S1P_2$−/− mice being deaf [144] and

Table 2: Synthetic inhibitors of SK and S1P receptors.

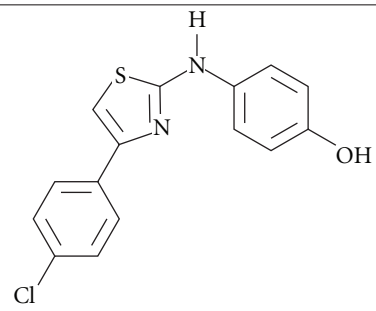
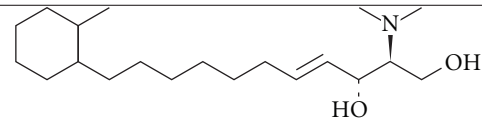
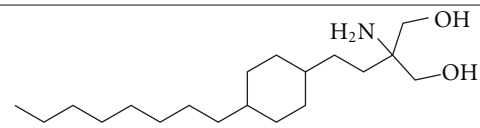
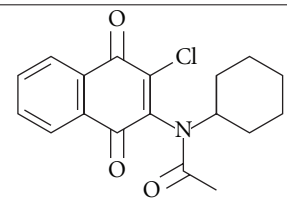
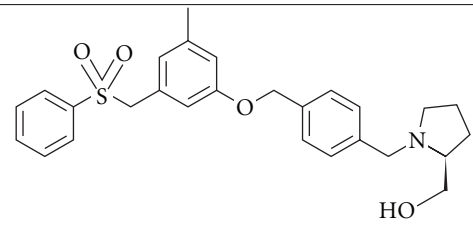
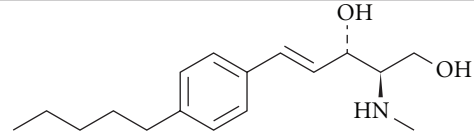
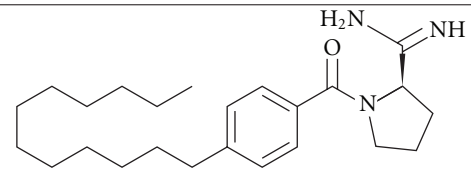
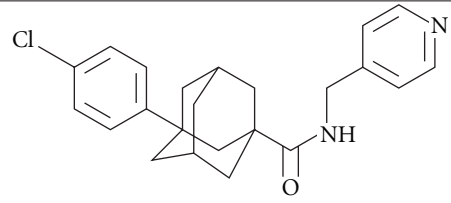
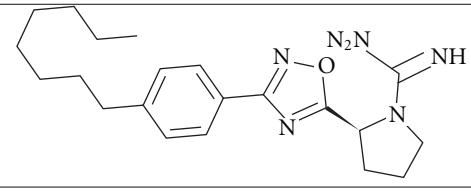
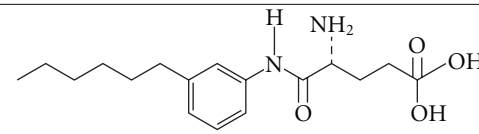
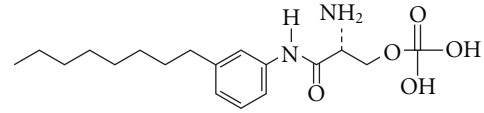
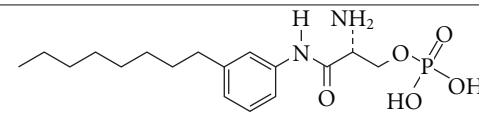
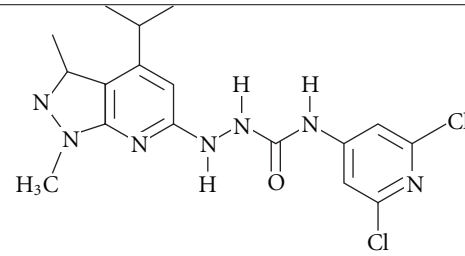
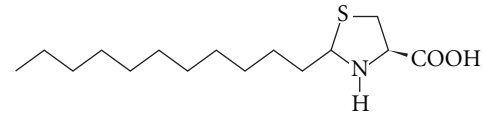
Compound	Inhibitory target(s)	Structure	Ref.
SKI-II	SK-1 SK-2		[75]
DMS	SK-1 SK-2		[76]
FTY720	SK-1 $S1P_{1,3,4,5}$		[77]
CB5468139	SK-1		[78]
PF543	SK-1		[79]
SK1-I	SK-1		[80]
Compound 1a	SK-1		[81]
ABC294640	SK-2		[82]
SLR080811	SK-2		[83]

Table 2: Continued.

Compound	Inhibitory target(s)	Structure	Ref.
W146	$S1P_1$		[84]
VPC44116	$S1P_{1\,\&\,3}$		[85]
VPC23019	$S1P_{1\,\&\,3}$		[86]
JTE013	$S1P_2$		[87]
CAY10444	$S1P_3$		[88]

experiencing occasional seizures [145]. The "side effects" of small molecule therapy that modulate the SK/S1P pathway may also raise concerns. For example, FTY720 at the clinical dose has been reported to cause transient bradycardia, atrioventricular block, macula oedema, hypertension, dyspnea, and elevated liver enzymes [146]. These symptoms are infrequent and manageable; however, compliance of this treatment can be discouraged by patients. In addition, treatment with FTY720 is also thought to increase the risk of infections as *Sphk1*−/− mice are more susceptible for endotoxin-induced lung inflammation than WT controls [147]. However, human preclinical data showed that FTY720-treated patients have no increased risk of infections in 2-year treatment when compared to the placebo group, except a small increased risk of lower respiratory tract and lung infections [128]. Notably, although the regulation of SK/S1P looks promising for controlling disease development, high specificity and potency of the pharmacological agents are preferable to avoid the undesirable off-target effects.

12. Strategy for Targeting Sphingolipids as a Therapeutic Approach

An effective approach to target sphingolipids for allergic inflammation diseases and avoid adverse effects is to better understand "when" and "where" such that specific SK/S1P inhibitors can be administrated appropriately. In ECs, we and others have demonstrated that the SK/S1P pathway regulates the expression of adhesion molecules to control neutrophil recruitment *in vitro* and *in vivo* (Figure 4). For example, during the early phase of allergic inflammation, histamine-induced SK-1 activity (but not SK-2 activity) rapidly exocytoses P-selectin to the surface of ECs to initiate neutrophil rolling in the postcapillary venules of WT mice, a process shown to be S1P receptor independent [66]. As expected, this histamine-induced neutrophil recruitment does not occur in *Sphk1*−/− mice [66]. Furthermore, TNFα-induced SK-1 activates $\alpha_5\beta_1$ integrin on human umbilical vein ECs (HUVEC) to promote the adhesion of neutrophils under shear stress, again the events appear to be S1P receptor independent and can be inhibited by FTY720 [148].

By contrast in the late phase of allergic inflammation (>4 hours), S1P receptor-activated pathways promote vascular adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1, and E-selectin gene and protein expression on HUVEC in response to TNFα [67], globular adiponectin [149], or histamine [150]. Exposure of ECs to S1P can also increase Weibel Palade body (WPB) exocytosis of vWF in a PLC-γ-induced calcium-dependent manner. However, prolonged exposure of S1P enhances PI3K-induced nitric oxide production resulting in reduced WPB exocytosis by ECs [151]. Taken together, these studies suggest that increased SK-1 activity is predominantly involved in the early phase of allergic inflammation whilst S1P/S1P receptors are primarily involved in more delayed immune responses.

$S1P_{1–5}$ are distributed in different tissues with $S1P_{1–3}$ being widely expressed and at high levels in brain, lung, spleen, heart, liver, skeletal muscle, and kidney with addition

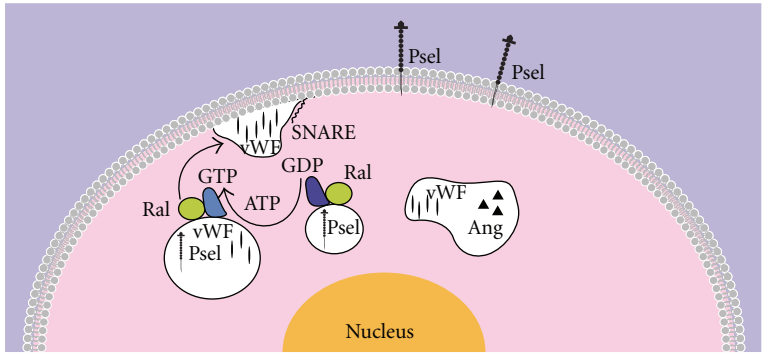


Figure 4: . Exocytosis of P-selectin by ECs. P-selectin is preformed and stored in Weibel Palade bodies (WPBs). It is found to be solely present or co-stored in WPBs with von Willebrand Factor (vWF) or angiopoietins (Ang). Upon extracellular stimulation, WPBs exocytose to the cell surface via the activation of Ral-GTP from Ral-GDP. WPB-containing vWF is also driven and translocated to the plasma membrane by SNARE. The rapid surface expression of P-selectin mediates the initial recruitment of leukocytes to ECs by rolling and tethering, which is important during the early development of allergic inflammation.

of $S1P_1$ in lymphoid and $S1P_3$ in testis; $S1P_4$ is restricted to lymphoid and lung tissue and $S1P_5$ is only expressed in brain, skin, and spleen (reviewed in [152]). These divergent tissue distributions of S1P receptors may provide some insight into which specific S1P receptor inhibitors should be administered in relation to the development of inflammation and disease. Notablty, FTY720-P binds to $S1P_{1,3,4,5}$ and may result in multiple side effects; thus other selective $S1P_1$ inhibitors (ONO-4641 and CS-0777) have been generated and undergone Phase 1 and 2 clinical trials for MS and psoriasis (reviewed in [125, 153]). Different methods of administration can be used to deliver the inhibitors/drugs for local inhibitory effects as evident by *in vivo* studies where the inhalation of SK inhibitor can attenuate airway inflammation [127], the administration of FTY720 in the eyes can prolong corneal graft survival [154], and nanoparticle-mediated delivery of drugs can enhance the therapeutic outcomes in hindlimb ischemic mice [155]. However, many questions remain to be answered, such as whether this nanotechnology is effective enough to deliver SK/S1P inhibitors to specific sites of the body and whether it is safe to be used in humans.

13. Conclusion and Future Perspectives

Early allergic reactions and recruitment of inflammatory cells are key to allergic disease formation and progression. An effectual therapeutic approach is lacking amongst the current treatment options, and most treatments (e.g., H_1 antagonists) are ineffective in their regulation of the early phase of allergic inflammation. Thus a better therapeutic strategy is urged for a rapid control of allergic symptoms to prevent tissue damage and development of severe conditions. The SK/S1P pathway has been shown to be important in cell survival, migration, differentiation, and immune responses. Herein, we discuss its role in allergic inflammation, both the early and late phases as well as chronic inflammation. Further studies involving the manipulation of SK/S1P pathway and its impact on a variety of diseases as well as the early phase of allergic inflammation will culminate to provide better insight into how we can translate animal studies into a new clinical treatment for human allergic inflammation.

Based on these *in vitro* and *in vivo* studies, sphingolipids are clearly involved in the regulation of adhesion molecule expression on the vasculature and as such may be a biological marker for attenuating leukocyte recruitment and subsequent allergic inflammatory reactions. The next step is to translate these animal models into human clinical studies with the ultimate goal of developing new treatments to tackle allergic diseases. Herein we propose that the current sphingolipid compounds may be effective in attenuation of allergic inflammation. For example, FTY720 or new small molecular inhibitors could be further investigated for their drug adverse effect profile to then determine their suitability for long-term use as prophylaxes.

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Appendix 4:

Neutrophil interactions with the vascular endothelium.

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Neutrophil interactions with the vascular endothelium ☆

David Dimasi [a], Wai Y. Sun [a,b,c], Claudine S. Bonder [a,b,c,d,*]

[a] Centre for Cancer Biology, SA Pathology, Adelaide, South Australia, Australia
[b] School of Medicine, University of Adelaide, Adelaide, South Australia, Australia
[c] Co-operative Research Centre for Biomarker Translation, La Trobe University, Victoria, Australia
[d] School of Molecular and Biomedical Sciences, University of Adelaide, Adelaide, South Australia, Australia

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ABSTRACT

Neutrophils are a key mediator of the innate immune system and are pivotal in the inflammatory response to infection or tissue damage. Fundamental to the role that neutrophils play in host defence is their interaction with the vascular endothelium. From the initial mobilisation of neutrophils out of the bone marrow to their ultimate transmigration through the vasculature, endothelial cells are a vital component of the inflammatory process. This review focuses on the interactions that take place between neutrophils and the vascular endothelium during the various stages of the inflammatory response. The role of the vascular endothelium in detecting the initial episode of infection or tissue damage is explored and how this ultimately leads to mobilisation of the neutrophils from the bone marrow and into the bloodstream. The recruitment and adhesion of neutrophils to the vascular endothelium is also discussed, with particular emphasis on the various discrete stages of the adhesion cascade and what molecules mediate these steps. In addition, a novel role for the lipid kinase sphingosine kinase in neutrophil adhesion is examined. With the advent of improved imaging techniques and the development of new animal models, this is a dynamic area of research and this review aims to summarise some of the more recent findings.

1. Introduction

Neutrophils form a major component of the human body's innate immune system, providing a first line of defence against invading pathogens such as bacteria and fungi, as well as responding to episodes of sterile tissue damage. The phagocytic and cytotoxic capabilities of neutrophils are integral to their role in host defence and they possess a range of mechanisms with which they can eliminate pathogens and dead or dying host cells [1]. The effectiveness of neutrophils in fighting infection is also reliant on their ability to leave the vasculature and migrate into the extracellular space and ultimately, to the site of infection or tissue injury. Indeed, if the process of neutrophil egress from the vasculature is impaired through a genetic deficiency then recurrent life-threatening infections will develop [2,3]. The initial recruitment of neutrophils to the vascular endothelium is vital to the migratory process and is mediated by an intricate array of adhesion molecules and chemoattractants. The process of neutrophil adhesion and migration has been extensively studied and although significant progress has been made in understanding the mechanisms involved, much is still to be determined. This review aims to summarise the current understanding of neutrophil interaction with the endothelium during inflammation and emerging evidence for the role of the lipid kinase sphingosine kinase in this process.

2. Activation of the vascular endothelium

Detection of a microbial infection or tissue damage by the host is one of the earliest events in the inflammatory response and is undertaken by sentinel cells located within the compromised tissue, including dendritic cells, macrophages, mast cells and vascular endothelial cells [4,5]. Exposure of these cells to stimuli such as pathogen-associated molecular pattern (PAMP) and damage-associated molecular pattern (DAMP) molecules, induces secretion of the cytokines interleukin-1β (IL-1β), IL-6, and tumour necrosis factor-α (TNF-α) and a range of different chemokines [6,7]. Pattern recognition receptors found on the surface of these sentinel cells are responsible for recognising structures conserved amongst microbial species (PAMPs) or endogenous molecules released from damaged cells (DAMPs) [5–8]. In response to the release of these inflammatory mediators, the adjacent vascular endothelium becomes activated, thus inducing profound changes in gene expression and function that allow the endothelial cell to participate in various inflammatory processes. Upon activation, the vascular endothelium begins to secrete a range of cytokines, chemokines and colony stimulating factors, as well as up-regulating expression of adhesion molecules on the luminal surface [9]. These changes support one of the key functions

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* Corresponding author at: Centre for Cancer Biology, SA Pathology, Frome Road, Adelaide, SA 5000, Australia. Tel.: +61 8 8222 3504; fax: +61 8 8232 4092.
E-mail address: claudine.bonder@health.sa.gov.au (C.S. Bonder).

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of an activated vascular endothelium, which is to promote mobilisation and recruitment of neutrophils to the inflammatory site.

3. Neutrophil mobilisation

Neutrophils are the most abundant immune cell type in humans, with up to 10^{11} new neutrophils formed in the bone marrow each day [10]. Under normal physiological conditions, the vast majority of neutrophils remain within the bone marrow, with less than 2% found in the bloodstream [11]. Upon entering the bloodstream, neutrophils have a very short half-life of approximately 6.5 h, thus necessitating in a continual release of mature neutrophils from the bone marrow storage pool [12]. During an inflammatory episode such as microbial infection or tissue injury, neutrophils can be rapidly mobilised from the bone marrow, resulting in a dramatic increase in their circulating number. Levels of neutrophils in the bloodstream can increase 10-fold within a few hours of an inflammatory insult, [13] and this represents a critical first step in the trafficking of neutrophils to the site of inflammation.

An array of chemokines, cytokines and their receptors are responsible for the maintenance of neutrophil homeostasis in the bone marrow and for their subsequent egress into the bloodstream. Fundamental to the retention of neutrophils in the bone marrow is CXC chemokine receptor 4 (CXCR4), a G-protein coupled receptor expressed on the surface of mature neutrophils. The major ligand for CXCR4 is CXC ligand 12 (CXCL12), a chemokine produced predominately by osteoblasts within the bone marrow. Interaction between CXCR4 and CXCL12 retains neutrophils within the marrow environment [14–16]. A second member of the CXC receptor family expressed on the surface of mature neutrophils, CXCR2, is used to facilitate the egress of neutrophils into the bloodstream through binding of its ligands CXC ligand 1 (CXCL1) and CXCL2, the major source of which is the endothelial cells of the bone marrow [15,17]. As shown in Fig. 1, in an ongoing struggle for control, the CXCR4/CXCL12 complex usually dominates, thus retaining most neutrophils in the bone marrow. Disturbance of this equilibrium, through the action of chemokines and cytokines released during an inflammatory episode, shifts the signalling in favour of CXCR2 and its ligands, thus promoting neutrophil egress into the bloodstream.

One of the key mediators responsible for mobilising neutrophils from the bone marrow is the hematopoietic cytokine granulocyte colony-stimulating factor (G-CSF), which acts by indirectly shifting the balance in favour of the CXCR2 ligands (Fig. 1). This is achieved through down-regulation of CXCR4 expression on the surface of neutrophils and by reducing the number of CXCL12 secreting osteoblasts whilst simultaneously increasing expression of CXCL1 and CXCL2 in the bone marrow endothelial cells [18–20]. In response to an acute inflammatory episode, serum levels of G-CSF increase and bacterial products and/or secondary inflammatory mediators are known to be potent stimuli for the production of G-CSF [21–23]. Along with fibroblasts [24,25] and mononuclear phagocytes, [26,27] vascular endothelial cells play an important role in secreting G-CSF following activation by cytokines such as TNF-α or IL-1 [9,28,29]. In addition to G-CSF, other chemotactic factors such as leukotriene B4, complement factor C5a and the chemokine IL-8 (KC in mice) also play an important role in mobilising neutrophils from the bone marrow [13].

4. Neutrophil recruitment to the vascular endothelium

Following mobilisation from the bone marrow during an acute inflammatory episode, circulating neutrophils are rapidly recruited (*i.e.* within minutes) into peripheral tissues and represent the most abundant immune cells at the inflamed site for many hours. The process of neutrophil migration from the bloodstream and into the inflamed extravascular tissue involves a complex interaction between the neutrophil and the adjacent vascular endothelium, a mechanism known as the adhesion cascade. As shown in Fig. 2, the adhesion cascade describes a series of discrete events from the initial capture and tethering of the neutrophil to the endothelium, to the ultimate migration of the neutrophil out of the vasculature and into the inflammatory site of the parenchyma.

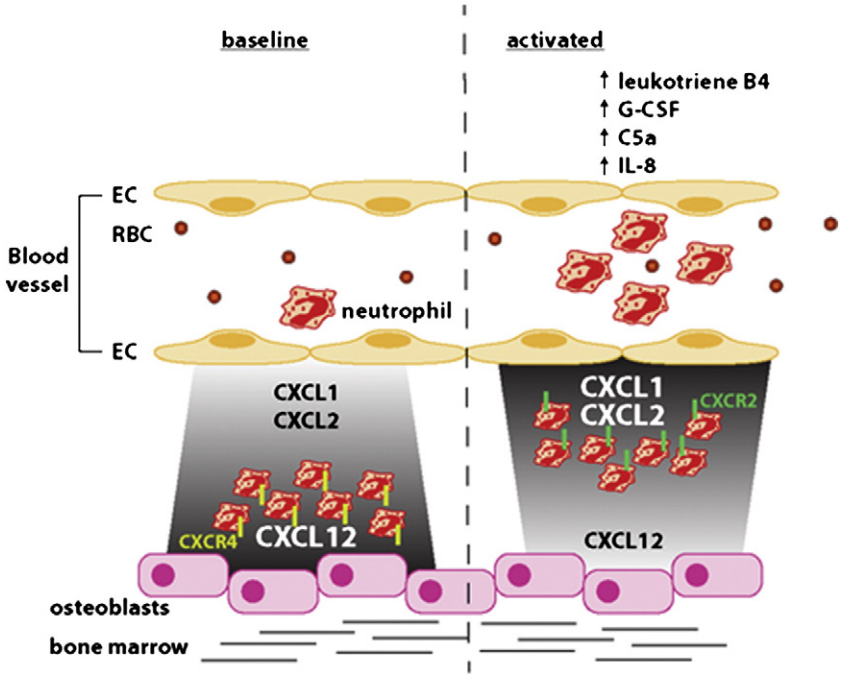

Fig. 1. Neutrophil retention in the bone marrow and their mobilisation into the peripheral circulation are regulated by neutrophil expression of chemokine receptors (CXCR4 and CXCR2) and their interaction with chemokines (CXCL12, CXCL1 and CXCL2). During an inflammatory response activation mediators (*e.g.* leukotriene B4, G-CSF, C5a and IL-8) alter chemokine expression for subsequent neutrophil release and function as immune regulators.

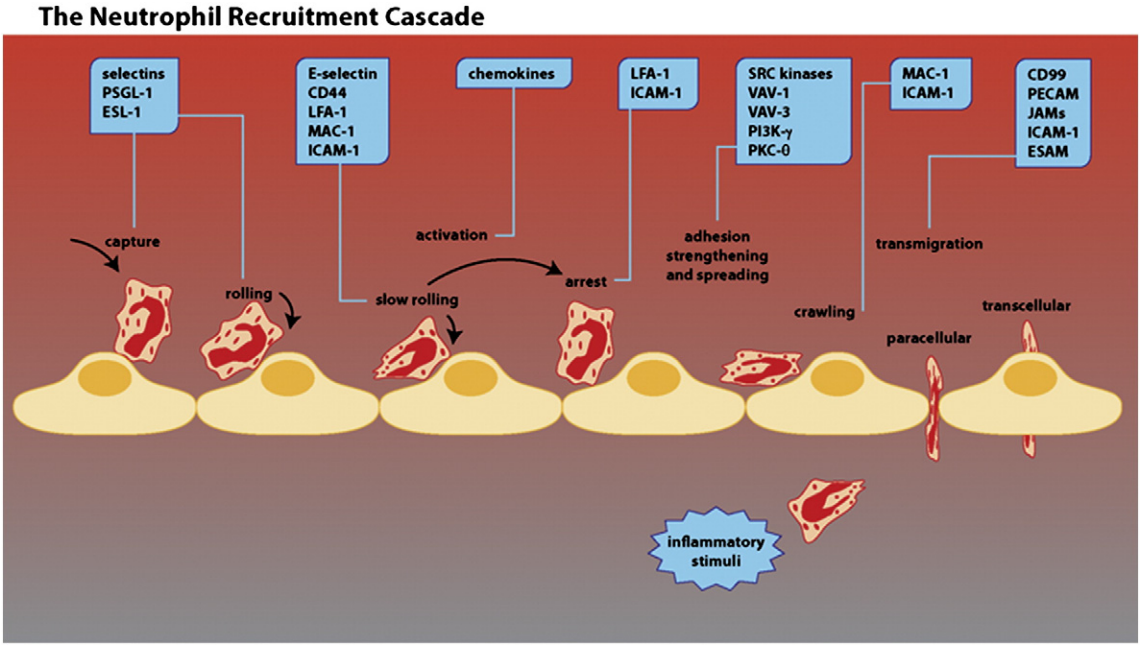

Fig. 2. The recruitment of neutrophils to a site of inflammation is governed by a cascade of adhesion events which include, capture, rolling, adhesion, arrest and transmigration. These adhesive events are regulated by families of adhesion molecules expressed by both the circulating neutrophils and the vascular endothelium.

4.1. Capture or tethering

As mentioned previously, endothelial cells activated by inflammatory cytokines and other mediators express adhesion molecules and synthesise chemokines and lipid chemoattractants that are presented on their luminal surface. The upregulation of adhesion molecules facilitates the first step in the adhesion cascade, involving the physical interaction of the neutrophil with the vasculature, known as capture or tethering. A family of three cell surface transmembrane glycoproteins, termed E-, L- and P-selectin, play an integral role in mediating this initial step of the adhesion cascade and studies using knock-out mice or antibody blockade of selectin function have demonstrated how ablation of these proteins can severely impact the recruitment of neutrophils to the endothelium [30,31]. L-selectin is constitutively expressed amongst the majority of leukocytes, whereas both P-selectin and E-selectin are expressed by activated vascular endothelial cells [32]. After activation, L-selectin is shed from the cell surface and a functionally active soluble form of L-selectin can be found in the blood stream [33]. Notably, shedding of L-selectin from activated T cells has been shown to prevent their re-entry into peripheral lymph nodes [34]. Within resting endothelial cells, pre-formed P-selectin is stored in secretory vesicles termed Weibel–Palade bodies, but upon activation by inflammatory mediators is rapidly translocated to the plasma membrane [35,36]. Conversely, E-selectin is synthesised *de novo* and is transcriptionally upregulated upon endothelial cell activation [37,38]. Following expression of E- and P-selectins on the endothelial surface, the process of capturing circulating neutrophils is enabled by binding of the selectins to P-selectin glycoprotein ligand-1 (PSGL-1), a homodimeric transmembrane protein expressed on all leukocytes. The involvement of E- and P-selectin and PSGL-1 in mediating neutrophil capture has recently been reviewed by Zarbock et al. [39]. Whilst the interaction between E- and P-selectin and PSGL-1 occurs under the strain generated by the blood flow, these conditions actually support adhesion. This phenomenon is related to the catch bond character of selectins, which strengthens each bond as shear stress is applied [40,41].

The role of L-selectin in neutrophil capture has been the subject of some controversy, with varying hypotheses on its function. Deficits in leukocyte adhesion have been observed with L-selectin knock-out mice and studies incorporating function ablating antibodies, indicating a critical role for the protein in this process [30,31]. It was initially believed that L-selectin bound binds to an endothelial expressed ligand, although identifying this ligand has proved elusive [42]. Recent studies still support the existence of an endothelial ligand for L-selectin, [43] although others maintain that no such ligand exists [44]. However, as with E- and P-selectin, L-selectin does bind PSGL-1 expressed on other leukocytes [45]. The interaction between L-selectin and PSGL-1 promotes leukocyte-leukocyte contact, which is an important mechanism for mediating secondary neutrophil capture, a process whereby neutrophils that have already adhered to the vascular endothelium can subsequently attract additional neutrophils through binding to their exposed surface. This secondary capture is an important mechanism for increasing the number of neutrophils that are recruited to the site of inflammation [46].

The rapid expression of P-selectin on the endothelial surface is enhanced by co-translocating the Weibel–Palade bodies with other intracellular molecules, such as von Willebrand factor (vWF), [47] small GTP-binding protein Ral, [48] N-ethylmaleimide-sensitive factor attachment protein (SNARE) and angiopoietin-1 and -2 [49]. Interestingly, *in vivo*, vWF-deficient mice exhibit a reduction in cytokine-mediated P-selectin expression which leads to reduced recruitment of neutrophils and an attenuated inflammatory response [50]. The small molecule Ral is activated by GDP converting to GTP reversibly *via* ATPase to form the active GTP-Ral, which is then associated with calcium and calmodulin for the translocation of Weibel–Palade body-containing vWF. Through this mechanism GTP-Ral is also suggested to mediate the rapid expression of P-selectin [48]. Notably, SNARE is also associated with the exocytosis of Weibel–Palade bodies in endothelial cells and the inhibition of SNARE by nitric oxide reduces vascular inflammation. Whether SNARE has a direct role for P-selectin translocation in endothelial cells remains to be examined [51]. P-selectin mRNA is also transcribed during inflammatory responses [52,53]. Notably, the promoter region of the P-selectin gene differs between humans and mice, where NFκB binding region is present in the upstream of murine P-selectin gene but not in that of the human *SELP* gene promoter [54–56]. In mice, TNFα, IL-1 and lipopolysaccharide (LPS) activates NFκB causing

phosphorylation and hydrolysis of IκBα, which then promotes the translocation of NFκB to nucleus for P-selectin gene transcription [57,58]. This does not occur in humans where, in contrast, IL-4 [59,60] oncostatin M, [52] IL-13[61] and substance P [53] promote the transcription of P-selectin.

4.1.1. Sphingosine kinase, a mediator of endothelial adhesion molecule expression

Sphingosine kinases, of which there are two isoforms in mammalian cells (SK-1 and SK-2), mediate the phosphorylation of sphingosine to form sphingosine-1-phosphate (S1P) (reviewed in Pitson [62]) and increasing evidence suggests that SKs can regulate adhesion molecule expression on the endothelium. Firstly, histamine rapidly activates SK-1 which promotes P-selectin surface expression on endothelial cells and subsequent neutrophil recruitment *in vitro* and *in vivo* [63]. Second, TNFα-induced SK-1 activity causes $\alpha_5\beta_1$ integrin activation on endothelial cells, which mediates adhesion of neutrophils under shear stress through binding to angiopoietin-2 [64]. Third, TNFα-induced up-regulation of E-selectin, vascular cell adhesion protein 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) expression on endothelial cells is an SK-1 dependent process [65,66] and S1P has been shown to synergize with histamine during a 4 hour exposure to promote gene expression of these same adhesion molecules [67]. Taken together, these studies have shown that SKs can regulate adhesion molecule expression for acute and chronic stages of neutrophil recruitment.

4.1.2. Anti-selectin therapy to attenuate the recruitment of neutrophils

Given that selectins are involved in the early capturing and rolling of neutrophils, antagonism of these molecules has been targeted as a therapeutic approach to control the excessive recruitment of these cells [68,69]. For example, the first selectin antagonist CY1503 (Cylexin) is known as an analogue of sialyl-Lewis X and inhibits E-, P- and L-selectins. Lefer et al. showed that canines treated with CY1503 exhibited a significant reduction in the degree of myocardial infarct size associated with coronary artery ischemia and reperfusion, which is a result of a reduced leukocyte accumulation at the sites at 4.5 h post-operation [70]. However, the effects of CY1503 remain controversial, as a similar study failed to consistently reduce myocardial injury and neutrophil accumulation followed by a prolonged period of reperfusion for 48 h [71]. Notably, most of the selectin antagonists have failed in phase III clinical studies because the potency of selectin blockade is insufficient [69]. The levels of neutrophil rolling need to be reduced by at least 90% in order to prevent the later firm adhesion events [72,73]. Therefore, a novel mechanism for controlling selectin expression awaits to be investigated for the development of anti-selectin therapy.

4.2. Rolling

Following capture, the next step in the leukocyte adhesion cascade is referred to as rolling. In this stage, the neutrophils physically roll along the endothelium in the direction towards the inflamed site, as evidence suggests that the initial capture event does not occur at a location immediately adjacent to the infected or damaged tissue [7]. As with neutrophil capture, rolling is predominately mediated by interactions between endothelial selectins and their associated ligands. Studies employing P-selectin deficient mice have demonstrated a pivotal role for this protein in neutrophil rolling, mediated through binding to PSGL-1 [74–77]. In addition, both E- and L-selectin have been implicated in facilitating rolling [75,77]. There is also evidence that binding of E-selectin to an alternative ligand termed E-selectin-ligand-1 (ESL-1) is important in mediating neutrophil rolling. Interaction between E-selectin and ESL-1 is necessary to convert the initial capture into a stable rolling event, thus engaging the neutrophil in a more intimate contact with the endothelial surface [78]. Ultimately, this helps to facilitate the subsequent arrest and recruitment of neutrophils into the extracellular space.

4.3. Slow rolling

A reduction in the velocity of the rolling neutrophil is indicative of the next stage in the adhesion cascade, termed slow rolling. A role for E-selectin is still evident at this point, whereby ligand recognition switches from ESL-1 to another plasma membrane protein termed CD44 [78]. Both PSGL-1 and ESL-1 reside on microvilli protrusions found on the neutrophil surface, [76,79] whereas CD44 is exclusively found on the planar surface [80]. It is thought that during the transition from rolling to slow rolling, the microvilli recede, exposing CD44 and permitting binding to endothelial E-selectin. Engagement of CD44 initiates intracellular signalling events that result in the redistribution of PSGL-1 and L-selectin to the exposed surface of the neutrophil, a process that is believed to promote secondary capture of circulating neutrophils [78].

Another family of surface receptors that participate in slow rolling and subsequent steps in the adhesion cascade are the integrins. Localised on the surface of many cell types, including leukocytes, integrins are a class of 'activatable' receptors that can greatly increase their ligand-binding ability *via* intracellular signalling pathways generated from cell-surface molecules such as G-protein-coupled receptors. Of particular relevance to slow rolling are the β_2-integrins lymphocyte function-associated antigen 1 (LFA-1; also known as $\alpha_L\beta_2$-integrin) and macrophage receptor 1 (MAC-1; also known as CD11b–CD18 or $\alpha_M\beta_2$-integrin), both of which interact with the endothelial ligand ICAM-1. *In vivo* studies conducted in the mouse have demonstrated that slow rolling not only requires E-selectin, [81] but also LFA-1 or MAC-1 [82,83]. Integrins are believed to adopt a transient and low-to-intermediate binding affinity to ICAM-1 at this stage of the adhesion cascade, thus permitting a reduced rolling velocity without arrest of the neutrophil on the endothelial surface [84].

4.4. Arrest

Neutrophil arrest on the vascular endothelium proceeds from the slow rolling stage and is mediated by activation of β_2-integrins. Binding of neutrophil G-protein-coupled receptors to chemokines, which have been immobilised on the inflamed endothelial surface by glycosaminoglycans, triggers intracellular signalling pathways that almost instantaneously activate surface integrins [84]. In addition to chemokines, integrins can also be activated through selectin-mediated signalling pathways [85]. Activation of LFA-1 is critical for neutrophil arrest, allowing it to establish a higher-order binding to its endothelial ligand ICAM-1 [84,86,87]. Activation increases the strength of the receptor–ligand interaction through modulation of integrin affinity and avidity [84,88,89]. An upregulation in integrin affinity is associated with a conformational change in the structure of the receptor, which increases ligand binding and decreases ligand dissociation [90]. Such a conformational change has been demonstrated with LFA-1 on activated leukocytes [91]. The absolute requirement for integrin activation in facilitating arrest has been verified by observing the behaviour of neutrophils isolated from patients suffering from a form of leukocyte adhesion deficiency known as LAD-III. Caused by mutations in the CalDAG-GEFI gene, individuals with LAD-III exhibit impaired neutrophil arrest despite displaying normal selectin-mediated rolling, the cause of which is failed activation of β_2-integrins [92].

4.5. Adhesion strengthening and spreading

In order to remain in stationary contact with the endothelium, neutrophils undergo an adhesion strengthening phase following arrest. Adhesion strengthening prevents neutrophil detachment from the endothelium and also permits morphological changes, known as

spreading, that polarise the neutrophil and prepare it for intravascular crawling. The processes of adhesion strengthening and neutrophil spreading are mediated by intracellular signalling molecules that are activated by outside-in signalling mechanisms. Outside-in signalling is the process by which ligation of a cell-surface receptor, such as a β_2-integrin, activates signalling pathways inside the cell. It is thought that these signalling pathways increase adhesion strength and induce spreading by facilitating cytoskeletal arrangements that result in integrin clustering and/or increases in integrin binding affinity [93,94]. Studies in knock-out mice have demonstrated that ablating the function of intracellular signalling molecules such as the SRC family kinases FGR and HCK, [95] the guanine nucleotide exchange factors VAV1 and VAV3, [96], PI3Kγ [97] and PKC-θ [98] greatly accelerates the detachment of adherent neutrophils under flow by disrupting LFA-1 binding. These observations provide convincing evidence for a post-arrest phase of neutrophil adhesion stabilisation. In addition, PKC-θ and mammalian actin-binding protein 1 have also been shown to be essential for neutrophil spreading [98,99].

4.6. Intravascular crawling

Prior to crossing the vascular endothelium and entering the extracellular space, neutrophils crawl along the inside of the blood vessel seeking preferred locations for transmigration. These are generally represented by tricellular endothelial junctions, as it is at these sites that neutrophil transmigration through the endothelial layer can occur most efficiently [100,101]. There is also evidence to suggest that neutrophils will select an area for transmigration based on the structural properties of the underlying basement membrane [102]. The direction of neutrophil crawling is dependent on shear forces, as adherent neutrophils crawl perpendicular to the blood flow [103]. The β_2-integrin MAC-1, through its binding to endothelial ICAM-1, is essential for intravascular crawling and neutrophils in MAC-1 deficient mice exhibit a profound defect in crawling proficiency [86]. In addition, MAC-1 deficient mice also display delayed transmigration as neutrophils fail to crawl to the preferred transmigratory sites [86]. The guanine nucleotide exchange factor VAV1, which has been implicated in supporting firm adhesion, is also necessary for neutrophil crawling, where it is believed to play a role in LFA-1 mediated outside-in signalling that ultimately results in MAC-1 activation and induction of the crawling phenotype [103].

4.7. Transmigration

The final stage in the adhesion cascade is the ultimate migration of the neutrophil from the vasculature into the inflamed tissue. Following the initial adhesive and crawling events, neutrophils emigrate from the vascular lumen by passing between (paracellular) or through (transcellular) the endothelial cells. The paracellular route is the predominant and best characterised form of transmigration and accounts for approximately 85–95% of neutrophil egress from the vasculature [104,105]. In contrast, transcellular migration appears to be limited to specific tissues such as the bone marrow, pancreas and thymus and has also been demonstrated to occur when intraluminal crawling is prevented [86,106]. Both forms of transmigration may occur rapidly (within minutes), [107,108] with *in vitro* data demonstrating that transcellular migration takes place in under a minute [109]. It is still unclear however, as to what mechanisms determine whether a neutrophil transmigrates *via* the paracellular or transcellular pathway.

Despite their vastly different modes of action, both the paracellular and transcellular pathways share some common early interactions with the vascular endothelium. The binding of neutrophil integrins to their endothelial ligands activates signalling events within the endothelium that assist with the transmigration process. Leukocyte adhesion induces the formation of pro-adhesive sites at the plasma membrane termed endothelial adhesive platforms, which are specialised tetraspanin-enriched microdomains that express high levels of ICAM-1 and VCAM-1 [110]. In addition, endothelial cells form 'docking structures' or 'transmigratory cups', which are projections rich in ICAM-1, VCAM-1, cytoplasmic ERM (ezrin, radixin and moesin) proteins and cytoskeletal components (such as viniculin, α-actinin and talin-1) [104,111]. Fig. 3A shows that these 'docking structures' help to facilitate transmigration through either a transcellular or paracellular pathway by physically surrounding the neutrophil and generating redistribution of surface integrins into a conformation that assists with the migratory process [104].

4.7.1. Paracellular migration

Whereas the mechanisms that determine whether a cell will undergo paracellular or transcellular migration are not well understood, one important determinant is the display of specific endothelial markers that indicate the most efficient method of transmigration under the prevailing conditions (Fig. 3B). For example, there is evidence that the endothelial expressed molecule ICAM-2, most likely through binding to LFA-1 serves to direct neutrophils towards the inter-endothelial junctions, thus promoting paracellular migration [112]. Another important feature of the early stages of the paracellular migration pathway is the reduced strength of the inter-endothelial junctions, which is primarily mediated through redistribution of the molecule vascular endothelial (VE)-cadherin [113,114]. In addition, the integrity of the inter-endothelial junctions is compromised by increased levels of intracellular endothelial Ca^{2+}, the consequence of which is endothelial cell contraction *via* activation of the myosin light-chain kinase [115].

Upon localising to the site of paracellular migration, a range of molecules expressed within the endothelial junction are involved in facilitating passage of the neutrophil through the endothelium. These include the junctional adhesion molecule (JAM) family members JAM-A, JAM-B and JAM-C, platelet/endothelial-cell adhesion molecule 1 (PECAM-1), endothelial cell-selective adhesion molecule (ESAM) and CD99 [107,108,116,117]. Evidence from antibody and knockout mouse studies has demonstrated the fundamental role that these molecules play in transmigration, with ablation of their function resulting in inhibition of neutrophil passage through the endothelium. Using mice deficient in JAM-A or PECAM-1, Woodfin et al. revealed that these molecules act sequentially to mediate transmigration, with JAM-A directing the neutrophil through the endothelial junction *via* interaction with neutrophil expressed JAM-A or LFA-1, while PECAM-1 supports migration through the vascular basement membrane *via* an interaction with $\alpha_6\beta_1$ integrin [112]. Recently, with the use of blocking antibodies in a mouse model, JAM-C was shown to not only regulate transmigration of neutrophils from the vasculature and into the extracellular space, but to also prevent reverse migration of neutrophils back into the blood stream [118]. Studies have also demonstrated the importance of ESAM and CD99 as components of the paracellular migration pathway, with both molecules supporting homophilic interactions on the neutrophil surface [119–121].

Recently, data has also emerged about an intracellular endothelial structure, known as the lateral border recycling compartment (LBRC), which is critical to the transmigration process [122,123]. The LBRC is a reticulum of interconnected tubulovesicular structures that lies just beneath the plasma membrane adjacent to the endothelial cell borders. The vesicles are enriched for JAM-A, PECAM and CD99, all molecules known to be implicated in paracellular migration. In fact, it is likely that many of the observations described above involving JAM-A, PECAM and CD99 were mediated as part of the LBRC. During transmigration, membrane from the LBRC is targeted to the endothelial junction where it surrounds the leukocyte and establishes interactions between the respective surface molecules [122,123]. The LBRC membrane is thought to assist the passage of the leukocyte through the endothelial junction by removing structural barriers to transmigration, such as VE-cadherin and associated catenins. The importance of the LBRC is illustrated by the observation *in vitro* that disruption of LBRC trafficking to the migration site blocks leukocyte transmigration [123].

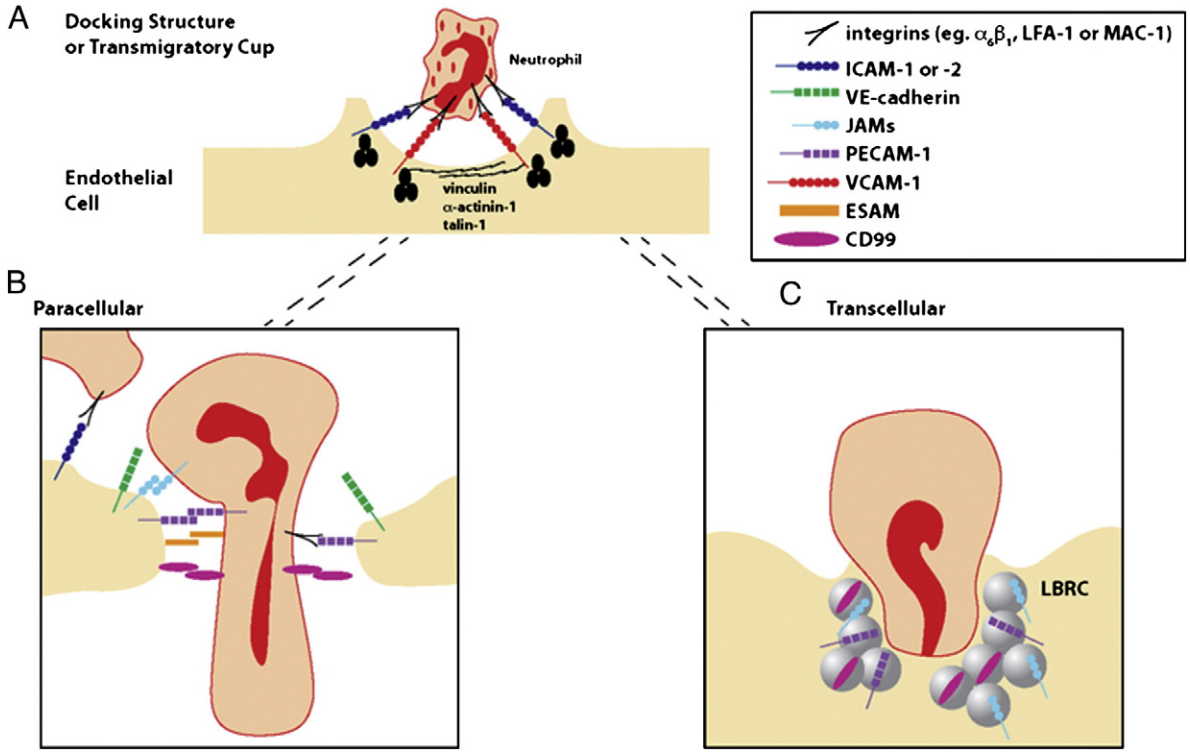

Fig. 3. Transmigration of neutrophils begins with the development of transmigratory cups (A) and can occur *via* two mechanisms, paracellular (B) or transcellular (C) migration. The processes underpinning these events are regulated by families of adhesion molecules expressed by both the neutrophils and the activated endothelium.

Whilst there is conclusive evidence supporting the involvement of the LBRC and associated molecules in neutrophil transmigration, aspects of their roles in the overall pathway remain to be defined. In addition, numerous other molecules expressed on the endothelial surface have been implicated in neutrophil transmigration, indicating that many facets of the paracellular migration pathway are yet to be understood.

4.7.2. Transcellular migration

As previously discussed, the factors that determine whether a neutrophil will undergo paracellular or transcellular migration have not been resolved. It has been speculated that neutrophils will migrate *via* the transcellular pathway in vascular areas where endothelial junctions are tight, such as the blood–brain barrier, although this is inconsistent with the rates of transcellular migration that occur in regions with leaky junctions such as the postcapillary venules [124]. As with paracellular migration, where the neutrophil is directed towards the inter-endothelial junctions, transcellular migration also appears to involve selection of appropriate migration sites by the neutrophil. *In vitro* studies have demonstrated that migrating leukocytes probe the endothelial surface in search of permissive sites for trancellular migration, using structures known as invadosome-like protrusions (ILPs) [109,125]. The ILPs invaginate the surface of the endothelium in search of an area with minimal resistance, as this will provide the most efficient route for passage through the cell. Upon locating such a site, the ILPs progressively extend until they ultimately breach the basal endothelial membrane. Intracellular membrane vesicles located within the endothelial cytoplasm, such as vesiculovacuolar organelles, have been observed both *in vivo* and *in vitro* to become enriched around the site of the invaginating ILPs and are thought to assist in leukocyte migration by creating a 'gateway' through the body of the endothelial cell [125,126]. Mamdouh et al. also recently reported that the LBRC is critical for transcellular migration of neutrophils (Fig. 3C), similar to its involvement in paracellular migration with the recycling of CD99, PECAM-1 and JAMs involved in this process [127]. This illustrates that despite possessing vastly different modes of action, paracellular and transcellular migrations share many similarities in terms of molecular interactions and mediators, although much remains unknown about the complete mechanisms that drive both pathways.

5. Therapeutic applications

Despite the immense amount of research devoted to inflammatory disorders, there is an urgent need to develop novel anti-inflammatory therapeutics as many of the current medications have limited clinical benefit. Aberrant activation of the immune system can often have detrimental effects and excessive neutrophil influx has been implicated in inflammatory disorders such as psoriasis, rheumatoid arthritis, inflammatory bowel disease and asthma [128]. A promising strategy to develop new anti-inflammatory drugs is to target the mobilisation and recruitment of neutrophils to the inflammatory site, particularly given the increasing knowledge of the mechanisms that underlie this process. Individual molecules involved in the mobilisation and recruitment pathway, such as selectins, integrins and chemoattractants, offer unique pharmacological targets and several have already been investigated for their therapeutic potential. One of the first anti-inflammatory agents to employ this approach was efaluzimab, a humanised monoclonal antibody against LFA-1 [129]. Produced by Genentech, efaluzimab was approved for the treatment of psoriasis in 2003 but was withdrawn from the market in 2009 due to its association with progressive multifocal leukoencephalopathy, a potentially fatal side effect thought to be related to the drug immunosuppressive properties [130]. Another integrin inhibitor, natalizumab, which targets integrin $\alpha_4\beta_1$, is approved for use against multiple sclerosis [131] and Crohn's disease [132] but has also had its use restricted due to the increased risk of developing progressive multifocal leukoencephalopathy [130]. Whilst demonstrating improved efficacy against several inflammatory disorders, these integrin blocking approaches carry significant risks related to impaired host response and future work will need to focus on minimising these side effects.

As previously discussed, blockade of selectins or PSGL-1 has been demonstrated in animal models to inhibit several inflammatory conditions and this strategy has shown some promise in human trials. An example is Bimosiamose, a pan-selectin inhibitor developed by Revotar Biopharmaceuticals, that has reported positive results from human phase IIa clinical trials for chronic obstructive pulmonary disease and asthma [133,134]. Inhibition of chemoattractants and their receptors is also an approach that has been successfully employed in animal studies and has led to the development of several agents that are now undergoing human clinical trials. The complement factor C5a, which acts through its neutrophil expressed receptor C5aR, plays an important role in mobilising neutrophils from the bone marrow and dysregulation of C5a or C5aR has been identified in numerous inflammatory disorders [135]. This pathway has been targeted by a monoclonal antibody against C5aR developed by G2 Therapies and Novo Nordisk, which is currently in a phase I clinical trial for rheumatoid arthritis [136]. In addition to inhibiting molecules such as integrins, selectins and chemoattractants, another area that may yield promising targets for anti-inflammatory therapies is the sphingolipid pathway. In a recent study, Sun et al. demonstrated that inhibition of SK-1 significantly reduced the number of rolling neutrophils in both an *in vitro* and *in vivo* model of allergic inflammation [63]. With many of these therapies showing promise in both pre-clinical models and early phase clinical trials, it is anticipated that the strategy of inhibiting neutrophil mobilisation and recruitment will identify effective anti-inflammatory treatments for many conditions. However, a critical aspect of any potential therapies is that they must avoid compromising the host response, which may entail the use of highly specific inhibitors or combinatorial treatments.

6. Conclusion

The complex and multi-faceted relationship that exists between neutrophils and the vascular endothelium forms the basis of the innate inflammatory response that is an integral part of the human body's defence mechanism. From the initial mobilisation of neutrophils from the bone marrow, to the ultimate transmigration of the neutrophil through the endothelial cell layer, both the neutrophil and vascular endothelium are involved in a series of interactions that orchestrates this vital systemic response. Recent advances in imaging and molecular biology techniques, along with the advent of a range of new *in vivo* models, has allowed significant advances in our understanding of the mechanisms involved in neutrophil mobilisation and recruitment. In particular, new insights have been gained into the molecular interactions involved in the adhesion cascade, which has not only included the identification of additional discrete steps in the cascade, but also recognising the intricacy of established responses such as transmigration. This is well illustrated by the recent finding that sphingosine kinase, a molecule never previously associated with the acute inflammatory response, mediates surface expression of P-selectin and the subsequent adhesion of circulating neutrophils [63]. Whilst progress has been made, there are still many aspects of the neutrophil–endothelial relationship that need to be further investigated, such as what factors determine whether a neutrophil undergoes paracellular or transcellular migration. The benefits of understanding these processes could be immense and could lead to the identification of novel pathways and targets that could be utilised in the search for treatments to promote host defence or mitigate inflammatory tissue injury.

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