Regulatory Role of Semaphorin 3E on Human Neutrophils Migration

by

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List of Abbreviations

Arf6: ADP-ribosylation factor 6

APAAP: Alkaline Phosphatase - Anti-Alkaline Phosphatase

APC: allophycocyanin

ANOVA: analysis of variance

BAL: bronchoalveolar lavage

C5a: complement component 5 alpha

CCR9: C-C receptor 9

CD4: cluster of differentiation 4

CD8: cluster of differentiation 8

CDC42: cell division control protein 42 homolog

CI: chemotactic index

CCL25: C-C motif chemokine 25

CRs: complement receptors

CNS: central nervous system

COPD: chronic obstructive pulmonary disease

cPLA2: cytosolic phospholipase A2

CXCR-4: C-X-C chemokine receptor type 4

CXCR-1: C-X-C chemokine receptor type 1

CXCR-2: C-X-C chemokine receptor type2

Dex: dexamethasone

Dll4: c-jun N-terminal Kinase (JNK) Delta-like 4

DOCK2: dedicator of cytokinesis 2

DP: double positive thymocyte $CD4^+$ $CD8^+$

ECM: extracellular matrix

ESL-1: E-selectin ligand 1

FACS: fluorescence-activated cell sorting

FAK: Focal Adhesion Kinase

Fc: fragment crystallizable

FcεRI: Fc epsilon receptor I

FcαR: Fc-alpha receptor

FcγRI: Fc-gamma receptor I

FITC: fluorescein isothiocyanate

fMLP: N-formyl-methionyl-leucyl-phenylalanine

GAP: GTPase Activating Protein

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

G-CSF: granulocyte colony stimulating factor

GDIs: GDP dissociation inhibitors

GEFs: guanine nucleotide exchange factors

G-ELISA; GTPase Enzyme-linked immunosorbent assay

GM-CSF: granulocyte-macrophage colony-stimulating factor

GTPases: guanosine triphosphatases

HRP: horseradish peroxidase

ICC: immunocytochemistry

IPT : Immunoglobin-like fold shared by
Plexins and Transcription factors domains)

ICAM: intracellular adhesion molecule

IL-8/CXCL-8: interleukin-8

LFA-1: Lymphocyte function-associated antigen 1

LPC: lysophosphatidylcholine

LPS: lipopolysaccharide

LTB4: leuktriene B4

mAb: monoclonal antibodies

Mac-1: Macrophage-**1 antigen**

MFI: mean fluorescence intensity

MPO: myeloperoxidase

MMP9: matrix metalo-ptoteinases-9

MRS: MET-related sequence

NOD: nucleotide-binding oligomerization domain protein

NLRs: NOD-like receptors

NETs: neutrophil extracellular traps

Nrp1: neuropilin-1

NADPH: nicotinamide adenine dinucleotide phosphate

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

TNF : tumor necrosis factor

PAMPs: pathogen associated molecular patterns

PBMC: peripheral blood mononuclear cells

PBS: Phosphate buffered saline

PE: phycoerythrin

PGN: peptidoglycan

PI3Ks: phosphatidylinositol 3-kinases

PKCζ: protein kinase Cζ

PLC-g: phospholipase C g

PMNs: polymorphonuclear leukocytes

PNS: peripheral nervous system

PRRs: pattern recognition receptors

PSGL-1: P-selectin glycoprotein ligand-1

PSI: plexin-semaphorin-integrin

PVDF: polyvinylidene fluoride

RBD: GTP biding domain

RPM: revolutions per minute

ROS: reactive oxygen species

RT-PCR: reverse transcription-PCR

SDS: sodium dodecy1 sulfate

SDS-PAGE: polyacrylamide gels

SDF-1: stromal cell -derived factor-1

Sema3E: semaphorin3E

SP: single positive cells $CD4^-$ $CD8^+$ or $CD4^+$ $CD8^-$

TBST: Tris-Buffered Saline and Tween 20

TM: transmembrane domain

Th1: T helper 1 cells

VCAM: vascular cell adhesion molecule

VEGFR2: vascular endothelial growth factor receptor-2

VEGF: vascular endothelial growth factor

Uncl- Sema3E: uncleavable 87 kDa of Sema3E

ABSTRACT

Semaphorin3E (Sema3E) is a secreted protein that was originally implicated in the development of the nervous system. However, its role in processes other than neuronal guidance is not fully understood. Sema3E interacts with the receptor PlexinD1 with high affinity. Furthermore, differential expression of PlexinD1 with neuropilin-1 (Nrp1) and Vascular Endothelial Growth Factor Receptor-2 (VEGFR2) determines pro-migratory or anti-migratory property of Sema3E. Recent studies demonstrated that semaphorins exhibit an inhibitory effect in most of inflammatory diseases. Among all inflammatory cells, neutrophils are an indispensable component of innate immunity and they are the foremost cells migrating to the site of inflammation. Substantial evidence indicated that the number of neutrophils is elevated in many inflammatory diseases. The aim of this study is to determine the expression pattern of Sema3E and its receptors, PlexinD1, Nrp1 and VEGFR2 in human neutrophils and to investigate the role of Sema3E on neutrophils' migration.

Here we found that isolated human neutrophils, from peripheral blood of healthy volunteers, constitutively express Sema3E and its receptors PlexinD1 and VEGFR2 at both protein and mRNA level; however, Nrp1 expression was not detected in these cells. Additionally, Sema3E display a potent ability to inhibit CXCL8/IL-8 induced neutrophils migration as determined by transwell *in vitro* system and microfluidic device coupled to real-time microscopy. Our data showed that Sema3E modulates the migration of neutrophils induced by the most potent chemoattractant stimuli, CXCL8/IL-8, suggesting an important regulatory role of this pathway in inflammatory diseases associated with neutrophilia.

1. INTRODUCTION

1.1 Chapter 1: Neutrophils

Neutrophils or polymorphonuclear leukocytes (PMNs), are an indispensable component of innate immunity and the foremost cells that migrate to the site of inflammation [1]. Their ability of rapid surveillance and eradication of foreign antigens was described early in the twentieth century. Neutrophils released from the bone marrow have been considered as fully differentiated cells with rapid turnover rate in blood, short tissue life span, limited metabolic capacity and confined ability to synthesize new proteins [2-3]. Paradoxical to these classical findings, recent studies demonstrated that neutrophils have a potent transcription capacity and strong ability to synthesize a large variety of proteins in response to various stimuli [4-5]. Thus, neutrophils elicit diverse functions as regulatory agents of the immune function through their production of various cytokines and chemokines such as IL-1α, IL-1β, IL-18, and CCL2 [6]. The proper perspective of neutrophils is gradually expanding as an important component involved in serious human diseases characterized by tissue neutrophilia and/or defective neutrophil function.

Neutrophils are released from pluripotent hematopoietic stem cells in the bone marrow where 60% of leukocytes are granulocyte precursors [5,7]. Neutrophil maturation occurs in multiple steps in a long process which takes several days. For instance, differentiation from myeloblasts to myelocytes requires ~7.5 days, whereas differentiation from myelocytes to mature neutrophils or granulocytes takes ~ 6.5 days[8, 9, 10].

During the maturation steps, cellular machinery is precisely regulated in order to develop into well-characterized mature neutrophils. This includes improving phagocytic capacity followed by enhancing oxygen-dependent microbicidal activity, elevating adhesiveness, cell motility, and promoting chemotactic activity [8, 9, 10]. Interestingly, the number of mitochondria and ribosomes decline during maturation, thereby equipping the cytoplasm of mature neutrophils with glycogen granules, which are their primary source of energy[3,11]. Four subsets of neutrophil granules that are initiated during the maturation process exist in mature neutrophils (Figure1.1)[12]. The first granules generated during granulopoiesis are called primary or azurophilic granules. These granules contain several types of proteolytic enzymes which encompass cathepsins, proteinase-3, elastase and myeloperoxidase (MPO), and also contain antimicrobial agents which are characterized by bactericidal activity [3,13, 14]. Azurophilic granules are known to be the most potent microbicidal compartment activated during phagocytosis[15]. The second major granules are named as secondary or specific granules. They are developed late during maturation in the bone marrow. These secondary granules comprise of several types of important proteins that act as receptors for distinct immune cellular components including chemotactic peptides, cytokines, opsonins, adhesion proteins, and extracellular matrix proteins. Also, secondary granules contain proteins that are localized in the plasma membrane such as lactoferrin and collagenase[3, 13, 16, 17, 7] The third granules to emerge are known as tertiary granules (gelatinase granules). Lastly, the fourth granules are similar to lysosomal membrane markers and called as secretory granules present only in fully differentiated neutrophils[18].

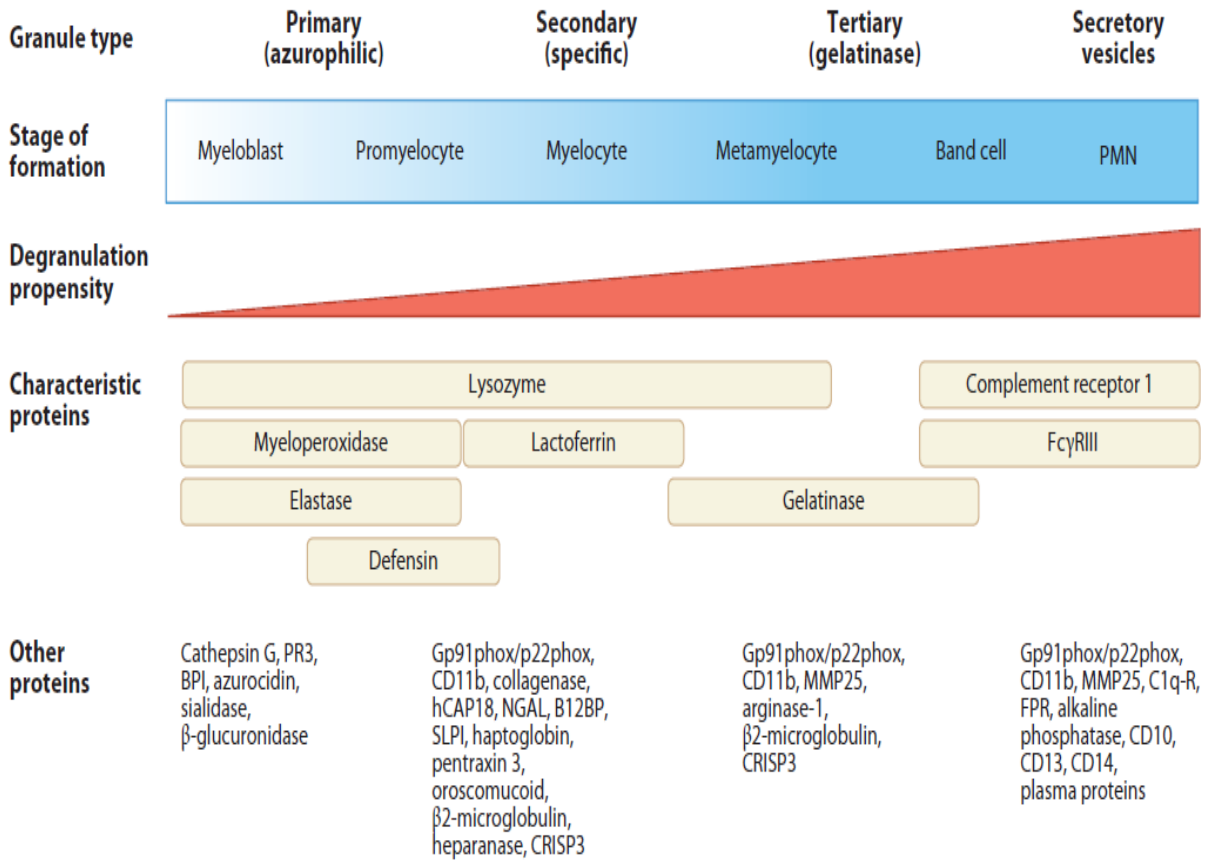


Figure1.1. Granules biosynthesis during neutrophils maturation in the bone marrow[19]

Neutrophils granules synthesis in the bone marrow during maturation step. They are divided into four types (primary or azurophilic, secondary or specific, tertiary or gelatinase and secretory vesicles). Granule biosynthesis is determined by the time point of neutrophil haematopoiesis in the bone marrow[19].

1.1.2 Neutrophils' role in fighting against invading pathogens

In inflamed organs, neutrophils recognize a wide range of surface-bound and secreted microbial products which are also known as pathogen associated molecular patterns (PAMPs) such as CpG-containing DNA and lipopolysaccharide (LPS). These molecules are directly recognized by receptors, pattern recognition receptors (PRRs), expressed on the surface of neutrophils. PRRs include toll-like receptors (TLRs) [20, 21], nucleotide-binding oligomerization domain protein (NOD), NOD-like receptors (NLRs) and the collectin family [22]. Activation of PRRs induces downstream signalling events that mobilize numerous cellular functions of neutrophils. They prolong neutrophil life span, release chemokines and cytokines, promote phagocytosis and enhance antimicrobial activity. Among all PRRs, TLRs are becoming widely regarded as critical receptors in the pathogen-mediated priming process. The vast majority of TLR members are expressed on human neutrophils and mediate several cellular activities. They promote neutrophil degranulation, a process in which cells release their granules' contents to the surrounding environment, and enhance reactive oxygen species (ROS) production and release to the surrounding areas [13, 7].

Similar to some of the TLRs, NLRs are located in the cytoplasm to detect intracellular microbial products. For instance, NOD2, a member of the NLR family, recognizes muramyl dipeptide derived from *Staphylococcus aureus* peptidoglycan (PGN). Activation of this receptor induces a downstream signalling cascade that involves targeting NF-κB and enhances neutrophil activation. In addition, collectins are another type of PRR receptors that are expressed on the cell surface and

recognize carbohydrate moieties. Their function is to enhance neutrophil recognition of microbes, and some studies indicate that PRRs directly induce phagocytosis and may act as co-receptors [32].

Neutrophils destroy invading pathogens using two different strategies: (1) oxygen-dependent activity [23], and (2) oxygen independent activities [24, 7]. In the oxygen-dependent process, the elimination of microbes by neutrophils mainly occurs through phagocytosis or by taking up the entire pathogen. This process is associated with the generation of microbicidal activity and an increase in reactive oxygen species (ROS) production [25, 26]. Notably, the efficient phagocytosis ability of neutrophils occurs with opsonized microbes via two different receptor subclasses: complement receptors or/and Fragment crystallizable (Fc) receptors [27, 28]. The recognition of complement-opsonized microbes is mediated by complement receptors (CRs) that are expressed on the surface of neutrophils including CR1[29, 30], CR3 [31, 32], and CR4 [33], while antibody-coated microbes detected by specific receptors recognize the Fc-region of antibody coated microbes [34]. These receptors are expressed on the surface of neutrophils and they include FcεRI [35], FcαR [43], FcγRI [7], FcγRIIA [36], and FcγRIIIB [37, 38]. Among all these receptors, FcγRII and CR3 are the most functional phagocytic receptors, whereas CR1 and FcγRIIIB primarily act as co-receptors to promote the activity of PRRs and finally lead to the full activation of the phagocytic process [12].

When neutrophils are fully activated, they synthesize a wide variety of genes encoding immunomodulatory mediators that modulate subsequent neutrophil functions. These factors include IL-1α, IL-1β, IL-1ε, IL-1RN, IL-6, IL-8, IL-10, IL-12β, IL-15, IL-18, CCL2, CCL3, CXCL1, CXCL2, CXCL3, CXCL12, CCL20, tumor necrosis factor (TNF) and vascular endothelial growth factor (VEGF). Moreover, during inflammatory circumstances, these agents

play an important role in modulating the early responses of other innate immune components such as monocytes, macrophages, dendritic cells, and lymphocytes. Neutrophils also play a prominent role in linking innate immunity cells with adaptive immunity cells and as a result priming adaptive immunity cells to amplify their proper response [6]. During this process, neutrophils produce cytokines and chemokines which influence dentritic cell maturations and, in turn, T cells proliferation and polarization [6].

Neutrophils can also phagocytose invading pathogens via a different process called neutrophils degranulation, which is an oxygen independent activity. In this procedure, neutrophils fuse with the plasma membranes of the target pathogen (exocytosis) or with phagosomes to release their granule contents, thus killing the target microorganism [39]. About 50% of azurophilic granules proteins contain α- -defensins [40], which is considered as the most powerful antimicrobial peptide [41, 42, 43]. Thus, the fusion of neutrophil azurophilic granules with phagosomes results in enriching vacuole lumen with plenty of anti-microbial peptides, α-defensins in particular [34]. Thse anti-microbial peptides are released by neutrophils and contribute to several functions of innate host defence such as chemotaxis and wound repair [44]. Furthermore, degranulation activity equips phagosomes with specific granule molecules such as flavocytochrome *b* [45] and lactoferrin [46], which has several important functions. For instance, lactoferrin mediates numerous important activities contributing to the killing of target pathogens. This include iron deposition that is necessary for microorganism growth; generation of iron in order to supply required neutrophil hydroxyl radicals [47] and increase membrane permeabilization to modulate direct anti-microbial activity [48, 21].

In addition to ROS production and neutrophil degranulation, there is another mechanism of killing target microbes by the formation of neutrophil extracellular traps (NETs) that contain decondensed chromatin structures filled with antimicrobial molecules including azurophilic granule proteins and cytosolic proteins. This process provides neutrophils with a potential capacity of killing a wide range of invading pathogens such as bacteria and fungi [49]. Moreover, recent studies have indicated that NETs formation by mobilized neutrophils require nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase [50, 51]. NADPH oxidase is an electron transport enzyme that converts oxygen to superoxide anions and induce superoxide production. During "respiratory bursts", neutrophils are able to generate enormous amounts of activated NADPH oxidase which are synergized with other molecules including NETs to eradicate the target microbe [52].

Under normal circumstances, approximately 10^9 neutrophils/kg body weight are released from the bone marrow into the body each day [53]. Since neutrophils contain a huge amount of cytotoxic agents, it is critical to clear excessive numbers of these cells in the site of inflammation to prevent tissue damage. Thus, neutrophils undergo a programmed cell death (apoptosis) or express proapoptotic molecules that could be recognized by tissue macrophages present in the same inflamed area to perform their phagocytosis activity of apoptotic neutrophils [21, 54]. Interestingly, it has been observed that senescent neutrophils can circulate back to the bone marrow as well [65]. Mature neutrophils express chemokine receptors such as CXCR2 and CXCR1 (receptors for intrelukin-8 (CXCL8/IL-8)). These receptors direct neutrophils to rapidly migrate to the site of infection. In senescent cells, the expression of these receptors is downregulated, whereas other chemokine receptors, such as CXCR4, are highly expressed in order to direct neutrophils back to the bone marrow where stromal cell derived factor-1 alpha (SDF-1a), the CXCR4 ligand, is

constitutively expressed. Collectively, neutrophil accumulation and migration to inflamed organs is reduced via a feedback mechanism dependent on CXCR4 expression. The fate of senescent neutrophils upon reaching the bone marrow remains unclear and needs further investigation [55].

1.1.3 Neutrophils migration and chemoattractant factors

Mature neutrophils are released into the circulatory system with a short half-life from 6 to 8 hours [8, 56, 57]. Under some stimuli circumstances and chemoattractant factors, neutrophils migrate to inflamed organs and therefore prolong their life span by 1 to 2 days [3, 8, 58, 59, 19, 11]. In normal human adults, the production of neutrophils is generated from 1 to 2 x 10^{11} cells per day [60]. During inflammation, their number increases further under the influence of granulocyte colony stimulating factor (G-CSF) so that there are sufficient cells to fight against specific microorganisms [61, 60]. Interestingly, most of the chemoattractant factors behave as priming molecules that reinforce neutrophils capacity to response efficiently to the second stimuli. In inflammatory circumstances, several cell types such as monocytes, endothelial cells and epithelial cells release a range of chemoattractant stimuli including complement component C5a, Leuktriene B4 (LTB4), bacterial peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP), and the neutrophil chemoattractant factor (CXCL8/IL-8). These factors have a potential capacity to enhance neutrophil chemotaxis activity, and promote rapid recruitment to the inflamed organ [62, 63]. At the site of inflammation, endothelial cells, when responding to stimuli, express selectins and integrin-mediated cellular adhesion, intracellular adhesion molecule (ICAM), and vascular cell adhesion molecule (VCAM) [64]. These molecules allow neutrophils to perform rolling, tethering, firm adhesion, spreading and finally engage in diapedesis (Figure1. 2). Moreover,

various chemoattractant factors that released by activated endothelial cells create a gradient. Based on the chemokine gradient, several receptors including the above mentioned, are up regulated and eventually assist neutrophils to sense the signal and facilitate directional migration towards target tissue [12, 65, 66] .

The initiation contact between neutrophils and endothelial cells occurs through the binding of both selectins (E- and P-selectin) to the constitutively expressed P-selectin glycoprotein ligand-1(PSGL-1) on the tips of neutrophils. Binding of E-selectin to E-selectin ligand 1 (ESL-1) then allow slow rolling of neutrophils [67].This binding process provides sufficient strength to enhances neutrophils attachments to the endothelial cells during the shear stress of the laminar blood flow in vessels (Diagram.2) [68]. Binding of PSGL-1 ligands induces signalling pathways that mediate the activation of phospholipase C □ (PLC-□), Phosphoinositide 3-kinase (PI3K), and p38 mitogen-activated protein kinase, molecules that induce integrin activation and cytoskeleton rearrangements in neutrophils [69-71]. Then adhesion molecules β2 integrins, lymphocyte function-associated antigen 1 (LFA-1), (α2 β2) and Mac-1 (αmβ2) are present on activated neutrophils while their ligands [72], members of the immunoglobulin superfamily, ICAM-1 and ICAM-2, are present on endothelial cells [55,56].

The transition process from rolling to adhesion of neutrophils is entirely regulated by LFA-1 that is expressed on the surface membrane of endothelial cells [73]. Although E-or P-selectin extends the rolling of neutrophils, they do not exhibit the high affinity form of LFA-1 [74]. PSGL-1 induces the activation of LFA-1 mediated rolling through binding to ICAM-1[75]. The full activation of LFA-1 is provided by activated chemokine receptors, which bind to their chemokine ligands that

are secreted by endothelial cells [76]. As a results, the process of rolling and firm adhesion of neutrophils occurs through the fully activated LFA-1[69]. Then, neutrophils polarize and gain entry to the endothelial cells through the concentrated chemokine and phagocytosis receptors at the leading edge [77-78]. The activity of neutrophil chemotaxis is organized and controlled entirely by F-actin and G1 type G protein coupled receptors respectively (GPCRs) as explained below [77-78].

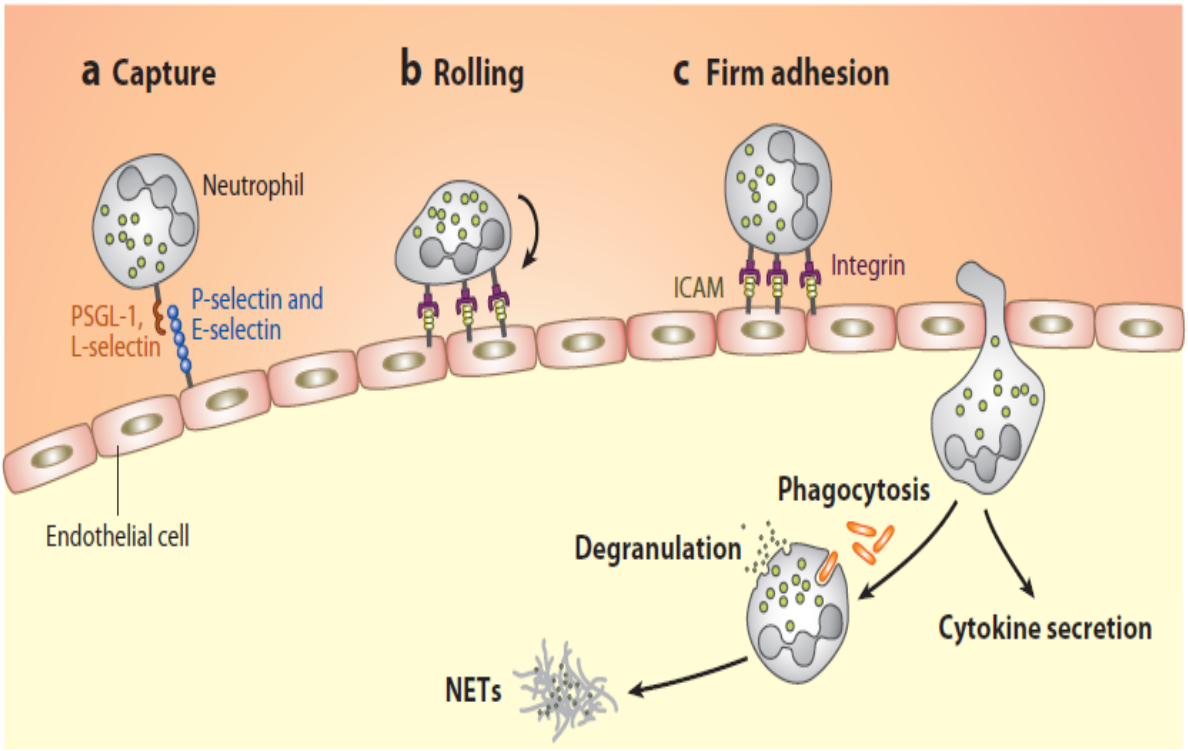


Figure1.2. Neutrophils migration to the inflamed organ [19]

Activated endothelial cells express P- and E-selctin and other adhesion molecules ICAM and VCAM. Thus, activated neutrophils, which express ligands of these receptors, perform rolling, tethering and firm adhesion with endothelial cells. Finally, in response to chemokine gradients, neutrophils emigrate through endothelial cells toward chemokine sources[11,19].

1.1.4 CXCL8/IL-8 signalling in human neutrophils

During inflammation, neutrophils are rapidly recruited and migrate to the injured tissues. The fast response of neutrophils requires multiple cellular processes to enhance cell adhesion and chemotaxis activity as indicated above. These processes induce the mobilization of downstream signalling events of several membrane receptors including adhesion molecules such as E-selectin P-selectin, β2 integrin and chemokine receptors[12]. Upon activation of specific chemokine receptors expressed in neutrophils, cells begin chemotactic activity toward the source of the target chemokine. Chemotaxis activity of neutrophils occurs due to actin cytoskeleton rearrangement and the expression of adhesion molecules, therefore facilitating their movement within the extracellular matrix toward the chemokine gradient. Following gradient recognition, neutrophils rapidly establish a leading edge towards an area of high chemokine concentration. Following receptor/ligand interaction, the receptor transmits the extracellular signals to the intracellular cytosol and triggers the downstream signalling required to induce complex mechanisms (restriction of highly stimulated regions). Actin polymerization and accumulation at the leading edge is the last and the main target of chemotaxis activity [77].

As mentioned previously, CXCL8/IL-8 is the most potent chemoattractant factor and recruits neutrophils to the site of inflammation. It interacts with two distinct receptors that are expressed in human neutrophils, CXCR1 and CXCR2. These two receptors consist of seven transmembrane receptors known as GPCRs [79, 80] (Figure1.3). In neutrophils, the activation of GPCR induces the mobilization of a trimeric G protein to release Gβγ heterodimer from the inhibitory activity induced by Gαi [81]. Gi trimeric protein is essential for neutrophil chemotaxis. Active Gαi subunits

may not be directly involved in chemotaxis but it terminates the activity of Gβγ, a key regulator of many pathways which occur in response to chemoattractant activity in leukocytes [82]. Substantial evidence indicates that inhibiting Gβγ or their downstream effectors ameliorate the leukocyte response to chemoattractant stimuli. These effectors include PI3Ks, guanosine triphosphatases of the Rho family (Rho GTPases), and Rac GTPases, protein kinase Cζ (PKCζ), cytosolic tyrosine kinases, and cytosolic phospholipase A2 (cPLA2). Upon GPCR activation, Gβγ subunit activates and accumulates phosphatidylinositol 3-kinases (PI3Ks). The accumulation of PI(3,4,5)P3 triggers the translocation of Dedicator of Cytokinesis 2 (DOCK2) , a specific GDP to GTP exchange factor for Rac. The activation of DOCK2 then activates Rac GTPase and Cdc42 GTPase (family members of Rho GTPase) to mobilize actin polymerization at the leading edge of neutrophils. Moreover, PI(3,4,5)P3, Rac, and polymerized actin provide new signals as a positive feedback to further enhance the leading edge of neutrophils. Accumulation of PI3K in the leading edge is a key step in directing neutrophils towards a chemokine source [77].

1.2 Chapter 2 Semaphorins

1.2.1 Semaphorins

Guidance molecules are the key players in shaping mature patterns of neuronal connectivity[83, 84]. Their entire function is constructed to direct neuronal cells to their proper target through highly specialized structural molecules at the leading edge of neuronal cells known as growth cones. The interpretation of whether these growth cones should function in repulsion or attraction depends on the receptors and the intracellular signalling pathways involved [85].

Among all guidance cues, semaphorins comprise one of the largest protein families. They are key players during the development of the nervous system [85], particularly in the structures of several neuron cells which encompass cerebral cortex, hippocampus, olfactory bulb, the visual system, cerebellum, and the spinal cord [86, 87, 88, 89]. Although the semaphorin family was known initially to act as a repulsive cue, several studies indicate that, like other guidance cues, they could function as both repellent and attractant cues at the same time [90].

Nowadays, the vast majority of studies regarding semaphorins or their signalling establish the function of this family in a wide variety of cells outside the nervous system, and play fundamental roles in many physiological and pathological events [91] including angiogenesis [92, 93], tumor progression [94, 95], osteoclastogenesis [96], cardiogenesis [97, 98], and the regulation of immune responses [99, 86, 91]. Additionally, their central role in many cellular aspects has been well established in cell survival, apoptosis, proliferation, and directional cell migration [100, 101]. Semaphorins exist in two different forms: 1) secreted protein and 2) membrane binding protein.

Up until now [102, 103], more than 20 types of semaphorins have been identified and classified into eight subclasses on the basis of their sequence similarity and structural elements of their amino acids [102]. Classes 1, 2 and one of class 5 (Sema5c) have been found in invertebrates. while classes 3 to 7 have been identified in vertebrates, and class 8 in viruses only. Members of classes 1, 4, 5 and 6 are transmembrane proteins whereas class 2 and 3 are secreted proteins [102]. Class 3 semaphorins are the only proteins found in vertebrate in a secreted form and can be distinguished by a basic-charged domain at the C-terminus (Figure1.3) [104]. The terminology of semaphorins appears with the prefix "Sema" followed by a number referring to the subclass of the protein and finally followed with a capital letter to indicate the individual member within the subclass. For example, Sema3E refers to semaphorin subclass 3 member E [102].

The structure of different semaphorins is distinguished by several types of domain including immunoglobulin-like, thrombospondin, basic C-terminal domains, sema domain and plexin-semaphorin-integrin (PSI) domain. The function of semaphorins is regulated entirely by the extracellular sema domain that includes 500 N-terminal amino acids, followed by the receptor binding domain, a cysteine-rich motif PSI domain [94].

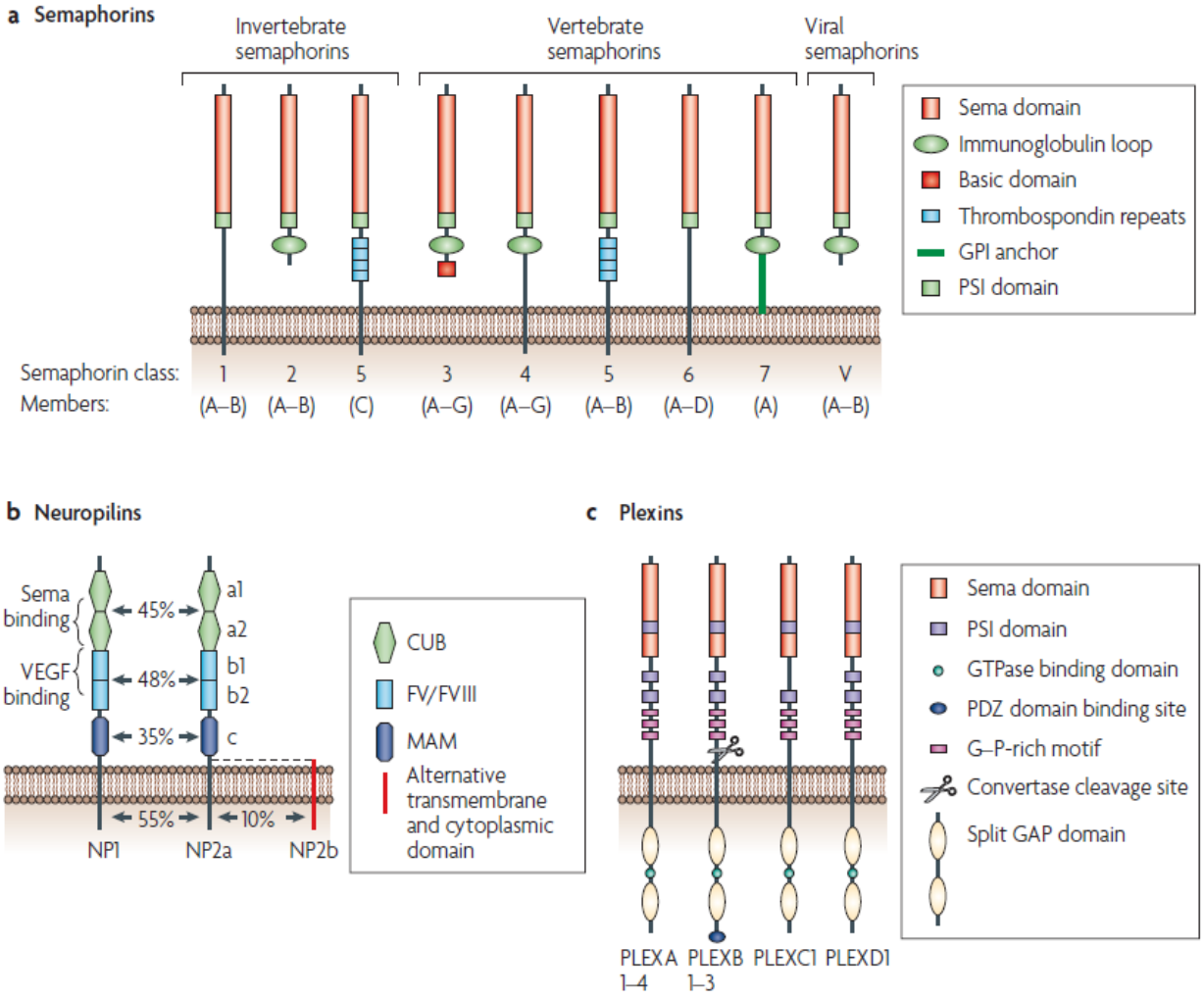


Figure1.3. Semaphorin family and their receptors (Neuropilins and Plexins)

(A) Illustration of the sequence similarity and structure feature of different semaphorin subclasses. (B) Two members of Neuropilins family transmembrane receptor. (C)Four family members of Plexins transmembrane receptors (A-D) characterized in the bases of their structure feature and sequence similarity[105].

1.2.2 Semaphorins' role in endothelial cells

As mentioned earlier, semaphorins are involved in various physiological aspects including the development of vascular system. Different classes of semaphorins have direct and indirect effects on the motility of endothelial cells, repelling blood vessels and promoting blood vessel formation. In addition, they might have an indirect effect through the modulation of angiogenesis of endothelial cells [106]. For example, Sema3A, one of the best studied class 3 semaphorins, exhibits both direct and indirect influence during vascular development. It impairs endothelial cell migration through inhibitory signals triggered by of recognition of its ligand in the extracellular matrix. This binding then enhances de-adhesion processes that are required for vascular remolding [93, 107]. Embryonic Sema3A-/- mice have shown a serious vascular defect in the head and abnormal blood trunk vessels [108]. Other members of class 3 semaphorins, Sema3E [109, 110] Sema3F [111] and Sema3B [112], exhibit anti-angiogenesis properties, while some class 4 semaphorins exhibit opposite roles in angiogenesis. For example, Sema4D has been found to enhance endothelial cell migration, proliferation and organization into new blood vessels [113]. In contrast, Sema4A, expressed in endothelial cells, inhibits VEGF-induced Rac-1 activation and integrin dependant adhesion [114]. Overall, each member of the semaphorin family can exhibit different roles even in the same cell type, such as endothelial cells [90]. Studies have indicated that semaphorins also play a pivotal role in the cardiovascular system. For example, through Sema3A-/- cardiac impaired mice, Sema3A has been found to generate cardiac sympathetic innervations and thus playing an important role in controlling the heart rate [115, 116]. Furthermore, Sema3C-/- mice display extensive cardiac dysfunction causing those mice to die within hours after birth due to congenital cardiovascular defects [117]. Moreover, morphological abnormality of the cardiac tube is associated with either suppression or over expression of Sema6D in chick

embryos, indicating that strict regulation of Sema6D expression is critical for cardiac tube formation [118, 90].

1.2.3 Immune semaphorins

The nervous and immune systems have several aspects that overlap and link with each other. For instance, it has been indicated that Ephrine, an axon guidance molecule, is involved in the regulation of immune cells [119, 120]. Also, immunological synapses that occur in antigen presentation by T cells are similar to the enological synapse which occurs in the neuron system. Semaphorins, as mentioned earlier, were initially discovered as repulsive axon molecules that guided neuronal axons to their proper targets. More recent evidence indicates that semaphorins are involved in the regulation of the immune system as well [91]. They play an important role in cell to cell interaction as well as in immune cell trafficking[121].

Sema4D is the first semaphorin that has been identified with an immunoregulatory function. Sema4D is expressed on T cells, B cells, activated B cells and mature dendritic cells (DC) [122, 123, 124]. It promotes production of antibodies and antigen specific T cells through the activation of B cells and DCs [122, 125, 126, 90]. Also, Sema4A expression has been detected constitutively in DCs, causing T helper 1 cell (Th1) polarization [127, 128]. It plays an important role in T cell priming via T cell-DC-cognate interaction[128] and production of Sema4A by T cells promote cell differentiation. Sema7A, another class of semaphorins, is also involved in immune cell interaction. It is released by activated T cells and stimulates monocytes/macrophages to induce proinflammatory cytokine production [129].

Semaphorins are also involved in immune cell trafficking. They do so by regulating cytoskeletal dynamics through the modulation of integrin mediated adhesion and actin contractility [87]. They also regulate immune trafficking in both primary and secondary lymphoid organs [91]. In the thymus, semaphorin3E promotes thymocyte differentiation and contributes to thymus development [130].

Other immune semaphorins that have been implicated in immune cell migration include Sema3A, Sema7A and Sema4D. Sema3A impairs chemokine CXCL-12 induced T cell and monocyte migration, whereas sema3A directed in the opposite site against chemokine gradient, it will enhance cell migration toward chemokine gradient [131]. Interestingly, Sema7A has been suggested to promote monocytes migration [129] while Sema4D has been found to inhibit chemokine induced monocyte migration [132]. All this evidence indicates the semaphorins' appreciable role in immune cell trafficking [91] .

1.2.4 Semaphorins role in the diseases

A huge body of evidence indicates that altered semaphorin expression is associated with pathophysiological consequences leading to neuronal diseases and malignancies. For example, in Alzheimer disease, Sema3A accumulation precedes a progressive mental dysfunction, causing degenerative effect in hippocampal neurons [133]. Also, the upregulation of Sema3A concidess with a decline in perception leading to mental disorder, whereas the downregulation of the same protein is associated with motor and sensory defects causing traumatic injury of the peripheral nervous system (PNS). The deregulation of semaphorins expression or their receptors causes

serious neurological defects of the nervous system, such as traumatic injury of the central nervous system (CNS), traumatic injury of PNS, ischemic stroke, neuroblastoma and many other neuronal diseases. The alteration of semaphorin expression in neuronal diseases is a fundamental therapeutic target in neurological disorders [90].

1.2.5 Semaphorin role in cancer cell migration

In addition to their function in neurological diseases, semaphorins are also key regulatory mediators in some tumor diseases. Semaphorins are released by cancer cells or by other cell types in tumor microenvironments. They exert multiple cellular functions in an autocrine manner, including cell survival, proliferation, apoptosis, cell adhesion and the most important aspect of tumor progression, cell migration [94]. It is well known that tumor cells have a potential capacity to migrate and invade surrounding tissues, thereby requiring the breakdown of cell matrix adhesion as well as the maintenance of some adhesion molecules at the leading edge of these cells. In this process, integrins play an important role in directing tumor cell migration by linking tumor cells with the extracellular matrix (ECM) and the intracellular cytoskeleton [85]. Since semaphorins can potentially regulate integrin expression, tumor progression can also be regulated by semaphorins. Thus, upon semaphorin binding to plexin receptors, conformational changes occurs and leads to rapid integrin focal adhesion disassembly prior to actin depolarization and cytoskeleton remodelling [134]. For example, it has been found that Sema3A inhibits the migratory property and invasion capacity of breast tumor cells [135] and can act as a suppressor of breast tumor metastasis through the mobilization of $\alpha 2\beta 1$[136]. However, this finding remains contradictory. Also, depending on the receptor complex expressed by tumor cells, Sema4D can inhibit or promote cell

migration; whereas plexin B1 alone impairs cell migration [134, 137], the involvement of the oncogenic PTKs Met and ErbB2 alter the inhibitory property of Sema4D and instead promote tumor growth and invasion [138, 95, 137]. Moreover, several other types of semaphorins, most likely class 3 semaphorins, mediate the regulation of several types of tumor migration including Sema3C, Sema3E, and Sema3F. In some cases, overexpression of these proteins might promote cell invasion and migration and finally, prolong tumor progression [94].

1.2.6 Semaphorin receptors

The high affinity receptors of semaphorins are plexins and neuropilins [139, 140, 141]. Plexins were discovered in 1995 in humans [142] and their first clone was completed in 1996. Plexins are comprised of a single type I transmembrane protein with calcium dependent homophilic cell adhesion properties [142, 143] Sema-plexin signaling plays important roles in axonal pathfinding and patterning in several organs and tissues [90, 89] . Four subclasses of plexins have been identified, including plexin-A, plexin-B, plexin-C and plexin-D (Figure1.3), whereas nine members of these subclasses have been discovered in vertebrates. The structure of plexin receptors is similar to semaphorin structure in which there is a sema domain in their extracellular matrix and two to three repeats of plexins, Semas and Integrin (PSI) domains and three of other additional domain Ig-like fold shared by plexins. Followed by immunoglobulin-like fold shared by plexins and transcription factor domain (IPT). However, the cytoplasmic tails of plexins are different as they contain two amino acids that are similar to GTPase activating proteins (GAPs)[144]. The neuropilin family of proteins is another group of semaphorin co-receptors (Figure1.3), and they have been

observed as co-receptors of both class 3 semaphorins and vascular endothelial growth factors (VEGF). Two neuropilin family members have been identified in vertebrate Nrp1 and Nrp2, each consisting of single transmembrane glycoproteins that share the same structural domain [139], [140, 106].

1.2.7Other Semaphorins co receptors

Dependent on both cell types and semaphorin subclass, several co-receptors for semaphorins exist in addition to the high affinity plexin family receptors. For instance, while membrane bound semaphorin interact directly with plexins, secreted class 3 semaphorins, with the exception Sema3E [101], require neuropilins as co-receptors. Also, CD27 and Tim2 (T-cell immunoglobulin and mucin domain-containg protein 2) were found to interact functionally with Sema4D and Sema4A in immune responses [92, 122].

1.2.8 Semaphorins' regulation of integrin through small GTPase

The involvement of semaphorins in the modulation of several cellular aspects, including cell survival, apoptosis, cell growth and cell motility [145, 146], requires the engagement of integrin in semaphorin signalling [147, 145, 146]. Several studies have indicated that the heterodimeric cell-surface receptors forming integrin are regulated by semaphorins. This activity triggers the downstream cellular events through intrinsic GAP activity found in the intracellular tail of PlexinD1. In normal circumstances, the activation of GTPase R-Ras promotes focal-adhesion

formation (Figure1.4), cell adhesion and might regulate cell spreading and cell migration through phosphatidylinositol-3-kinase. The optimal outcome of semaphorin/plexin interaction leads to GTPase R-Ras inactivation and sequesters it away from integrin, and therefore inhibits integrin-mediated adhesion to the extracellular matrix (ECM) and other downstream events. In addition, plexins regulate the activity of small GTPase RhoA through its intracellular transducer that is associated with the cytoskeleton domain. Thus, the activation of RhoA induces cell repulsion and finally impairs directional cell migration [148, 149, 150].

In some circumstances, one semaphorin could display both positive and negative regulation of cell adhesion and migration by binding to more than one plexin receptor. For instance, Sema7A exhibits opposite functions through binding to two different receptors [94]; it impairs cell-substrate adhesion when binding to plexin C1[151] but enhances cell spreading in melanocytes via β1 integrin[152]

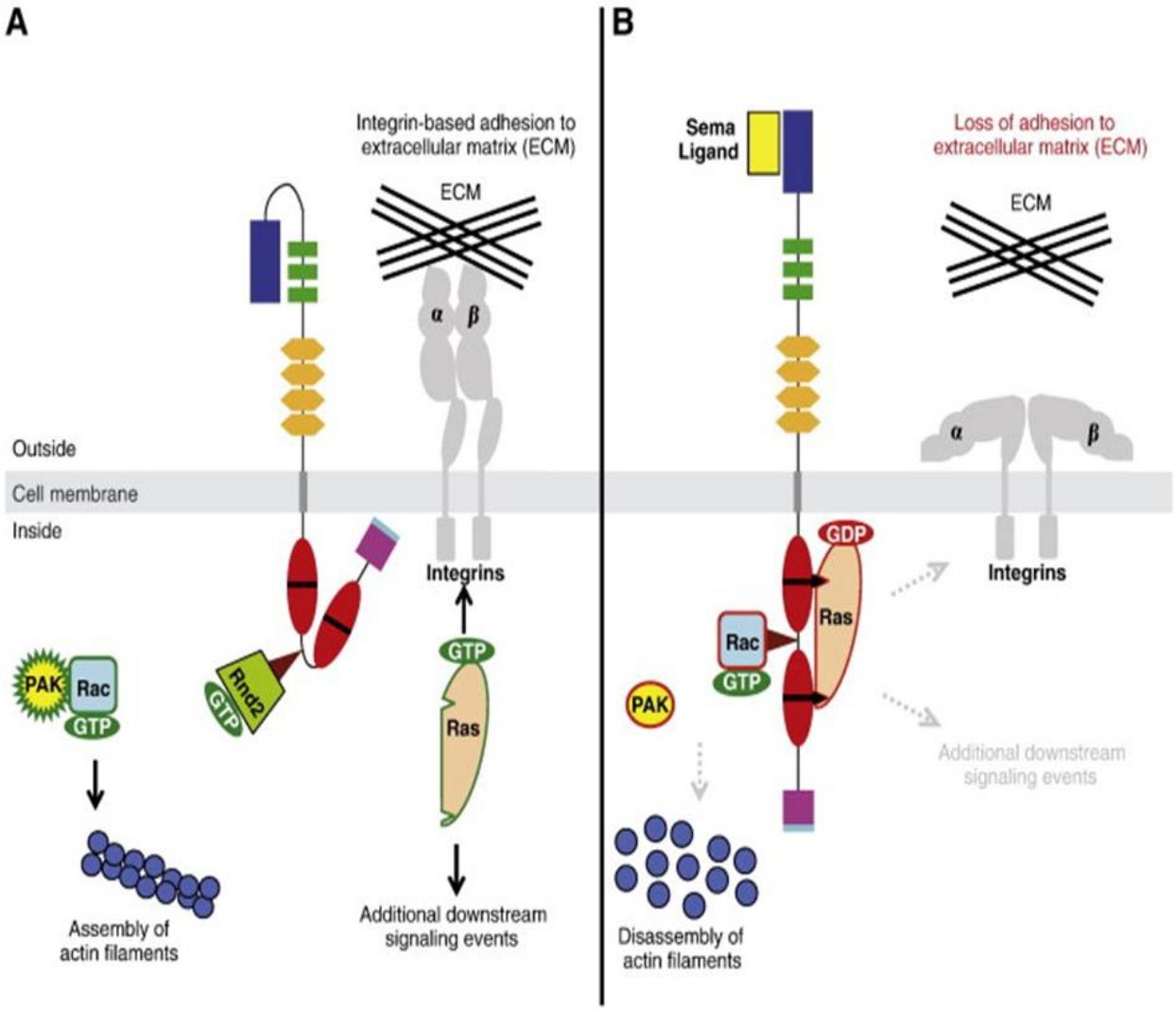


Figure 1.4. Schematic representative of the common model of Sema3E \ PlexinD1 interaction.

(A) In the absence of the ligand Sema3E, PlexinD1 loses function of GTP bound Rnd2 that located on its intracellular tail, allowing it to intact with active Rac and R-Ras. (B) Sema3E binding to PlexinD1 induces conformational changes that leads GTP bound Rnd2 interaction with the active form of both Rac and R-Ras [153].

1.3 Chapter 3 Class 3 Semaphorins

1.3.1 Class 3 Semaphorins

Class 3 semaphorins, originally identified in the nervous system as axon guidance factors, exhibit their function through binding to neuropilin-plexin complexes on the surface membrane. However, recent studies have shown wide spread expression of these molecules in several tissues and organs, displaying important function in endothelial cell patterning, tumour biology and the regulation of immune responses [154]. Seven soluble proteins (Sema3A -3G) with approximately 100 kDa have been discovered as Sema3s family. In general, Sema3s bind to plexins in a paracrine manner through a highly conserved amino-terminal with 500-amino acid called Sema domain. After binding to plexins, Sema3s induce intracellular signalling by binding to neuropilins. After forming a complex with Nrp, Sema3s mobilize a particular plexin subclass [155], with the exception of Sema3E that, in most of the cases, binds directly to PlexinD1[109]. With respect to their function as apoptosis inducers, the Sema3s' role in the vascular system has been well established; they inhibit angiogenesis, tube formation, sprouting, branching, survival, and endothelial cell adhesion. Also, in some tumor cells, Sema3s including Sema3A, 3B, 3D, 3F, and 3G inhibit angiogenesis and tumor growth as seen in breast cancer and lung cancer. However, this inhibitory function is not seen in all Sema3 members as some of them induce tumor angiogenesis, growth and metastasis. For example, Sema3C has been found to control vascularisation of tumours and promote their migration and metastasis spreading [156]. Sema3E expression has also been found to be associated with tumour progression; in vivo experiments showed that Sema3E enhances lung cancer metastasis, but not invasiveness. This process is

entirely regulated by Sema3E isoform p61 kDa that is known to alter the repellent activity of full length Sema3E [90].

1.3.2 Class 3 Semaphorins in Different Organs

In addition to their role in the vascular system, semaphorin class 3 proteins are important in the development of a variety of organs, including the lung, kidney, bone, and the tooth. During lung development, Sema3s display vigorous functions. For instance, upon binding to Nrp1, Sema3A displays an inhibitory function in foetal lung branching morphogenesis. Blocking the binding site of Nrp1to Sema3A prevents the inhibitory influence of branching morphogenesis induced by Sema3A [157]. On the other hand, Sema3C and Sema3F manifest the opposite function during lung development. *In vitro* experiments indicated that branch formation of the E11.5 lung was stimulated by Sema3C and Sema3F, exhibiting both negative and positive regulation of class 3 semaphorins in the lung [158]. Moreover, during kidney ontogeny, the expression of Sema3A and Sema3F, and their receptors Nrp1 and Nrp2 are deregulated, which might be important during kidney morphogenesis [159]. For example, the presence of recombinant Sema3A showed a reduction in ureteric bud branching whereas this branching was elevated by low expression of Sema3A[160]. Also, in immortalized podocytes, Sema3A regulates the expression of plexins, and it may induce podocyte apoptosis through an autocrine mechanism [161]. Furthermore, Sema3A is also involved in bone development as Sema3A-/- mice exhibit fusion of cervical bones, partial duplication of ribs and weak alignment of the rib-sternum junction [116]. Sema3B transgenic mice also manifest deficiencies in cancellous and cortical bone mineralization [162]. Furthermore, during tooth organogenesis in mouse embryos, most class 3 semaphorins, including Sema3A,

3B, 3C, and 3F and their receptors, Nrp1 and Nrp2, display a distinct developmentally regulated expression pattern [86,90]. For example, Sema3A controls the time duration of tooth innervations, dental axon navigation and patterning [163]. Overall, these studies provide insight into the pivotal role of the semaphorin family, particularly class 3 semaphorins, during developmental processes and in many pathophysiological aspects [90] .

1.3.3 Sema3E

Class 3 semaphorins in vertebrate are secreted proteins and encompass 7 members (sema3A-3G) [164]. Sema3E is a member of the class 3 semaphorin subclass, and binds to the receptor PlexinD1 with high affinity[165]; neuropilins (NRPs) can also interact with Sema3E as a co-receptor to modulate the downstream signalling events [166]. More recently, vascular endothelial growth factor receptor-2 (VEGFR2) has been identified as an additional Sema3E co-receptor [148]. Depending on the receptor complexes involved, the downstream signalling of Sema3E induces either cell repulsion or attraction (Figure1.5) [153]. For example, in the nervous system, some of the neuronal cells such as corticofugal and striatonigral axons, express PlexinD1 alone , thus displaying cell repulsion upon Sema3E binding (Figure1.5B left), while other neuronal cells, such as subiculo-mammillary neurons, express co-receptors Nrp1 and VEGFR2 in addition to PlexinD1, and exhibit cell attraction upon Sema3E interaction (Figure1.5B right) [148]. Therefore, differential expression of these receptors has a significant impact on Sema3E signalling and function [153].

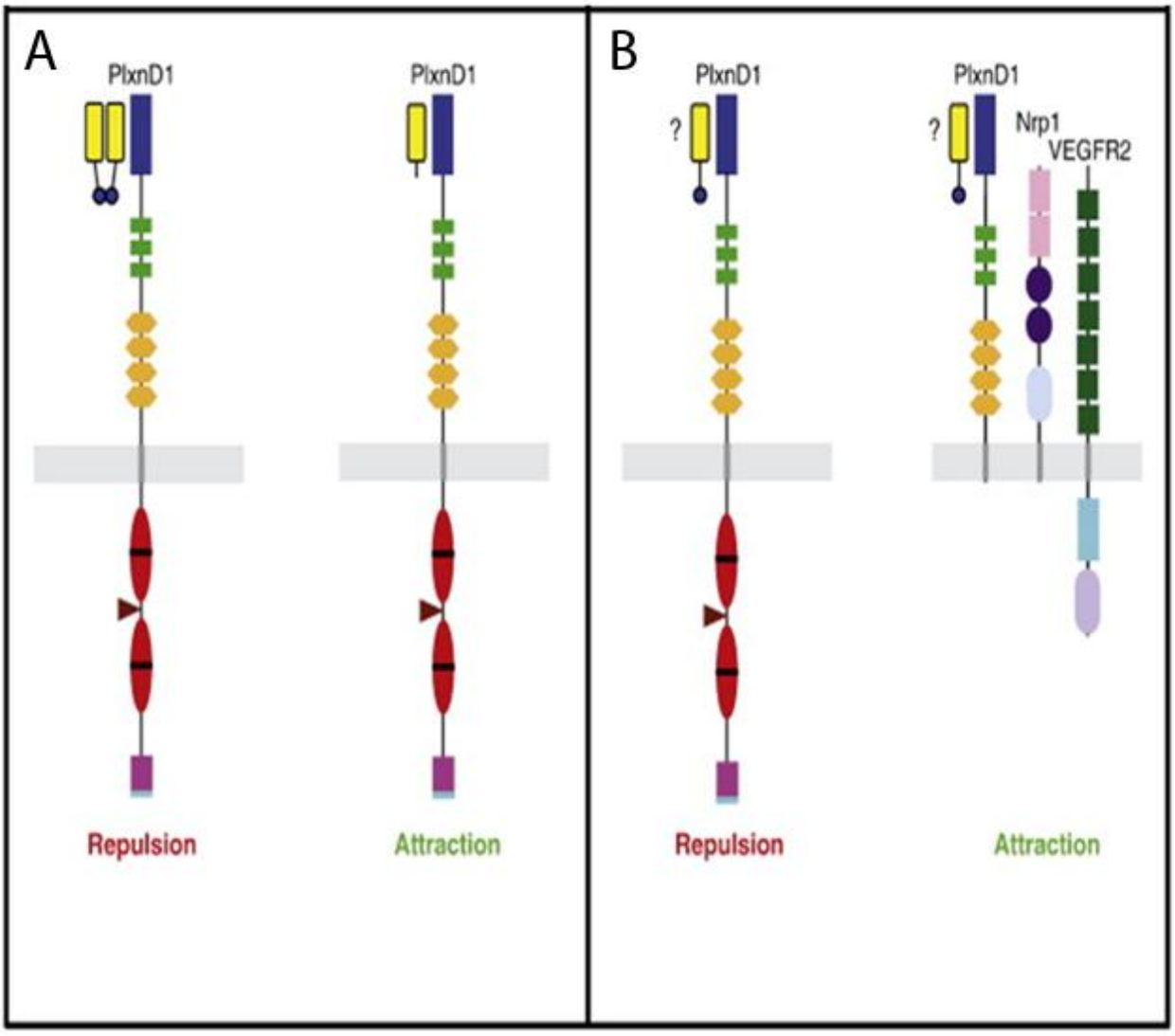


Figure1.5. Sema3E/PlexinD1 interaction induces cell repulsion and attraction.

(A) Full length Sema3E dimers induce cell repulsion upon PlexinD1 binding in the vascular system (left) whereas Sema3E p61 fragment induces cell attraction upon binding to PlexinD1 expressed on endothelial cells (right). (B) In the nervous system, full length 87 kDa [148,167] Sema3E induces cell repulsion on neuron cells that express PlexinD1 alone (left). However, Sema3E promotes cell attraction in other neuron cells that express PlexinD1 and co-receptors VEGFR2 and Nrp1(right) (B) [153] .

1.3.4 Sema3E Role in Cancer

Sema3E expression and function remain debatable in cancer cells [94, 168]. PlexinD1 receptor expression has been found in high levels in vessel and tumor cells in many human tumor diseases. This expression was associated with elevated levels of Sema3E expression as well. Increased levels of both the receptor and the ligand were associated with tumor progression suggesting their role in tumor progression. Whereas over expression of exogenous Sema3E induced metastatic spreading, invasiveness and transendothelial migration of cancer cells, it inversely correlated with invasion property of some tumor diseases [168]. For instance, in metastasis melanoma, PlexinD1 expression was elevated with the invasion level, whereas Sema3E expression was negatively regulated with the progression of the disease. Over expression of Sema3E in a xenograft model of metastatic melanoma has shown a dramatic decrease of metastatic spreading of these cells [169]. Moreover, it has been observed that tumour metastasis was impaired upon knocking down either endogenous Sema3E or its receptor PlexinD1[168].

1.3.5 Sema3E isoforms

The phenomena of underlying mechanisms by either promoting or inhibiting metastasis spreading of cancer cells was found to be through proteolytic maturation via furin enzymes that were released by cancer cells [170]. Blocking the furin enzyme has been found to eliminate the tumorigenicity of cancer cells [171, 172]. Full length Sema3E (p87-Sema3E) is approximately 87 kDa and may be cleaved in a furin-dependent manner to two fragments, p61-Sema3E that comprises sema domains and p25-Sema3E, (Figure1.6) which contains Cys residue.

Additionally, the monomer p61-Sema3E has shown ability to bind PlexinD1 in mouse xenografts and promote lung metastasis. Also, it has been observed that adenocarcinoma cells lacking endogenous Sema3E and exclusively secreting p61 form tumors upon transient transfection of both the full length Sema3E or the p61 isoform. Furthermore, the autocrine signalling of p61 Sema3E-PlexinD1 is found to enhance the ECM degradation and thus increase cell migration and metastasis spreading of tumors [170, 153].

It has been reported that the activation of cancer metastasis by p61-Sema3E associated with the transactivation of oncogenic tyrosine kinase ErbB2 that synergizes with PlexinD1 to switch cell repulsion to attraction [169]. However, more recent studies have discovered a new Sema3E isoform, furin-resistant or Uncleavable Sema3E (Uncl-Sema3E), which is also found to bind PlexinD1, impair its association with ErbB2 and its ability to induce metastasis spreading of tumor cells. *In vitro* experiments have shown that Uncl-Sema3E compete with 61kDa to bind PlexinD1. Moreover, the same group has reported that the new isoform Uncl-Sema3E acts as a powerful anti-angiogenic factor in endothelial cells and, as a result, inhibit several aspect of those cells including cell adhesion to extracellular matrix, cell survival, and directional migration. Also, *in vivo* experiments have observed that the pro-metastatic activity, angiogenesis, and tumor growth were impaired by both local and systemic delivery of Uncl-Sema3E compared to conventional drugs that target vascular endothelial growth factor. These properties make furin-resistant isoform a promising therapeutic target for several cancer diseases [173].

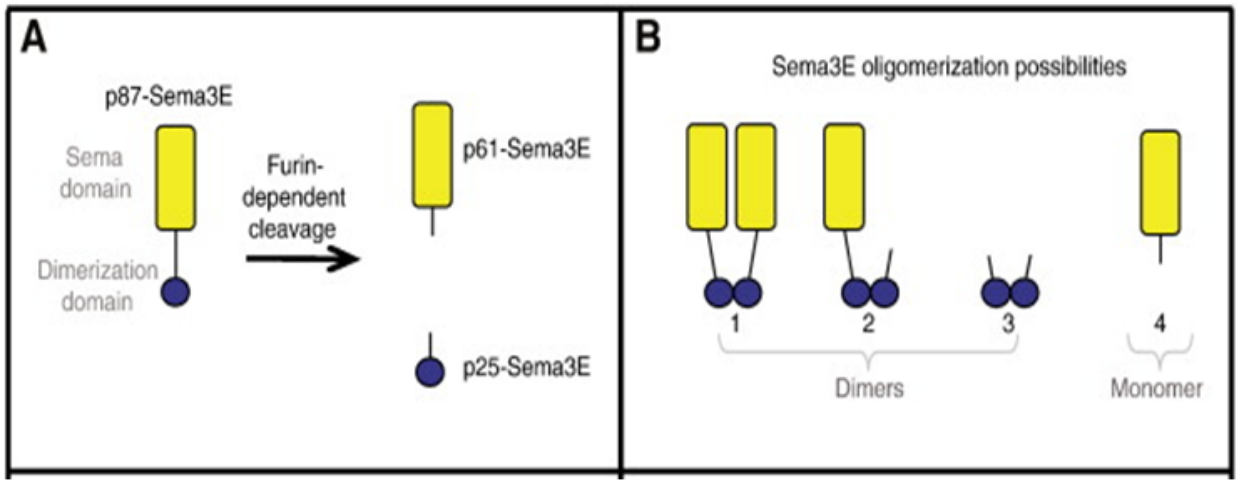


Figure1.6. Sema3E isoforms induces cell repulsion and cell attraction upon binding PlexinD1.

(A) In cancer cells, the full length 87kDa of Sema3E cleaved in furin dependent manner to generate two products, long isoform contains sema domain 61 monomer, and short product contain dimer 25. (B) Three different outcome of Sema3E that released by cancer cell as result of proteolytic activity [153].

1.3.6 PlexinD1

PlexinD1 is a family member of plexins and the only receptor found in vertebrates. The ectodomain size of PlexinD1 is about 870-1400 aa. This region contains two different domains; the first is the Sema domain (amino terminal) consisting of about (500 aa); the second domain consists of three cysteine-rich motifs known as MET-Related Sequences (MRS repeats or PSI-Plexin, Sema and Integrin-domain) with 50 aa long for each. The last region of PlexinD1 ectodomain contains four IPT domains, each of which have glycine-rich repeats (Figure1.7). The Sema domain, located in the extracellular region of PlexinD1, interacts entirely with the ectodomain of Nrp and their interaction is Sema3E-independent [174, 141, 175, 176, 153] . The intracellular tail consists of 630 aa and contains a "Sex and plexins" SP domain that anchorages two highly conserved regions C1 and C2 [141], known as the Ras-GAP (Guanosine triphosphatase (GTPase)-Activating Proteins (GAPs)) [177]. Interestingly, the plexins family in general and PlexinD1 in particular are the only receptors associated with small GTPases. Following GTPase, Rho GTPase-Binding Domain (RBD) is located to link between the two C regions [178, 179, 180, 144, 181, 182, 153]. C-terminal including COOH terminus with stretch of between 40-60 aa is followed by the C2 region [153]. Although Sema4A has been viewed as a PlexinD1 primary ligand [92,150], only Sema3E has been identified as a PlexinD1 ligand in the immune system [92]. PlexinD1 expression is found to be essential for various physiological and pathological aspects in several cell types including neo-angiogenesis, neurogenesis and regulation of the immune response [153]. PlexinD1 is ubiquitously expressed in endothelial cells of mouse embryonic tissue, and it is found to be particularly implicated in the development of the vascular system [183, 150, 109, 176]. PlexinD1-deficient embryos display severe defects in angiogenic segmental arteries that are associated with atypical long filopodia formation and inappropriate branching [184, 110]. Also, PlexinD1-/- mouse

embryos reveal hypervascularization of the heart's epicardium [150, 109], increased ectopic blood vessel branching in the hindbrain [185] and reductions of the fourth and sixth aortic arch arteries [153]. Moreover, endothelial-specific deletion of PlexinD1 in mice exhibit defects in retinal vasculature arrangement [186]. Accordingly, all of this evidence manifests the role of PlexinD1 in orchestrating vascular systems in different cell types[153].

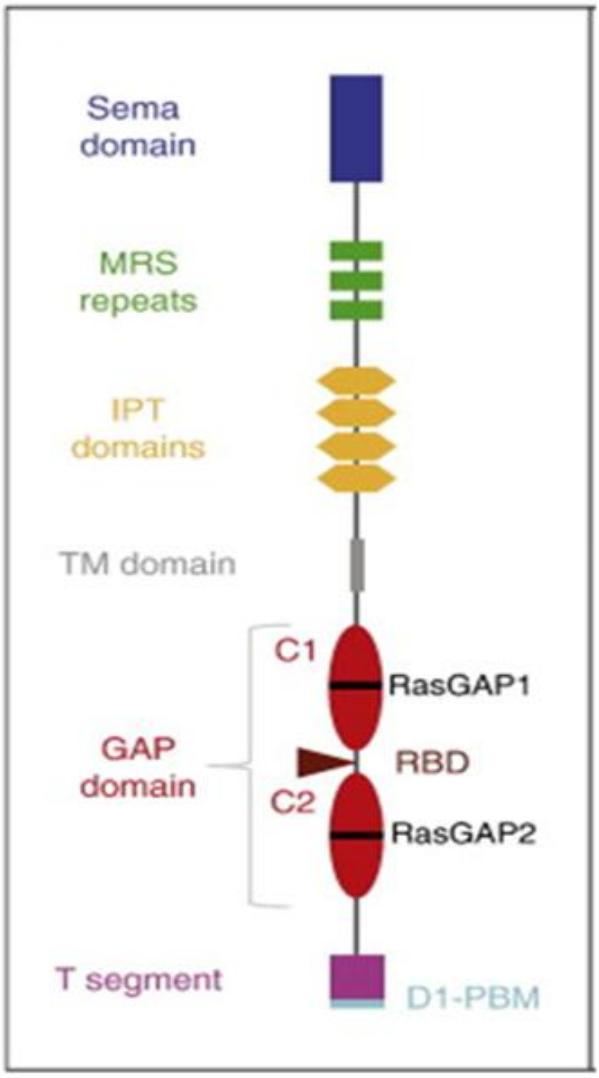


Figure1.7. Schematic representing PlexinD1 structure.

PlexinD1 consists of a N-terminal protein in the extracellular region containing the sema domain, followed by MET-related sequence (MRS), four IPT and a transmembrane domain (TM). The cytosolic tail of PlexinD1 contains GTPase Activating Protein (GAP) with two highly conserved C regions C1 and C2, followed by the Terminal (T) segment [153].

1.3.7 Neuropilin (Nrp)

Neuropilins (Nrps) are transmembrane proteins with a molecular weight of 120kDa and consist of two families, Nrp1 and Nrp2. Both families share similar structures. They contain four different domains: two repeats of complement-binding-like domains, homologous with C1r and C1s (a1 and a2), two repeats of FV/VIII (b1/b2) domain, a MAM (c) domain, and the last domain (d) which contains a transmembrane region and a short cytoplasmic tail with 40-43 amino acids. The length of the human Nrp1 gene is approximately 157 kb and contains 19 exons [89]. The most common feature of class3 semaphorins is their binding to Nrps through a1/a2 and b1/b2 domains that forms the entire semaphorin binding region. The C domain is known to be essential for interaction with plexins as well as dimerization [187]. Moreover, Nrps also acts as a co-receptor of VEGF, which has a distinct structure of Sema3s, forming a complex with VEGFR. It has been observed that Sema3s competes with VEGF to bind Nrps in the b1/b2 region and therefore inhibit the activity of VEGF [188]. Nrps are widely expressed in several cell types including neurons, endothelial cells, inflammatory cells, artery cells, vascular smooth muscle cells, and tumor cells. The wide expression of Nrp receptors allow both Sema3s and VEGF to exhibit their function in most of these tissues [189]. The roles of Nrps in angiogenesis have been well observed. It has been reported that knocking-down Nrp1 expression in zebrafish displays angiogenesis formation[190], meanwhile knocking-down Nrp2 expression exhibits a reduction of small lymphatic vessels, some vascular regions and blood vessel size [191]. In addition to PlexinD1, Nrp1 also binds to Sema3E generating two distinct functions: cell repulsion and cell attraction. For example, Sema3E induces cell repellent effects on corticofugal and striatonigral neurons that express PlexinD1 alone. In other neuronal cells such as subiculo-mammillary cells that express both PlexinD1 and Nrp1 receptors, Sema3E induces cell attraction. Thus Nrp1 could

switch cell repulsion to attraction upon gaining an interaction with a Sema3E-PlexinD1 complex[149].

1.3.8 Sema3E/PlexinD1 interaction

During the last few years, the vast majority of researchers focused their effort to understand the role of Sema3E-PlexinD1 interaction and function in several cells types. For instance, studies have elicited their function during synaptic formation in cortico-thalamo-striatal circuits, in which Sema3E is secreted by thalamo-striatal axons, and PlexinD1 expressed by specific postsynaptic neurons. The interaction between the ligand of Sema3E and its receptor PlexinD1 determines connectivity in a highly specific manner that is required for the proper balance of excitatory synapses and, as a result, leads to proper behaviour[192]. Additionally, in the immune system, Sema3E-PlexinD1 signalling is involved in orchestrating thymus trafficking. During thymocyte maturation, chemokine gradients promote the migration of double positive thymocyte $CD4^+$ $CD8^+$ (DP) from the cortex of the thymus gland into the medulla and form single positive cells $CD4^-$ $CD8^+$ or $CD4^+$ $CD8^-$ (SP). PlexinD1 is highly expressed in DP, and this expression is influenced by the presence of a T cell receptor TCR. The Sema3E-PlexinD1 interaction impairs chemokine CCR9/CCL25 signaling induced DP thymocyte trafficking into the medulla[130]. Moreover, Sema3E-PlexinD1 signalling also exhibits their function during vascular system development, and function as a potent anti-angiogenic factor that inhibits vascular endothelial growth factor (VEGF) path-finding which, as a result, suppresses cell growth and tube formation. On the other hand, PlexinD1 is highly expressed and localized at the apical end of sprouting blood vessels. This expression is precisely regulated by VEGF that released by the surrounding

tissues. Upon Sema3E binding to PlexinD1, the signalling of Delta-like 4 (Dll4) Notch induced by VEGF is negatively regulated and thus affects the decision of cell fate between tip and stalk cells [193]. Another study indicated that, in growing blood vessels, the expression of PlexinD1 is also enhanced via VEGF which is released by astrocytes. VEGF induced disoriented projection of endothelial filopodia impaired upon increased PlexinD1 and Rho J expression in the extraretinal vessel [194]. Moreover, recent studies have also exhibited a critical role of Sema3E-PlexinD1 signalling in osteoblast formation. This signalling displays a potential capacity to couple between osteoblast and osteoclast formation, which is needed to maintain the local bone homeostasis [195]. *In vivo* experiments demonstrated inhibitory function of the Sema3E-PlexinD1 complex on osteoblast migration, and decrease in osteoblast formation [195].

1.4 Chapter 4: Small GTPase

1.4.1 Small GTPase

Over the last few years, the functions of the Rho family of GTPases (Rho GTPases) have been well documented. They are involved in the regulation of multiple receptors that control numerous cellular processes including cell movement, morphology, and proliferation [145-146]. Rho GTPases are the key players that convert extracellular signalling of a wide variety of membrane receptors such as tyrosine kinase receptors, integrins, T, and B lymphocyte receptors [146]. The activity of the Rho family is facilitated by several proteins that interact with the active Rho GTPases known as effector proteins. These effectors initiate the signalling cascade to control several other cellular aspects which encompass the organization of actin, gene expression and vesicular trafficking. It has been reported that c-jun N-terminal Kinase (JNK) and p38 MAP kinase pathways are activated by Rac and Cell division control protein 42 homolog (Cdc42). Also, RhoA, Rac, and Cdc42 have been involved in the regulation of serum factor and nuclear factor (NF-kB) [196]. The capacity of the Rho family to interact with numerous types of proteins including upstream and downstream targets provides a more complex view of the signalling network of these family members[197].

The Rho family of GTPases are genes encoded with small monomeric Ras-related proteins[198]. Based on their sequence similarity in terms of protein domain and function, these proteins have been classified into 20 distinct proteins. Accordingly, Rac1, RhoA, and Cdc42 are the best characterized and the most studied members of this family[199]. Rho GTPase shuttles between the active GTP-bound and inactive GDP-bound positions (Figure1.4) [153,200]. Rho GTPase transmits

the downstream signal through the conformational changes that are created by GTP-bound, enabling its interaction with effecter molecules with high affinity. Interestingly, three distinct families are precisely regulating the shuttle between GTP/GDP: cyclic guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and the guanine nucleotide dissociation. The activation of Rho by GEFa occurs through catalyzing the exchange of GDP for GTP, thus elevating the levels and the formation of GTP-bound in the cell [201], whereas the activity of both Rho GAPs and Rho GDP dissociation inhibitors (GDIs) are negatively regulated. The Rho GTPase protein's main function is to increase GTP hydrolysis rates that enhance the formation of GDP-bound state[200], whereas GDIs impair the disassociation activity of the nucleotide, thus interfering with GDP/GTP exchange and GTP hydrolysis [202-203].

1.4.2 Rac 1 activity and neutrophils

Rac GTPases are one of the best studied members of the Rho GTPases. As with any other GTP-ases, their function is entirely focused on switching the cycle between an inactive GDP-bound state to an active GTP-bound state. As mentioned earlier, a wide variety of effector proteins control the activity of GTPases. Mainly, the induction of Rac GTPases triggered by DbI-family GEFs which are activated dependently by receptor kinases [204]. Moreover, the regulation of GAPs on the GTPase occurs through the elimination of γ-phosphate and returns the active GTPase to the GDP-bound inactive state, whereas GDIs stabilize the inactive GDP-bound protein and sequester them within the cytoplasm [202-203]. Three isoforms of Rac proteins have been identified in mammals: Rac1, Rac2 and Rac3. These proteins share a 90% similarity in their sequences and approximately 89% similarity in AA composition. The entire function of the majority of Rho

GTPases, specifically Rac GTPase, is the regulation of cell adhesion and migration through stimulation of F-actin polymerization and changes in cytoskeleton structure that are important during immune cells trafficking [197].

It has been demonstrated that Rac1 and Rac2 regulate both distinct and overlapping cellular function in hematopoietic cells. The homology of Rac1 and Rac2 in their amino acids creates some difficulties in understanding the role of each protein in several cell types [205-206]. For example, mice deficient in both Rac1 and Rac2 exhibited notable defects in neutrophil migration and adhesion in response to chemoattractant stimuli compared to Rac2 deficient mice alone [207] whereas Rac1 deficient mice do not show any defect in neutrophil migration or oxidase production in response to fMLP [208].

Later on, an *in vitro* experiment indicated that Rac2 has the ability to modulate neutrophil migration through the mobilization of F-actin polymerization and polarization[209]. In contrast, Rac1 displays a pivotal role in tail retraction during cell movement and in the regulation of cell spreading [207-208]. Marie and her colleagues have indicated that Rac1 activity plays an important role during neutrophil migration through the modulation of cell-body contraction and uropod formation. Also, hematopoietic cell deficient Rac1 has been shown to impair fMLP induced neutrophil migration into the lungs [210]. This effect was through the accumulation of Akt in the leading edge towards the chemoattractant gradient[211]. Moreover, recent studies have demonstrated that defects in either P-Rex or Vav expression (family members of DbI-type GEFs) is associated with the defect in GPCR dependent neutrophil response and Rac1 activation [212]. And this defect in Rac1 activity was sufficient to induce the impairment seen in fMLP-stimulated

P-Rex1-/- Vav1-/- (P1V1) neutrophils. All this evidence indicates that Rac, particularly Rac 1, is essential for neutrophils migratory function [210].

1.4.3 PlexinD1 as effector protein

Cumulative evidence shows that PlexinD1 is also capable of acting as Ras GTP, thus antagonizing both integrin-mediated cell adhesion to the extracellular matrix and PI3K signalling. In endothelial cells, the phosphorylation of Focal adhesion Kinase (FAK), that regulates the turnover of integrin-containing focal adhesions, was decreased upon Sema3E treatment. Also, the collapsed activity abrogated upon Sema3E treatment in PlexinD1 transfected COS-7 cells grown in collagen or fibronectin (integrin ligands), whereas the same cells when grown in poly-L-lysine or expressing PlexinD1, failed to collapse upon Sema3E treatment[213]. Furthermore, several studies have proposed that Rac 1, 2, and 3 are able to bind the intracellular tail of murine PlexinD1. *In vivo* experiments indicated that Rac2 played an important role in mediating RasGAP activity of PlexinD1 [214].These signalling induces integrin disassembly from extracellular matrix through the activation of small GTPases ADP-ribosylation factor 6 (Arf6), which regulates the intracellular trafficking of β1 integrin through GEP 100 effector proteins[213,215]. Overall, all of the evidence supports the theory in which Sema3E-PlexinD1 signalling induce cell collapse through the regulation of small GTPases (Figure1.4)[153]

2- RATIONALE OF THE STUDY

2.1 Why are neutrophils the target of this study?

Neutrophils, known as polymorphonuclear leukocytes (PMNs), are an essential part of the host defence of innate immunity[1]. They play an important role in fighting invading microorganisms[11]. However, in acute phases of inflammatory diseases neutrophils are becoming pathological [216] for the following findings;

1- During inflammation, neutrophils are the first cells that are rapidly released and subsequently migrate from the bone marrow towards the site of inflammation [77,217]. The accumulation of neutrophils in the injured organ increases the amount of proteolytic enzymes and other granules content whereby damage is induced in the surrounding tissues [216].

2- Among all chemoattractant factors, CXCL8/IL-8 is the most powerful pro-inflammatory CXC chemokine that attracts and activates neutrophils during inflammation. Under stimulatory circumstances, the production of this protein occurs by epithelial cells and various immune cells [218-220]. Activated neutrophils itself can also synthesize CXCL8/IL-8 [12].

3- High level of CXCL8/IL-8 during inflammation leads to additional neutrophil recruitment to the site of inflammation and prolongs their activation, and finally increases their transmission within epithelial cells [220]

4- Blocking CXCL8/IL-8 activity can also block neutrophil migration *in vitro* and neutrophil-mediated tissue injury in inflammatory disease [221].

2.2 Why Study the Sema3E-PlexinD1 function in human neutrophils?

Several studies have indicated that the Sema3E-PlexinD1 interaction exhibits an inhibitory signal in several cell types [153]. Four independent studies demonstrated that Sema3E exhibits inhibitory signals of growth and cell migration upon binding to PlexinD1. These papers clarified that:

1- Sema3E-PlexinD1 signaling is found to be a potent anti-angiogenic factor through the inhibitory signals of VEGF path-finding, which suppress cell growth and tube formation [193].

2- PlexinD1 is highly expressed in DP thymocyte and upon Sema3E binding, it suppresses chemokine CCR9/CCL25 that is mainly stimulating DP thymocyte migration into the medulla[130].

3- *In vivo* experiments demonstrated inhibitory function of Sema3E-PlexinD1 interaction on osteoblast migration, and they exhibited decrease in osteoblast formation[195].

4- Furin-resistant or Un-Cleavable Sema3E (Uncl-Sema3E) that also binds PlexinD1 impair its association with ErbB2, inducing the metastatic spreading of tumor cells[173].

The suppressive capacity of cell migration that is seen by Sema3E in several cells, enhances our efforts to investigate its role in human neutrophils, which is characterized mainly by rapid migration to the inflamed organ. The presence of Sema3E in the normal human tissue suggested its inhibitory effect on the inflammatory cells which is seen in severe asthma cases. Whilst neutrophils are the main inflammatory cells that are involved in severe asthma, and CXCL8/IL-8 is elevated as well, we hypothesised the inhibitory affect of neutrophil migration as following.

3. HYPOTHESIS & AIMS

3.1 Hypothesis

Sema3E impairs basal and chemokine CXCL8/IL-8 mediated human neutrophil migration

3.2 Aims

1- Examine the expression pattern of Sema3E in human neutrophils at the mRNA level, and verify whether Sema3E expressed in human neutrophils at the protein level.

2- Investigate PlexinD1 expression in human neutrophils at mRNA level, and determine the expression pattern of PlexinD1 at protein level.

3- Identify the expression pattern of Sema3E co-receptors Nrp1 and VEGFR2 at both mRNA and protein level.

7- Clarify the functional activity of Sema3E on the basal human neutrophil migration.

8- Examine the function of Sema3E on CXCL8/IL-8 (chemokine) induced human neutrophil migration.

9- Analyze the underlying mechanism of Sema3E inhibitory function on CXCL8/IL-8 induced human neutrophil migration.

4. MATERIAL & METHODS

4.1 Subjects

This study was approved by the Ethics Committee of the Faculty of Medicine, University of Manitoba. Adult non-smoker non-allergic healthy subjects volunteered to donate blood. Between 30-80ml of venous blood was taken from each donor, between 9-11am on weekdays only. EDTA coated tubes from RD biosciences were used to store the blood, which was immediately transferred to a cell culture hood for further processing.

4.2 Human peripheral blood neutrophil isolation and purification

Blood was collected from the peripheral vein of healthy donors using sterile syringes. Every 30ml of blood was then mixed with 30 ml of normal saline stored at room temperature. 10ml of the mixture was added to 3.5ml of Ficoll (Amersham) in 15ml Falcon tube. These tubes were rotated for 30min in a centrifuge at room temperature at 1000 RPM with the brakes off. The upper layer of plasma, Ficoll and peripheral Blood Mononuclear Cells (PBMC) of the layered tubes were disposed, while the bottom layer of the granulocyte-erythrocyte was kept. After this, about 6ml of dextran-saline was added to the bottom layer and mixed well to allow sedimentation of red blood cells and left at room temperature for 30min. Following the incubation, the resulting clear layer on top containing granulocytes, was removed and transferred to a 50ml tube while the bottom deep red layer was disposed. The granulocyte mixture in the 50ml tube still contained residual erythrocytes that affect purity. As such, further purification by hypotonic lysis was required. The

50ml tube was filled up with normal saline 0.85% kept at room temperature and 2% volume of patient's own plasma kept on ice, further centrifugation at 1200RPM at 4ºC was done for 5min. After this step, granulocytes with the residual erythrocytes were left at the bottom of the tube while the supernatant was gently poured out. Immediately, the cell layer at the bottom was mixed in 12 ml of hypotonic ice-cold 0.2% saline for 45sec. Right after this 45sec period, 12ml of hypertonic ice-cold 1.6% saline was added followed by 25ml of 4ºC normal saline.

Cell pellet was visually inspected for the amount remaining erythrocytes. The above hypotonic shock procedure was repeated if necessary; otherwise the pellet was re-suspended in proper amount of 4 ºC RPMI 1640 medium supplement with 100units/ml penicillin and 100ug/ml streptomycin. This step was usually followed by repeated centrifugation and re-suspension in the medium to ensure a clean population of cells. Samples were then taken for manual counting and viability assessment under a light microscope (40x) using trypan blue die according to standard laboratory protocol. Subsequently, neutrophils were re-suspended in the same medium but at a fixed density of 5million/ml. Furthermore, cytological examination of stained neutrophills by the Wright-Giemsa method was carried out on a sample of 100,000 neutrophills spread on a glass slide by a cytospin, routinely yielding neutrophil that was between 95-98%.pure

4.3 RNA isolation and RT-PCR analysis

RNA was purified from primary human neutrophils using TRIzol (invitrogen) according to manufacturer's protocols. RNA concentration and integrity were evaluated using a spectrophotometer. Reverse transcription was performed with 2ug of total RNA using a high

capacity reverse transcription kit (Applied Biosystems) according to manufacturer's protocols. DNA standards were prepared from PCR using complementary DNA (cDNA) of nuetrophils. The amount of extracted DNA was quantified by a spectrophotometer and expressed as copy numbers and serial dilutions were used to generate the standard curve. The forward and reverse-specific primer sequences were used, and the size of the amplified fragment and the annealing temperature for PlexinD1 were 5'-CCCCAACCCACAGTTCTCTA-3' and 5'-CAAGTAAGCTGCGACATCCA-3', 58ºC, 156bp, and for Sema3E were 5'-CTGGCTCGAGACCCTTACTG-3' and 5'- CAGTTTGTTGGGGATGCTTT-3', 40ºC, 155 bp, and for Nrp1 were 5'-TATTCCCAGAACTCTGCCC-3 and 5'-TGGGATTGCTGTGGATGACA3' 58 ºC, 250 bp , and for VEGFR2 were 5'-CCTCTGTGGGTTTGCCTAGT-3' and 5'- TGGATGTCGCAGCTTACTTG-3' 60 ºC , 350bp.. In parallel, human PBMCs (used as a positive control for Sema3E and PlexinD1) were cultured in RPMI 1640 (HyClone Laboratories), and reverse transcription was performed as mentioned above. Primers for housekeeping gene GAPDH and standard controls were developed in our laboratory. Detection of the fluorescent product was obtained at the end of the 72ºC extension period. Product specificity was determined by a melting curve analysis and by visualization of the PCR products on agraose gel.

4.4 Cell culture

Neutrophils were either used fresh (time 0) or cultured with/without Granulocyte-macrophage colony-stimulating factor GM-CSF (10ng/ml) for 18 hours at 37 ºC in 5% CO2. Following neutrophils cell culture, a conditioned medium was collected, filtrated, concentrated 50X using

centrifugal filter units (Amicon Ultra-4, Milipore). In parallel, neutrophils grown in the same medium were lysed using M-PER mammalian protein extraction reagent containing protease inhibitor cocktail (Roche, Canada). Conditioned medium and protein lysate were stored at-80C˚

4.5 Western bolts

$5x10^{-6}$ of isolated neutrophils were washed with PBS and subsequently the pellet was lysed with 150 ul of Nonident P-40 (NP-40) or Triton-X lysis buffer supplemented with a protease inhibitor cocktail (Roche). Following a 10 min lysis on ice, the tube containing lysed cells was centrifuged at 12,000RPM at 4C for 10min. The DNA containing the sticky pellet was disposed off. Protein lysate for each sample was measured for concentration and immediately aliquot to 10 or 15ul volume in 0.2 ml centrifuge tubes and restored at -80C until each aliquot was used for blotting.

Thawed protein sample aliquots were adjusted for differences in concentration and then mixed with the appropriate amount of lysis buffer to reach an equal volume for all samples run in the same gel. The samples were then mixed with a reducing 4x sample buffer containing Sodium Dodecy1 Sulfate (SDS) and B-mercaptoethanol. Prepared samples were boiled at 100C for 5min, centrifuged shortly and then transferred to polyacrylamide gels (SDS-PAGE) under denturing conditions. The gel was run in an electrophoresis apparatus at a range of voltages starting from 80 to 100V until the proteins were completely resolved

Proteins were then transferred to charged PVDF (polyvinylidene difluoride) membranes either at 100V for 90min (<60kDa) or overnight at 30V (>60kDa). The PVDF membrane was stained with ponceau red after being transferred to ensure transfer integrity and equal loading. Subsequently,

the membranes were blocked from incubating with 5% non-fat milk/bovine serum albumin in TBS/0.1% Tween 20 (TBST) for 1h at room temperature or overnight at 4C on a shaker. Blocked membranes were incubated with primary antibodies diluted in non-fat milk/bovine serum albumin in TBST overnight at 4C or for 2h at room temperature on a shaker. The membranes were washed three times for 15min each with copious amounts of TBST, and then incubated with the appropriate secondary antibodies for 1h at room temperature, followed by extensive washing at short intervals for 2h with TBST. Bands were visualized by the ECL plus/Advance Western Blotting Detection System (Amersham) according to manufacturer's instructions using AlphaEase FC software v3.1.2 on a Fluorchem 8800 Alpha Innotech).

Antibody concentrations used for western blotting were: Sema3E polyclonal antibody (1/1000), secondary antibody anti rabbit (1/5000), PlexinD1 polyclonal antibody (1/1000)

4.6 Immunocytochemistry

Immediately, after neutrophil isolation, a sample of 100,000 neutrophils were spread on a glass slide by a cytospin and fixed with 4% paraformaldhide by adding drop of 50 ul directly to the circle of spared cells and left for 15 min. Then, slides were washed twice with l% peroxidise blocking solution (1x PBS) for 10 min each and left to dry over night at room temperature. .

To determine whether human neutrophils express either Sema3E or its receptors (PlexinD1, VEGFR2 and NRP1), Alkaline phosphatase–antialkaline phosphatase immunocytochemistry was used to examine above proteins immunoreactivity with primary antibody. Briefly, 30 min prior to starting an immunocytochemistry analysis, slides must be retrieved from the freezer to allow the

temperature to equilibrate to room temperature. Slides were ladled with a solvent resistant pen by drawing a circle around the area of spared cells and rinsed 3 times in 1xPBS for 10 min each time. Slides were blocked with normal serum blocking solution (5% donkey serum or 5% rabbit serum and 5% human serum mixed with 1x PBS) for 1 hour at room temperature by adding 50ul drops in the labelled area. All antibodies were diluted in (1%PBS) with the following concentration Sema3E (1ug/ml, R&D), PlexinD1 (1ug/ml R&D), VEGFR-2 (1ug/ml R&D), and Nrp1(1ug/ml R&D). After the primary antibody incubation, slides were washed extensively three times for 10 min each with TBS cyto to reduce the chance of getting background. The surrounding area of the ladled spot was dried carefully using kemwiped avoiding attachment to the labelled spot (the same process must be followed between each incubation step). Immediately the slides were incubated with the secondary antibody biotinlated antibody (rabbit–anti-Goat IgG 1:200 dilution in PBS or biotinlated donkey–anti-sheep IgG 1:200 dilution in PBS) for 1 hour at room temperature by adding drops in the labelled areas as indicated earlier. After extensive washing with TBS cyto, the slides were then incubated with streptavidin-alkaline phosphatise (10ul/ 1ml of 1% PBS) for 30 min at room temperature. Again after extensive washing with TBS cyto, the slides were developed with Fast Red dissolved in the alkaline phosphatise substrate. The slides were filtered through a 0.2 mu filter prior to use and incubated for 10-20 min maximum, generally never incubate more than 20 min. During this process, slides were checked under the microscope until the red positive color appears; further incubation was stopped and immediately rinsed with water. Excessive water was removed and counterstained with Mayer's hematoxylin for 3-5min. Slides were then rinsed well with tap water and left to dry over night at room temperature. On the second day, a thin layer of premount medium was placed over the labelled area and covered with coverslip. Positive cells were stained in red after

development with Fast Red (Sigma-Aldrich Canada). Isotype-matched control mAb was used for negative control.

4.7 Flow cytometry (extracellular staining)

Approximately, $1x10^{-5}$ cells of isolated human neutrophils were transferred to FACS tubes and washed twice with 2 ml of ice cold 1XPBS. In between the washing steps, those tubes were rotated for 5 min at 4ºC, 1000RMP. After centrifugation, the supernatant was gently discarded and cells were re-suspended in 100 ul 1%PBS. Then while tubes were placed in ice, cells were incubated with the primary antibodies (Polyclonal anti-PlexinD1, mAb anti-VEGFR2, or Goat IgG1 isotype control (both 10 ug/ml). mAb anti-Nrp1 or Sheep IgG) in the shaker for 1 hour . Cells were then washed twice with 1XPBS as indicated earlier and incubated with a secondary antibody Alexa Fluor 488 conjugated donkey anti-goat IgG (1:100) (R&D) or Alexa Fluor conjugated donkey anti-sheep in the dark for 1 hour. This was also on ice. Cells were then washed once with 1XPBS, and fixed with 200ul of 2% paraformaldehyde for 2 min, and analyzed on FACSs san. The results were presented as specific mean fluorescence intensity (MFI) and as a percentage of positive cells using flowjo software

4.8 Flow cytometry (intracellular staining)

To measure Sema3E intracellular expression in human neutrophils, $1x10^{-5}$ of fixed cells with 2% paraformaldehyde were transferred to FACS tubes and washed with 2 ml flow buffer as indicated earlier . After washing and centrifugation, cells were re-suspended with 100ul of

saponin buffer and incubated on ice for 15 min to make the cells more permeable and allow antibodies to penetrate through the plasma membrane (0.1ng in 100ml flow buffer). After washing, cells were blocked for 15 min with 100ul of blocking buffer to reduce chances of getting non-specific binding. Cells were washed twice with a 2 ml flow buffer. After 5 mins of centrifugation, the supernatant was discarded and cells were re-suspended in 100ul flow buffer. Then, the cells suspension were incubated on ice with secondary antibody (mAb) mouse anti human Sema3E conjugated allophycocyanin (APC) (R&D), or (anti mouse IgG1 conjugated allophycocyanin (APC) isotype control (both 10 ug/ml) (R&D) for 30 min in complete darkness. After washing twice with a 2 ml flow buffer, FACS analysis was performed with CellQuest software (BD Biosciences, San Jose, CA).The results are presented as specific MFI and as a percentage of positive cells using flowjo software.

4.9 Neutrophil migration using Transwell system

Neutrophils migration activity was assessed ex vivo in 24 Transwell plates with insert 3 μm pore-sizes (Corning Costar, Lowell, MA, USA). This plate consists of upper (insert) and lower chambers. Using an autoclaved tweezer, the insert was removed and placed on an empty well in the same 24 well plate. 600 μl RPMI 1640 was added to the lower chamber alone or with CXCL8 (10 ng/mL). While isolated, the neutrophils (5×10^6 /ml) were added into the insert in 100 μl RPMI 1640. Using the same autoclaved tweezer, the insert was then transferred and positioned carefully in its proper place on the lower chamber while avoiding making bubbles in between the chambers (this was the last step after adding Sema3E and CXCL8/IL-8). Two different experiments have been done using the 24 well plates. In the first experiment, different

concentrations of Sema3E (1ng\ml, 10 ng\ml, 50ng\ml to 100ng/ml) were added to the cells in the insert combining with/without CXCL8/IL-8 that were added in the lower chamber. In the second experiment, different concentrations of Sema3E were added to the lower chambers also with/without CXCL8/IL-8. After 1 hour of incubation at 37° C, the inserts were carefully extracted. The cells which migrated to the lower chambers were subjected to a cell count using a hemocytometer.

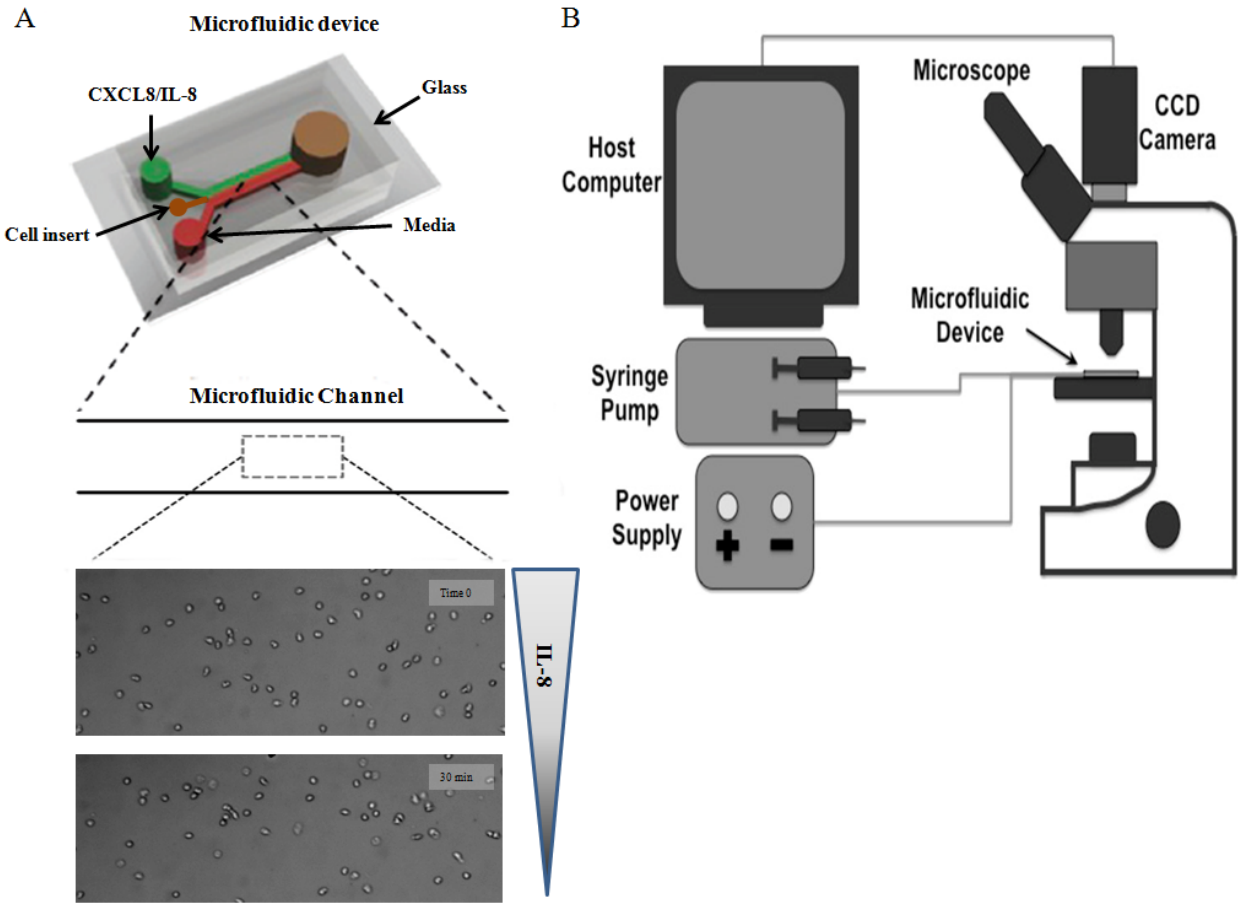
4.10 Neutrophil chemotaxis using microfluidic device

This experiment has accomplished in collaboration with Dr.Francis Lin, (Physics department, University of Manitoba). The flow-based microfluidic gradient-generating devices, a ‘‘Y’’ type device is designed with specialized computer programs such as Freehand (Macromedia, CA) and AutoCAD (Autodesk, Inc., CA). The ‘‘Y’’type design consist of a main gradient channel and two fluidic inlets (Fifure1.8 A). The width of the device is 350 um and the depth around 100um. The main gradient channel was coated with 0.25mg/ml fibronectin (BD Bioscience, CA) for 1 hour at room temperature prior to adding cells to provide substrate for cell adhesion and migration, followed by blocking with 0.4% BSA in RPMI for another hour at room temperature. Chemical gradients were created in the main channel and controlled by the continuous infusion of the chemokine (10ng/ml CXCL8/IL-8) or 100ng/ml of Sema3E in single gradient. In the combined gradient (10ng/ml CXCL8/IL-8) chemokine + 100ng/ml of Sema3E were added either co-existing in the same side or in the opposite sides. FITC-Dextran 10 kD that has approximate molecular weight of the chemokine molecule was added to the chemokine solution CXCL8/IL-8 to measure the gradient. The migration medium (RPMI) and chemokine solutions were infused

into the device and controlled by syringe pumps through tubing and the inlets of the device. The total flow rate was 0.2 µL/min[222].

Migration medium was prepared using RPMI-1640 with 0.4% BSA. Before applying the gradient, 500.000-1,000,000 cells were loaded into the device within the cell inlet and allowed to flow through the main channel for 5-10 min until 1000-2000 of the cells attached to the channel. Chemokine gradients were generated in the main channel at the flow rate of 0.2 ul min^{-1} (0.095 mm s^{-1}). The ‘‘Y’’ device was positioned under the microscope (Model No. BX60, Olympus) approximately 3 mm below the junction area of the channel chosen for imaging. (Figure1.8 B) Then migrating cells of a chosen field were recorded at6 frames min^{-1} for 30 minutes. Utilizing time-lapse microscopy, cell migration was recorded by 6 frames/min for 30 min using a CCD camera (Model No. 370 KL 1044, Optikon, Canada), and image acquisition was controlled by NIH ImageJ (v.1.34s).The gradient was checked before and after each migration experiment[222].

Using manual tracking (Image J software), approximately 25–50 cells were tracked from three independent experiments and analyzed for each experimental condition. Cells that migrated within the microscope field during the 30 min were selected for tracking within the microscope field. Chemotactic movement of cells was quantitatively evaluated by percentage of chemotaxing cells (%) and chemotactic index (CI), which is the ratio of the displacement of cells toward the gradient (y) to the total migration distance (Δd) (for more information see Francis Lin 2009 [223] and Francis Lin 2006 [224]



Fugere.1.8 Schematic representation of the microfluidic device [121]

3D of the ”Y” shape microfluidic device; green color indicates CXCL8/IL-8 solution and orange indicates medium. Pictures were taking at time0 and after 30min. Migration cells of a chosen field were selected for tracking (A). Microfluidic device was positioned on a microscope stage. Chemical gradients of chemokine and medium solution were generating through tubing from syringe pumps connected to the device. Cell chemotaxis was recorded utilizing time-lapse microscopy (B) [121]

4.11 Measurement of Rac1 GTPase activity

Rac1 GTPase activity was measured in snap-frozen cell lysates harvested from unstimulated or Sema3E (100ng/ml) ± CXCL8/IL-8 (10ng/ml) stimulated human neutrophils ($5x10^{-6}$/ml) at the different time points starting from 0.30s, 1:00min, and 3.0min to 5.0 min using a luminometric-based G-LISA Rac1 Activation Assay Kit (Cytoskeleton, Inc. Denver, CO). Briefly, cell lysates were subjected to Rho binding domain of Rac1 in a Rac-GTP affinity 96-well plate. Prior to this process, wells were incubated for 30min with an antigen presenting buffer on a cold orbital microplate shaker (400 rpm) at 4ºC for exactly 30 min, followed by washing the wells with 200ul of washing buffer using multi channel pipette. The washing buffer was removed completely onto paper towels with 5-7 hard pats. Then immediately, the pipette of 200 ul of room temperature antigen presenting buffer went into each well using multi channel pipette for exactly 2 min of incubation. Rac1-GTP was detected using anti-Rac1 primary antibody after 45mins of incubation at room temperature, followed by the washing step as mention earlier. Subsequently, HRP-conjugated secondary antibody adds to the wells and incubated at room temperature for 45 min in the shaker at 400 rpm. After washing and vigorous drying, wells were incubated and developed with a chemiluminescent reagent A&B with 1:1 dilution. A constitutively active Rac1 provided in the kit was used as positive control in all experiments and a blank well was also used as a positive control.

4.12 Statistics

Data were analyzed by one-way ANOVA first to determine if any significant differences may generally exist among various experimental groups. Tukey's test was then performed to detect

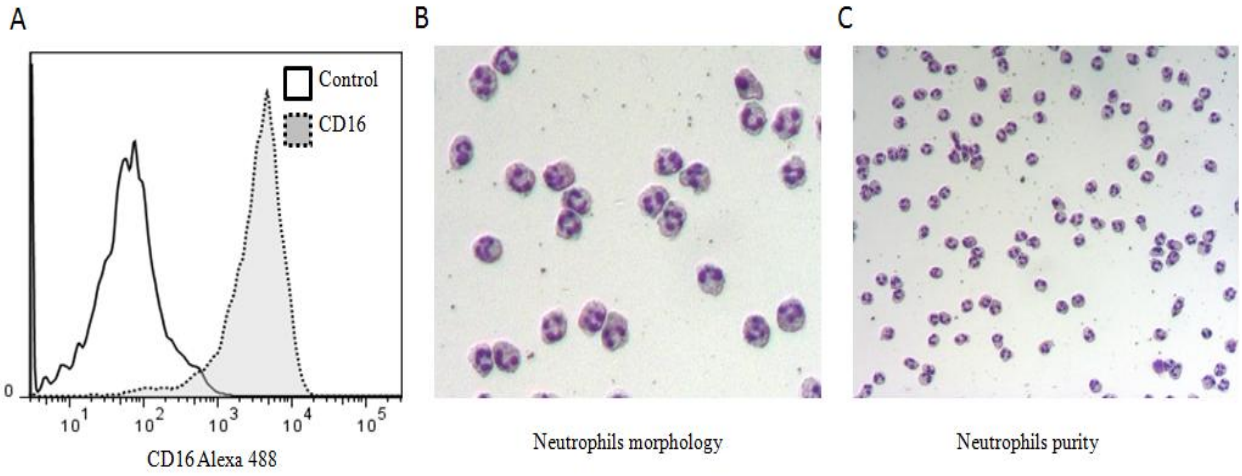
statistically significant differences to compares all possible pairs of means. GraphPad Prism 5 was the software of choice and $p<0.05$ was considered statistically significant. Microfluidic data were analyzed using Student's two-sample (two-tailed) t-test to assess the significance of the difference between the parameters relative to the critical p value (p, 0.05).

5-RESULTS

5.1 CD16 expression on the surface membrane of human neutrophils

Isolated human neutrophils in the steady state highly express CD16 in the surface membrane, whereas activated neutrophils loss the expression of CD16[225-226]. Thus to ensure the purity of neutrophils, freshly isolated cells were stained with anti CD16-Alexa 488 conjugated antibody. As indicated in Fig2.1A, neutrophils highly express CD16. Moreover, neutrophil morphology is another alternative method that can be used to test cell purity (Fig2.1B).

The method of neutrophil isolation using Ficoll/dextran also isolated eosinophils that exist in circulation. Although blood samples were taken from healthy volunteers, the purity of neutrophils was examined using cytospin slides (Fig2.1C)**.**



Figuer2.1. CD16 expression on the surface membrane of freshly isolated neutrophils.

CD16 expression in isolated human neutrophils was examined utilizing FACS analysis. $5x10^5$ of neutrophils suspension resuspended in 100ul of PBS were blocked with Fc blocking buffer for 30 min. After two times washing, cells were incubated with anti human CD16-Alexa 488 conjugated for 30 min on ice. As a negative control, cells were labeled with isotype-matched goat IgG (A). Neutrophils morphology direct after isolation, indicating healthy non-stimulated cells (B). Neutrophils purity always above 96% (C)

5.2 PlexinD1 expression at mRNA level in human neutrophils

The expression of PlexinD1 have been identified in a wide variety of cells including endothelial cells[109], bone-marrow-derived cells[195] and the thymus gland[130]. However, no reports have observed the expression of PlexinD1 in primary human neutrophils. Here, in order to verify the functional responses of neutrophils to Sema3E, we first investigated the expression of PlexinD1, the high affinity receptors of Sema3E[153], in human neutrophils. Human neutrophils were isolated from healthy donors using Ficoll. Purified mRNA was subjected to RT-PCR using PlexinD1-specific primers. Amplification products were analyzed by 2% gel electrophoresis. As shown in (Fig2.2), human neutrophils expressed PlexinD1 at the mRNA level, similarly to our positive control PBMC as it have been observed in previous work[130]. GAPDH, a house keeping gene, was used as a mRNA quality control.

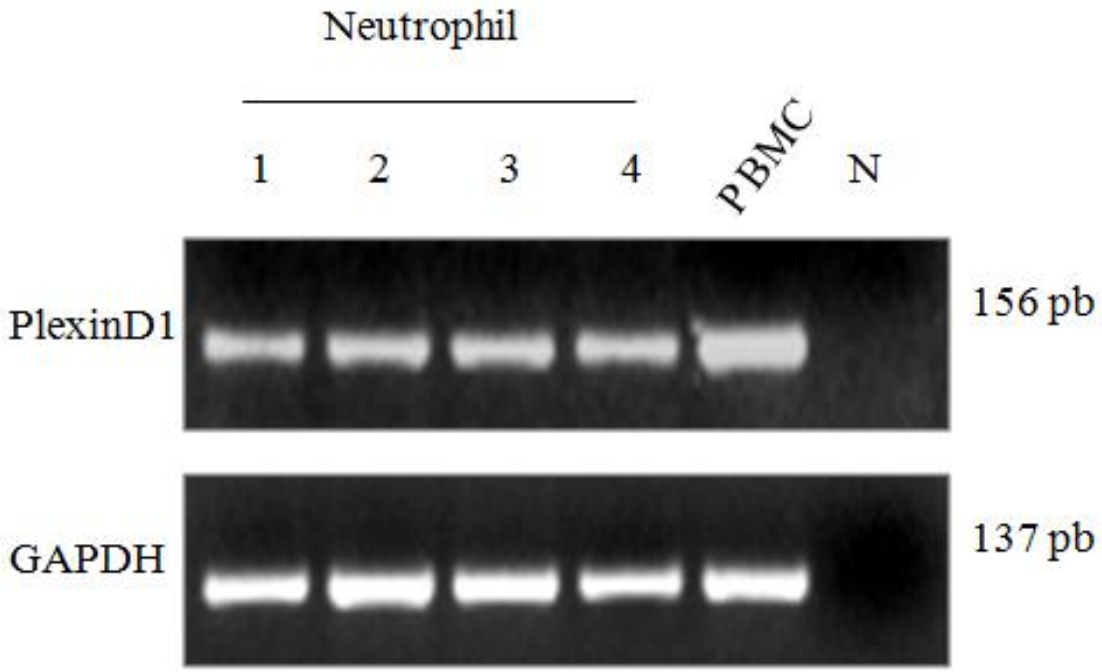


Figure2.2. Human neutrophils express, Sema3E high affinity receptor, PlexinD1 at m RNA level.

Utilizing the TRIzol method, RNA was extracted from freshly isolated human neutrophils. RT-PCR analysis was performed using specific primers of PlexinD1. PCR amplification was performed at 60ºC for 40 cycles, and PCR products were run in 2% agraose gel. Neutrophil samples of random human population are showing consistent strong expression of PlexinD1 compared to the positive control (PBMC). GAPDH is served as a housekeeping gene, and (N) is referring to the negative control. It has been observed that PlexinD1 is highly expressed in T lymphocytes[130], thus in this experiment we utilized PBMC as-positive control, N-negative control that include water.

5.3 Human neutrophils express surface PlexinD1 receptor

Freshly isolated peripheral blood neutrophils were then analysed by FACS analysis using polyclonal goat anti-PlexinD1 antibody (R&D) followed by rabbit anti-goat-conjugated Alexa488 Ab. PBMC which are known to express PlexinD1 were used as positive control (Fig2.3C) [130]. As shown in Fig2.3A, PlexinD1 is highly expressed in human neutrophils from healthy donors. Fig2.3B represents the mean fluorescence intensity (MFI) of 12 samples analysed.

Then we sought to confirm PlexinD1 expression in primary human neutrophils utilizing immunocytochemistry (ICC) analysis. As showed in Fig2.4, PlexinD1 immunoreactivity was detected in 99% of human neutrophils, No staining could be detected with isotype control (i). It is noteworthy to add that ICC reflects staining of both extracellular and intracellular proteins.

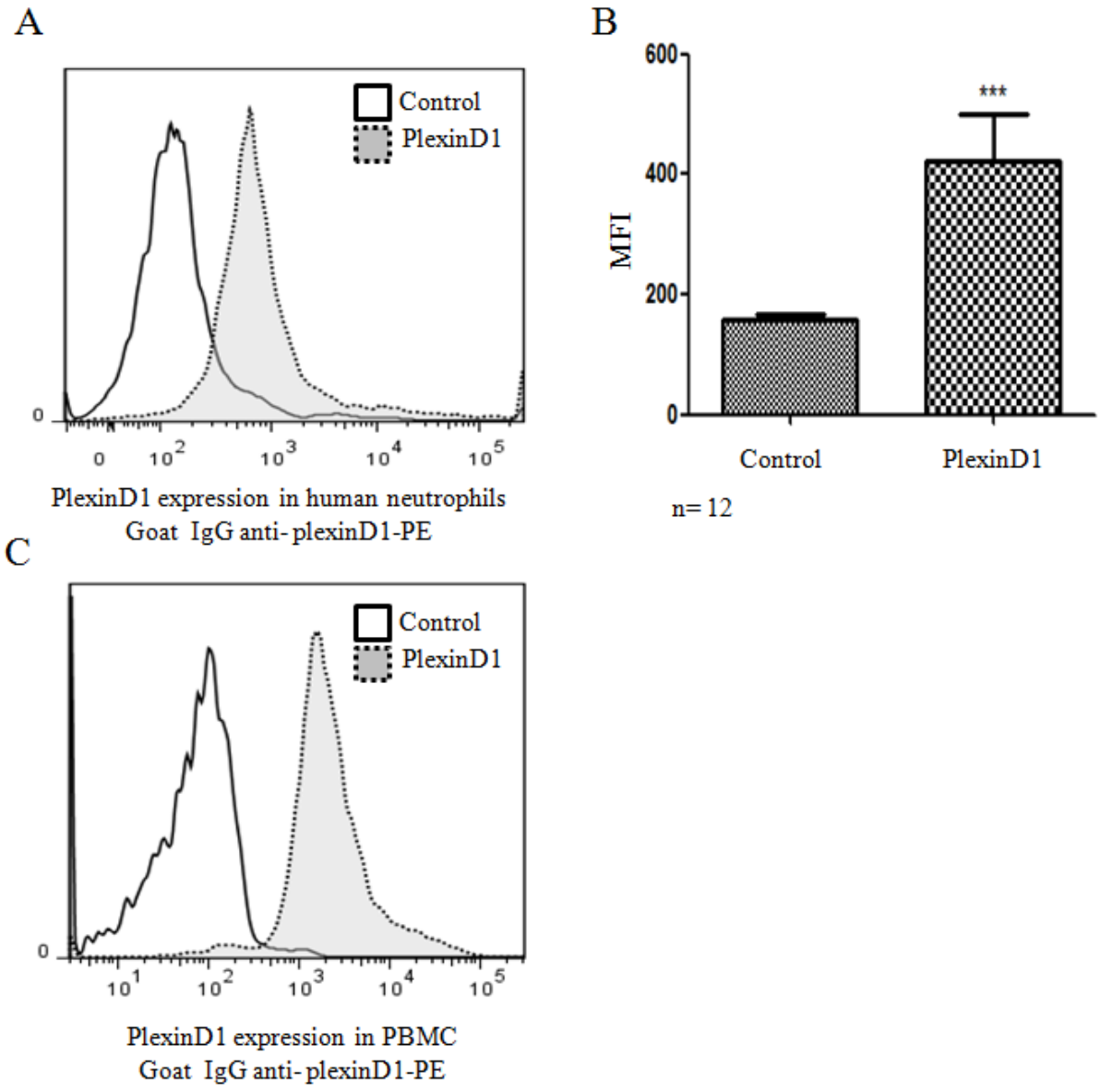


Figure2.3. PlexinD1 expression at protein level in human neutrophils (FACS).

In this experiment we utilized FACS analysis to examine the expression pattern of PlexinD1 on the surface membrane of neutrophils. Cell preparation of $5x10^5$ neutrophils were resuspended in 100ul of PBS and incubated with primary goat anti human PlexinD1 for 30 min on ice followed by anti-goat Alexa488 conjugated antibody for 30 min incubation on ice with avoiding light exposure. As a negative control, cells were labeled with isotype-matched goat IgG (A). Representative experiment of 12 human samples is shown. The MFI was calculated by subtracting the isotype control. The bar graph on the left hand represents the MFI average among 12 different samples (B). PlexinD1 expression in PBMC as a positive control for the antibody (C)

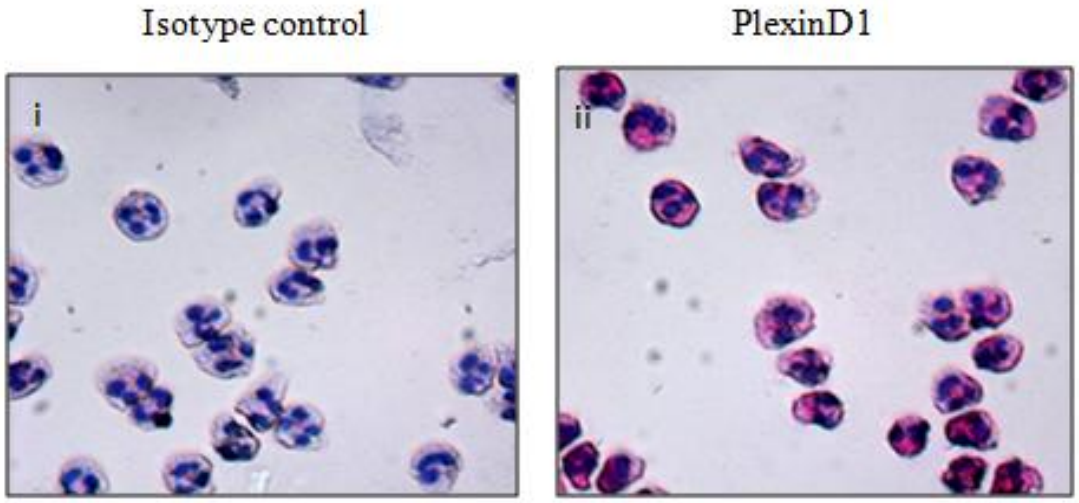


Figure2.4. PlexinD1 expression at protein level (ICC analysis).

Utilizing ICC analysis, we looked at PlexinD1 protein expression in human neutrophils. Cytospin slides containing fixed neutrophils were stained with goat anti human PlexinD1 followed by biotin conjugated rabbit anti goat and streptavidin alkaline phosphatase. Staining was revealed using (i) isotype control stained with goat IgG primary antibody. (ii) 90% of neutrophils are stained with red color, indicating strong expression of PlexinD1, in contrast to the negative control.

5.4 Human peripheral blood neutrophils express Sema3E at mRNA level

The expression of Sema3E has been indicated in most of normal human tissues, and this expression was ranged from moderate to strong in the cytoplasm and some occasional membrane including central nervous system (CNS)[149] and blood vessels[109]. Notably, this expression was negatively correlated with most of the developmental stages of several tissues; for instance, Sema3E has been found at high level in mice embryonic tissues compare to mice in the late stage of age[153]. Moreover, recent work from our laboratory observed a negative correlation of Sema3E expression between normal and asthmatic lung tissues (Hesam. M, personal communication 2012). However, no study has demonstrated the expression of Sema3E in primary human neutrophils. Thus in order to define the expression pattern of Sema3E in human neutrophils, we utilized RT-PCR analysis using specific primer sequence of Sema3E. These two ends primers were located between 15, 16, and 17 axons of the human chromosome 7q.21.11. As indicated in (Fig2.5), neutrophils expressed Sema3E at the mRNA level. It is well known that neutrophils released from the bone marrow enter into the circulation as fully differentiated cells[10], suggesting that Sema3E gene expression might be regulated during neutrophil maturation in the bone marrow. And this regulation might be important to some extent to determine cell fate. Overall, our study demonstrated for the first time that primary human neutrophils expressed Sema3E at gene level.

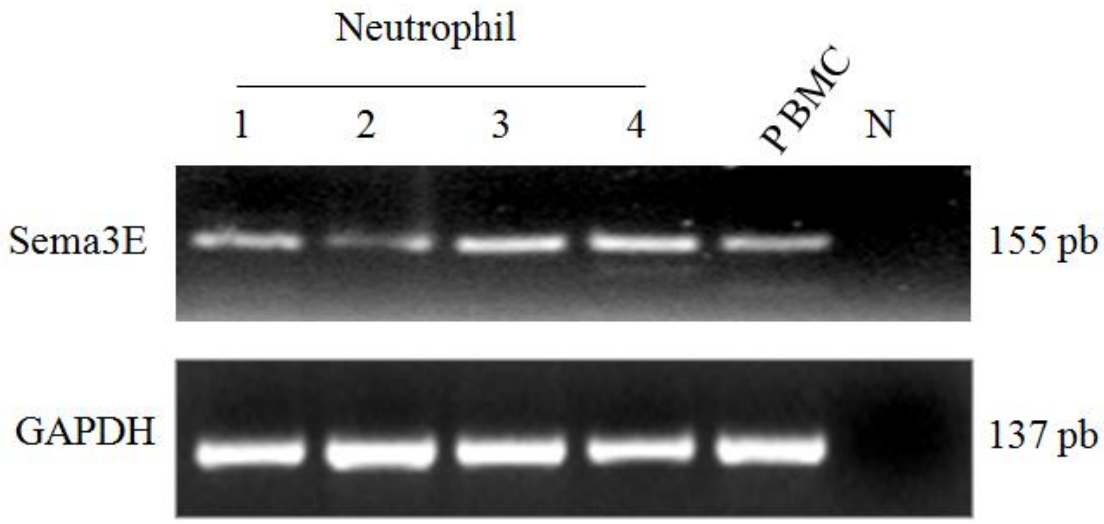


Figure2.5. Sema3E expression at mRNA level.

Total RNA was isolated from human neutrophils of four different subjects using TRIzol method. 2 µg of total RNA was subjected to MultiScribe^{TM} Reverse Transcriptase to synthesize cDNA. PCR amplification was done with Sema3E specific primers. PCR products were then loaded in a 2% agarose gel and run at 100 V for 30 min. P (Positive control) indicated the expression of Sema3E in PBMC and N is a negative control containing water. GAPDH as a housekeeping gene reflects sample quality

5.5 Sema3E protein expression in human neutrophils (FACS)

As mention earlier, Sema3E, as a member of the semaphorin class 3 is the sole protein that exists as a secreted form. This property of Sema3E promoted us to examine the expression of this molecule in the cytoplasm of human neutrophils. In order to test that, we utilized FACS analysis by performing intracellular staining of Sema3E in fresh isolated primary human neutrophils. Cells were permeabilized first utilizing saponin buffer to allow antibody penetration to the cytoplasm followed by detection of target protein. Using mouse IgG1 anti-human Sema3E APC-conjugated antibody, intracellular store of Sema3E was detected in human neutrophils (n=5). However, the surface expression of Sema3E was negative utilizing the same antibody. No staining could be detected with anti-mouse IgG1 APC-conjugated Ab used as an isotope control (Fig2.6).

Utilizing polyclonal goat IgG anti-Sema3E, human neutrophils also exhibit positive expression of Sema3E on the surface membrane,. This expression is reflecting the binding of Sema3E to PlexinD1 on the surface membrane. In this case Sema3E might be secreted by neutrophils in an autocrine manner or secreted by the surrounding tissues in paracrine fashion and bind to PlexinD1 to perform its function in human neutrophils (Fig2.7).

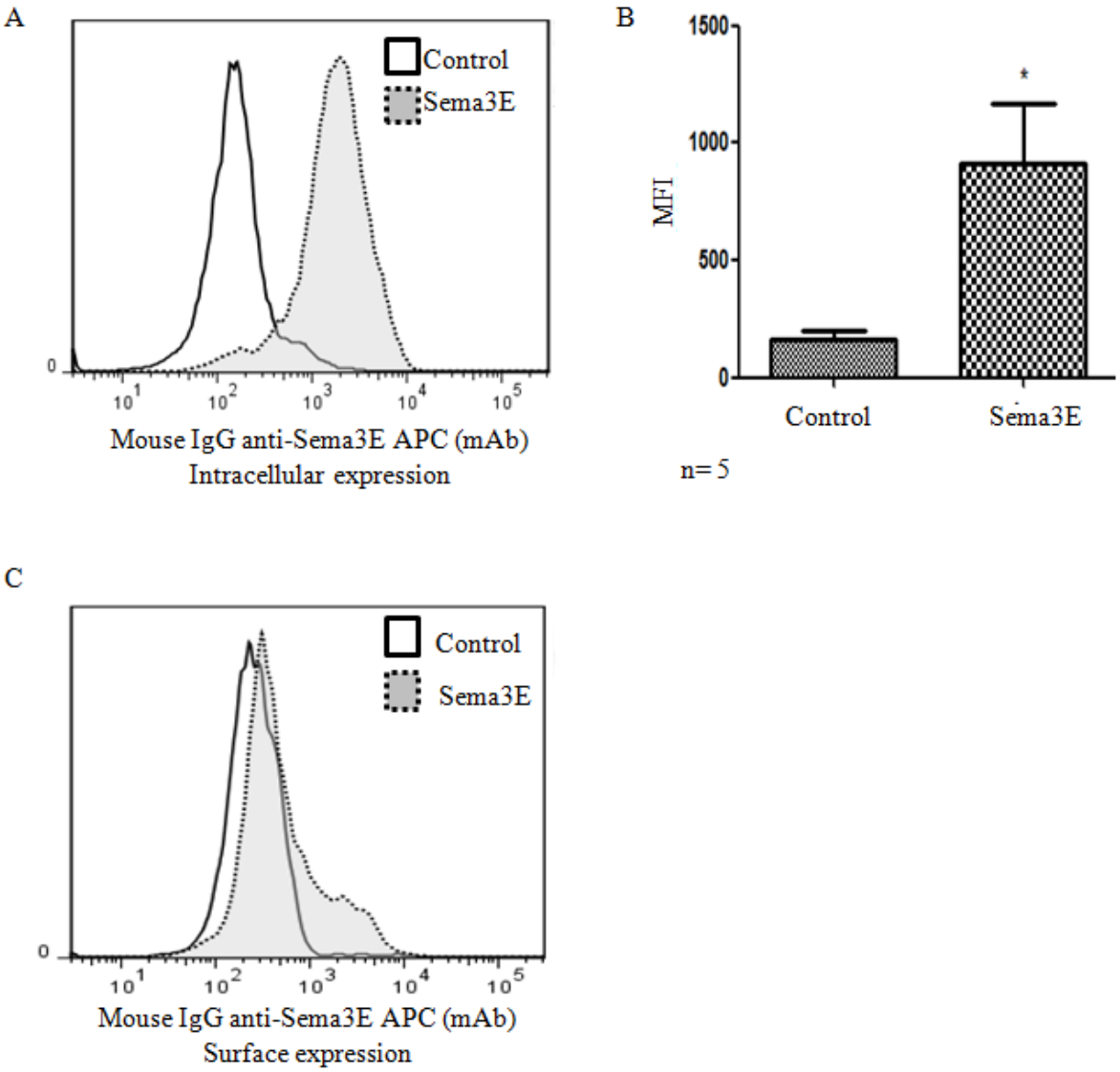


Figure2.6. Sema3E expression in human neutrophils (FACS).

We investigated the intracellular expression of Sema3E protein in human neutrophils utilizing FACS analysis. Fixed neutrophils were resuspended in 100ul flow buffer, and were permeabilized with 100ul of saponin buffer for 15 min, followed by incubation with Fc blocking buffer for 30 min. After two times washing, cells were incubated with mouse anti-human Sema3E-APC conjugated antibody, whereas the control tube was stained with primary anti mouse-APC conjugated as an isotype control (A). Mean florescent intensity of 5 different samples was shown in the left side (B). Surface expression of Sema3E utilizing the same antibody mouse IgG anti-Sema3E (C).

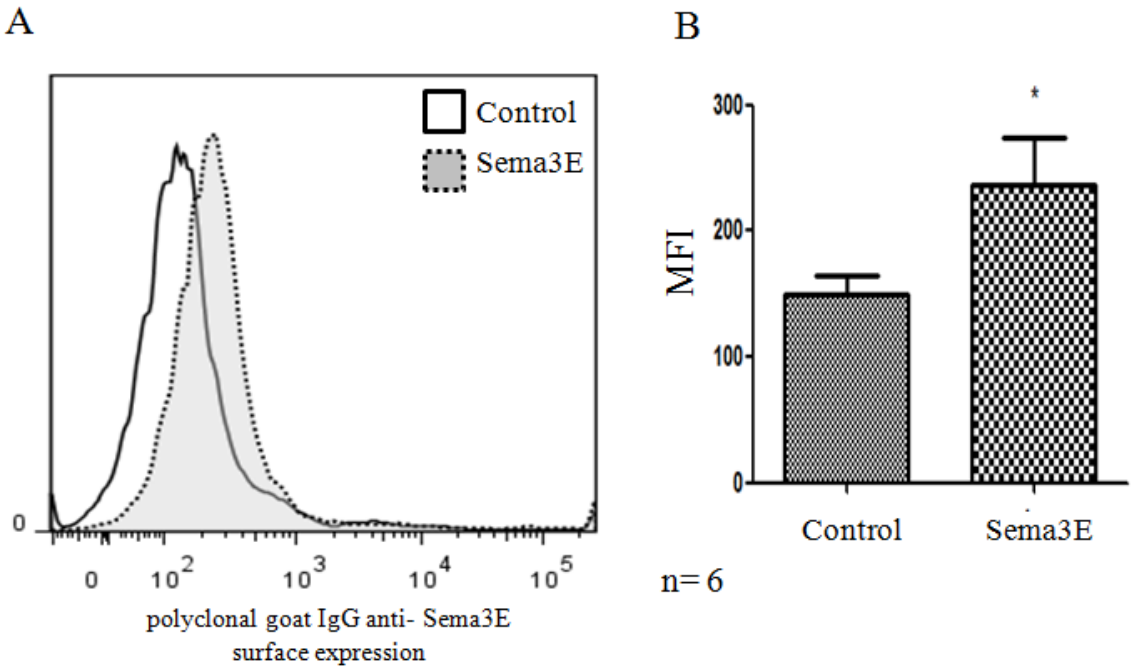


Figure2.7. Surface expression of Sema3E in human neutrophils (FACS).

We investigated the extracellular expression of Sema3E protein in human neutrophils utilizing FACS analysis. Freshly isolated neutrophils were resuspended in 100ul flow buffer. Cells were incubated with goat anti-human Sema3E antibody (R&D) for 30 min on ice, followed by secondary antibody anti-goat Alexa488 conjugated antibody staining for 30 min incubation on ice while avoiding light exposure. The control tube cells were labeled with isotype-matched goat IgG (A). Mean florescent intensity of 6 different samples was shown in the left side (B).

5.6 Sema3E expression in human neutrophils (ICC analysis)

We utilized ICC analysis to investigate the percentage of Sema3E expression in these cells and to examine whether Sema3E is expressed in all isolated populations. ICC staining confirms Sema3E expression at protein level as it indicated in (Fig2.8). However, cells are not equally expressing Sema3E. Some of these cells express high amount of Sema3E, while others showed low expression or no expression for this protein.

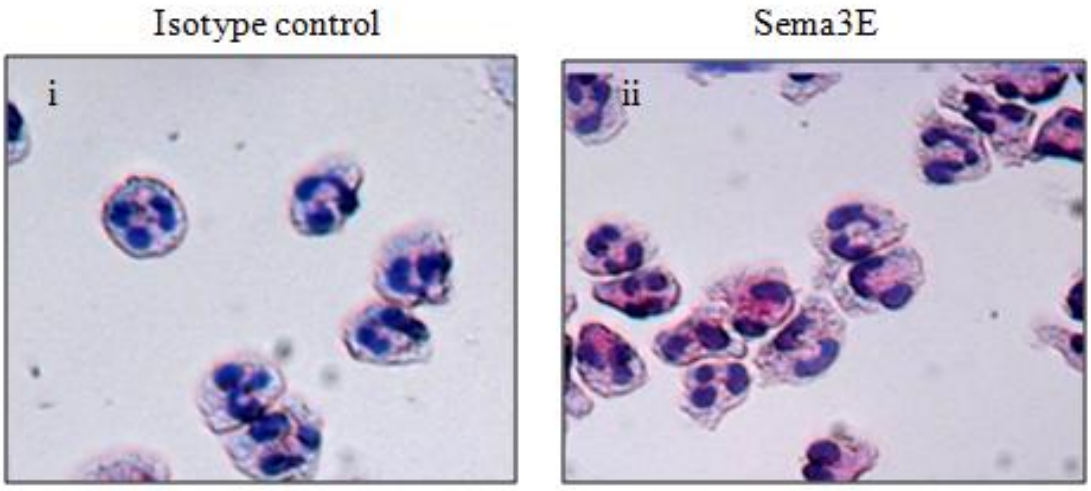


Figure2.8. Sema3E expression at protein level in human neutrophils ICC.

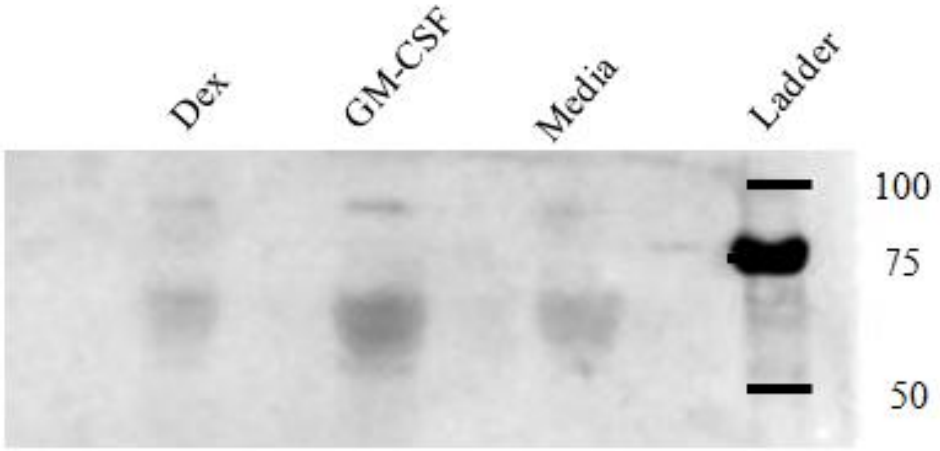
Isolated human neutrophils from healthy individuals were subjected to ICC analysis. Cytospin slides containing fixed cells were incubated with primary goat anti Sema3E for 30 min followed by biotin conjugated rabbit anti goat antibody, and streptavidin alkaline phosphatase. Cells stained with red color reflect the expression of Sema3E. (ii) 50% of cells are showing the expression of Sema3E. (i) Stained with goat isotype control. Cells that are not expressing Sema3E may be synthesizing this protein and releasing it extracellularly or they might be in different stages in term of lifespan.

5.7 Sema3E isoform detection in human neutrophils (Western blot)

Under some proteolytic activity as is seen in some cancer diseases, the full length Sema3E (87kDa), is cleaved to provide two isoforms: 61 kDa and 25 kDa [170]. There is a distinct function for each Sema3E isoform. Upon binding to PlexinD1 the furin resistant (Uncl- Sema3E) 87 kDa fragment, that was designed by Casazza and his group, was able to impair cancer cell migration and prevent its metastatic spreading, whereas the 61 kDa fragment has shown to enhance cell migration and metastatic activity[153]. Since Sema3E is produced and released as a secreted protein outside the cells[94], we investigated whether primary human neutrophils express any Sema3E isoform in the supernatant of cultured neutrophils. We cultured isolated neutrophils in media with/without GM-CSF and Dexamethasone (Dex. 10^{-7}M), and after 18 hours of incubation, collected the supernatants and concentrated them to 100X by ultracentrifugation using filtering tubes. Interestingly, we found that all stimulated/non-stimulated cells release both isoforms 87 kDa and 61 kDa of Sema3E (Fig2.9). For the same amount of protein loaded, GM-CSF stimulated neutrophils showed more production of Sema3E isoform compared to media alone or with Dex stimulated cells (Fig2.9). Our data suggest that the production of Sema3E 87 kDa isoform by peripheral blood neutrophils might be under influence of some proteolytic activity of neutrophils granules including matrix metalloproteinases-9 (MMP9) and elastase that are released under the inflammatory circumstances by activated neutrophils[216]. Thus, these enzymes might cleave the 87kDa fragment of Sema3E to the 61 kDa isoform.

To some extent both isoforms might be important for neutrophil migration as both could bind to PlexinD1 and display their distinct functions[153]. During inflammation where proteolytic enzymes have been found at a high level, the 61 kDa fragment might promote neutrophil recruitment to the

inflamed tissue. On the other hand, as it has been shown that the furin resistant fragment (Un-clev87 kDa of Sema3E) in cancer diseases[173], the 87 kDa fragment might have vigorous ability to maintain neutrophil function within the circulation in the normal circumstances and thus limit neutrophils migration to the normal tissues or organs.



n=3

Figure2.9. Sema3E isoforms in human neutrophils (Western blot).

Isolated neutrophils were cultured in the presence/absence of 10ng/ml GM-CSF, Dex 10^{-7}M at 37˚C for 18 hours. Supernatant was collected and concentrated to 100x using ultracentrifugation RAM $15x10^{5}$. 12ug of protein lysates were resolved on polyacrylamide gels (SDS-PAGE) and were transfer onto PVDF membranes. After blocking with 5% non-fat milk, membrane were incubated with primary anti-Sema3E overnight at 4˚C, followed by anti-rabbit antibody incubation for 1 hour at room temperature. Human neutrophils expressed both 61 kDa and the full length of Sema3E 87kDa as a secreted form in the supernatant of cultured neutrophils. This expression is increased in GM-CSF treated cells.

5.8 Nrp1 is not expressed in primary human neutrophils

It has been demonstrated that the presence of Nrp1 in some neuron cells switches cell repulsion to attraction upon gating the Sema3E-PlexinD1 complex[149]. Since the presence of Sema3E co-receptors exhibit two distinct functional outcomes, we investigated the expression pattern of Nrp1 in primary human neutrophils. We examined Nrp1 mRNA expression using RT-PCR analysis in four different samples. We found that compared to PBMC used as positive control, human neutrophils do not express Nrp1 at the mRNA level (Fig2.10A). This result suggests that the Sema3E-PlexinD1 complex do not require Nrp1 gating in primary neutrophils. Furthermore, FACS analysis confirmed our finding. As indicated in (Fig2.10B) sheep anti-Nrp1 antibody (R&D) was not able to detect the Nrp1 receptor in the surface of human neutrophils (n=3).

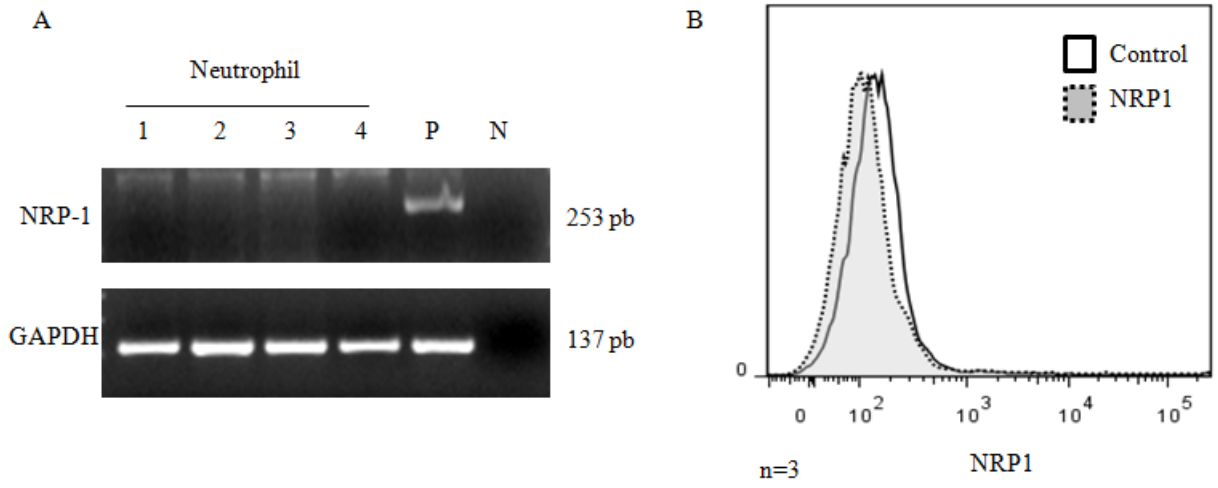


Figure2.10. Nrp1 is not expressed in human neutrophils.

Nrp1 at mRNA signal is absent in human neutrophils as indicated in all four samples in contrast to positive control (A). PCR amplification was performed at 60ºC for 40 cycles using specific Nrp1 primers located between exon 2 and 3. Human neutrophils do not express Nrp1 at the cell surface (B) as detected by FACS analysis.

5.9 VEGFR2 expression in human neutrophils

Previous studies indicated that, together Nrp1, VEGFR2 and PlexinD1 cooperate to induce cell attraction to promote cell adhesion, actin polymerization and thus enhanced cell migration[153]. In this study, we also investigated the expression pattern of VEGFR2 in primary human neutrophils. Interestingly, we found that these cells express VEGFR2 at mRNA level, (Fig2.11A). FACS and immunocytochemistry analysis further confirmed our finding. As indicated in (Fig2.11C), VEGFR-2 expression was significantly detected in the surface membrane of human neutrophils. The mRNA expression of VEGFR2 was detected consistently in all samples, whereas its protein expression was found in 70% of the total samples taken from different individuals, suggesting that the negative population of VEGFR2 in the other 30% samples might be due to some stimuli in the circulation that activate the binding of VEGF ligand to the receptor and thus internalized VEGFR2. We further confirmed protein expression of VEGFR2 utilizing ICC analysis. As indicated in (Fig2.11B), VEGFR2 express at protein level in 99% of total cells of the same sample. With the consideration of ICC ability as a technique that provide both intracellular and extracellular staining of any protein, the 99% cells that expresses VEGFR2 might reflect both intracellular and extracellular expression of VEGFR2. Although human neutrophils express VEGFR2, it might not be involved in the Sema3E-PlexinD1 complex. In most cases VEGFR2 engages with the Sema3E-PlexinD1 complex only in the presence of Nrp1[153]. VEGFR2 with its long intracellular tail form a complex with Nrp1 that is lacking the intracellular tail, and thus transduce the extracellular signalling to the cells[148]. Overall, our results showed that human neutrophils express VEGFR2 at both the protein and mRNA levels, suggesting that VEGFR2 may be involved in Sema3E-PlexinD1 complex.

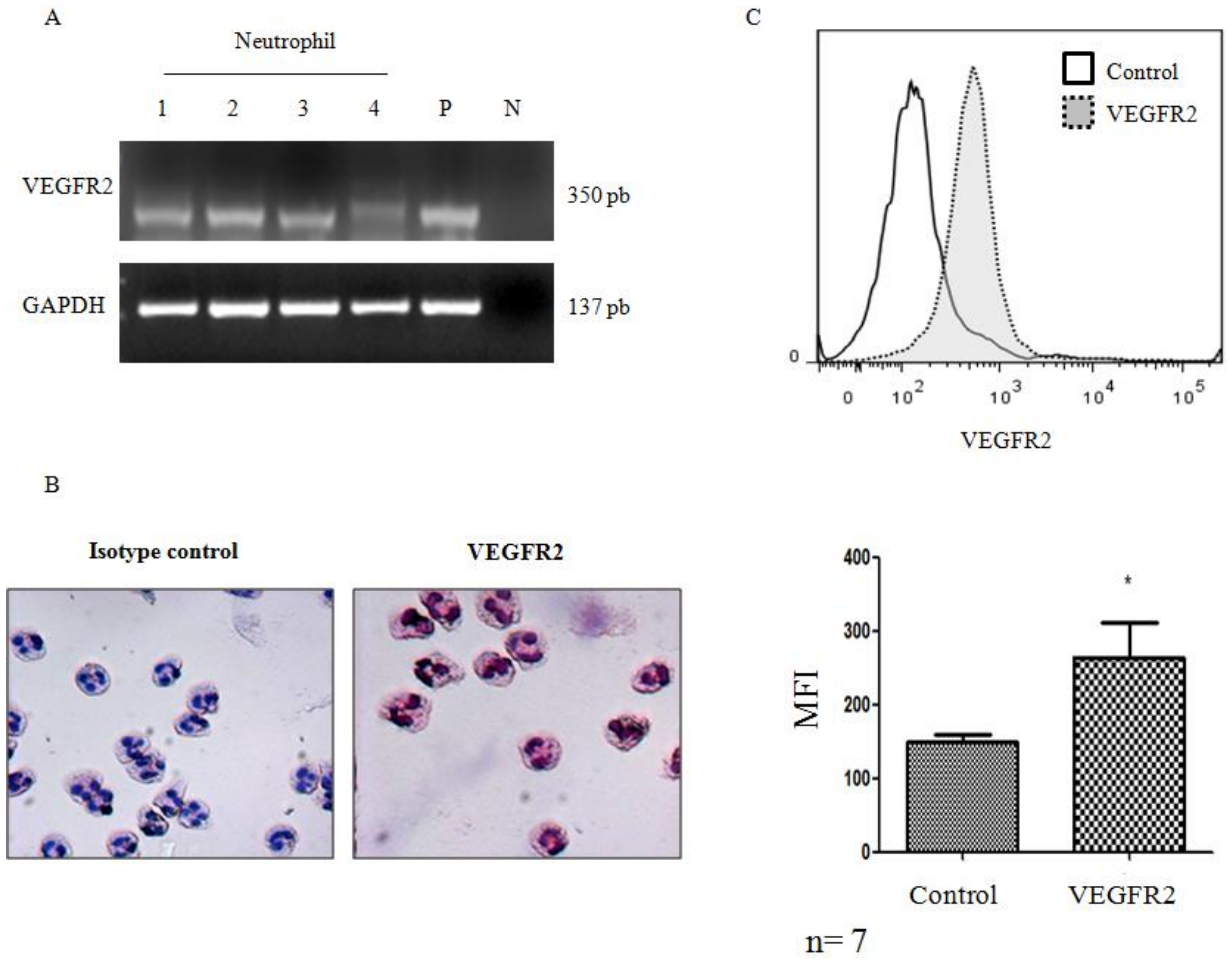


Figure2.11. VEGFR2 expression in human neutrophils.

Utilizing TRIzol method, RNA extracted from freshly isolated human neutrophils as explained in material and methods. RNA samples were subjected to RT- PCR analysis using primers located between exon 2, 3 and 4. Human neutrophils express VEGFR2 at mRNA level as indicated in four different samples. (A) P indicated the expression of positive control, Human Universal Gene (HUG) and (N) is negative control. GAPDH is a housekeeping gene that reflects quality of samples. (B) ICC staining indicated the expression of VEGFR2 at protein level. 90% of cells are showing the expression of this protein in human neutrophils. (C) FACS analysis confirms the expression of VEGFR2 in the surface membrane of human neutrophils. A significant expression of this protein has found in human neutrophils, bar graph indicated the average of MFI of 7 different human samples.

5.10 Sema3E alone does not change human neutrophil migration

Several semaphorin subfamilies do not display any functional affect on the baseline in cell migration of several tissues. Sema3A does not exhibit any affect on the baseline of thymocyte migration[131] or their migration into the medulla[130]. Here, we investigated whether Sema3E affects the basal migration of isolated human neutrophils. Utilizing the transwell system, we demonstrated that administration of Sema3E into the upper chamber of the well does not have any significant effect on neutrophil migration compared to the negative control. Although there was a slight reduction of neutrophil cell number upon 1ng administration of Sema3E alone, however this reduction was not significant in 5 different samples as indicated in (Fig2.12). Overall, these results suggested that Sema3E has no significant effect on basal human neutrophils migration in vitro.

5.11 Sema3E inhibits CXCL8/IL-8 induced human neutrophils migration

It has been observed that Sema3A and Sema3E were able to inhibit chemokine induced thymocyte migration into the medulla during maturation[130-131]. Whether Sema3E may have an effect on chemokine induced human neutrophil migration is not yet established. In this study, we investigated the function of Sema3E on CXCL8/IL-8 induced human neutrophil migration. Interestingly, 1ng/ml of Sema3E was able to significantly inhibit CXCL8/IL-8 induced neutrophil migration. High concentration of Sema3E 100ng/ml also significantly reduce cell number and impaired CXCL8/IL-8 pro-migratory activity by 5 fold compared to CXCL8/IL-8 alone (Fig2.12)

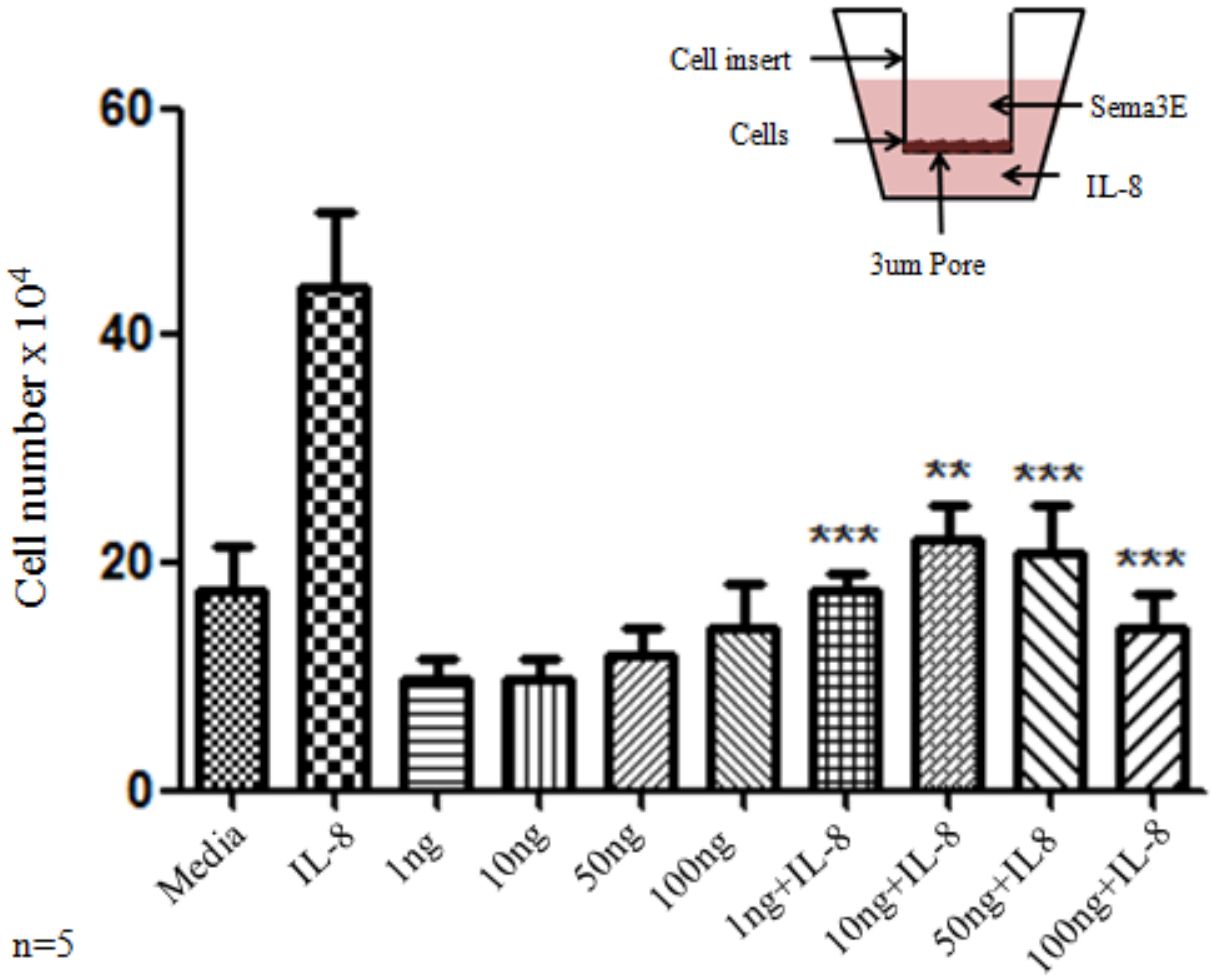


Figure2.12.Sema3E inhibits CXCL8/IL-8 induced human neutrophils migration (Sema3E+cells).

Migration assays were performed in 24 Transwell plates with a 3 □m pore size. Cells were added to the insert and indicated chemokine was added to the bottom chamber. Human recombinant Sema3E was added to the top insert with the cells. After 1h incubation, migrated cells to the bottom chamber were collected and subjected to cell count. Cells incubated in media alone are considered as a negative control, while cells incubated with CXCL8/IL-8 alone in the bottom is the positive control. This data represent five different experiments repeated in duplicate.

5.12 Sema3E administration in the lower chamber with CXCL8/IL-8

Administration of Sema3E in the upper chamber might not allow neutrophils to migrate into the lower chamber toward CXCL8/IL-8. Thus, we investigated neutrophils migration when Sema3E administrated in the lower chamber in the presence of CXCL8/IL-8. In contrast to the first experiment when Sema3E was in the upper chamber there was no reduction in cell number upon 1ng/ml administration of Sema3E combined with CXCL8/IL-8 in the lower chamber. Instead, there is a minor reduction in cell number at high concentration (50ng/ml) compared to CXCL8/IL-8 alone. Using Thkeys test, this different was however not statistically significant. Again a significant reduction in cell number was showed at 100ng/ml of Sema3E when mixed with CXCL8/IL-8, which is comparable to the effect observed in Fig.2.12. All together 100ng/ml of Sema3E is consistently impairing CXCL8/IL-8 induced human neutrophils migration in both conditions (Fig2.13).

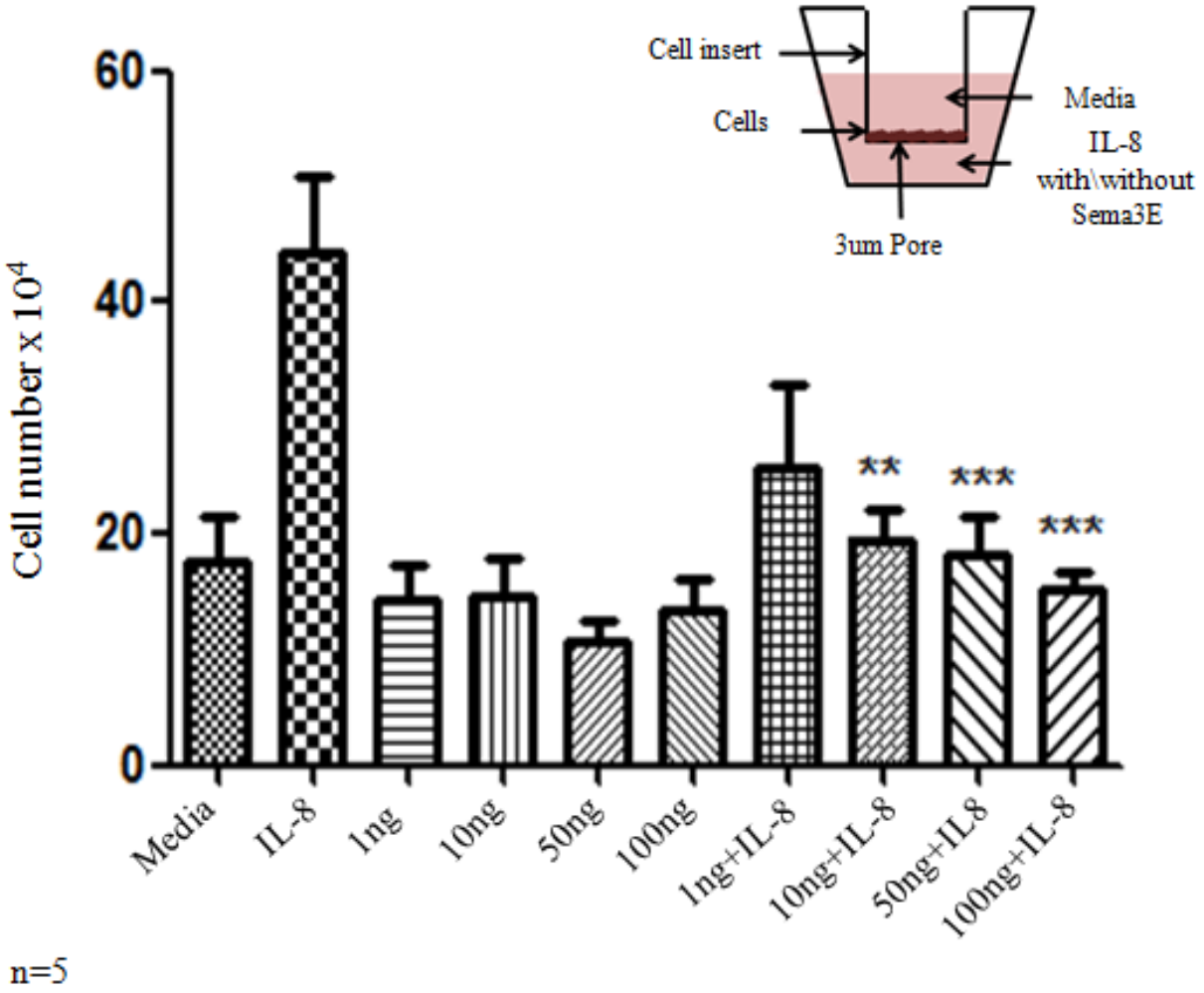


Figure2.13. Sema3E inhibits CXCL8/IL-8 induced neutrophils migration (in the bottom chamber).

Contrary to the previous experiment, we measured Sema3E function on human neutrophils migration where Sema3E is administrated with CXCL8/IL-8 in the bottom well. Isolated fresh cells were added to the insert, whereas Sema3E was added to bottom well with/without CXCL8/IL-8. After1h incubation, migrated cells were collected and counted using haemocytometer. No differences in cell number have seen of 1ng/ml in both baseline and CXCL8/IL-8 combination. However, significant reduction of cell number have found in presence of CXCL8/IL-8 upon 100ng/ml of Sema3E administration. This date represent five different experiments repeated in duplicate.

5.13 Neutrophils chemotaxis against Sema3E gradients in the microfluidic device

We confirm transwell data further by utilizing the microfluidic device that optimized mainly test cell chemotaxis in response to chemokine gradient (see material and methods). As expected, neutrophils exhibited robust chemotaxis activity toward CXCL8/IL-8 gradient (Fig2.14). In contrast, neutrophils moved randomly in Sema3E gradient. The microfluidic based results confirm our transwell based finding. Also, we calculated the CI for microfluidic based experiments as mentioned in material and methods section. The CI for IL-8 based positive control is 0.23 indicating robust chemotaxis. The CI for Sema3E alone is -0.05, indicating random migration of neutrophils in response to Sema3E gradient, however neutrophils speed was comparable to the positive control CXCL8/IL-8 experiment (Fig 2.16).

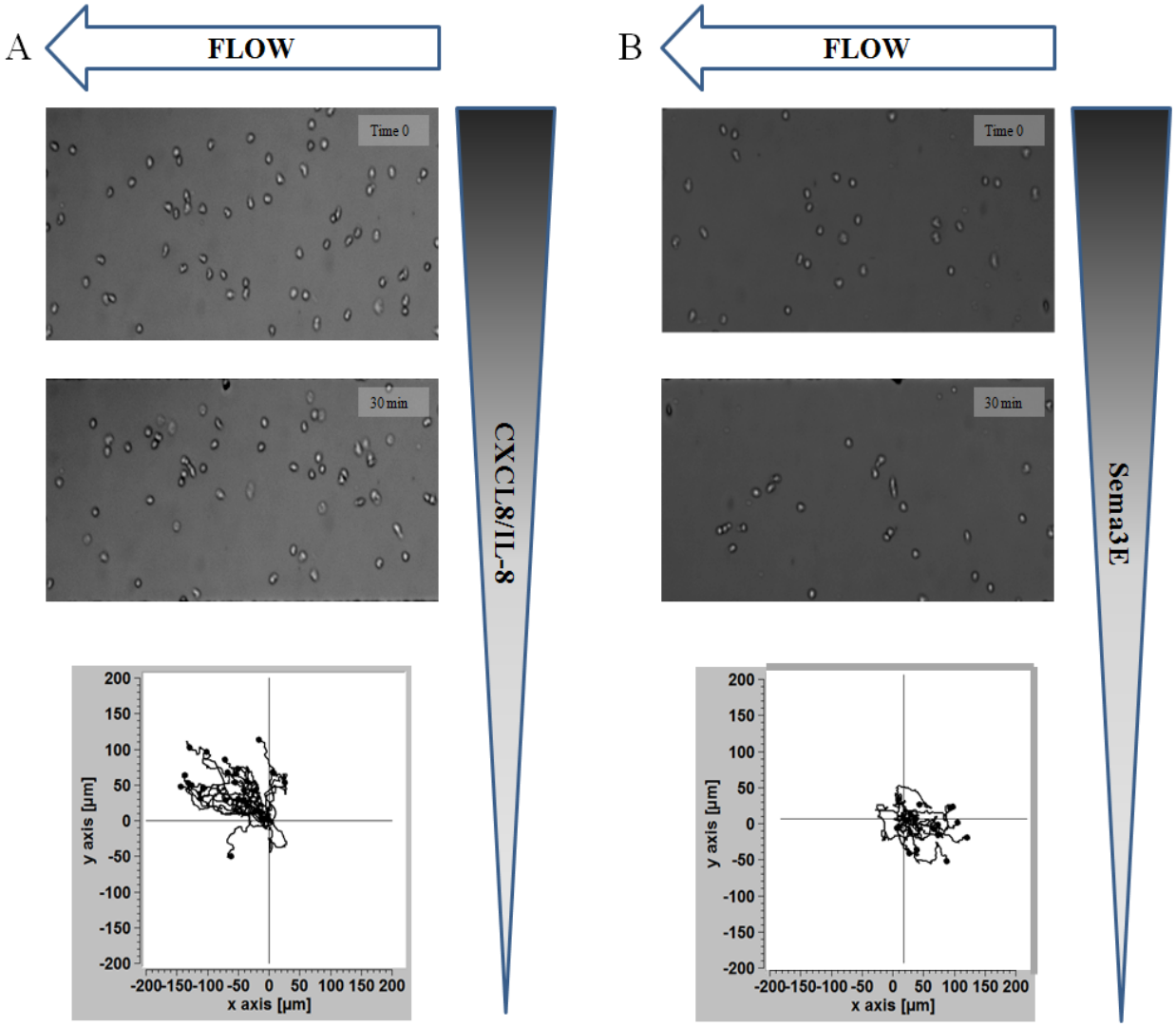


Figure2.14. neutrophils migration against Sema3E gradient.

Pictures of migrating cells in the positive control 10ng/ml CXCL8/IL-8 gradient are showed in (A). And (B) indicated neutrophils migration in response of 100ng/ml Sema3E gradient (See material and methods). The indicated picture represents the event distribution of the cells at time0. After 30min most of the cells were moving toward CXCL8/IL-8 gradient. The migrating cells were calculated from x-y assorts at the beginning and the end of the cell tracks. x-y curve in the bottom is indication the pathway and the distance of each individual cells starting from time 0 up to 30min. (B) In Sema3E gradient however neutrophils were moving randomly.

5.14 Random migration of neutrophils in response to competing gradients CXCL8/IL-8 and Sema3E

In addition, we also assessed neutrophils migration in response to co-existing gradients by microfluidic device. In this experiment, we created competing gradients in which 10ng/ml of CXCL8/IL-8 was configured in one side and 100ng/ml of Sema3E along the opposite directions. Our data indicated that neutrophils migration toward CXCL8/IL-8 gradient was significantly altered in presence of Sema3E gradients compare to CXCL8/IL-8 gradient alone. Moreover, a continuous reduction in chemotaxis index was observed, and this defined random migration of neutrophils in different directions (Fig2.15B). Moreover, when we configured CXCL8/IL-8 and Sema3E in the same side of microfluidic channel, neutrophils were randomly moved within the microchannel and negatively respond to CXCL8/IL-8 gradients (Fig2.15A). In term of cell velocity, only few cells of the total cell number were moving in both experiments compared to CXCL8/IL-8 or Sem3E alone experiment. Also, the CI in both experiments was significantly reduced compare to the positive control (Fig2.16). All together, these experiments demonstrated that Sema3E has a potential ability to inhibit CXCL8/IL-8 chemokine induced neutrophils migration.

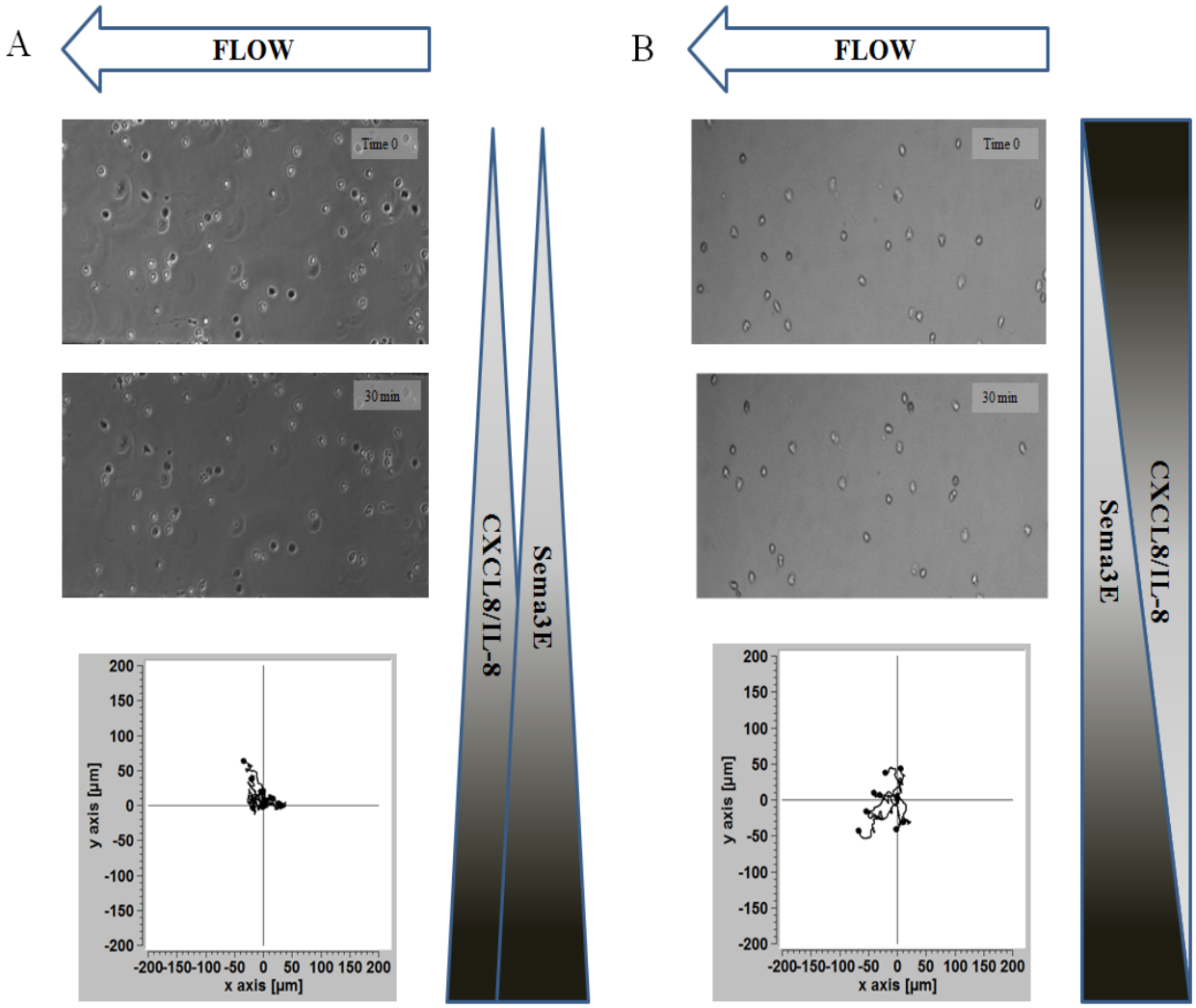


Figure2.15.Neutrophils random migration in CXCL8/IL-8 and Sema3E competing gradients.

(A) Co-existing Sema3E and CXCL8/IL-8 in the same side resulted in neutrophils random migration within microchannel of microfluidic device. (B) Indicated neutrophils chemotaxis in response to Sema3E competing gradients with CXCL8/IL-8 in opposite side of the microchannel.

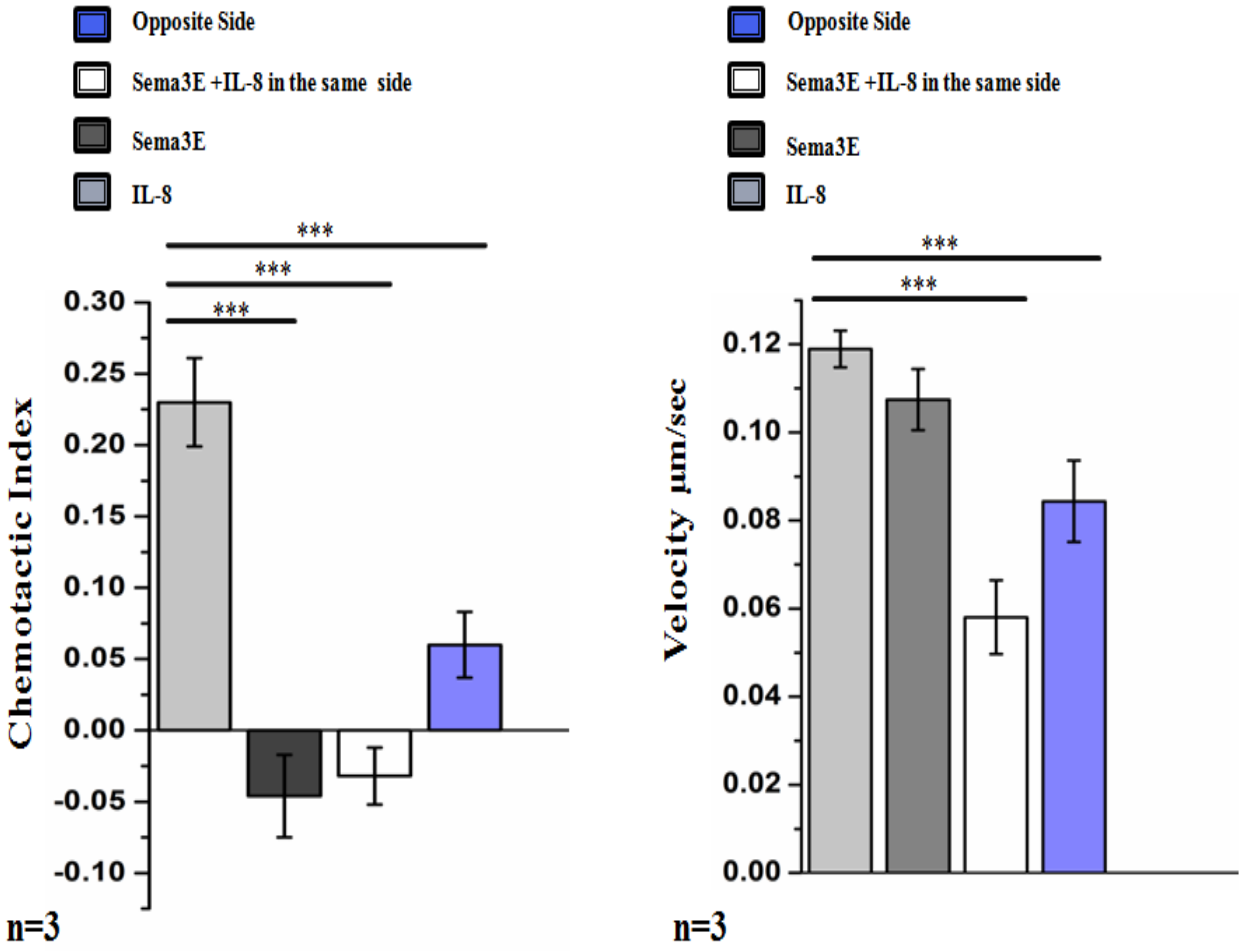


Figure 2.16. Comparison of chemotactic index CI and cell velocity of all four experiments.

CI and speed are showing cell migration in response to CXCL8/IL-8 gradient. However, competing gradients experiments CI and cell velocity were significantly decreased. In Sema3E gradient alone CI was alos decreased, but the cell velocity was comparable to CXCL8/IL-8 experiment.

5.15 Sema3E suppresses Rac1 activity in activated human neutrophils

It have been demonstrated that Rac1 GTPase exhibits important role during neutrophils chemotaxis by regulate cell-body contraction, uropod formation and cell spreading[210]. *In vivo* experiments indicated that neutrophils deficient in Rac1 failed to move toward fMLP induced cell migration into the lung[210]. The regulation of Rac1 activity, a family member of Rho small GTPases, is also essential in Semaphorins/Plexins signalling[153,227]. F-actin polarization is also regulated entirely by the mobilization of Rac1 activity[77,153]. Therfore, we investigated whether Swma3E influence Rac 1 activity in neutrophils. Our preliminary data shown suppression of Rac1 activity upon Sema3E interaction with PlexinD1. Unstimulated neutrophils, which is refer to (Time 0) (Fig2.17A), exhibits high activity of Rac1 that was comparable with the positive control. Although the source of activation is not known, Sema3E was able to suppress Rac1 activity in time dependant manner starting at 0:30s to 3min. Rac1 activity is gradually decreases up to 1 min in CXCL8/IL-8 stimulated cells, and this activity increases after 3min (Fig2.17B). In the combination of Sema3E+CXCL8/IL-8, the activity of Rac1 was comparable to the positive control and (Time 0) (Fig2.17C). Cells that incubated with CXCL8/IL-8 alone and Sema3E+CXCL8/IL-8 combination are under stress. Thus, these cells were not able to response to neither CXCL8/IL-8 nor Sema3E stimulation. This data is very preliminary and need further study of Rac1 activity upon Sema3E stimulation in presence of CXCL8/IL-8 in steady state of isolated human neutrophils

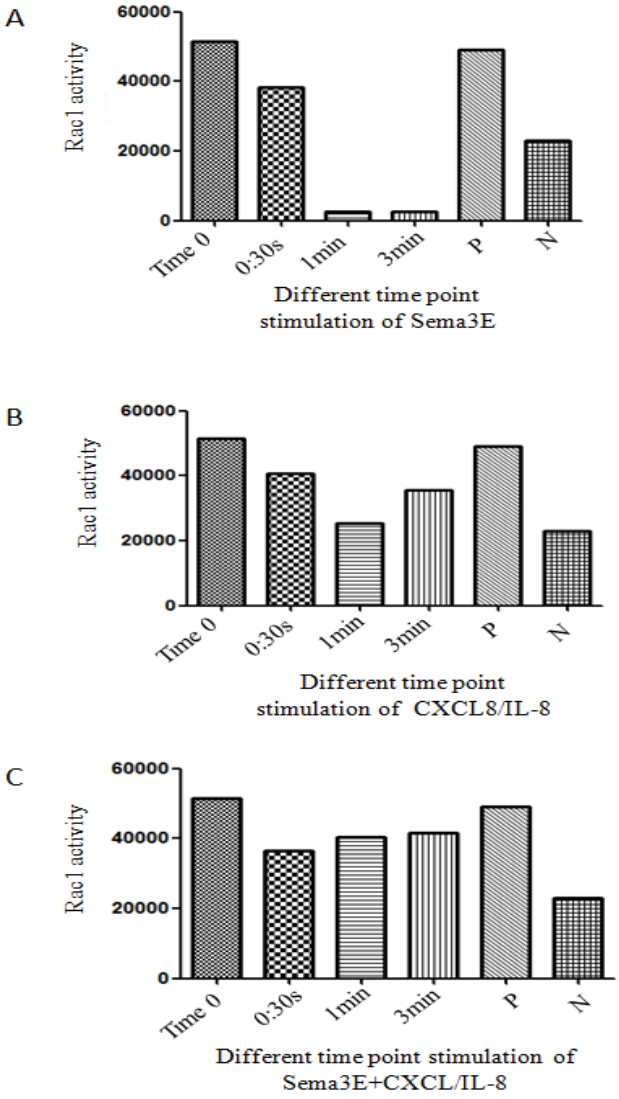


Figure2.17. Measurement of Rac1 GTPase activity.

Snap-frozen neutrophils cell lysates harvested from unstimulated (Time 0) or Sema3E (100 ng/ml) stimulated cells for different time points 0:30s, 1 and 3 min (A). Human neutrophils stimulated with CXCL8/IL-8 (10ng/ml)alone (B) and CXCL8/IL-8 +Sema3E (C) at the indicated time points using a luminometric-based G-LISA Rac1 Activation Assay Kit (Cytoskeleton, Inc. Denver, CO). Briefly, cell lysates were subjected to Rho binding domain of Rac1 in a Rac-GTP affinity 96-well plate. Then, Rac1-GTP was detected using specific primary Ab followed by HRP-conjugated secondary Ab incubation and development with a chemiluminescent reagent. A constitutively active Rac1 provided in the kit was used as positive control (P) in all experiments. The negative control refers to blank well (N)

6- DISCUSSION

6.1 Excessive amount of neutrophils leads to tissue damage

During inflammatory responses, neutrophils are rapidly mobilized to the target organ and release their mediators to fight invading microorganisms[21]. Excessive amount of neutrophils causes damage to the surrounding tissues[64]. Since neutrophils numbers are increased in most of inflammatory diseases such as asthma and chronic obstructive pulmonary disease (COPD) [216,228], it is important to understand the mechanisms and the factors involved in limiting excessive amount of these cells during inflammation. In this study we provide the first evidence that axonal guidance molecule Sema3E inhibits neutrophils migration in response to CXCL8/IL-8 chemokine.

6.2 The role of class 3 semaphorins during inflammatory disorder

The role of class 3 of Semaphorin have been shown in several inflammatory diseases and autoimmune disorders[154, 229] .For example, $CD4^+$ T cells isolated from rheumatoid patients display defective expression of Sema3A, whereas over expression of Sema3A in mice challenged with collagen-induced arthritis remarkably reduce the symptom of the disease[229]. Furthermore, Sema3A mediates $CD4^+$ T cells production of IL-10 that has suppressive activity on autologous $CD4^+$ T cells[229]. Another finding suggested that Sema3C expression in macrophage and fibroblast found in the synovial tissue of rheumatoid arthritis (RA) patients could be one of the factors that reduce sympathetic innervations of RA tissue[230]. Moreover, spinal cord

demyelination of rats injected with Lysophosphatidylcholine (LPC) induced multiple sclerosis, showed upregulation of Sema3A and Sema3F expression in motor cortex[231]. All together, these observations suggested a role for semaphorins in inflammatory diseases which is characterized by inappropriate immune response including rapid migration of leukocytes to the inflamed organ.

6.3 Sema3E expression in human neutrophils

Sema3E, a member of semaphorin family, is a widely expressed protein in several human organs such as central nervous system and thymic gland[130,148]. Sema3E binds to PlexinD1 with high affinity[165] and induces cell repulsion or inhibit cell migration in most of cell types[109,173,192-193,195]. However, no report has indicated the expression pattern of this protein in human neutrophils. Here, we observed for the first time, that Sema3E expression at both mRNA and protein levels is found in human neutrophils isolated from healthy donors.

6.4 Sema3E isoforms expression in human neutrophils

Two opposite functions of Sema3E can be induced to determine directional cell migration; one is the presence of the co-receptors (Figure1.6); and the other, the presence of Sema3E isoforms (Figure1.7). Based on this scenario, Sema3E could induce either cell repulsion or attraction[153]. Recombinant Sema3E (87 kDa) displays negative regulation of thymocyte migration into medulla during thymocyte maturation[130], whereas in cancer cells, Sema3E induces cell attraction, promotes cancer cell migration and metastasis spreading[170]. The pro-migratory property of

Seama3E in cancer cells is due to proteolytic activity induced by furin enzyme that is released by these cells. In furin dependant manner, the full length of Sema3E (87 kDa) that is released by cancer cells is cleaved to 61 kDa and 25kDa isoforms[94,153]. Through its binding to PlexinD1, 61kDa isoform enable ErbB2 oncogene engagement with PlexinD1, triggering intercellular signalling cascade to promote cell migration and metastatic spreading of cancer cells[169]. Contrary to this finding, a recent study by the same group showed that furin resistant Sema3E (Uncl-Sema3E) altered the inhibitory function of 61 kDa and showed competitive binding to PlexinD1, triggering intracellular cascade that sequesters it away from ErbB2. Thereby, inhibit cancer cell migration and preventing its metastasis and spread[173]. Interestingly, in this study we demonstrated that human neutrophils express both isofoms, the full length 87 kDa and 61 kDa suggesting that a proteolytic activity might be induced by neutrophils granules, which contains a wide range of proteolytic enzymes such as MMP9 and elastase [216]. Further investigation into the effects of granular contents of human neutrophils on Sema3E (full length) will provide clearer understanding on whether the proteolytic mediators have any influence on the full length Sema3E and its subsequent induction of expression of Sema3E 6kDa in human neutrophils. Autocrine action of semaphorin class 3 has been observed in several cell types, Sema3A for example has been found to stimulate cell adhesion in breast cancer cells[136] and regulate angiogenesis in endothelial cells in an autocrine fashion [232]. Migration of osteoblasts was also inhibited by Sema3E production in an autocrine manner[195]. Thus, Studying the function of 61kDa Sema3E in human neutrophils will provide clearer understanding of its role during inflammation.

6.5 PlexinD1 and VEGFR2 expression in human neutrophils

PlexinD1 is ubiquitously expressed on a wide variety of human tissues such as endothelial cells[110], bone marrow derived cells[195] and cancer cells[135]. Since PlexinD1 is the functional high affinity receptor of Sema3E[165], we investigated the expression pattern of this receptor on human neutrophils. We found consistent high expression of PlexinD1 at both mRNA and protein levels in human neutrophils.

It have been demonstrated that the expression of the other co-receptors VEGFR2 and Nrp1 in the same tissue alters the function of PlexinD1. For example, it has been reported that gating Sema3E/PlexinD1 complex with Nrp1 switches axonal repulsion to attraction [149]. Also, Bellon et al had indicated that VEGFR2 engagement with Nrp1/PlexinD1 complex induces cell attraction and increases axonal growth[148]. Although Sema3E binds only PlexinD1 in tissues outside the nervous system[165], we investigated the expression pattern of other semaphorin co-receptors Nrp1 and VEGFR2 in human neutrophils. The negative expression of Nrp1 in human neutrophils as indicated in Fig.10 suggests independent interaction of Sema3E /PlexinD1 complex.

Contrary to Nrp1, our study indicated the expression of VEGFR2 at mRNA and protein levels in human neutrophils. VEGFR2, with its long intracellular tail, engaged with Nrp1/PlexinD1 complex to transduce the extracellular signal to the intracellular part of the cells[153]. However, Sema3E/PlexinD1 did not require Nrp1 in developing vasculature[109]. Based on above observations, we speculate that the inhibitory function of Sema3E on CXCL8/IL-8 stimulated human neutrophils migration due to Sema3E /PlexinD1 interaction which may be independent of the co-receptors, Nrp1 and VEGFR2. Further investigation utilizing VEGFR2 neutralizing

antibody will illustrate the function of this receptor on human neutrophils migration in response to Sema3E.

6.6 Suppressive activity of semaphorins on chemokine induced cells migration

Several studies have investigated the role of class 3 semaphorin in cell migration. Negative regulation of thymocyte migration has seen by Sema3A on CXCL12 induced thymocyte migration into medulla. The ability of Sema3A to inhibit recruitment activity of thymocyte triggered by CXCL12 is through the down regulation of CXCR4 receptor that expresses in all thymocyte subsets [131]. In addition, Choi et al demonstrated the role of Sema3E in orchestrating thymocyte trafficking during maturation within the thymus [130]. Activation of PlexinD1 upon binding to its ligand Sema3E impairs the activity of CCL25 chemokine enhanced thymocyte migration into medulla[130]. Here, we have observed the function of Sema3E on CXCL8/IL-8 induced human neutrophils migration. Similar to the finding by Choi et al, we reported for the first time that Sema3E inhibits neutrophils migration induced by CXCL8/IL-8. We observed significant reduction in cell number at a concentration of 100ng/ml, which is similar to the concentration used by Choi et al (10ug/ml). Our data is also in agreement with another report that showed the effect of Sema3A on thymocyte migration [131].

We further confirmed the function of Sema3E in human neutrophils migration by utilizing the microfluidic devices. Although the traditional Transwell system indicated the number of migrated cells in response to chemokine stimuli, it does not provide real time visualization of cell movement, requires a large number of cells (100μl of $5x10^{-6}$ /ml), and does not reflect a proper

control of gradient generation. Microfluidic device on the other hands provides a considerable improvement of Transwell system and is becoming a more useful tool in studying neutrophils chemotaxis. It requires less cell number, better control of the chemokine gradient and able to visualize cells movement during chemotaxis activity [223]. Thus, in this project we utilized microfluidic devices to have a clear view of Sema3E affect on human neutrophils migration in response to CXCL8/IL-8 gradient. Our microfluidic data confirmed what have seen in Transwell system.

6.7 Is neutrophils proper target for Sema3E?

Very recent study has demonstrated the functional role of Sema7A on neutrophils migration. In this study, Sema7A knockout mice showed to decreased neutrophils transmigration to the endothelial cells during hypoxia [233]. In support of this, our data also shows the functional role of Sema3E in human neutrophils migration (Figure2.16).However, in contrast to Sema7A, Sema3E role in human neutrophils can inhibit cell migration induced by CXCL8/IL-8 chemokine. Due to this recent finding of Sema7A function in neutrophils migration, we hypothesis that Sema3E could be a therapeutic target that might decrease or limit neutrophils migration to the inflamed organ in acute inflammatory responses such as asthma. Further in vivo studies are required to validate this finding.

6.8 CXCL8/IL-8 chemokine and semaphorins

Several studies have indicated the involvement of semaphorin family in CXCL8/IL-8 production. CXCL-8 is one the most powerful chemoattractant factors that induces neutrophils recruitment, and it can be produced by activated neutrophils itself. Sema7A for example, has been found to stimulate monocytes production of CXCL8/IL-8. Co-culturing keratinocytes expressing Sema7A with monocytes enhances CXCL8/IL-8 production by these cells [234]. Interestingly, Sema3B, with respect to its suppresser capacity of tumor growth, enhances CXCL8/IL-8 production and as a result increases metastatic spreading of tumor cells [235]. Moreover, Sema5A that secreted by pancreatic cancer cells has the ability to enhance angiogenesis and migration of these cells through CXCL8/IL-8 production by endothelial cells [236]. In this study, we are demonstrating the functional activity of Sema3E in CXCL8/IL-8 induced human neutrophils migration. Although no change was seen on the base line of Sema3E on human neutrophils migration whereas significant reductions of cell number was observed in the combination of Sema3E and CXCL8/IL-8.

6.9 Rac1 activity in response to Sema3E stimulation in human neutrophils

We hypothesised that the inhibitory affect of Sema3E upon binding to PlexinD1 in human neutrophils is through the regulation of small GTPases Rac1 in human neutrophils[227, 211, 209]. The repulsion activity that induced by Semaphorins/Plexins signalling is rely on small GTPase (Rho and Rac1); Rac1 activity is important to induce cell migration through the regulation of actin polymerization and integrin adhesion to the ECM [153]. For example, Sema5A binding to plexinB3

has showed inhibitory function of glioma cell migration through Rac1 inactivation [237]. Interestingly, Recent study has found that Sema3F expression is regulated by Rac1 activity in Neurofibromatosis type 2 (NF2). Merlin, the NF2 gene product, act as a tumor suppresser gene in schwannoma cells. The over expression of Rac1 in schwannoma cells that is lacking merlin/NF2 down regulate the expression of Sema3F [238].

Several studies have indicated the role of Rac1 activity on human neutrophils migration. Under inflammatory circumstances, the adhesion molecules on neutrophils are activated to enhance transmigration of these cells through endothelial cells [65]. It has been documented that small GTPase Rac is involved in neutrophils movement and transmigration to site of infection within endothelial cells. In a filamin-dependent manner, the Rho-guanine nucleotide exchange factor (Trio) was able to induced ICAM clustering and recruitment to adhesion site of leukocyte through Rac1 activity [77]. Furthermore, *in vivo* study indicated that Rac1 deficient neutrophils failed to sense the response of chemoattractant stimuli that triggers actin assembly. Although neutrophils in these mice exhibit normal chemokinesis, they failed to migrate toward the source of chemoattractant stimuli [211]. Interestingly, loss of Rac1 activity in neutrophils display significant reduction of neutrophils recruitment to the lung in response to inflammatory stimuli [210]. Here, our preliminary data is showing the inhibition of Rac1 activity upon Sema3E stimulation of human neutrophils. Since Rac1 activity is regulated by both signaling Sema3E/PlexinD1 and CXCR1, which is CXCL8/IL-8 receptor in human neutrophils, our future study is to investigated the activity of Rac1 in human neutrophils induced CXCL8/IL-8 migration upon Sema3E binding to PlexinD1.

In conclusion, it is becoming increasingly appreciated that Sema3E as an axon guidance molecules in the nervous system also plays a role in human neutrophils function. Our study indicated that Sema3E and its receptor PlexinD1 are expressed in human neutrophils, and upon Sema3E-PlexinD1 interaction, human neutrophils migration in response to CXCL8/IL-8 were attenuated. The inhibitory signalling of Sema3E in neutrophils migration occurs through the regulation of small GTPase Rac1 that has been found to mediate actin rearrangement. Our novel finding provides rationale for putative therapeutic approach of many inflammatory diseases in which neutrophils are becoming pathologica

7-FUTURE DIRECTIONS

1- Clarify whether the inhibitory affect of Sema3E is PlexinD1 dependent. That could be examined utilizing anti-PlexinD1 neutralizing antibody or recombinant PlexinD1 Fc fragment.

2- Examine the regulation of Sema3E gene expression in human neutrophils in response to GM-CSF, and CXCL8/IL-8.

3- Investigate whether the involvement of VEGFR2 is required in Sema3E-PlexinD1 complex utilizing anti-VEGFR2 neutralizing antibody.

4- Studying the regulation of CXCL8/IL-8 gene expression of neutrophils cultures in presence of Sema3E.

5- Investigate the expression of Sema3E 61kDa in the supernatant of human neutrophils cultures in response to elastase, a proteolytic enzyme stored in neutrophils granules.

6- Verify Sema3E function on human neutrophils migration in presence of both CXCL8/IL-8 and elastase.

7- Define the regulation of CXCR1 and CXCR2, which are CXCL8/IL-8 receptors, upon Sema3E stimulation on human neutrophils in presence of CXCL8/IL-8

8- Investigate the activity of Rac1 in human neutrophils upon Sema3E interaction with PlexinD1 in asthmatic and COPD patients.

9- Explore the inhibitory effect of Sema3E in neutrophils migration during acute asthma response. Sema3E knock mice are a proper animal model to investigate the role of Sema3E in neutrophils migration. The same mice will challenge by LPS for 3 to 4 hours and sacrificed to collect BAL fluid. The effect of Sema3E will be measured based on the neutrophils number in BAL fluid.

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