Semaphorin-3A, a regulator of the immune system

Maryam Heidari

Department of Anatomy & Cell Biology

McGill University

Montreal,Quebec

April 2016

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

DEDICATION

Dedicated to the latest savior of pure descendant of Prophet Muhammad (peace be upon him), MAHDI, who will come and will save the world from tyranny and injustice

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

I am very grateful to GOD ALMIGHTY for without His graces and blessing, this study would not have been possible.

As Prophet of mercy and kindness, Muhammad (peace be upon him) said: "Do not thank GOD who does not thank people", I wish to express my appreciation and sincere thanks for the guidance of my thesis supervisor Dr. Stephanie Lehoux. I am grateful for giving me the opportunity to join her laboratory, for her expertise and advices during my PhD and her editorial help with this thesis.

I would also like to thank Dr. Hojatollah Vali, one of the members of my supervisory committee, for his supports at the beginning in my admission process and later on for his financial supports without any expectations.

In addition, I would like to thank Dr. Craig A. Mandato, chair of the department of Anatomy & Cell Biology and the other member of my supervisory committee, for many invaluable moral supports.

Non of this could have been happened without having a great team in the laboratory. First of all, I am very grateful to Dr. Talin Ebrahimian, the Research Associate of our laboratory, for all her guidance and help with my project and experiments and without her this adventure would not finish.

My thanks are also go to Daniel Rivas and David Simon for excellent technical assistance, especially David for challenging my project all the time!

I would also like to thank my thesis committee members, Dr. Nathalie Lamarche-vane, Dr. Jean-Francois Cloutier for their comments and recommendations during

my thesis advisory meetings. In addition I would like to especially thank my mentor Dr. Elaine Davis for her supports and advices. She was there for me whenever I needed and also financially supported me in part.

The path toward this dissertation has been circuitous. Its completion is thanks in large part to the special person, my husband Hassan, who understood, supported, and stuck with me along the way. I am tremendously fortunate to have him beside me. In addition my many thanks are also addressed to my great children Sana and Mohammad Mahdi who understood and tolerated a busy mother.

Also in this very special moment, I would also like to express my deepest thanks to my Parents, Afagh and Reza, for their love encouragement and supports that made this journey more easier.

PREFACE AND CONTRIBUTION OF AUTHORS

This dissertation is written in accordance with the guidance for thesis preparation from faculty of graduate studies and research of McGill University. It is written as a manuscript-based thesis and comprises of three chapters.

I am the author of this thesis and the primary scientist of the research project presented. I was involved in all aspects of the project and I have primarily performed all the research experiments and analysis except for some certain parts as mentioned below.

The experiments were designed by Dr. Stephanie Lehoux who is also provided mentorship, guidance, and editorial help. In addition Dr. Talin Ebrahimian, the research associate of the laboratory, was involved in part in designing the experiments and helped me in some *in vitro* experiments and analysis(figures 2.1A & D, 2.3, 2.5B & C, 2.7, 2.8 2.11B). Daniel Rivas and Davis Simon, technical assistant of the laboratory, provided technical support and helped me in some experiments (figures 2.4, 2.9, 2.10, 2.13, table 2.1). Catherine A Lemarie performed the experiments depicted in 2-11B.

All research elements of this thesis are considered original scholarship and distinct contribution to knowledge.

ORIGINAL CONTRIBUTION TO KNOWLEDGE

1- "Semaphorin-3A reduces atherosclerotic plaque formation in apoE$^{-/-}$ mice through regulation of M2 type macrophage migration"

Maryam Heidari, Talin Ebrahimian, David Simon, Hojatollah Vali, Craig A. Mandato, and Stephanie Lehoux

In this study, we provide the first evidence of an effect of semaphorin-3A on atherosclerosis plaque formation. We demonstrate for the first time that semaphorin-3A decreases atherosclerosis plaque formation probably through increases M2 macrophage migration.

2- Semaphorin-3A and acute inflammation

Maryam Heidari, Talin Ebrahimian, David Simon, Hojatollah Vali, Craig A. Mandato, and Stephanie Lehoux

In this study, we have started to study the impact of semaphorin-3A on local, acute inflammation. So far there is no evidence of an anti-inflammatory role of semaphorin-3A in innate immunity. Our preliminarily data demonstrate that semaphorin-3A decreases LPS-induced inflammation probably through regulation of macrophages and T cells.

List of Abbreviations

AGE	Advanced Glycation End
AHA	American Heart Association
AKI	Acute Kidney Injury
APC	Antigen Presenting Cells
ApoB-LP	Apolipoprotein B-containing Lipoproteins
$ApoE^{-/-}$	Apolipoprotein E knock-out
Arg I/II	Arginase I/II
ATP	Adenosine triphosphate
B.C.	Before Christ
BCA	Brachiocephalic Artery
CCL	chemokine C-C motif Ligand
CCR	chemokine C-C motif Receptor
CD	Cluster of Differentiation
Cdc42	Cell division control protein 42
CTRL	Control
CUB	C1r/C1s, uEGF, and BMP1
CVD	Cardiovascular Disease
CX_nCL	C-X_n-C motif Chemokine Ligand

CX_nCR	C-X_n-C motif Chemokine Receptor
DC	Dendritic Cell
DRG	Dorsal Root Ganglion
EAE	Experimental Autoimmune Encephalomyelitis
EC	Endothelial Cell
ECM	Extracellular Matrix
ERK	Extracellular signal-regulated kinases
FACS	fluorescence-activated cell sorting
FAK	Focal Adhesion Kinase
FAO	Fatty Acid Oxidation
GAP	Guanosine triphosphatase Activating Protein
GDP	Guanosine diphosphate
GPI	Glycosylphosphatidylinositol
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatase
ICAM-1	Intercellular Adhesion Molecule 1
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin
iNOS	inducible Niitric Oxide Synthase
IPT	Ig like Plexin and Transcription factors

JNK	c-Jun N-terminal kinase
kDa	kilo Dalton
LFA1	Lymphocyte Function-associated Antigen 1
LDL	Low Density Lipoproteins
$LDLR^{-/-}$	Low Density lipoprotein Receptor knock-out
LPS	Lipopolysaccharide
LRP1	LDL Receptor-Related Protein 1
Ly6C	Lymphocyte antigen 6C
MAM	Meprin/A5-protein/PTPmu
MAP	Mitogen Activating Protein
MAPK	Mitogen Activating Protein Kinase
MCP-1	monocyte chemotactic protein 1
MCSF	Macrophage Colony-Stimulating Factor
MD-2	Lymphocyte antigen 96
MHC	Major Histocompatibility Complex
MMP	Matrix Metalloproteinase
MyD88	Myeloid Differentiation primary response gene 88
NF-κB	Nuclear Factor kappa light-chain enhancer of activated B cells
NK cells	Natural Killer cells
NO	Nitric Oxide

Nrp	Neuropilin
OCT	Optimal Cutting Temperature
Otk	Off-track receptor
Ox-LDL	Oxidized LDL
Ox-RBC	Oxidized Red Blood Cell
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
Plx	Plexin
PRR	Pattern Recognition Receptor
PSGL-1	P-selectin Glycoprotein Ligand-1
PSI	Plexin, Semaphorin, and Integrin
qRT-PCR	Quantitative Real-time Polymerase Chain Reaction
ROS	Reactive Oxygen Species
Sema	Semaphorin
TCR	T Cell Receptor
TF	Tissue Factor
TGF-β	Tissue Growth Factor β
Th cell	T lymphocyte helper cell
TIMP	Tissue Inhibitors of Metalloproteinase
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor

Treg	Regulatory T cells
VCAM-1	Vascular Cell Adhesion Molecule 1
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptors
VEH	Vehicle
VLA4	Very Late Antigen 4
VSMC	Vascular Smooth Muscle Cell
WHO	World Health Organization

ABSTRACT

Background:

Atherosclerosis, a global health issue and first leading cause of myocardial infarction and strokes, is an immunity-related disease. Recent data indicate that neural guidance molecules including Semaphorin-3A (Sema-3A) have immune regulation function. It has been shown that in some inflammatory diseases such as rheumatoid arthritis, the serum levels of Sema-3A is lower in patients [19, 121], and administration of Sema-3A in animal models of these diseases showed a protective effect in the progress of diseases [19]. Both Sema-3A and its receptors are expressed by most of the immune cells involved in atherosclerosis such as monocytes, macrophages, and T cells and regulate the function of these cells [62, 135, 97]. However, the effect of Sema-3A on atherosclerotic plaque progression is not known. We hypothesize that Sema-3A may have atheroprotective effects by regulating macrophage function and inflammation resolution.

Methods:

In vivo: ApoE$^{-/-}$ mice were electroporated with Sema-3A or control plasmid to overexpress the protein (plasma Sema-3A increased levels were verified by ELISA), and kept on high fat diet for 9 and 13 weeks. Plaque size was determined with oil red O staining in aortic sinus, brachiocephalic artery (BCA), and aorta. Plaque characterization was performed by immunostaining. To investigate the effect of Sema-3A on acute inflammation C57BL/6 mice were treated with Sema-3A or control plasmids.

Five days later LPS or VEH were injected into the skin of the ears. LPS-induced-inflammation was studied 48 hours later.

In vitro: Bone-marrow-derived monocytes were obtained from C57BL/6 mice and differentiated into macrophages by M-CSF. Macrophages were polarized to M1 or M2 types using IFN-γ or IL-4 respectively. Western blotting was performed to determine the expression of Sema-3A receptors on macrophages. M1 and M2 macrophage migration was assessed by boyden chambers assay. mRNA levels of integrins was performed by qRT-PCR and activation of some integrins were measured on human PBMCs derived from healthy donors, by flow cytometry.

Results:

Our findings show that atherosclerotic plaque is significantly smaller in Sema-3A overexpressing as compared to control mice in BCA, aortic sinus, and aorta. The plaque stability was comparable in both groups. However, plaque macrophage content was two-fold lower in Sema-3A overexpressing mice. This was associated with less circulating monocytes in this group. Moreover, we found that even though the monocyte recruitment to the plaque was not influenced by Sema-3A *in vitro*, M2 macrophage migration was increased by Sema-3A, which could explain lower macrophage content in the plaques of these mice. Further analysis showed that increased M2 macrophage migration by Sema-3A was associated with higher activation of β1-integrins and phosphorylation of focal adhesion kinase in these cells. The negative immuno-regulatory effect of Sema-3A was also confirmed by decreased

LPS-induced acute inflammation by Sema-3A. LPS-induced ear thickening was significantly decreased in Sema-3A overexpressing as compared to control mice. Our preliminarily data showed that Sema-3A prevented LPS-induced infiltration of macrophages and T cells in the ears.

Conclusion:

Our data show that Sema-3A reduces inflammation by regulating immune cell function. In a chronic inflammatory disease, such as atherosclerosis Sema-3A prevents plaque development possibly by increasing M2 macrophage mobility through regulation of β1-integrins. And in the acute inflammation model, Sema-3A protective effect seems to be due to the regulation of macrophages and T cells.

RÉSUMÉ

Contexte:

L'athérosclérose est un problème de santé mondial et est la première cause de l'infarctus du myocarde, une pathologie impliquant l'immunité. Des données récentes indiquent que des molécules guides des neurones telle que la Semaphorin-3A, ont une fonction de régulation du système immunitaire. Il a été démontré que, dans certaines maladies inflammatoires telles que la polyarthrite rhumatoïde, les niveaux de Sema-3A plasmatiques sont inférieures chez les patients [19, 121] et l'administration de Sema-3A dans des modèles animaux a démontré un effet protecteur dans la progression de ces maladies [19]. Sema-3A et ses récepteurs sont exprimés par la plupart des cellules immunitaires impliquées dans l'athérosclérose telles que les monocytes, macrophages, lymphocytes T et Sema-3A peut réguler la fonction de ces cellules [62, 135, 97]. Cependant, l'effet de Sema-3A sur la progression de la plaque d'athérosclérose n'est pas connu. Nous faisons l'hypothèse que Sema-3A peut avoir un effet athéroprotecteur en régulant la fonction des macrophages et en diminuant l'inflammation.

Méthodes:

In vivo: Des souris ApoE$^{-/-}$ ont été électroporées avec un plasmide Sema-3A ou contrôle afin de surexprimer la protéine (l'augmentation des niveaux plasmatiques a été vérifiée par ELISA). Les souris ont été maintenues sous un régime riche en cholestérol pendant 9 et 13 semaines. La taille des plaques d'athérosclérose a été évaluée avec coloration à l'huile rouge dans le sinus aortique, le TABC et l'aorte. La caractérisation des plaques a été réalisée par immunohistochimie. Afin d'étudier

l'effet de Sema-3A sur l'inflammation aiguë, des souris C57BL/6 ont été traitées avec le plasmide Sema-3A ou contrôle, et cinq jours plus tard du lipopolysaccharide (LPS) ou du PBS ont été injectés dans la peau des oreilles. L'inflammation induite par le LPS a été étudiée 48 heures plus tard.

In vitro: Des cellules monocytiques extraites de la moelle osseuse ont été obtenues à partir de souris C57BL/6 et différenciées en macrophages par M-CSF. Les macrophages ont été polarisés afin d'obtenir des types M1 ou M2 en utilisant l'IFN-γ ou l'IL-4, respectivement. L'expression des récepteurs de Sema-3A sur les macrophages a été déterminée par immunobuvardage. La migration des macrophages M1 et M2 a été évaluée dans des chambres de Boyden. L'expression génique des intégrines a été mesurée par qRT-PCR et la cytométrie de flux a été utilisée pour mesurer l'activation de certaines intégrines exprimées par des PBMC humaines provenant de donneurs sains.

Résultats:

Nos résultats indiquent que les plaques d'athérome étaient significativement plus petites chez les souris surexprimant Sema-3A par rapport aux souris témoins dans le sinus aortique, le TABC et l'aorte. La stabilité des plaques était comparable chez les deux groupes. Cependant, le contenu des plaques en macrophages était diminué de deux fois chez les souris surexprimant Sema-3A. Ceci était associé à une diminution des monocytes circulants dans ce groupe. De plus, nous avons constaté que bien que le recrutement des monocytes était identique chez les deux groupes de souris, Sema-3A augmentait la migration des macrophages M2 *in vitro*. Ceci pourrait expliquer la diminution du contenu en macrophages des plaques chez ces souris.

Nous avons également montré une augmentation de l'activation des intégrines-β1ainsi que la phosphorylation de la kinase impliquée dans les adhérences focales. Le rôle immuno-regulateur negatif de Sema-3A a été également confirmé par la diminution de l'inflammation aiguë induite par le LPS avec Sema-3A. L'épaississement des oreilles induit par le LPS était significativement diminué chez les souris surexprimant Sema-3A vs les contrôles. Nos résultats préliminaires ont montré que Sema-3A empêche l'infiltration des macrophages et des cellules T dans les oreilles injectées avec LPS.

Conclusion:

Nos résultats montrent que Sema-3A réduit l'inflammation en régulant la fonction des cellules immunitaires. Dans l'athérosclérose qui est une maladie inflammatoire chronique, Sema-3A empêche le développement de la plaque, en augmentant la mobilité des macrophages de type M2, via la régulation des intégrines β1. Lors d'inflammation aiguë l'effet protecteur de Sema-3A semble être dû à la régulation des macrophages et des cellules T.

Literature Review

CHAPTER 1
Literature Review

1.1 The vasculature

The human cardiovascular system is composed of the heart and blood vessels. The earliest known writing on the cardiovascular system was found in the Ebers Papyrus, 1500 B.C. The ancient Egyptian medical papyrus is 20 meters long and 30 centimeters wide containing over 700 prescriptions and remedies, both physical and spiritual. Interestingly the anatomical-physiological section of this document begins with this phrase: "The beginning of the physician's secret: knowledge of the heart's movement and knowledge of the heart" and the Egyptians continued by acknowledging the connection of the heart to the arteries. The Egyptians thought air came in through the mouth and into the lungs and heart. From the heart, the air travelled to everywhere through the arteries. Although this concept of the cardiovascular system is only partially correct, it represents one of the earliest accounts of scientific thought [42, 25].

The large blood vessels (that are the focus of this thesis) consist of three layers. The most innermost layer is called tunica intima, a single layer of squamous endothelial cells (ECs) facing the lumen and covering the vessel. This layer plays a unique role in vascular biology and its dysfunction gives rise to cardiovascular disease. Underlying this layer is the basal lamina and the internal elastic lamina which support the ECs. The second layer (the thickest layer in arteries) is the tunica media, which

mainly contains vascular smooth muscle cells (VSMCs) that control the caliber of the vessel. This layer is usually surrounded by, the external elastic lamina which separates it from the third layer, the tunica advantitia (the thickest layer in veins). This outermost layer is entirely made of connective tissue, fibroblasts, and immune cells. It also contains nerves and capillaries, called vasa vasora, which supply the large vessels.

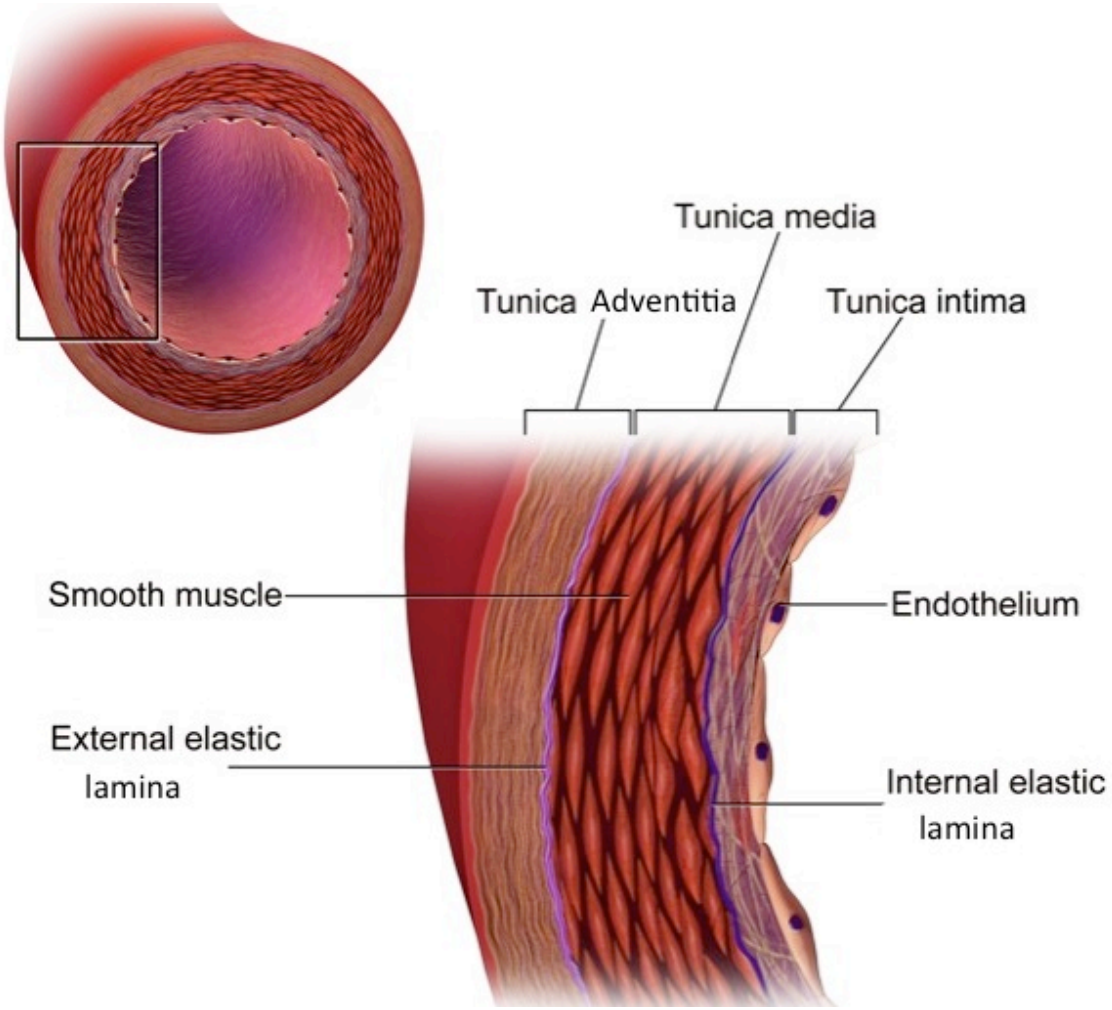


Figure 1–1: Structure of the wall of artery. The large blood vessels consist of three layers: tunica intima, tunica media, and tunica adventitia. (Modified from picture by Bruce Blaus.)

Blood vessels are involved in almost every medical condition from simple inflammation to cancer. In the present study we focus on one of the most common diseases of the blood vessel wall, cardiovascular disease specifically atherosclerosis.

1.2 Atherosclerosis

Cardiovascular disease (CVD) is a category of diseases including atherosclerosis, stroke, hypertension, venous thrombosis, and cardiomyopathy. These all involve the heart and/or blood vessels. It is a world-wide disease no longer limited to developed countries. CVDs are reported to be the leading cause of death in north America. In the past, CVDs were considered to be an older adult disease. However, now according to American Heart Association (AHA) reports, more than 40% of patients are between 45-69 years old [59, 67]. There are many risk factors associated with CVDs such as life style, diet, physical inactivity, gender, age, and socioeconomical factors. Some of these factors could be changeable, but there are numerous involved factors that are not under control [84].

In this study we focused on atherosclerosis at the cellular and molecular level. Although it has been extensively studied, failure of risk factor management and therapeutic approaches and strategies to effectively abrogate the atherosclerosis risk makes it a necessity to find new clinical targets.

Atherosclerosis is a chronic inflammatory disease in which plaque forms at the sites of disturbed laminar flow, particularly, arterial branch points and bifurcations. It is a very dynamic and complex process that its accompanied by inflammation and goes through different stages.

In 1829, Jean Lobstein introduced the term atherosclerosis for the first time. A few years later two theories were postulated by two pathologists called Rudolf Virchow and Carl Von Rokitansky. Virchow emphasized cellular pathology as a critical point in atherosclerotic plaque formation, while Von Rokitansky believed that mechanical injury and toxins led to endothelial dysfunction and further inflammation. 200 years later Mayerl *et al.* analyzed human samples in Von Rokitansky's collections and showed accumulation of T cells in early plaque [74].

There are many traditionally found risk factors associated with this disease such as smoking, male gender, hypertension, diabetes, age, and the most important one, hypercholesterolemia. High serum lipid levels especially the elevated level of low density lipoproteins (LDL) have been shown to be strongly related to the development of atherosclerosis. It is generally accepted that the atherosclerotic lesions are initiated by infiltration of LDL particles into the subendothelial space. However, there is a growing body of evidence that shows that the immune system is the master regulator of the atherogenic process. There are a variety of cells found in the subendothelial space of an atherosclerotic artery such as macrophages, dendritic cells (DCs), foam cells, lymphocytes and other inflammatory cells, and VSMCs. In addition, under proatherogenic conditions nitric oxide (NO) production by ECs is decreased and the burden reactive oxygen species (ROS) and advanced glycation end (AGE) products

are increased [32]. So these features of atherosclorosis show that it is a complex disease and that many components of the vascular, metabolic, and immune systems are involved in this process (Figure 1–2).

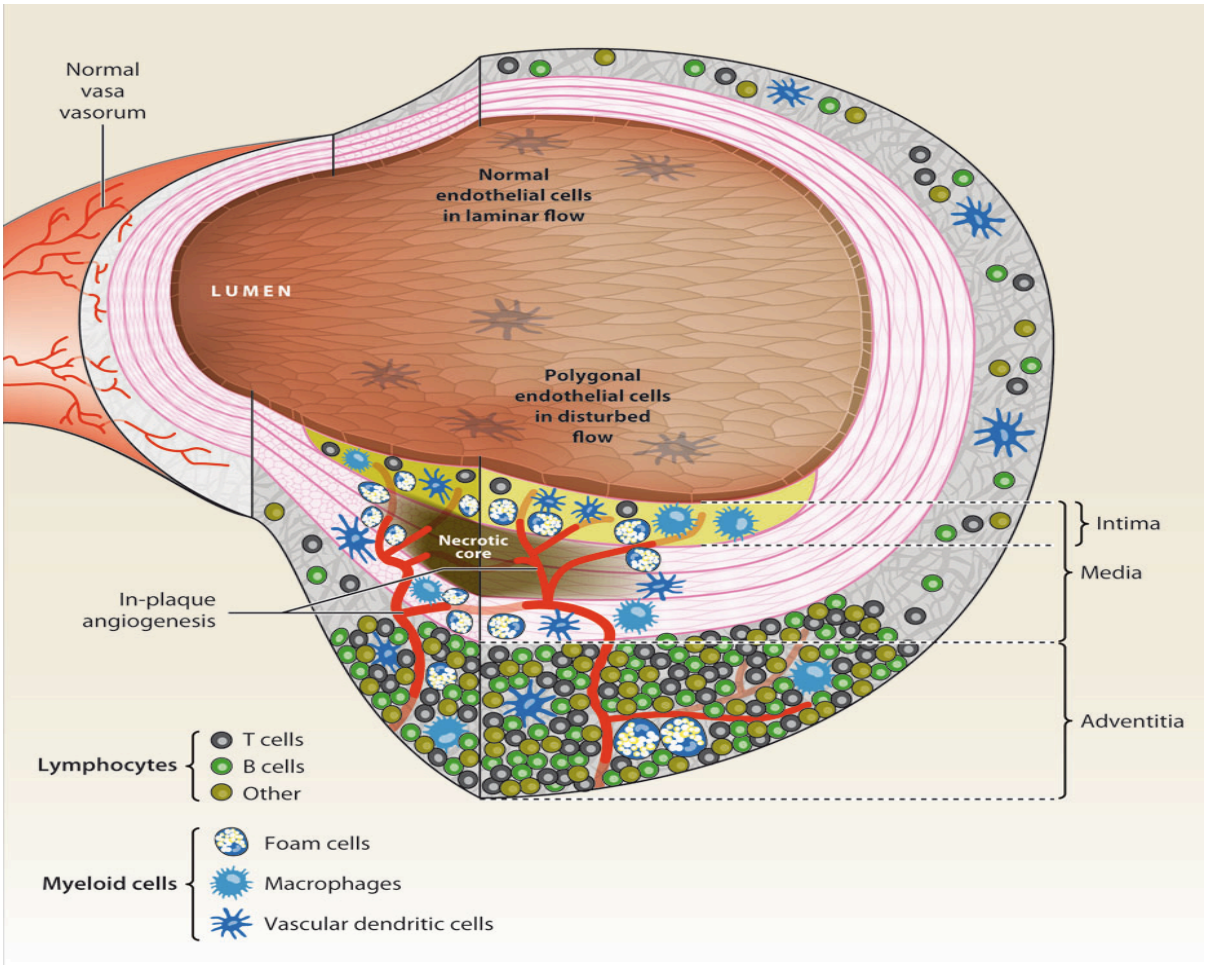


Figure 1–2: Atherosclerosis is an inflammatory disease. Many types of immune cells like vascular dendritic cells, macrophages, and T cells accumulate in the subendothelial space. As plaque formation progresses macrophage and DCs invade the tunica media in the lesion area. In a healthy vessel, the adventitia contains sparse T cells, B cells, and DCs, but in the lesion area the lymphocytes populate the lesion site [32]. (No permission required from publisher)

1.2.1 Mouse models of atherosclerosis

A diverse range of small and large animal models have been used to study the atherogenic process. No model is ideal as each has its own advantages and limitations with respect to manipulation and modeling of the human atherosclerosis process. Useful large animal models include pigs, rabbits, dogs, and nonhuman primates. Due in large part to the relative ease of genetic manipulation and breeding, low cost of maintenance, and the relatively short time frame for the development of atherosclerosis, murine models are currently the most extensively used. The two most frequently used murine models of atherosclerosis are the apoE$^{-/-}$ model and the LDLR$^{-/-}$ model. ApoE, primarily synthesized in liver, is one of the apolipoproteins that coats the surface of lipoprotein particles and serves as a ligand for receptor-mediated removal of lipoproteins from plasma, so lacking apoE results in elevated cholesterol level and atherosclerosis. In human and mice, mutations in the LDL receptor causes markedly increased total and LDL cholesterol levels [35, 102, 89]. Although not all aspects of murine atherosclerosis are identical to humans, studies using murine have provided insight into the fundamental mechanisms driving early atherosclerosis.

1.2.2 Atherosclerotic plaque formation

Human and animal studies show that the key initiating step in the atherosclerotic process is subendothelial accumulation of apolipoprotein B-containing lipoproteins (apoB-LPs). ApoB-LPs are made by liver and intestinal cells, and they contain a core of neutral lipids, markedly cholesteryl fatty acyl esters and triglycerides, covered by a layer of phospholipids and protein. When they enter into the circulation these apoB-LPs are converted into atherogenic compounds (LDL and remnant lipoproteins respectively) and upon entry into the subendothelial space, undergo various modifications such as oxidation, enzymatic and non-enzymatic cleavage and aggregations. On the one hand, this initiates the inflammatory response, resulting in activation of overlying endothelial cells to secrete chemokines that promote directional migration of inflammatory cells [78]. On the other hand VSMCs which are usually in a quiescent, contractile phenotype in a healthy vessel, change phenotype to enter a proliferative state and migrate into the subintimal space. In the course of normal inflammatory response, immune cells infiltrated into the lesion are expected to leave the site after participating in inflammation resolution and healing process. However, in the case of atherosclerosis these cells get trapped in the plaque and undergo apoptosis. So loss of migratory capacity and inefficient inflammation resolution are major factors contributing to atherosclerotic plaque formation [105]. The resulting accumulation of LDL particles, cell debris, and continuous inflammation drive the activation of adaptive immunity in response to chronic inflammation [70].

1.2.3 Monocytes

Monocytes are one of the first immune cells present in lesion sites in response to activated ECs. They play an essential role in plaque initiation and progression. They can originate from bone marrow or spleen, but the mechanism of rolling and entering the subintimal space is independent of their origin.

Different references list different steps for the leukocyte adhesion cascade, but the main steps are capture, rolling, firm adhesion, and transmigration as shown in figure 1–3. Monocytes are attracted by chemokines like CCL5 and CXCL1 immobilized on endothelial glycosaminoglycans and then roll on P-selectin expressed at the luminal surface of activated endothelial cells. The next step is to strengthen the adhesion which is done by firm interaction between integrins on monocytes (VLA4 and LFA1) and the adhesion molecules on ECs (VCAM-1 and ICAM-1 respectively).

Upon adhesion, monocytes spread and crawl to transmigrate into the vessel wall in response to chemokines released by ECs and VSMCs. It has been shown that the principal chemokines and their counterpart ligands in this step are CX3CR1/CX3CL1, CCR2/CCL2, and CCR5/CCL5. Also it has been reported that these chemokines play crucial role in recruitment of monocytes from bone marrow (CCL2) and survival of these cells (CX3CR1). Elimination of these proinflammatory molecules reduced the plaque size in apoE$^{-/-}$ mice by 90% [77]. In addition to the above mentioned chemoattractants, recent data show that neuronal guidance cues like semaphorins, netrins, and ephrins, independent of other chemokines, act as chemoattractants or chemorepellents on immune cells. This will be discussed more in detail later.

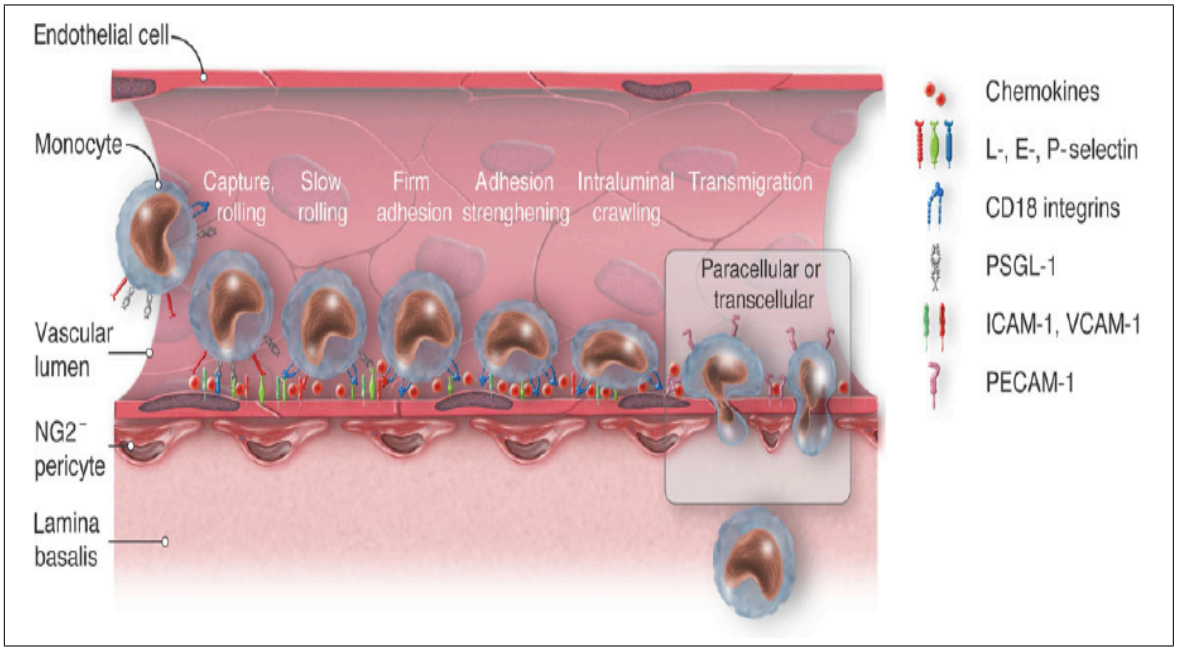


Figure 1–3: Schematic view of mechanism of monocyte recruitment into the atherosclerotic plaque: Activated ECs express adhesion molecules such as ICAM-1, VCAM-1, PSGL-1 and chemokines that interact with monocytic ligands and mediate the different steps of the adhesion cascade. Reprinted from reference [34] with permission from the Oxford University Press.

The role of different monocyte subsets in the atherosclerotic process has recently become a topic of interest. In ApoE$^{-/-}$ mice on a high fat diet, the number of inflammatory monocytes referred to as Ly6c^{hi} is increased. This subset of monocytes is shown to be involved in acute inflammatory responses and to produce higher levels of P-selectin glycoprotein ligand-1 (PSGL-1), which contributes to their homing to and rolling on ECs. The other subtype of monocytes , patrolling monocytes or Ly6c^{lo}, is associated with inflammation resolution [78, 6]. Although human monocytes don't express Ly6c, there is a proinflammatory monocyte population in humans that have

high expression of PSGL-1 [6].

1.2.4 Macrophages

Macrophages are innate immunity cells involved in primary response to pathogens. However, they have well-established role in tissue homeostasis, coordination of the adaptive immune response, inflammation resolution and repair. So it is not surprising that they are also the main contributor in atherosclerotic lesion formation, progression, and vulnerability. Although macrophage clearance of lipoproteins and apoptotic cells is beneficial in terms of plaque formation, diminished capacity of macrophage migration contributes to their failure to resolve inflammation and to progression of advanced plaques. Even in the advanced plaque, macrophages continue to be the major sponsor for the inflammatory response through secretion of proinflammatory mediators such as chemokines, cytokines, ROS, NO, Matrix Metalloproteases (MMPs), and even through their apoptosis. Notably, dying macrophages release their lipid content and tissue factors which form the prothrombotic necrotic core, characteristic of an unstable vulnerable plaque [77].

1.2.4.1 Macrophage polarization

Macrophage function is strongly influenced by exposure to cytokines which determine their phenotype. Conversely both the quantity and the phenotype of macrophages influence the inflammatory state of plaques. Macrophages show high plasticity and heterogeneity, and as the stimuli from the micro-environment change,

they can switch their phenotype. For instance Lee *et al.* using an ischemia-reperfusion-induced kidney injury model, showed that at early phase of injury pro-inflammatory macrophages were recruited to the site of lesion, but later on more reparative macrophages were detected. Using cell-tracing methods, they showed that rather than recruiting reparative macrophages, proinflammatory macrophages switched their phenotype due to change in the environment [61].

Upon entry of monocytes into the atherosclerotic lesion, the majority of them become dendritic cell-like and/or macrophages. Macrophages exhibit a plasticity in phenotype, ranging from macrophages involved in inflammatory processes (M1) to macrophages involved in healing processes (M2, M2a, M2C) (refer to figure1–4) [78]

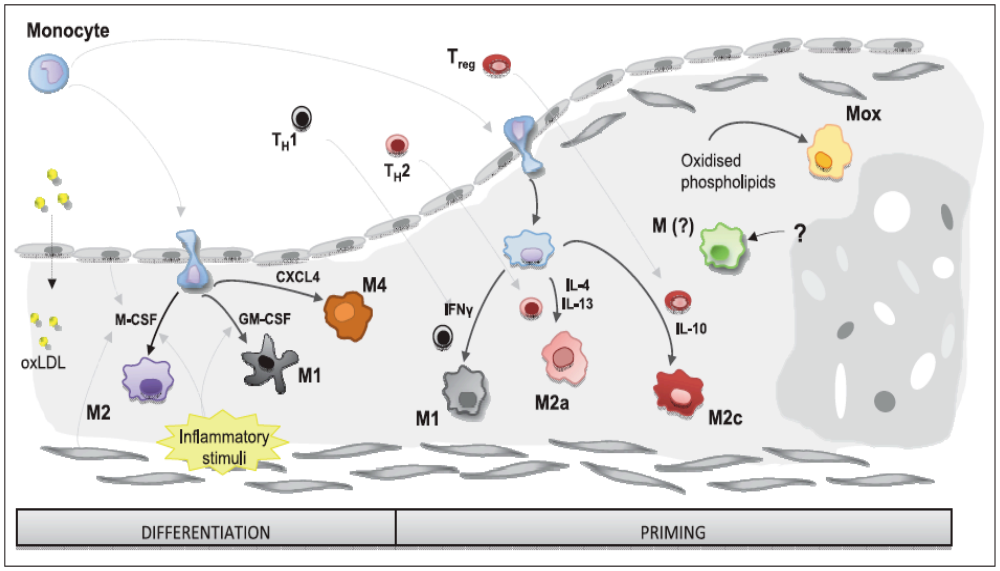


Figure 1–4: Macrophages heterogeneity in atherosclerosis: Depending on various stimuli from the microenvironment, macrophages can polarize towards different phenotypes from inflammatory M1 to reparative M2. This polarization could be in response to differentiation factors such as MCSF or T cell derived cytokines like IFN-γ, IL-10, IL-4/IL-13. Reprinted from reference [128] with permission from the publisher Schattauer GmbH.

1.2.4.2 M1 vs. M2 macrophages

Found in the 1970s, the classically known macrophage activation (M1) is induced by IFN-γ produced by $CD4^+$T cells, $CD8^+$T cells, and natural killer (NK) cells. IFN-γ gives M1 cells increased antigen capacity, increased synthesis of proinflammatory cytokines, toxic mediators, and amplified phagocytic capability.

Soon after M1 macrophage classification, in 1982, the immunological counterpart of IFN-γ, interleukin (IL)-4 was identified. However, it took 10 years of investigations until the concept of alternative activation was proposed. Several independent, parallel studies showed that macrophages also undergo alternative activation by IL-4 and IL-13, which trigger a different phenotype resulting in upregulation of mannose receptor, induction of major histocompatibility complex (MHC) class II antigens, inhibition of NO production, and increased arginase activity [73]. There are two isoforms of arginase, arginase I and II (ArgI/II). They both catalyze L-arginine to L-ornithine which is a precursor of polyamines (involved in cell growth, proliferation, and differentiation) and proline (the key component of collagen). Since the expression of ArgI is higher in M2 macrophages than M1, M2 cells are classified as reparative or healing macrophages. The expression of inducible NO synthase (iNOS) is higher in M1 macrophages. It catalyses L-arginine and produces NO. Macrophage NO upregulates VSMC cell surface Fas priming them for apoptosis. Since VSMCs promote

plaque stability, VSMC apoptosis may increase plaque rupture, so M1 macrophages are considered to be inflammatory macrophages [51, 10].

Recent evidence designates an essential role for cellular metabolism in macrophage activation. In particular, M1 and M2 macrophages use different metabolic pathways to fuel their functions. M1 macrophages use aerobic glycolysis to quickly supply energy needed for intense proinflammatory responses, whereas M2 macrophages use a more efficient ATP-generating program of fatty acid oxidation (FAO), capable of being conserved for longer periods of time [85].

The presence of M1 and M2 macrophages is documented in both human and murine atherosclerotic plaques. Khallou-Laschet *et al.* showed that in early atherosclerotic plaques, M2 macrophages are more predominant and are associated with smaller plaques. As the plaques progress, M1 macrophages over take the M2 population [51]. Studies in mouse models of atherosclerosis have shown that conditions that increase macrophage polarization toward an M1 phenotype [12] or diminish polarization toward M2 [64] promote atherosclerotic plaque formation [105], whereas provision of the M2-polarizing factor inhibits disease progression [13] and even promotes plaque regression [120].

1.2.4.3 Foam cells

LDL is the main source of excess cholesterol deposited in plaques. Native LDL uptake is subject to negative feedback regulation, so it undergoes some modification. Imbalance of modified LDL influx and efflux leads to lipid-laden macrophages or foam cells. Macrophages express several scavenger receptors including scavenger receptor

A and B, CD36, CD68, and scavenger receptor for phosphatidylserine and oxidized lipoproteins responsible to uptake ox-LDL. Cholesterol up taken by these receptors is delivered to lysosomes, where it is hydrolyzed to free cholesterol and fatty acids.

As intracellular cholesterol level increases, endogenous cholesterol biosynthesis and LDLR expression are suppressed. However in the condition of continued cholesterol uptake by scavenger receptors, this inhibitory mechanism is not sufficient to protect the cell from excess cholesterol. Therefore excess cholesterol must be exported to extracellular acceptors to be transported to the liver [63].

ATP-binding cassette A1 and G1, membrane-bound proteins, are major cholesterol efflux regulatory proteins that initiate reverse cholesterol transport in macrophages. Deficiency of these two transporter proteins causes foam cell formation [32].

In addition, modified LDL induces cell death due to cytotoxicity and it stimulates ECs to recruit more inflammatory cells. It also modulates gene expression in macrophage-derived foam cells, upregulating proinflammatory IL-8 and ROS, and downregulating tissue inhibitors of metalloproteinases (TIMPs). This shifts stable plaques toward a more vulnerable phenotype. Macrophage-derived foam cells also stimulate SMC migration from the media into the intima. These SMCs highly express LRP1 (LDL Receptor-Related protein 1) due to hypercholesterolimia and contribute to LDL uptake, becoming foam cells themselves [100].

1.2.4.4 Efferocytosis

Successful resolution of the inflammatory disease process requires a diverse series of processes including inhibition of inflammatory cell recruitment, promotion of

inflammatory cell egress, and clearance of apoptotic cells by phagocytes. This latter process is known as efferocytosis [105]. Efficient efferocytosis has important consequences on plaque regression. For example, it has been found that efferocytosis leads to TGF-β secretion, which in turn can inhibit monocyte recruitment into the lesion [38].

It has been shown that efferocytosis is impaired in atherosclerosis, and in combination with apoptosis of advanced lesional macrophages it leads to necrotic core formation [78]. Understanding the principals behind defective efferocytosis is important and may help to find a suitable therapeutic solution. So far several possible mechanisms have been proposed in this regard. A) Oxidative stress exerted by ROS inhibits efferocytosis by macrophages. B) Ox-LDL is immunogenic and anti-ox-LDL autoantibody has been found in plaques. Upon binding of antibody, it prevents recognition of apoptotic cells by macrophages. C) Both ox-LDL and ox-RBC (Red blood cells) compete with apoptotic cells for the same epitopes on the surface of macrophages. D) Free cholesterol loading in foam cells downregulates proteins that facilitates efferocytosis. E) Accumulation of lipid droplets in foam cells causes macrophage stiffening preventing pseudopodia formation thereby decreasing efferocytosis [100].

Right now, finding a golden solution to overcome these defectives is a big challenge in the atherosclerosis regression studies. It seems that M2 macrophages are more likely to degrade phagocytic cargo in an attempt to maintain homeostasis. So increasing M2 macrophage activity looks like a promising path to follow.

1.2.5 Dendritic cells

Dendritic cells are another bone-marrow derived cells that are present in atheroprone regions before plaque formation and in established atheromata [88]. However, the exact origins of residential DCs and lesional DCs are disputed. They could result from local proliferation, recruitment from bone marrow, or both. A study in C57BL/6 mice show that the residential DCs are derived predominantly from the bone marrow [49]. Tacke *et al.* studied the origin of lesional DCs in $ApoE^{-/-}$ mice under high fat diet and found that lesional DCs originate from $Ly6C^{lo}$ monocytes [106]. DCs have multiple roles in plaques. These cells secrete chemokines like CCL19 and CCL21 that might accelerate naive lymphocyte recruitment into the plaque. Also they may present possible atherosclerosis antigens like ox-LDL to the T cells and promote T cell differentiation and proliferation.

1.2.6 T lymphocytes

T lymphocytes play a crucial role in the inflammatory process of atherosclerosis. They enter the intima by binding to VCAM-1 and in response to the IFN-γ-inducible chemokines that binds to CXCR3 receptor on T cells [65]. The $CD4^+$ T cells are the most prominent T cell population in both human and mouse lesions [94]. When activated they differentiate into several subtypes including effector Th1 and Th2 cells. Th1 cells produce proinflammatory cytokines such as IFN-γ, IL-12, IL-15, IL-18, and TNF, leading to M1 macrophage polarization, production of MMPs and tissue factors (TF) by macrophages, thereby increasing vulnerability and thrombogenicity of the

plaque lipid core. Th2 cells release cytokines like IL-10 to suppress inflammation and promote M2 macrophage polarization [6, 94, 73].

Study of early atherosclerotic lesions show a Th1 response [41, 11] which may be counterbalanced by Th2 response [75], so timely negative regulation of the immune system is critical to allow it to perform its duty while maintaining it under tight control to avoid overactivation.

The balance between Th1 and Th2 response is highly regulated by regulatory T cells (Tregs, $CD4^+CD25^+$). Tregs suppress effector T cell proliferation via secretion of inhibitory cytokines like IL-10 and TGF-β. In addition to regulating effector T cell functions, Tregs also induce M2 macrophages, inhibit foam cell formation, and influence cholesterol metabolism (figure 1–5) [29]. It has been shown that Tregs are decreased in the peripheral blood of all patients with autoimmune diseases including rheumatoid artheritis, type I diabetes, multiple sclerosis, systemic lupus erythmatosus, and acute coronary disease [27, 57, 125, 23, 96].

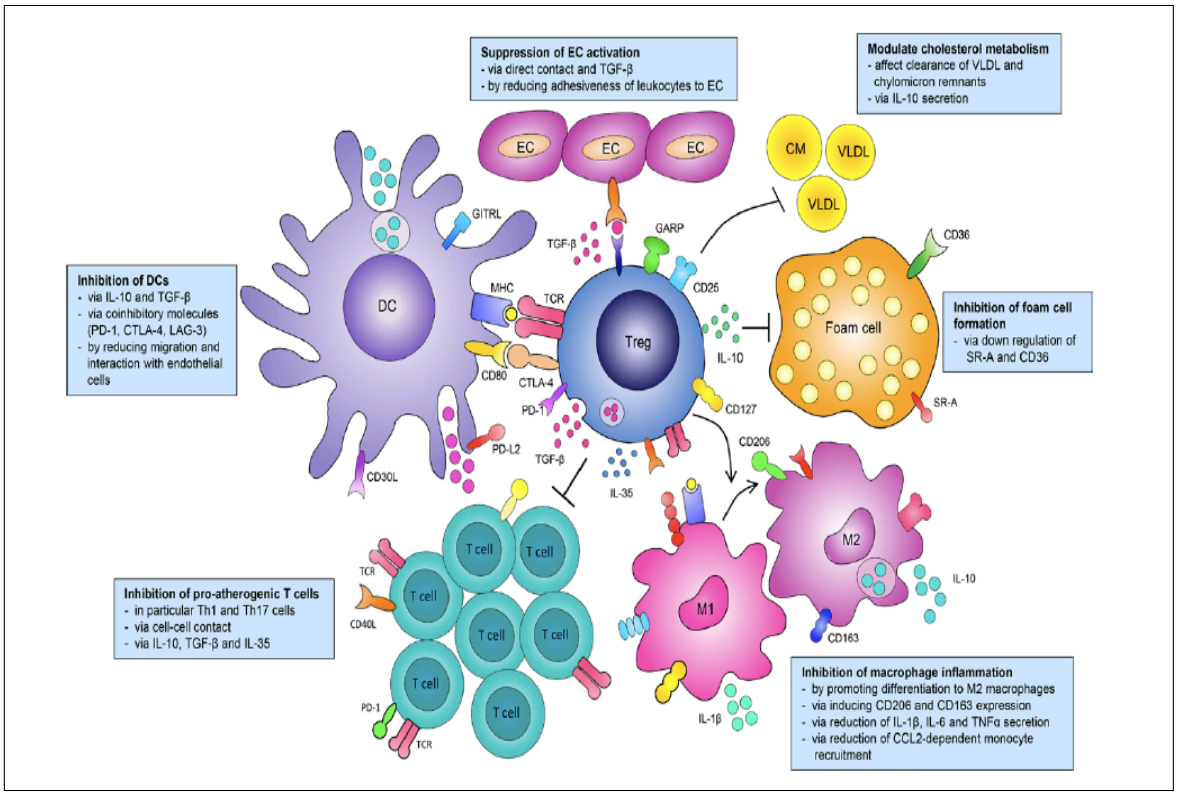


Figure 1–5: Mechanism of action of Tregs in atherosclerosis: Tregs have a key role in modulating immune cell function to suppress inflammation in atherosclerosis. They inhibit proatherogenic T cells either via direct cell-cell contact or via secreting cytokines. They also suppress DC and EC activation, foam cell formation, and promote M2 macrophage polarization. Reprinted from reference [29] with permission from the Wolters Kluwer Health, Inc.

In healthy vessels T cells and Tregs are virtually absent. However, they are present in all stages of atherosclerotic lesion, both in the intima and the adventitia. Compared with other inflammatory disease, the frequency of Tregs is significantly lower in atherosclerosis [24], which could be due to inhibition of FoxP3 expression by ox-LDL. FoxP3 is a transcription factor responsible for genes involved in Treg function[79]. When $CD4^+CD25^+$ Treg cells were transferred to mice susceptible to atherosclerosis, smaller plaques, less infiltration of T cells and macrophages, and increased collagen content were observed, showing that Treg play an essential role in controlling the inflammatory responses [3]. Thus restoration of this balance between effector T cells and Tregs may have a great therapeutic potential to prevent cardiovascular disease.

1.3 Neuronal guidance molecules & immune system

It has been very well established that similar molecular events govern cell-cell contacts in different cell types. Recent reports describing cellular interactions between immune cells such as T cells and DCs show similarities in processes that occur during synapse formation in the nervous system. Semaphorins, netrins, slits, and ephrins are four major guidance cues that regulate neural development and play roles in directional cell migration [108]. However, accumulating data support new roles for these proteins beyond the nervous system and axon guidance, and show their roles in vasculogenesis and angiogenesis, immune cell regulation, and tumor progression [123]. Now it becomes clear that several semaphorins, called "immune semaphorins" play crucial roles during immune responses.

1.4 Semaphorins

The word semaphorin consists of "semaphore + in". Semaphore means the system of flags and lights used in rail and maritime communication to signal the direction of movements (Merriam-Webster online dictionary) and that is exactly what semaphorin proteins do.

So far eight classes of semaphorins have been discovered. Class 1 and 2 were found in invertebrates, class 3-7 in vertebrates, and class V in viruses [56, 83]. Based on biochemical structure there are three types of semaphorins: secreted, membrane glycosylphosphatidylinositol (GPI) anchored, and transmembrane. Class 2, 3, and V are secreted, but all others are membrane bound proteins [114]. All semaphorins have a sema domain comprising of 400-1000 amino acids in the form of seven-blade $\beta-propeller$ fold similar to $\beta-propeller$ structure of α integrins. The sema domain is also found in other proteins such as plexins, and the receptor tyrosine kinases Met and Ron. Another main domain that all semaphorins have is a PSI (plexin, semaphorin, and integrins) domain, which is similar to sequences found in the extracellular domain of β integrins [9]. Depending on the class of semaphorins they may also have an immunoglobulin (Ig) like domain or thrombospondin domain or both (figure 1–6).

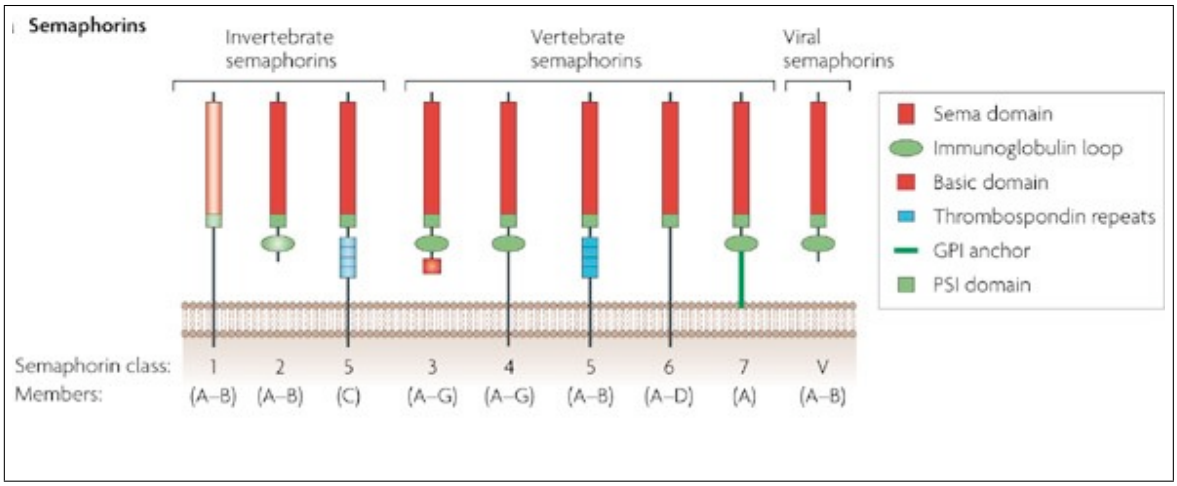


Figure 1–6: Semaphorin family members: All semaphorins have a large amino-terminal Sema domain that is essential for their signaling. There are 8 classes with a conserved PSI domain at the C terminus that is homologous to the N-terminus of β intergrins. Sema-3 family is the only secreted member in vertebrates. Reprinted from reference [83] with permission from the Nature Publishing Group.

1.4.1 Semaphorin-3A

Semaphorin-3A (Sema-3A) is secreted as a 95kDa monomer, that could undergo proteolytic cleavage through the removal of the 33kDa C-terminal domain and create a 65kDa isoform [54]. The phenotype of mice carrying a null mutation of Sema-3A is almost normal at birth, but they have axon guidance defects and develop multiple skeletal defects and die shortly after birth due to the dilation of the right ventricle and right atrium of the heart [8].

As it will be discussed more in detail later in this thesis, Sema-3A is secreted by several immune cells including macrophages and T cells. Several studies suggest that Sema-3A could be protective in the context of atherosclerosis. For example, in

LDLR$^{-/-}$mice after two weeks of high fat diet, expression of Sema-3A and netrin-1 were downregulated in endothelial cells in atheroprone regions. This suggested a new role for these guidance cues in endothelial barrier function preventing monocyte entry. Also, it was shown that expression of Sema-3A is highly downregulated in endothelial cells exposed to proatherogenic conditions including low shear stress flow, monocyte recruitment chemokines like MCP-1 and IL-8 or proinflammatory stimuli like TNF-α and LPS [123]. The mechanism by which Sema-3A exerts its action will be discussed later.

1.4.2 Semaphorin-3A receptors

The first receptors identified for semaphorins were neuropilins, but it soon became clear that neuropilins alone can not transduce the signal. They make a complex with another type of transmembrane receptor, plexins. The fact that each semaphorin can interact with diverse receptor complexes and that plexins and neuropilins may have different partners in the cell membrane made the study of semaphorin function quite complex. Moreover, the biological response to semaphorin strongly depends on the cellular context, meaning that it is not only modulated by the amount of receptor expressed or the availability of intracellular transducers, but also by the expression of coreceptors in the same cell.

1.4.2.1 Neuroplilin-1

Neuropilin, first named A5 neuron-specific cell surface antigen, was initially found in Xenopus embryos in 1991. Simultaneously, another neuropilin- like gene was

found in human and called neuropilin-2 (Nrp-2), so A5 neuron-specific cell surface antigen was renamed as neuropilin-1 (Nrp-1). Nrp-1 is a direct receptor for Sema-3A [81], for the 165-amino-acid isoform of vascular endothelial growth factor, VEGF [83], and for transforming growth factor beta-1, TFG-β1 [36]. Nrp-2 was shown to behave as a receptor for other members of the semaphorin-3 family [22].

Neuropilins are single pass transmembrane spanning proteins. As shown in figure 1–7 they have two complement-binding domains (CUB domains) which apparently serves as a semaphorin binding domain, and two coagulation factor V/VIII binding homology domains, essential to define the profile of semaphorin specificity, a MAM domain which is an adhesive domain and plays an important role in homo- and hetero-dimerization of neuropilins, a transmembrane segment, and a short cytoplasmic domain. Both neuropilins have differently spliced isoforms, but interestingly Nrp-2 has a variant with completely different transmembrane and intracellular domains indicating a different function for this form [114]. Nakamura and colleagues showed that the intracellular domain of Nrps is not essential for chemorepellent signaling since deletion of this domain did not prevent semaphorin repulsive effect [81]. Also the same group used the spliced-soluble form of Nrp-1 to show that although the soluble form of a receptor usually has an inhibitory effect on signaling by trapping the ligand, in this case soluble Nrp-1 did not prevent semaphorin signaling, proving the presence of a partner for neuropilins.

The biological function of neuropilins was basically first revealed by an *in vitro* assay showing Sema-3A repels growing tips on neuropilin-1 expressing dorsal root ganglion cell [69].

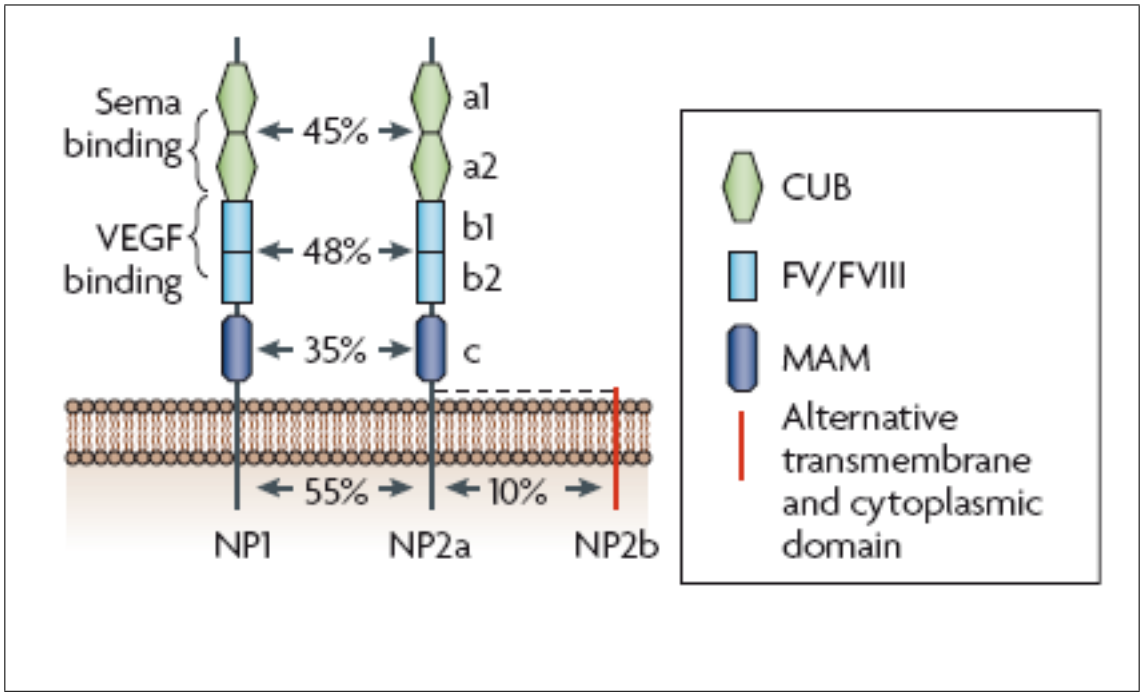


Figure 1–7: Nrp receptor family: The two neuropilins are single-pass transmembrane receptors. At the N terminus they both have two CUB domains which are semaphorin binding sites. At the C terminus they contain a MAM domain playing a role in the formation of the neuropilin complex, and in the middle there are two FV/FVIII coagulation factor-like domains that are the VEGF binding site. Reprinted from reference [83] with permission from the Nature Publishing Group.

The genetic knockout of Nrp-1 is embryonic lethal, but its deficiency alters vascularization in the brain and causes different morphogenetic defects in the heart and large vessels [114].

1.4.2.2 Plexin-A1/A4

In humans there are at least nine different plexin genes grouped in 4 subfamilies based on sequence similarities, classes A-D [114]. There are 4 members in class A, 3 members in class B, and one member in class C and D. As said before the main characteristic of plexins is a sema domain. In addition there are 3 PSI domains and 3 IPT (Ig like plexin and transcription factors) domains in the extracellular part of the plexins. The intracellular segment is highly conserved and consists of two segmented GAP (GTPase activating protein) domains with a linker domain of GTPase binding region [56, 113] which control cytoskeletal dynamics and integrin function through monomeric G proteins (figure 1–8) [116]. They can associate with different receptors to respond differently to the same ligand. For example, in the development of a chick embryo, plexin-A1 (Plx-A1) forms a complex with off-track (Otk) receptor and vascular endothelial growth factor receptor 2 (VGEFR2) receptor, both tyrosine kinase receptors, and mediates distinct biological activity in cardiac morphogenesis [104]. Plx-A1 knock-out mice show severe osteoprosis due to decreased bone resorption as a result of defective osteoclastogenesis [111]. Plx-A4 knock-out mice have enhanced T cell priming and T cell mediated immune responses, similarly to EAE (a murine model of human multiple sclerosis) [135].

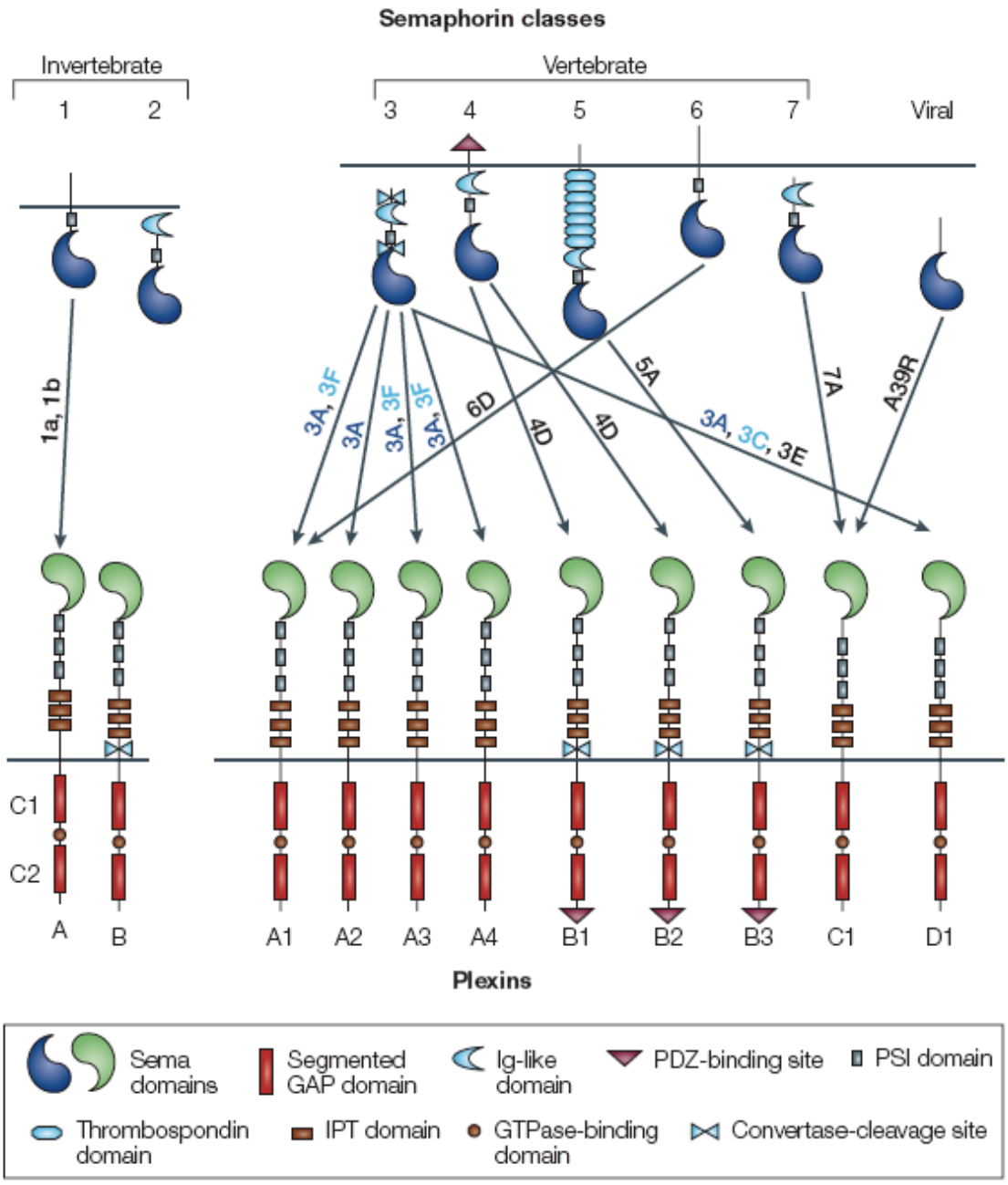


Figure 1–8: Semaphorins and Plexins interaction: Both semaphorins and plexins contain sema domain. Arrows indicate interacting partners and blue labels on arrows shows the necessity for Nrp-1 (dark blue) or Nrp-2 (light blue). Reprinted from reference [56] with permission from the Nature Publishing Group.

1.4.3 Semaphorins & Adaptive Immunity

The immune and nervous systems have some similarities. The interface between interacting antigen presenting cells (APCs), such as macrophages and DCs, and T cells resemble the contact between pre- and post-synaptic neurons. A lot of molecules first studied in neurobiology have been shown to play roles in adaptive immunity. For instance, agrin, essential for neuromuscular junction formation, is expressed by lymphocytes and is involved in formation of lipid raft clusters serving as a platform for signal transduction [52]. Accumulating evidence indicates some of the semaphorin family members are also found in both systems and have a role in the regulation of the immune system. Some suppress immune cell activation and proliferation, whereas the others stimulate immune-mediated responses. It has been shown that Sema-4A expressed by DCs enhances T cell activation [58] or Sema-4D expressed by T cells prolongs B cell activation through binding to B cell surface receptor CD72 and preventing its inhibitory function [104, 53].

In general, Sema-3A negatively regulates immune responses [107]. For instance it has been shown that down regulation of Sema-3A in the nasal epithelium contributes to hypersensitivity in animal models of allergic rhinitis. When recombinant Sema-3A protein was administrated intranasally to the mice, sneezing and nasal rubbing was diminished [98]. In another research the role of Sema-3A in psoriasis was investigated. Sema-3A expression was lower in skin biopsy specimens of patients compared to the healthy individuals and its level was negatively correlated with itch intensity and severity of the disease [122]. These examples as well as others indicate a potential regulatory role of Sema-3A in the immune system.

Sema-3A is not only expressed by immune cells, but coronary artery endothelial cells also express it. Its expression is down-regulated by pro-atherogenic factors including oscillatory shear stress and pro-inflammatory cytokines. It is well known that this negative guidance cue protein inhibits migration of human monocytes directed by chemokines and it has been shown that blocking the effect of Sema-3A and netrin-1 by blocking peptides increases leukocyte adhesion to the endothelium [123].

It has been shown that all Sema-3A receptors are expressed on monocyte and macrophages of human and mouse origin [129, 135], and the expression level of Sema-3A receptors significantly increased upon polarization to M2 macrophages [46]. Considering these studies, many recent investigations have focused on the role of the biological ligand Sema-3A in immunity.

1.4.3.1 Semaphorin-3A & T cells

Sema-3A is secreted by activated DCs and T cells at late time points of immune response. It inhibits DC-induced proliferation of T cells through modulation of actin polymerization and abrogation of T cell receptor (TCR) polarization and focal adhesion kinase (FAK) phosphorylation [62].

In vivo and *in vitro* studies have identified a regulatory role for Sema-3A and its receptors in immune responses, chemotaxis, and cytokine production of T cells and DCs, as well [109]. Nrp-1 is expressed by DCs and T cells and has a critical role in DC/T cell synapse formation [118]. Plx-A1 is expressed on DCs and it is required for the efficient generation of antigen-specific T cells. Plx-A4 is expressed by T cells, DCs but not B cells and NK cells [135], and PlX-A4$^{-/-}$ T cells show hyperproliferative

responses to stimulation *in vitro*. The same response was also observed in both T cells from Nrp-1 mutant mice and Sema-3A-deficient mice [135]. As reported by Catalano *et al.*, Sema-3A produced by DCs and several tumor cells is a negative regulator for T cells. Sema-3A inhibits T cell receptor-mediated proliferation and cytokine production via downregulation of mitogen activating protein (MAP) kinases [20] resulting in tumor cells escape from detection and clearance by immune cells. Also Garcia *et al.* have shown that Sema-3A inhibits CXCL12 chemokine-driven migration of all types of CD4 and/or CD8 T cells through downregulation of its receptor CXCR4 and FAK phosphorylation [33].

In addition Sema-3A induces Treg [20, 97], which as explained before suppress effector T cells, secrete M2-polarizing cytokines, and are generally considered to be atheroprotective [3]. For instance when $ApoE^{-/-}$ mice kept on high fat diet were treated with measles virus which is a potent inducer of Sema-3A secretion, the atherosclerotic plaque was smaller due to a significantly increased in Treg response [2, 119].

1.4.4 Semaphorin-3A & Cell Migration

Cell migration is a multistep process that is essential in embryogenesis, and throughout life. It contributes to tissue repair and regeneration, and is involved in progression of many diseases like cancer, mental retardation, atherosclerosis, and arthritis. Cell migration is not only an important process in humans, but also it extends to plants and even single-celled organisms.

Migration is a highly orchestrated process. The first step is cell polarization and extension of lamellipodia driven by polymerization of actin filaments toward

the source of chemoattractant under control of Rho GTPases. Then weak nascent adhesions are formed under these lamellipodia by binding the integrins to the ECM. Afterward internal organelles are moved forward by the tension created by stress fibers, and finally the cell retracts its rear edge by destablilizing and releasing focal adhesions. Growth-cone collapse upon contact with chemorepulsive cues like semaphorins is well studied which includes retraction of filopodia and lamellipodia and a localized rearrangement of the actin cytoskeleton suggesting that the intracellular signaling of semaphorins in controlling directional cell migration [114] should be the same as what has been found in growth cone collapse.

It is known that release of cell-substrate adhesion signals are normally required to start cell migration, whereas constant inhibition of integrin function blocks cell motility and passive retraction of pseudopodia. Considering this delicate balance, although semaphorins have been mainly described as inhibitory signals because they prevent cell migration and axon outgrowth, it has been shown that they could potentially act as permissive cues for lamellipodia extension and cell migration [90].

Semaphorins have established functions in regulation of cell motility and morphology through plexins. For instance, they control oligodendrocyte [103] and neural crest cell [28] migration. Moreover, they modulate leukocyte migration [33] and have crucial role in EC migration and angiogenesis as well [101]. Recently it was shown by Casazza *et al.* that Sema-3A/Nrp-1 interaction increased macrophage motility in hypoxic tumor area [18]. A recent imaging study has revealed that Sema-3A secreted by lymphatic ECs guides DCs to enter into the afferent lymphatics [109]. PlxA1$^{-/-}$

DCs and DCs with mutant Nrp-1 show an impaired trafficking to lymphatics pointing out a role for Sema-3A/Nrp-1/PlxA1 in DC transmigration [39].

As mentioned earlier, a landmark of atherosclerosis is the persistence of cholesterol-laden macrophages in the plaque. Although defective egress has long been recognized as a fundamental step in the progression of the plaque, the mechanisms that regulate this process are not well understood. Although there is no direct evidence of the effect of Sema-3A on macrophage migration in the context of atherosclerosis, the above mentioned studies show the effect of Sema-3A on cells closely related to macrophages.

Emerging evidence suggests two mechanisms by which semaphorin-plexin signaling influences these cellular functions. One is the regulation of the actin cytoskeleton through the Rho family GTPases and the other is the modulation of integrin-mediated cell migration involving R-Ras- a Ras-family GTPase [104].

1.4.4.1 Integrins

Cell interaction with extracellular matrix (ECM) and neighbour cells elicits several responses that have essential roles in the regulation of cell behavior. Regardless of the type of cells this interaction shares two common features: they are mediated by integrins and they interact with the actin cytoskeleton intracellularly. Integrins are heterodimers of α and β subunits that binds to ECM through a large extracellular domain, and inside the cells their intracellular domains interact with focal complex proteins. Depending on their functional state, they have different affinity to ECM

and a dynamic regulation is necessary to control this integrin plasticity throughout life.

Several aspects of immune responses like transmigration of leukocytes through ECs or macrophage egress from atherosclerotic plaque are profoundly dependent on cytoskeletal dynamics and integrin activity. As discussed earlier in the monocyte section (section 1.2.3), two integrins, $\alpha_4\beta_1$ (VLA) and $\alpha_L\beta_2$ (LFA) play vital roles in adhesion and firm attachment of monocytes to the activated ECs in atheroprone regions [31]. In addition leukocyte recruitment is not only via transmigration through ECs, however, there is an alternative trafficking through vasa vasorum and neovessels that form within the advanced plaque [130], and it has been shown that $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$ are the main integrins involved in neovessel formation [47]. So integrins are involved from very early stages of plaque formation, and integrin activity regulators may therefore be a good therapeutic target for atherosclerosis. Since semaphorins play roles in angiogenesis and immune cell regulation, it is reasonable to postulate that they may regulate integrins through their plexin receptors. In an attempt to find molecules downstream of Sema-3A/Plx-A signaling, Serini *et al.* showed that Sema-3A is colocalized with focal complexes in endothelial cells spread on fibronectin, suggesting a dynamic transient association between Sema-3A and integrin-containing focal complexes. This inhibits EC adhesion and migration by inhibiting integrin function [101]. The molecular mechanism by which Sema-3A regulates integrin function in different cell types, especially myeloid cells, is not fully understood yet. Sema-3A signaling is well studied in neurons and it is likely that binding of Sema-3A to Plx-A1 induces the intrinsic R-Ras GAP activity of Plx-A1

thus resulting in switching GTP-bound R-Ras to GDP-bound R-Ras [95]. As R-Ras is known to sustain integrin activation, the inactivation of R-Ras by Sema-3A/Plx-A1 may lead to inactivation of integrins, thereby inhibiting integrin-mediated cell adhesion [56] and increase migration in these cells (figure 1–9).

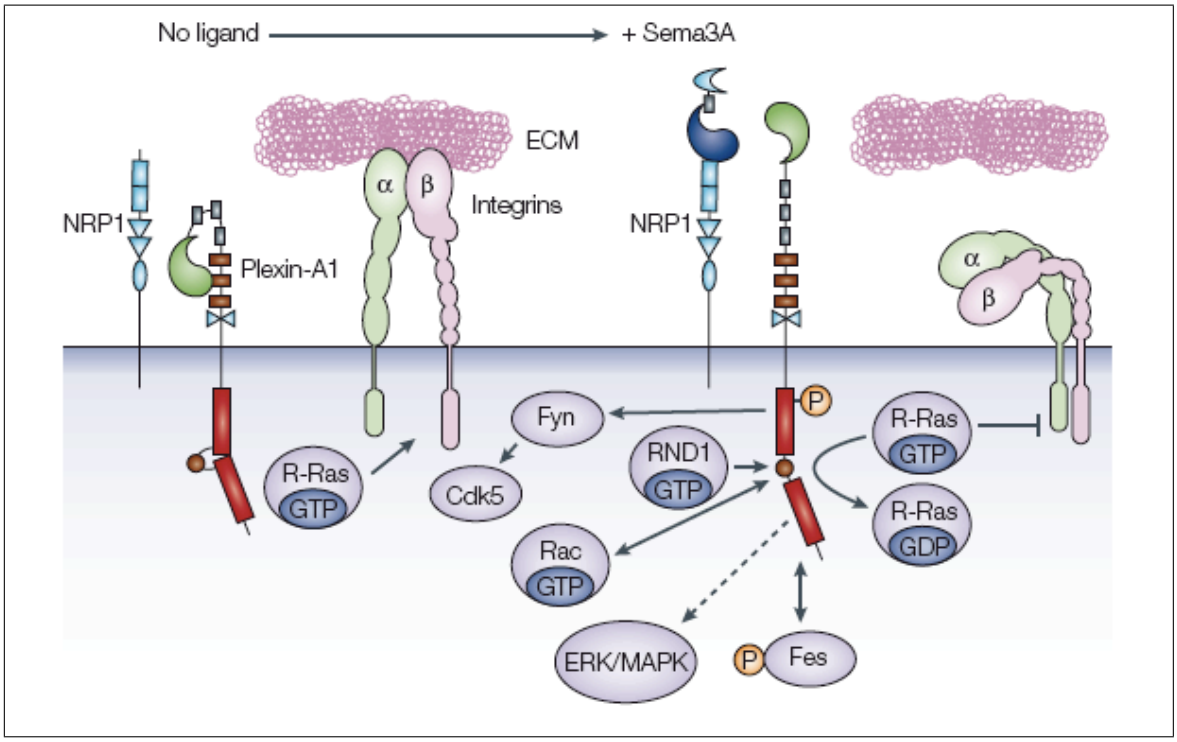


Figure 1–9: Proposed mechanism of regulation of integrin activity by Sema-3A in neurons: In no ligand condition R-Ras is active and GTP-bound, resulting in integrin-mediated attachment of cell to the ECM. Upon binding of Sema-3A to Nrp-1 and activation of Plx-A1, R-Ras becomes inactive and GDP-bound leading to inactivation of integrins. Reprinted from reference [56] with permission from the Nature Publishing Group.

In addition Pan *et al.* investigated the mechanism of suppression of tumor cell migration by Sema-3A, and found that in breast cancer cells, Sema-3A increases both expression and activation of $\alpha_2\beta_1$ integrin subunits. This stimulates tumor cell adhesion to matrix and prevents migration [86].

Regardless of some studies showing the effect of Sema-3A on the biological function of immune cells, the exact mechanism of these effects and how semaphorins regulate integrins in the immune system are not known yet.

1.4.4.2 Focal Adhesion Complexes

Focal adhesions are flat, elongated structures with a surface area of several square microns that often located at the cell periphery. This complex of proteins convey information across the cell membrane to regulate extracellular-matrix assembly, cell migration and morphogenesis, cell proliferation, differentiation, and death [16]. These structures are dynamic and rapidly turned-over. They form at ECM-integrin junctions to bring together cytoskeletal and signaling proteins during cell function. For the cells to process different extracellular stimuli correctly, there are essential intracellular signaling proteins that function as integrators which translate extracellular input as signaling pathway output. One of these unique integrators is focal adhesion kinase (FAK), a tyrosine kinase that plays a key role in cytokeletal rearrangment [76].

FAK contains a central catalytic domain with large N- and C- terminal non-catalytic domains. The C-terminus of FAK is rich in protein-protein interaction sites that direct FAK to other focal adhesion proteins. This region is necessary for

FAK signaling. FAK also has multiple serine/threonine phosphorylation sites which are like a switch allowing it to signal to multiple downstream pathways leading to different physiological outcomes [87].

The relation between Sema-3A signaling and FAK is extensively studied in the nervous system. For example, Chacn *et al.* have shown that upon stimulation of axons with Sema-3A, FAK was phosphorylated at tyrosine 925 which results in disassembly of focal complex protein paxillin from FAK and growth cone collapse [21]. In another study Schlomann and his team defined the molecular pathway underlying the chemoattractant activity of Sema-3A on rat hippocampal dendrites, and showed the engagement of $\beta 1$ integrin in this pathway. Also they found that FAK is phosphorylated downstream of activation of $\beta 1$ integrin by Sema-3A/Plx-A1 [99].

By finding new roles for semaphorins and their receptors outside of the nervous system, new efforts have begun to clarify the role of focal adhesion complexes in other cells. Serini *et al.* have shown that at the cell periphery of ECs, Sema-3A either colocalized with or surrounded focal complexes suggesting dynamic transient association. Sorting between Sema-3A and integrin containing focal complexes resulted in inhibition of ECM ligand recognition by EC integrins and prevented EC adhesion to ECM [101]. More investigation is necessary to clarify how Sema-3A regulates FAK outside the nervous system.

1.4.4.3 Rho Kinases

In this subsection the focus is on a particular family of proteins that seems to have a pivotal role in regulating the biochemical pathways relevant to cell migration, the Rho GTPases.

Rho GTPases are a family of small signaling G proteins and a subfamily of the Ras superfamily. They act as molecular switches to control signal transduction pathways by cycling between a GDP-bound (inactive form) and a GTP-bound (active form). So far 20 members have been found in mammals. The best characterized function of these proteins is the regulation of actin dynamics. Three members of this family have been extensively studied: Cdc42, Rac1, and RhoA. The *in vitro* studies of a wide variety of cells demonstrated that Cdc42 and Rac1 regulate the polymerization of actin filaments to form peripheral filopodia and lamellipodia, respectively, at the front of the migrating cells. RhoA regulates the assembly of actin:myosin contractile filaments at the back of the migrating cells. In addition, three regulatory proteins promote the assembly of integrin-based, matrix adhesion complexes [92].

The role of Rho-family GTPases in response to guidance cue stimulations has been well studied in terms of axon guidance, and it is well established that these proteins are important components of Sema-3A/Plx-A axon guidance signaling [56]. As it has been shown in figure 1–8, Plx-A has a Rho GTPase binding domain (GAP) in its cytoplasmic region suggesting a direct interaction with this family of proteins. The first indication that Rho GTPases were involved in Sema-3A signaling came from studies in chick dorsal root ganglions neurons (DRGs). When DRGs were treated with the dominant negative form of Rac1 they did not respond to Sema-3A, whereas

constitutively active Rac1 partially imitated Sema-3A-induced growth cone collapse. This suggested that Rac1 mediates the F-actin reorganization induced by Sema-3A [48]. In a study of local stimulation of the growth cone of a neuroblastoma cell with Sema-3A, it was revealed that Sema-3A activates Cdc42 and RhoA at the leading edge of this cell with different mechanisms and dynamics [92].

Sema-3A has an active role in the regulation of immune cell migration, and probably it uses the same machinery as in the nervous system. Takamatsu and his colleagues showed that during transmigration of DCs from the lymphatic endothelial junctions, Sema-3A constantly acts on the rear side of DCs, where Plx-A1 is localized. Sema-3A induces myosin light chain phosphorylation, resulting in actomyosin contraction and morphological changes in DCs to pass the EC barrier [109]. Many of the integrins that are involved in T-cell-mediated immunity have been found to be regulated by R-Ras, another Rho GTPases family member. These include $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, and $\alpha_M\beta_2$ integrins [56]. Also in macrophages, the R-Ras GTPase regulates activation of $\alpha_M\beta_2$ integrin and increases phagocytosis in macrophages [14]. So future studies are necessary to focus on how Sema-3A/Plx-A signaling implicates its effects on integrins via R-Ras and Rho GTPases in immune cells.

1.5 Semaphorin-3A and Acute Inflammation

The innate immune system is the first line of defence involved in rapidly detecting invading pathogens. Several recognition strategies have evolved to provide a reliable detection system. The pattern recognition strategy is based on the detection of a limited set of conserved molecular patterns that are unique to microbes and

similar among the entire class of pathogens. These patterns are detected by pattern recognition receptors (PRRs) that signal to the host defence system.

So far several classes of PRRs have been identified, but the best characterized one is Toll-like receptors (TLRs), a mammalian homologue of drosophila Toll receptor discovered in 1997. So far ten members of these receptors have been identified. These receptors are activated by molecular patterns associated with a broad range of pathogens including bacteria, viruses, fungi, and protozoa. In addition to ligand specificity, the individual TLRs differ in their expression patterns and the signal transduction pathways they activate. The intracellular signaling cascades elicited by these receptors result in transcriptional expression of a variety of overlapping and unique genes involved in the inflammatory and immune responses [5, 50, 112]. Activation of TLRs also leads to the development of antigen-specific adaptive immunity [110].

Many pathogen-associated molecular patterns have been defined that interact with particular TLRs. Thus TLRs are sensors that the innate immune system has devised to decode the type of invading pathogens and elicit an appropriate effective immune response. In 1998 TLR4 was shown to be involved in the recognition of lipopolysaccharide (LPS), a major cell wall component of Gram-negative bacteria. However, to induce the proper response, TLR4 also needs the help of other molecules. In the serum of the host, LPS binds to LPS binding protein and then this complex is recognized by CD14, a GPI-anchored molecule expressed on monocytes, macrophages, and neutrophils. The next step is association of CD14/LPS

with TLR4. In addition, MD-2 molecule, associated with the extracellular region of TLR4, increases TLR4 responsiveness to LPS [110].

Upon stimulation of myeloid cells with LPS, various signaling pathways including MAPK pathways (ERK, JNK, and p38), and NF-κB are activated. These pathways directly or indirectly phosphorylate and activate different transcription factors which induce expression of proinflammatory genes (figure 1–10).

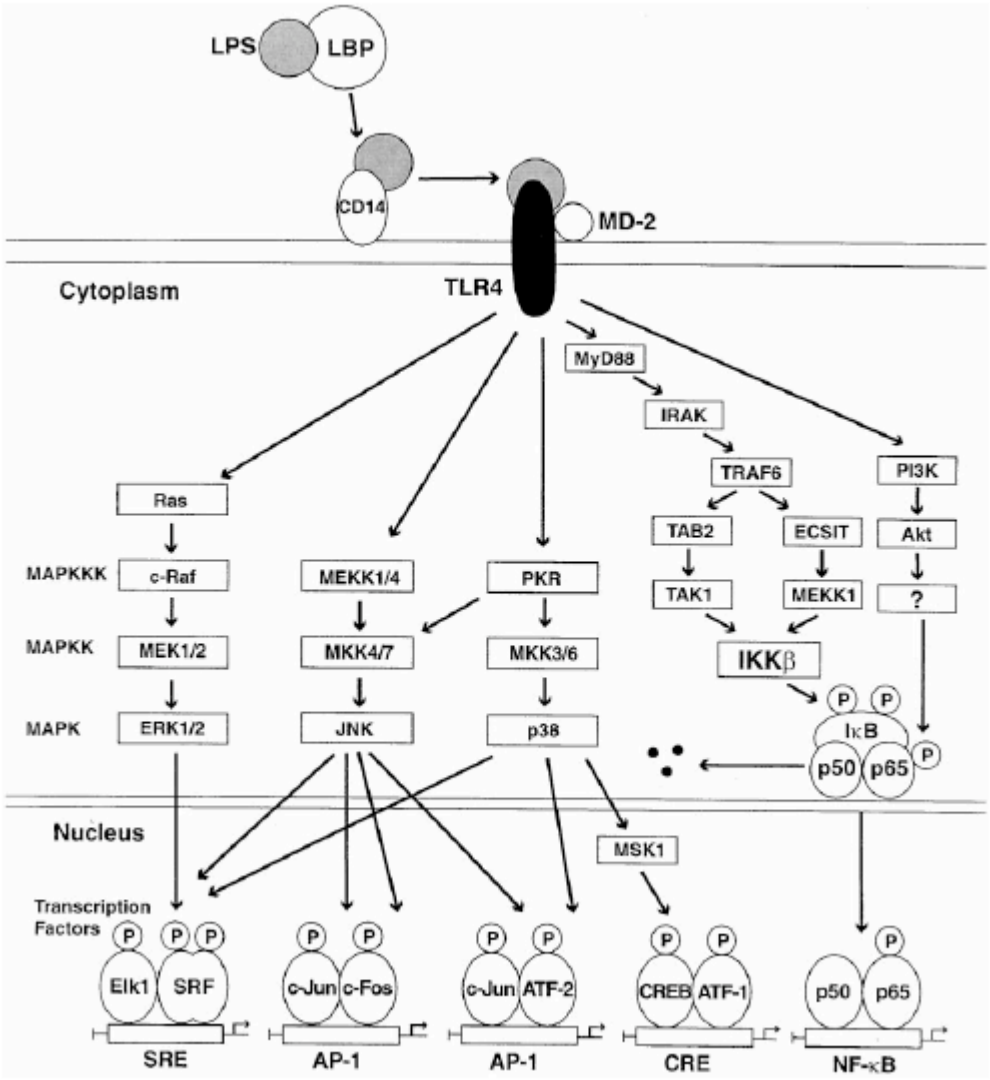


Figure 1–10: LPS stimulation of myeloid cells activates various signaling pathways and transcription factors: In the serum LPS binds to LBP and then is transferred to CD14. Interaction of LPS with TLR4 stimulates the activation of various signaling pathways including ERK, JUNK, p38, and also NF-κB. Reprinted from reference [40] with permission from Elsevier.

Studies in MyD88 deficient mice revealed that there are two separate pathways upon stimulation of macrophage TLR4 by LPS. The MyD88-dependent pathway was shown to be responsible for proinflammatory cytokine production, whereas the MyD88-independent pathway mediates the induction of Type I Interferons and interferon inducible genes (figure 1–11) [68].

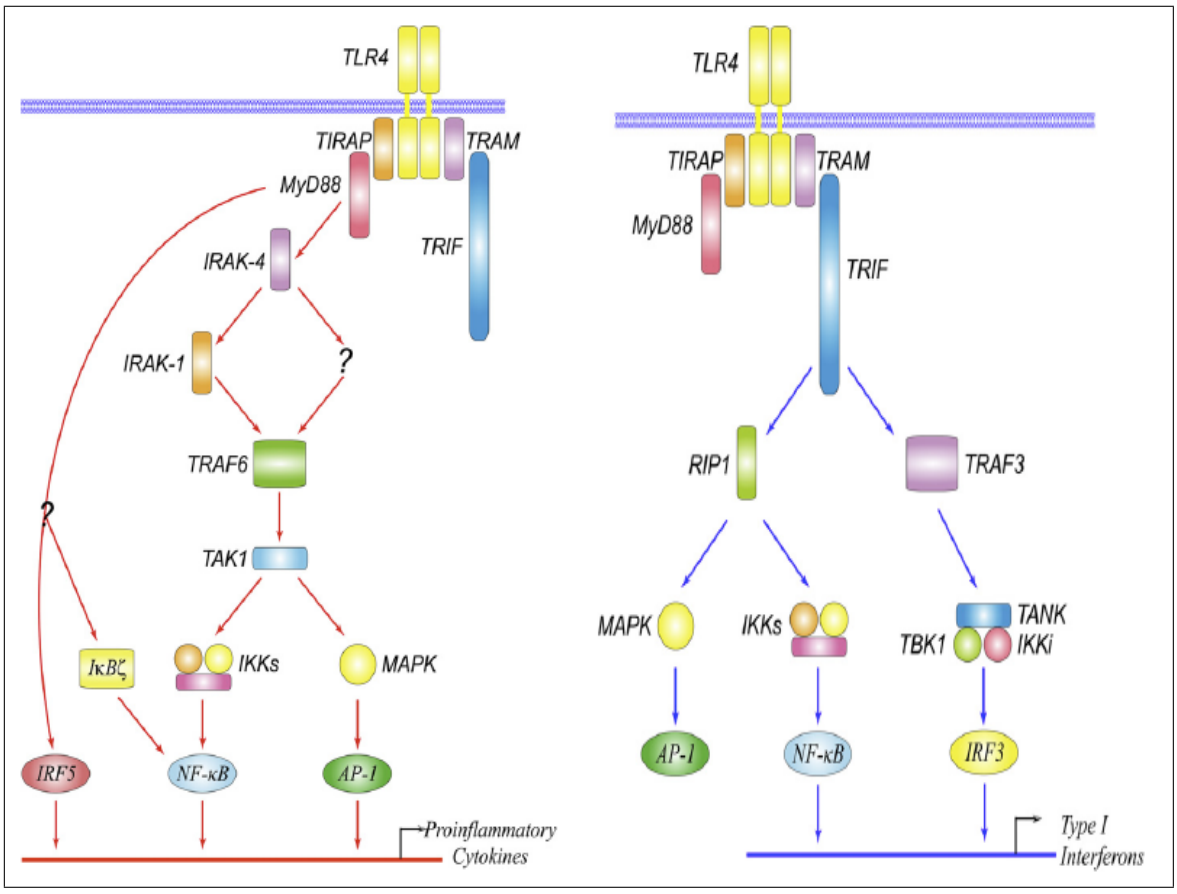


Figure 1–11: TLR4 signaling pathways: Depending on combination of different adaptor molecules downstream of TLR4 activation, the response is different. NF-κB is activated in both MyD88-dependent and -independent pathways. However in the former, the response is secretion of inflammatory cytokines and in the later, type I interferons are secreted. Reprinted from reference [68] with permission from Elsevier.

Despite numerous studies showing the immuno-suppressive effect of Sema-3A in the adaptive immune system, little is known about the role of Sema-3A in innate immunity and acute inflammation. What has been shown so far is that Sema-3A/Plx-A signaling has a complete opposite role in adaptive vs. innate immune system. In two *in vivo* attempts to identify the role of Sema-3A in acute inflammation, it was shown that it has proinflammatory function. In the first study, LPS was injected into adult rat brains to induce focal acute inflammation. 24 hrs later microgila activation was detected in response to acute inflammation. Further characterizations showed significant increase in Plx-A1 and Nrp-1 colocalized with activated microglia in LPS-mediated inflammatory lesions. Sema-3A expression was also upregulated compared to control and induced apoptosis in adjacent microglia [71]. In the second, acute kidney injury model, Ranganathan *et al.* showed that Sema-3A is upregulated in both humans and mice after acute kidney injury, contributing to tissue damage, increasing neutrophil infiltration, inducing epithelial cell apoptosis, and enhancing TLR4 mediated inflammation [93].

The same pattern was also found in *in vitro* experiments. Ito and his colleagues showed that in microglia cells treated with Plx-A1 siRNA, TLR-4-mediated generation of proinflammatory factors like iNOS, IL-8, and TNF-α were significantly lower compared to control due to defect in NF-κB and ERK1/2 activation indicating that

Plx-A1 is required for responsiveness of microglia cells to LPS stimulation. Also they found that stimulation of these cells with LPS increased expression of Sema-3A after 2 hours, and after 4 hours the proteolytic form of Sema-3A, 65 kDa fragment, was detected in cultured media [44]. But still there are a lot of details missing in the pathway of Sema-3A/Plx-A/TLR4 which needs more investigation to confirm whether Sema-3A has an proinflammatory role in innate immunity.

1.5.1 NF-κB signaling

Many studies have demonstrated that one of the pathways activated by LPS in leukocytes is NF-κB [45]. The NF-κB family of transcription factors has five members: p65 (REL-A), REL-B, cytoplasmin (c) REL, p50, and p52, which function as homo- and heterodimers. In the cytoplasm, NF-κB dimers are usually in an inactive form bound to the inhibitor of NF-κB (IκB). Upon phosphorylation and proteolysis of the IκB proteins, NF-κB is activated and translocates to the nucleus and binds to DNA and starts the transcription process [4].

Stimulation of Plx-A4$^{-/-}$ peritoneal macrophages with LPS showed defective p65 and IκB phosphorylation compared to WT macrophages. However, there was no defect in response to TNF, anti-CD40, and IFN-γ treatment indicating a specific role of Plx-A4 in TLR activation. Since there was no defect in Akt phosphorylation in response to TLR activation by LPS, Wen *et al.* concluded that Plx-A4 is involved specifically in NF-κB and JNK activation [126]. The same study showed that inflammatory cytokine production, including TNF, IL-1β, IL-6, CCL2, and CCL3 was reduced in response to LPS in Plx-A4 knock-out macrophages compared to control,

through abolished LPS-induced Rac1 activation. Thus Rac1 serves as a signal transducer downstream of Plx-A4 to modulate NF-κB and JNK activation through TLRs. In another experiment they showed that Sema-3A/Plx-A4 signaling is required to amplify the TLR signaling upon activation by LPS [126]. Unfortunately there is no evidence of the role of Nrp-1 and Plx-A1, the main receptors of Sema-3A in NF-κB activation which deserves more attention of investigators.

1.6 Rational and Objectives

Given the critical importance of the immune system in diseases such as atherosclerosis, the goal of this thesis is to contribute to understanding the role of semaphorin-3A in the regulation of the immune system. To find out the optimal treatment for immune-related diseases, it is essential to know how immunity regulates these diseases and find out new molecules that could be a target for future treatments. Furthermore it is well known that semaphorin-3A has an anti-inflammatory role in some diseases including rheumatoid arthritis and systemic lupus. However the role of this protein in atherosclerosis and acute inflammatory diseases is unknown.
Therefore, the specific objectives of this thesis are:

1. To investigate the role of semaphorin-3A on atherosclerotic plaque formation in a mouse model of atherosclerosis
2. To determine the impact of semaphorin-3A on innate immunity and acute inflammatory disease

Chapter II:First Paper

Hypothesis and Objectives

Although there is extensive knowledge of the mechanisms of plaque formation, the failure of regular risk factor management to effectively eliminate the risk of cardiovascular disease makes it a necessity to find new clinical targets. Still more than half of the patients in controlled clinical trials have heart attacks or strokes despite aggressive treatments.

The timing of treatment is crucial in the complex environment of the atherosclerotic plaque. For example increasing apoptosis could be beneficial at the early stage of plaque formation since efferocytosis is effective at eliminating debris. However, in advanced plaques where efferocytosis is impaired, increasing apoptosis leads to the release of inflammatory content of cells which creates a secondary necrotic core and more complications. Increased macrophage egress is however, a process which is beneficial in terms of plaque progression and regression whatever the stage of the plaque.

The first question this research tries to address is whether Sema-3A, being protective in some immune-related disease, also has the potential to decrease atherosclerotic plaque formation. So far Sema-3A has not been studied in terms of atherosclerosis, and our novel research aims to address this issue.

We hypothesize that Sema-3A prevents atherosclerotic plaque formation by regulating macrophage function.

Specific objectives:

- To determine the effect of Sema-3A on plaque formation *in vivo* in a mouse model of atherosclerosis

- To determine the effect of Sema-3A on macrophage function *in vitro*

CHAPTER 2

Semaphorin-3A reduces atherosclerotic plaque formation in apoE$^{-/-}$ mice through regulation of M2 type macrophage migration

[*]Maryam Heidari[1,2],[*]Talin Ebrahimian[1], David Simon[1], Hojatollah Vali[2], Craig A. Mandato[2] and Stephanie Lehoux[1]

[1] Lady Davis Institute for Medical Research, Jewish General Hospital, Montreal, Quebec, [2]Department of Anatomy & Cell Biology, McGill University, Montreal, Quebec

*Equal contribution

Running title: Sema-3A decreases atherosclerosis

Corresponding author: Stephanie Lehoux

stephanie.lehoux@mcgill.ca

Lady Davis Institute for Medical Research

3755 Cote Ste Catherine, Montreal, QC, H3T 1E2, Canada

Phone: +1-514-340-8222; fax: +1-514-340-8252

Keywords: Sema-3A, atherosclerosis, macrophages

Subject Codes: Atherosclerosis, Vascular Biology, Basic Science Research

Total number of figures and tables:6

2.1 Abstract

Recent data point to an important immunomodulatory role for neural guidance molecules, including Sema-3A, in inflammatory diseases. Sema-3A is a secreted member of the Sema family and is produced by several immune cells including macrophages. Nevertheless, the effect of Sema3A on atherosclerotic plaque formation has never been tested directly, and how Sema-3A may alter the function of major plaque inflammatory constituents, macrophages, is unknown. To investigate the role of Sema-3A in atherosclerosis, ApoE$^{-/-}$mice were administered with a Sema-3A overexpressing or a control plasmid and were fed a high fat diet for 9 or 13 weeks. We found that Sema-3A receptors are expressed in macrophages and in atherosclerotic plaques. Our results show that Sema-3A overexpressing mice had significantly smaller atherosclerotic plaques than control mice in the aortic sinus (0.3 ± 0.02 vs. 0.4 ± 0.03 mm^2), the BCA artery (0.04 ± 0.01 vs. 0.1 ± 0.01 mm^2) and the aorta (9.5 ± 1.4 vs $15.3 \pm 2.9\%$), assessed by oil red O staining. No differences were observed in plaque stability, measured by collagen and smooth muscle cell alpha-actin staining. However, there was significantly less (2-fold) macrophage content in the plaques of Sema-3A compared to control mice, associated with decreased circulating monocyte number determined by flow cytometry as cd11b positive and Gr-1 negative cells (4.97 ± 0.74 vs. $7.2 \pm 0.62\%$). To better define the involved mechanisms, we investigated macrophage function *in vitro* and found that recombinant Sema-3A increased by 4 fold migration of M2 but not M1 macrophages. mRNA levels of alpha and beta subunits of integrins were unchanged in bone-marrow-derived macrophages. In contrast, active beta-1 integrin expression was significantly enhanced (2-fold) by

Sema-3A in human M2 macrophages. Importantly, Sema-3A induced a significant increase (by 50%) of focal adhesion kinase phosphorylation. In conclusion, our data show that Sema-3A prevents atherosclerotic plaque formation in ApoE$^{-/-}$ mice. This may be due in part to enhanced motility and function of M2 macrophages through regulation of beta-1 integrin.

2.2 Introduction

Atherosclerosis is a chronic inflammatory disease and a leading cause of myocardial infarction and stroke, which account for the majority of morbidity and mortality in industrialized countries. Monocytes and macrophages are the most abundant cells in the atherosclerotic plaque. Monocyte recruitment is a crucial first step in atherosclerotic lesion formation, but retention of ensuing macrophages and foam cells contributes most significantly to the developing plaque. Macrophage function is strongly influenced by exposure to cytokines; this polarization process generates 2 macrophage subtypes broadly referred to as M1 and M2. M1 classically activated macrophages are induced by IFN- and drive proinflammatory responses [37, 73]. M2 alternatively activated macrophages differentiate in the presence of IL-10, IL-4 or IL-13 and express anti-inflammatory mediators [72], associated with wound healing. The progression and exacerbation of atherosclerosis are propelled by gradual lesional accumulation of M1 macrophages which secrete pro-inflammatory cytokines [105]. Conversely, the clearance of apoptotic cells by phagocytosis and plaque regression are associated with M2 macrophage function [120, 132].

One of the most important questions in atherosclerosis is how to curb lesion progression. Identifying new molecules which can enhance reparative macrophage

function would have a real beneficial impact in modulating the atherosclerotic process and answer this question. Semaphorins are a large family of secreted and membrane bound proteins first found to regulate axon guidance and neural development [55, 69]. Known roles of semaphorins have since expanded well beyond the nervous system, and include modulation of immune responses [115, 137]. At present, eight classes of semaphorins have been identified, with over 20 known members in vertebrates. Class 3 semaphorins are the only secreted vertebrate semaphorins [54]. Sema-3A is secreted by several immune cells including macrophages and T cells [19, 62, 126]. It exerts its action through a receptor complex formed from Nrp-1 [22, 43] and an A-type plexin, preferentially Plxn-A1 or Plxn-A4 [113]. Nrp-1 is the primary ligand binding site and Plxn-A proteins are the signal transducing components [30, 56]. Both receptor types are detected in monocytes and macrophages of human and murine origin [19, 133, 129, 46]. Although macrophages and T cells are abundant in atherosclerotic lesions, no link had been made thus far between Nrp-1 or plexins and atherosclerosis.

Evidence of association of Sema-3A with human inflammatory diseases has recently been uncovered. Sema-3A levels are abnormally low in patients with rheumatoid arthritis and systemic lupus erythematosus [121] and Sema-3A administration has a potent anti-inflammatory and reparative effect in animal models of such diseases [19]. Moreover, Sema-3A is a strong suppressor of T cells associated with inflammatory processes [20, 97]. Several studies suggest that Sema-3A could be protective in the context of atherosclerosis. Sema-3A dramatically inhibits proliferation of T cells and inflammatory cytokine production by effector T cells [20, 135], which

secretes cytokines that skew macrophages towards an M1 phenotype. On the contrary, Sema-3A induces regulatory T cells [19, 97], which suppress effector T cells, secrete M2-polarizing cytokines, and are generally considered to be the most active cells against atherosclerosis [3, 7]. In hyperlipidemic ApoE$^{-/-}$ mice, administration of measles virus promoted a strong regulatory T cell response and reduced the development of plaques[2]. Measles virus has since been shown to be a potent inducer of Sema-3A release [119]. Finally, Sema-3A is involved in dendritic cell transmigration [109], an important step in plaque regression. Nevertheless, the effect of Sema-3A on atherosclerotic plaque formation has never been tested directly, and how Sema-3A may alter the function of major plaque inflammatory constituents, macrophages, is unknown.

In the present study we treated ApoE$^{-/-}$ mice placed on a high fat diet with Sema-3A, administered in the form of an electroporated plasmid. This well established technique successfully increases serum levels of secreted proteins such as Sema-3A [26, 127]. We demonstrate that Sema-3A has a protective effect on atherosclerotic plaque formation through regulation of M2 macrophage mobility.

2.3 Material & Methods:

2.3.1 Animal Housing & diet

B6.129P2-Apoetm1Unc/J (ApoE/) male mice were obtained from Jackson laboratory (Bar Harbor, ME) and mated in house. The McGill University animal use

committee approved the experimental protocols and animals were handled in accordance with institutional guidelines.

2.3.2 Electroporation

One hour before electroporation, hyaluronidase was injected in each thigh muscle of 20 mice (30 $\pm$lμl/thigh) to increase electroporation efficiency by 50% (1$\pm$lμl in 70$\pm$lμl of 0.9% NaCl) (Calbiochem) under isofluorane anesthesia. After one hour, the mice were divided in tow groups, ten mice per group, and Sema-3A or GFP plasmid was injected into each thigh (50 μg/thigh dissolved in 50 $\pm$lμl of 0.9% NaCl) under anesthesia. Thigh muscles were then lightly massaged and then immediately electroporated using a previously described protocol [26]. Briefly, eight 10 millisecond electrical pulses at 200 V/cm with a frequency of 1 Hz were applied. The pulses were generated using an ECM 830 Square Wave Electroporator and administered using electrodes (BTX Harvard Apparatus). Then the animals were fed a high fat diet containing 15% fat (from cocoa butter) and 0.5% cholesterol (Harlan Laboratories) for 9 weeks.

2.3.3 Physiological parameter measurement

Body weight was measured at the initiation of the HFD, and at sacrifice. Blood was removed via a syringe from the Heart at the time of sacrifice and was collected in heparin-coated tubes (Sarstedt) or EDTA coated tubes (Sarstedt). Blood cell enumeration (white blood cells, lymphocytes, monocytes, granulocytes, red blood cells, % hematocrit, hemoglobin and platelets) was performed with a hematology

analyzer (scil vet abc animal blood counter, Vet Novations). Some of the blood was used for flow cytometry analysis and the rest was centrifuged to collect plasma and total cholesterol, high-density lipoprotein (HDL) was determined by using the HDL and LDL/VLDL quantitation kit (Sigma-Aldrich).

2.3.4 Atherosclerotic lesion measurement and characterization

At sacrifice the vasculature was washed out with 2% heparin in PBS, and periadventitial tissue was removed. The heart and aortic branch were placed in 4% paraformaldehyde overnight at 4 °C shaker and then rinsed with 1X PBS and changed to 30% sucrose (in PBS) overnight at 4 °C on a shaker, and again rinsed with 1X PBS. Aortic arches were stained with oil red O (Electron Microscopy Sciences) for 30 minutes and mounted using Immu-Mount (ThermoFisher Scientific) on VWR vista vision unimark slides (VWR) and photographed using a Leica dissecting microscope under bright light. Aortic sinus and BCA segments were embedded vertically in Tissue-tek (Sakura), and serial 6μm thick sections at 60 μm intervals were cut using a cryostat (Leica CM 3050 S, Leica Microsystems). Sections of aortic sinus and BCA were stained with oil red O for 45 minutes and mounted using Immu-Mount on superfrost plus slides (VWR). Images were acquired using a Leica microscope. Using ImageJ software (National Institute of Health) the lesion for the aortic sinus and the BCA were determined as shown in figure 2.1 and the mean lesion areas in mm^2 were calculated using ImageJ . Smooth muscle cell content of the plaque was evaluated by immunohistochemistry staining for smooth muscle cell α-actin (5%

BSA block, 1/100 monoclonal anti-actin, α-smooth muscle alkaline phosphatase antibody, Sigma-Aldrich) for 1 hour and mounted using Immu-Mount. Collagen content of the plaque was evaluated using the picrosirius red stain kit according to manufactures protocol (Polysciences) and mounted using Eukitt mounting medium (Electron Microscopy Sciences). Plaque macrophage content was determined by immunohistochemistry staining for monocyte/macrophage marker (10% goate serum block, 1/50 polycolonal rat MOMA-2 antibody (abcam). Immunofluorescent and bright light pictures were taken using Leica microscope. Percentage of plaque staining was calculated using ImageJ.

2.3.5 Flow cytometry

Blood was collected at sacrifice and stained for the surface markers CD11b, Gr1, Ly6C, F4/80 (eBioscience). Flow cytometry was performed on the BD LSR Fortessa (BD Biosciences). Fluorescence minus one controls was used to remove the fluorescent background. Data analysis was performed using Flow Jo software (Tree Star Inc.).

2.3.6 ELISA

The blood plasma level of Sema-3A was determined by using mouse Sema-3A ELISA kit following the instruction protocol (MyBioSource Inc.)

2.3.7 M0, M1, and M2 macrophage preparation

Macrophages were obtained by flushing the bone marrow of both femurs and tibias of C57/BL6 mice at 3 months of age. Mice were anesthetized and killed by CO_2. Bones were flushed with RPMI 1640 and centrifuged, after which cells were resuspended in RPMI with serum (10% FBS) (macrophage media), plated on a 10 cm petri dish and incubated at 37 °C. After 2 hours, cells were washed and differentiated in the presence of macrophage media with recombinant mouse macrophage colony stimulating factor (M-CSF) (50ng/ml, PeproTech) for 5 days. Macrophages were then scraped and used as M0 macrophages or polarized for 24 hours at 37 °C to M1 macrophages (Interfron-γ 50ng/ml) or M2 macrophages (IL-4 10ng/ml) and then scraped and used. To check the expression of cell surface markers of M1 and M2 macrophages, total RNA of cells was extracted using a total RNA mini kit (Geneaid) following instructed protocol. RNA was quantified using a spectrophotometer (NanoDrop ND-1000, Thermo Scientific). cDNA was synthesized from 0.4 g RNA by using qScript cDNA Supermix kit (Quanta Biosciences). The expression level of M1 and M2 macrophage markers (NOS-2, TNF-α, IL-6 and Arg-1, Fizz1, Ym-1 respectively) was assessed by quantitative real time polymerase chain reaction (RT-qPCR) using 7500 Fast PCR (Applied Biosystems) under standard conditions of 60 °C annealing temperature for 40 cycles. All primers (Table 2-1) were designed using Primer 3 Plus software.3. SYBR -green chemistry (SensiFAST SYBR Lo-ROX kit, BIOLINE) was used with specific primers (listed in Supplementary Table) for all genes. Results were analyzed using the $\Delta\Delta$Ct method as calibrator samples. The

analyzed genes were expressed relative to the murine Rps16 housekeeping gene.

2.3.8 Macrophage polarization

The effect of Sema-3A on polarization of macrophages was studied. Bone-marrow derived M0 macrophages was polarized to either M1 or M2 with or without Sema-3A (100ng/ml) for 12 hrs at 37 °C. Then the cells were washed and total RNA was extracted and quantified as above. The expression levels of M1 and M2 markers were analyzed by qRT-PCR and analysed as mentioned before.

2.3.9 M1 Macrophage phenotype switch

The effect of Sema-3A on M1 macrophage phenotype switch to M2 was studied. Bone-marrow derived M1 macrophages was polarized to M1 with IFN-γ for 24 hrs at 37 °C as mentioned before. Then the cells were washed and stimulated with Sema-3A (100ng/ml), IL-4 (10ng/ml), or both at the same time. Total RNA was extracted and quantified as above. The expression levels of M1 and M2 markers were analyzed by qRT-PCR and analysed as mentioned before.

2.3.10 Western blotting

M0, M1, M2 macrophages were stimulated with vehicle or Sema-3A (100ng/ml) for 24 hours 0r 5-30 minutes and then collected and total proteins (15-20 μg) were extracted, separated by SDS-PAGE, transferred to nitrocellulose membrane and incubated overnight with primary antibodies against anti-Nrp-1, -Plx-A4 (Cell signaling),

-Plx-A1 (Santa Cruz) and -phospho Y407-FAK (Invitrogen). Membranes were subsequently stripped and re-probed with a GAPDH antibody (Sigma) to verify equal loading.

2.3.11 Sema-3A receptor expression

To investigate if Sema-3A has any effect on expression of its receptors, Nrp-1, Plx-A1, and Plx-A4, M0, M1, and M2 macrophages were prepared as prevoiusly described. The cells were starved using macrophage starving media (0.5% FBS) overnight at 37 °C, and stimulated with either vehicle, Sema-3A 25ng/ml or 100ng/ml for 24 hours at 37 °C. The cell lysate was obtained and proteins were extracted. The expression of Sema-3A receptors was determined by western blotting using rabbit monoclonal anti- Nrp-1 (Cell Signaling), rabbit polyclonal anti- Plx-A1 (Santa Cruz), and mouse monoclonal anti- Plx-A4 (Cell Signaling).). The expression of receptors were quantified relative to GAPDH with ImagJ software.

2.3.12 Migration assay

The migration capacity of all types of macrophages was assessed using 24-well plate 8.0 microns boyden chambers (Corning Inc.). Different types of macrophages were prepared as described before. 10×10^4 cells were resuspended in macrophage starving media (0.5% FBS) and plated in the top chamber. Recombinant Sema-3A, 100 ng/ml or vehicle was added to the macrophage starving media in the bottom chamber to eliminate any proliferation and incubated overnight at 37 °C. Then the filter was washed with 1XPBS and fixed with 2% paraformaldehyde for 15 minutes

at 37 °C. The filter was then stained with dapi for nuclei and mounted on slide. Fluorescent pictures were taken using Leica microscope and the cells migrated through the filter were counted.

2.3.13 Activated integrins

Due to lack of antibodies for activated integrins in the mouse, presence of activated β_1 and β_2 integrins on the M1 and M2 macrophages were assessed using human cells obtained as follows. Human peripheral blood mononuclear cells were isolated from blood of healthy donors (age 20-45 years, male and female, no known disease or medication) using a Ficoll density gradient (Sigma-Aldrich). Cells were plated in macrophage media in a 37 °C incubator for 2 hours, washed and differentiated over 7 days using human M-CSF (50ng/ml, R&D Systems) to macrophages. Then the macrophages were polarized to either M1 macrophages using recombinant human IL-4 (30 ng/ml, R&D systems) or M2 macrophages using recombinant human IFN-γ (20ng/ml, R&D systems) for 24 hours at 37 °C. Macrophages were then starved using macrophage-starving media overnight and stimulated with vehicle or recombinant human Sema-3A (100ng/ml, R&D systems) for 4 hours. Then the cells were scraped, collected, centrifuged, and resuspended in FACS buffer (2% FBS in PBS) and stained for the surface markers, activated β_1 integrin (1/400, Millipore) and activated β_2 integrin (1/100, Hycult Biotech). The activation of integrins was quantified using flow cytometry (BD LSR Fortessa, BD Biosciences, CA and analyzed using Flow Jo software (Tree Star Inc.).

2.3.14 Focal adhesion kinase avtivation

Activation of FAK was aassessed by western blotting. M2 macrophages were prepared as explained before. Cells were starved overnight to eliminate any false positive signals and then stimulated with vehicle or Sema-3A 100ng/ml for 30 minutes at 37 °C. The cells were lysed and proteins were extracted. The effect of Sema-3A on phosphorylation of FAK was determined using rabbit polyclonal anti- phospho Y407-FAK (Invitrogen). The expression of p-FAK was quantified relative to GAPDH with ImagJ software.

2.3.15 Efferocytosis

To assess the effect of Sema-3A on phagocytosis of apoptotic bodies (efferocytosis), M0, M1, and M2 macrophages were prepared as described before. To prepare apoptotic cell RAW macrophages were kept in culture for 5 days. Then the cells were scraped and resuspended in macrophage media containing orange cell tracker to fluorescently label macrophages (10 μM, Invitrogen), incubated at 37 °C for 30 minutes and then washed and resuspended in fresh macrophage media and placed under the UV for 30 minutes and incubated at 37 °C overnight to induce apoptosis. Efferocytosis experiment was done by stimulating M0, M1, M2 bone marrow derived macrophages with vehicle or 100 ng/ml Sema-3A for 30 minutes and then adding apoptotic cells (ratio 5:1) and incubating again at 37 °C for 1 hour. After 1 hour the cells were washed at fixed with 2% paraformaldehyde for 15 minutes at 37 °C and stained with dapi. Fluorescent pictures were taken using Leica microscope and the

number of phagocytic cells was counted.

2.3.16 Ac-LDL uptake (Foam cell formation)

M0, M1, and M2 macrophages were prepared as before. The cells were stimulated with vehicle or 100ng/ml Sema-3A for 30 minutes at 37 °C and Dil Ac-LDL (5 μg/ml, Invitrogen) were added to the cells and incubated again for 4 hours. Cells were washed and fixed with 2% paraformaldehyde, and the pictures were taken by Leica microscope. The Ac-LDL uptake was determined by measuring fluorescent intensity using ImageJ.

2.3.17 Monocyte adhesion

In vitro 96- well plates were coated with either ICAM-1 (10 μg/ml, R&D System) or VCAM-1 (10 μg/ml, R&D System) at 37 °C. After 30 minutes the plates were washed with 1X PBS. Mouse bone marrow cells were extracted and incubated at 37 °C for 2 hours. The floating cells were aspirated and attached monocytes were incubated at 37 °C with monocyte starvation medium (RPMI + 0.5% FBS) overnight. The day after the cells were stimulated with vehicle or 100 ng/ml Sema-3A for 30 minutes, then washed, scraped and plated on ICAM-1 or VCAM-1 pre-coated plates and incubated at 37 °C for 30 or 60 minutes. After the time is over, the cells were washed three times with 1XPBS and fixed with 2% paraformaldehyde for15 minutes at 37 °C. The cells were washed again with 1XPBS and stained with dapi. Fluorescent pictures were taken using Leica microscope and the number of attached cells

was counted.

Ex vivo Mouse carotid arteries were isolated, cannulated at both ends, immersed in an organ culture bath with cell culture medium supplemented with 10% FBS, and mounted in a closed, *ex vivo* perfusion system. The system includes a reservoir filled with cell medium alone, or cell medium containing 100ng/ml Sema-3A, TNF-α (10 ng/ml), or Sema-3A+TNF-α. Then carotid segments were kept in sterile condition in an incubator containing 5% CO_2 at 37 °C for 6 hours. After 6 hours, fluorescently labeled monocytes (orange cell tracker, 10 μM, Invitrogen) were injected in the intraluminal compartment of the vessels and allowed to adhere for 30 minutes before washout. Cells attached to the vessels were visualized by Leica microscopy and counted.

2.3.18 Monocyte migration

The migration capacity of monocytes was assessed using 24-well plate 8.0 microns boyden chambers (Corning Inc.). Bone marrow derived monocytes were prepared as described before. 50×10^3 cells were resuspended in monocyte starving media and plated in the top chamber. Recombinant Sema-3A, 100 ng/ml or vehicle was added to the monocyte starving media in the bottom chamber and incubated overnight at 37 °C. Then the filter was washed with 1XPBS and fixed with 2% paraformaldehyde for 15 minutes at 37 °C. The filter was then stained with dapi for nuclei and mounted on slide. Fluorescent pictures were taken using Leica microscope

and the cells migrated through the filter were counted.

2.3.19 Statistical Analysis

Results are expressed as mean±SEM. All data was evaluated by ANOVA. To assess comparisons between multiple groups, data were analyzed using the Newman-Keuls test. A probability value of <0.05 was considered to be statistically significant. Data that was significant ($p<0.05$) was further assessed by Student t test using GraphPad Prism software (GraphPad Software).

2.4 Results

2.4.1 Semaphorin-3A reduces atherosclerotic plaque formation in ApoE$^{-/-}$ mice.

First of all we verified the expression levels of Sema-3A receptors in the vessel wall and in the atherosclerotic plaques. Nrp-1, plxn-A1, and plxn-A4 were all expressed in the endothelium of the healthy arterial wall as well as in the endothelial cells overlying the plaque (figure 2–1A). In addition, we observed a co-localization of Nrp-1 with the macrophage marker, MOMA-2, indicating that macrophages within the plaque express Nrp-1 (figure 2–1A). These results imply that Sema-3A could possibly play a role in atherosclerosis. Therefore we investigated the effect of Sema-3A over expression on atherosclerotic plaque formation at two different time points. ApoE$^{-/-}$ mice were injected with Sema-3A expression plasmid under control of the cytomegalovirus promoter or control plasmid and were given a high fat diet for 9 or 13 weeks starting at ages of 8 weeks. We have chosen these two time points to evaluate the effect of Sema-3A on early and more complex plaque formation. In

this model, Sema-3A is expressed in muscle cells and secreted by these cells. Serum levels of Sema-3A were verified by Elisa, 80% elevation was observed one week after intramuscular administration of the Sema-3A plasmid (52.4 ± 5 ng/ml with Sema3A vs 28 ± 1.5 ng/ml with control plasmid) (figure 2–7). Injections were therefore repeated every 4 weeks to have constant expression and secretion of Sema-3A. Body weight, lipids (total cholesterol, HDL) and triglycerides were equivalent between the two mouse groups (figure 2–6). Atherosclerotic lesions size was analyzed in the aortic sinus, the BCA and the aorta by Oil Red O staining. As shown in figure 2–1B-C, lesion sites were determined by using ImageJ and the lesion area was measured. We observed a significant reduction in plaque area in all vessel segments of Sema-3A overexpressing compared with control mice at 9 weeks (sinus: 0.3 ± 0.02 vs. 0.4 ± 0.03 mm2, BCA: 0.04 ± 0.01 vs. 0.1 ± 0.01 mm^2, and aorta: 9.5 ± 1.4 vs 15.3 ± 2.9 %) (Figure 2–1B-D) as well as at 13 weeks (sinus: 0.35 ± 0.07 vs. 0.6 ± 0.08 mm^2, BCA: 0.04 ± 0.01 vs. 0.11 ± 0.02 mm^2, and aorta: 11.8 ± 1.5 vs 21 ± 2.3%) (figure 2–8) of high fat diet.

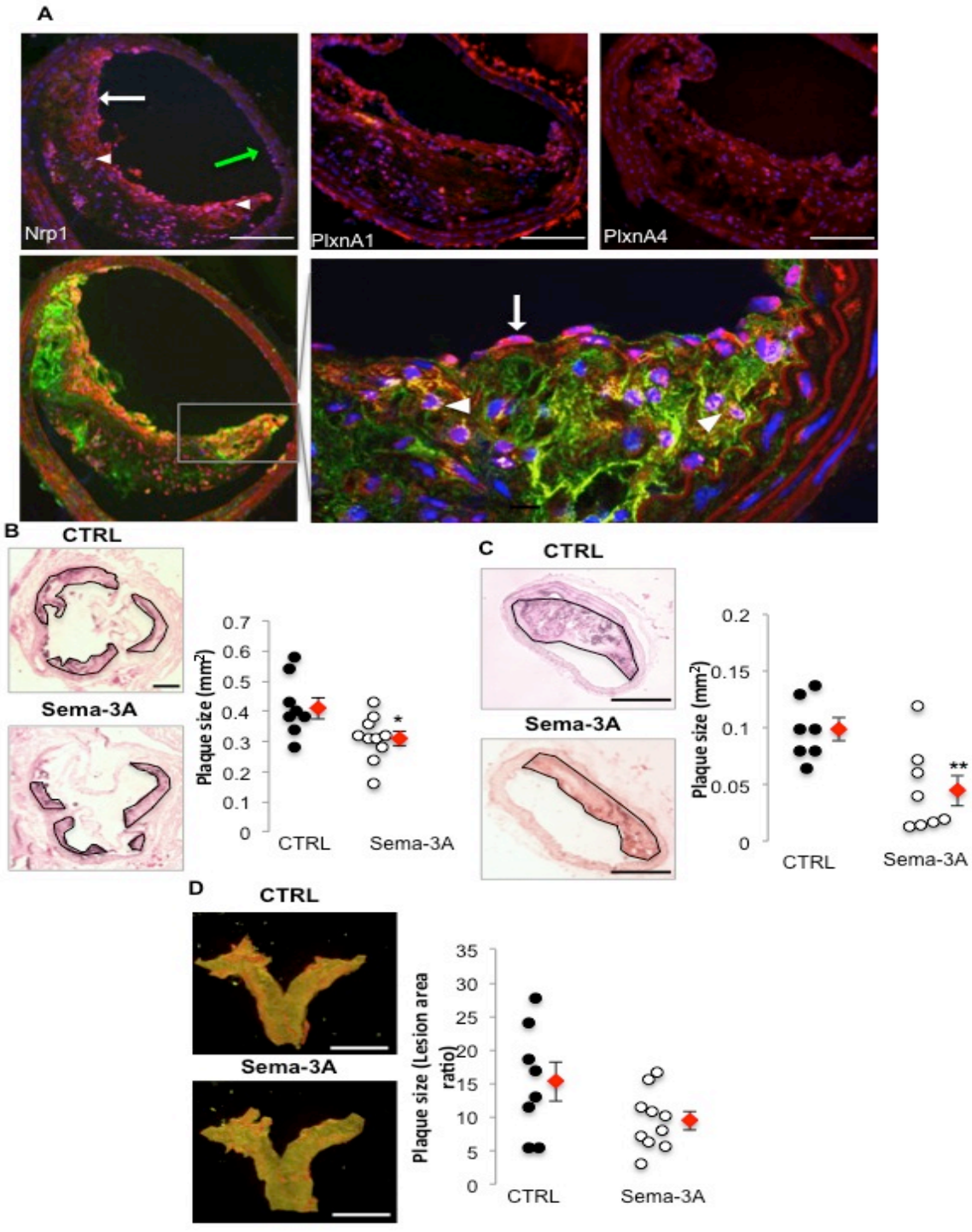
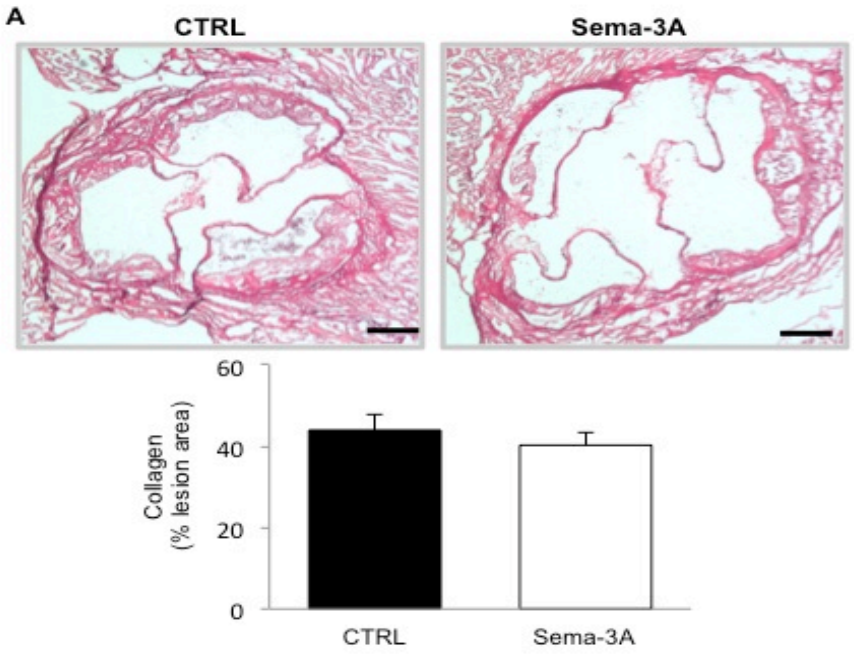

A
Nrp1
PlxnA1
PlxnA4
B
CTRL
Sema-3A
Plaque size (mm²)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
CTRL
Sema-3A
C
CTRL
Sema-3A
Plaque size (mm²)
0.2
0.15
0.1
0.05
0
**
CTRL
Sema-3A
D
CTRL
Sema-3A
Plaque size (Lesion area ratio)
35
30
25
20
15
10
5
0
CTRL
Sema-3A

Figure 2–1: Sema-3A overexpression reduces atherosclerotic plaque formation in ApoE-/- mice. ApoE$^{-/-}$ overexpressing Sema-3A protein and control (CTRL) mice were placed on a high fat diet for 9 weeks. A. Expression of Sema-3A receptors was assessed in atherosclerotic plaques by immunostaining. Nrp-1 (red) is detected in endothelial cells overlying the plaque (white arrows) and in the healthy vessel wall (green arrow). Nrp-1 is also detected in cells within the plaque (arrowheads), which are identified as monocytes/ macrophages by coimmunostaining with MOMA2 (green). Plxn-A1 is also abundant in the plaque. Plxn-A4 immunostaining is less marked. Nuclei are stained with DAPI. B,C,D. Lesion sites were determined as shown by black lines in (B) and (C). Lesion size was assessed with oil red O staining in the aortic sinus (B), the brachiocephalic artery (C), and the aorta (D).Data are presented as mean ± SEM of n=8-9. $*p<0.05$ vs. CTRL, $**p<0.01$ vs. CTRL. Scale bars = 50 μm (A, B, C), 25 μm (D).

To characterize plaque composition, we performed immunohistochemical stainings for collagen and smooth muscle cells and did not observe any significant differences between groups either 9 weeks or 13 weeks.(figure 2–2)


A
CTRL
Sema-3A
Collagen (% lesion area)
60
40
20
0
CTRL
Sema-3A

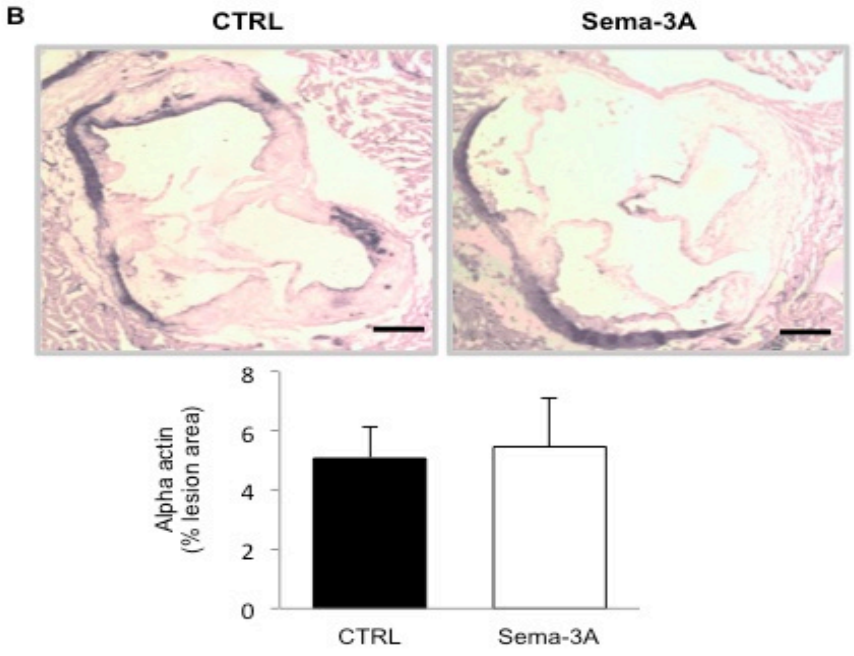

B
CTRL
Sema-3A
Alpha actin (% lesion area)
8
6
4
2
0
CTRL
Sema-3A

Figure 2–2: Plaque stability is not different between Sema-3A overexpressing and control mice. After 9 or 13 weeks of high fat diet, lesion collagen content (Sirius red staining) (A) and smooth muscle cell content (alpha-actin immunohistochemistry) (B) were quantified in the aortic sinus of Sema-3A overexpressing and CTRL mice . Data are presented as mean±SEM of n=8-9, $p>0.05$, Scale bars = 50 μm.

2.4.2 Overexpression of Sema-3A decreases plaque macrophage content associated with reduction of circulating monocytes.

To understand the mechanism underlying the decrease of atherosclerotic plaques by Sema-3A, we mainly focused on monocytes and macrophages that are the most abundant cells in the plaque. We characterized plaque macrophage content by MOMA-2 immunostaining that was significantly reduced in Sema-3A overexpressing vs. control mice (5.61±1.9 vs. 24.9±1.8) (figure 2–3B). This was associated with a reduction of circulating monocytes, determined by flow cytometry as $cd11b^{+}$ and $Gr\text{-}1^{-}$ cells (4.97±0.74 vs. 7.2±0.62%) (figure 2–3A). However we did not observe any difference in circulating $cd11b^{+}Gr1^{-}F4/80^{+}$ cells. These findings suggest that overexpression of Sema-3A decreases circulating monocytes which may be in part responsible for smaller plaque in Sema-3A overexpressing mice. In addition, less macrophages in the plaque of this group led to smaller plaque.

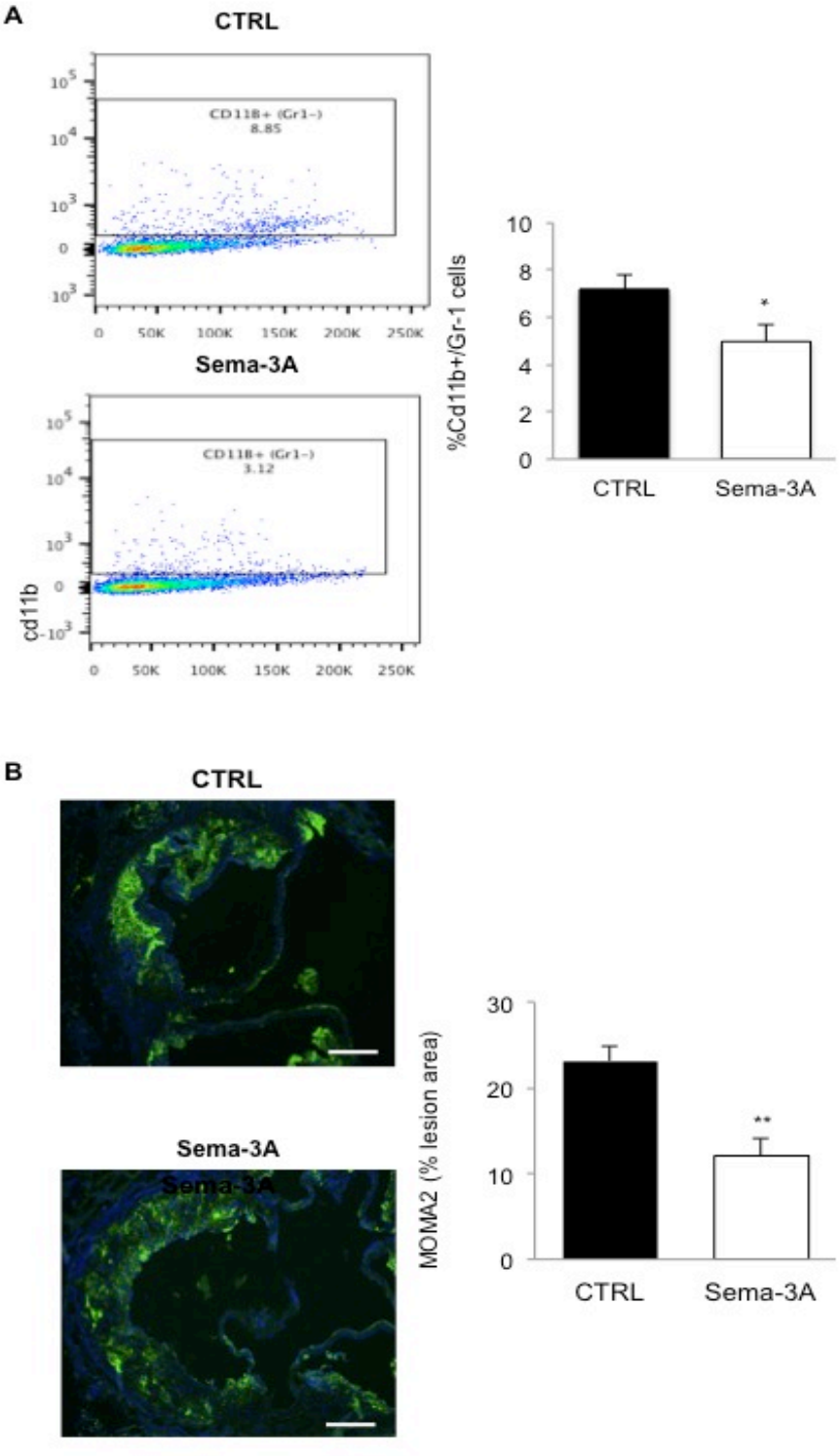

A
CTRL
CD11B+ (Gr1-)
8.85
Sema-3A
CD11B+ (Gr1-)
3.12
cd11b
0
50K
100K
150K
200K
250K
%Cd11b+/Gr-1 cells
10
8
6
4
2
0
*
CTRL
Sema-3A
B
CTRL
Sema-3A
Sema-3A
MOMA2 (% lesion area)
30
20
10
0
**
CTRL
Sema-3A

Figure 2–3: Decreased circulating monocytes is associated with decreased plaque macrophage content in Sema-3A overexpressing mice. After 9 weeks of high fat diet, circulating monocytes were assessed by flow cytometry (A), lesion macrophage content was assessed by MOMA-2 immunostaining, scale bar= 50 μm (B). Data are presented as mean±SEM of n=8-10. $*p<0.05$ and $**p<0.01$ vs. CTRL, Scale bars = 50 μm.

2.4.3 Sema-3A had no impact on monocyte function.

Since Sema-3A regulates circulating monocytes, we investigated monocyte function in terms of adhesion and migration (figure 2-9). Monocyte static adhesion to VCAM-1 and ICAM-1- as well as to activated endothelial cells under flow conditions were not influenced by Sema-3A (figure 2-9 A,B). In addition no difference was observed in basal and MCP-1-induced migration of monocytes in the presence of Sema-3A vs vehicle (figure 2-9 C).

2.4.4 Neuropilin-1 is highly expressed in M2 type macrophages.

Based on our *in vivo* findings, showing that monocyte, macrophage population seem to be regulated by Sema-3A; we first of all verified the expression levels of Sema-3A receptors in different type of macrophages extracted from the bone marrow. Nrp-1, plx-A1 and plx-A4 were all expressed in the 3 type (M0, M1, M2) of macrophages. Interestingly, Nrp-1 expression, the main receptor of Sema-3A; is very modest in M0 and M1 macrophages but was significantly higher (two fold) in M2 type macrophages and was decreased by Sema-3A, but still it was significantly higher than the other groups (figure 2–4). We next investigated if Sema-3A could influence macrophage polarization. We observed a significant increase of the mRNA levels of

Arg-1, an M2 marker and a decrease of IL-6, an M1 marker by Sema-3A. However Sema-3A did not influence Ym-1 and TNF-α mRNA levels. (figure 2–9). These results suggest that M2 type patrolling macrophages could be more responsive to Sema-3A overexpression than M1 inflammatory type macrophages.

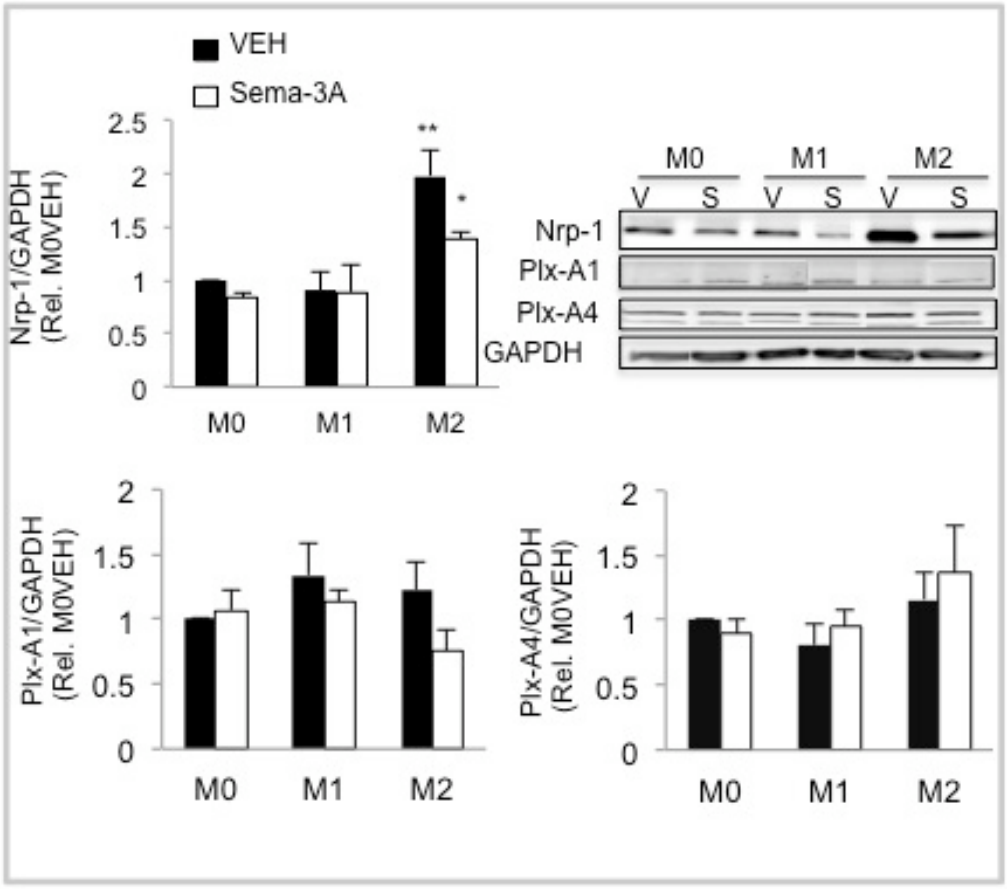


Figure 2–4: Neuropilin-1 is increased in M2 type bone marrow-derived macrophages. Bone marrow cells were extracted from C57BL/6 mice, differentiated into M0, M1 or M2 macrophages and stimulated for 24 hours with Sema-3A or vehicle (VEH). Relative protein expression of Sema-3A receptors is presented: Nrp-1, plx-A1, and Plx-A4 in M0, M1, M2 type bone marrow- derived macrophages. Data are mean±SEM of n=4-8. $*p<0.05$, $**p<0.01$ vs. M0VEH.

2.4.5 Sema-3A increases M2 type macrophage mobility partly through β_1-integrin activation.

We next investigated *in vitro* if Sema-3A alters M1 and M2 macrophage migration differentially. Boyden chamber assays revealed that Sema-3A significantly increased the migration of M2 and not M1 type bone-marrow-derived macrophages (145.5±19.8 Sema-3A vs. 31±8.3 VEH). (figure 2–5A) The same result was observed with thioglycollate-elicited peritoneal macrophages as well as human M2 type PBMCs. Differences in M1 and M2 cell responses to Sema-3A could be explained by differential integrin engagement or activation. We did not observe any effect of Sema-3A on mRNA levels of α or β integrin subunits in bone-marrow derived macrophages (figure 2–13). However, Sema-3A may influence integrin function. Since there is no antibody available for activated integrins in mouse, we probed control and Sema-3A-treated PBMCs for expression of active forms of β_1 (HUTS-4) and active β_2 (mAb24) by flow cytometry. The results indicated an increased number (2 fold) of cells expressing the active β_1 integrin only in M2 and not M1 PBMCs (figure 2–5B). There was no effect on active β_2 integrin. To further elucidate the role of integrins in Sema-3A-induced macrophage motility, we evaluated FAK activation, that is one of the most important pathways involved in cell motility, in M2 type macrophages. We observed a significant increase of 50% of FAK phosphorylation by Sema-3A (figure

2–5C). These results suggest that β_1-integrins possibly play a role in the migratory response of M2 macrophages to Sema-3A.

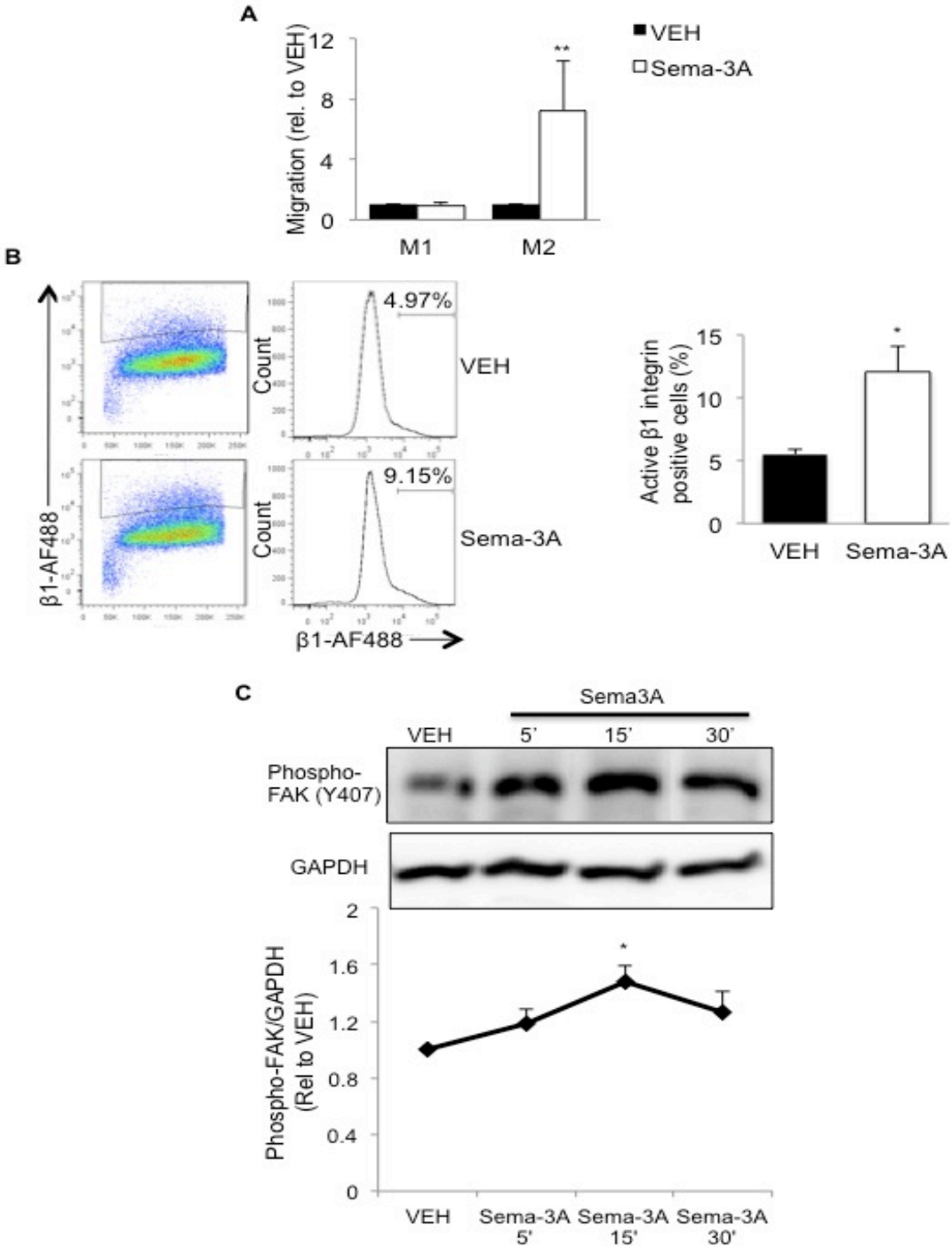

A
Migration (rel. to VEH)
12
8
4
0
**
M1
M2
VEH
Sema-3A
B
β1-AF488
Count
4.97%
VEH
9.15%
Sema-3A
β1-AF488
Active β1 integrin positive cells (%)
15
10
5
0
*
VEH
Sema-3A
C
Sema3A
VEH
5'
15'
30'
Phospho-FAK (Y407)
GAPDH
Phospho-FAK/GAPDH (Rel to VEH)
2
1.6
1.2
0.8
0.4
0
*
VEH
Sema-3A 5'
Sema-3A 15'
Sema-3A 30'

Figure 2–5: Sema-3A increases M2 type macrophage migration possibly through upregulation of β_1-integrin. A. Bone marrow cells were extracted from C57BL/6 mice, differentiated into M1 or M2 type macrophages and stimulated for 24 hours with Sema-3A or vehicle (VEH), Boyden chamber assays were used to investigate migration of cells after 16 hours incubation in starving media to eliminate proliferation possibility. B.PBMCs were extracted from peripheral blood of healthy humans, differentiated into M2 type macrophages and stimulated for 4 hours with VEH or Sema-3A. Flow cytometry was performed to detect active β_1 positive cells. C. Expression of phosphorylated FAK (Y407) was assessed in mouse raw M2 type macrophages stimulated with VEH or Sema-3A for 5, 15 or 30 minutes or. Data are mean±SEM of n=7-9. $*p<0.05$ vs. VEH.

2.5 Discussion

Sema-3A is secreted by several immune cells including macrophages and T cells [20, 62, 126]. Although they are abundant in atherosclerotic lesions, no link had been made between Sema-3A receptors such as Nrp-1 or plexins and atherosclerosis. In the present study, we demonstrate that Sema-3A reduces plaque development in the ApoE$^{-/-}$ atherosclerotic mouse model. Moreover, we provide evidence that this atheroprotective effect could be due, at least in part, to decreased circulating monocytes and increased mobility of M2 patrolling macrophages, possibly through regulation of β_1 integrin. Sema-3A exerts its action through a receptor complex NRP-1 and plexins. Both receptor types are detected in monocytes and macrophages of human and murine origin [20, 135, 129, 46]. Here we showed that all 3 receptors of Sema-3A are expressed in the healthy vessel wall as well as in the atherosclerotic plaque (figure 2–1A). Moreover, confocal microscopy revealed evident co-localization of Nrp-1 and macrophage staining, demonstrating that cells within atherosclerotic lesions, including macrophages, are disposed to being responsive to Sema-3A.

We used an original model to study the effect of Sema-3A on plaque formation. ApoE$^{-/-}$ mice were placed on a high fat diet with Sema-3A, administered in the form of an electroporated plasmid. GFP plasmid-injected mice served as controls. This well established technique successfully increases serum levels of secreted proteins such as Sema-3A [26, 127]. Sema-3A serum levels was measured every other week and stayed elevated as compared to mice injected with the control plasmid.

At 9 as well as 13 weeks, we found that Sema-3A overexpression decreased plaque formation in the BCA, the aortic arch, and the aortic sinus without having any effect on lipid levels (figure 2–1 B-D). These data support the notion of an atheroprotective effect of Sema-3A. Several studies suggest that Sema-3A could be protective in the context of atherosclerosis. In hyperlipidemic ApoE$^{-/-}$ mice, administration of measles virus promoted a strong regulatory T cell response and reduced the development of plaques [2]. Measles virus has since been shown to be a potent inducer of Sema-3A release [119]. Sema-3A is also involved in dendritic cell transmigration [119], an important step in plaque regression [120].

Regarding the stability of the plaques, collagen and smooth muscle cell contents were equivalent in Sema-3A over expressing and control mice plaques, suggesting that Sema-3A does not affect plaque stability. Interestingly, Sema-3A overexpressing mice had decreased circulating monocytes associated with decreased macrophage content within the plaques. Furthermore, thioglycollate-elicited monocyte recruitment was also significantly decreased with Sema-3A, without any effect on macrophages, suggesting that decreased circulating monocyte by Sema-3A could contribute to smaller

plaque as it was shown by others [91]. However, monocyte function in terms of adhesion and migration was equivalent between Sema-3A and VEH treated groups. These results indicate that Sema-3A regulates only the number of monocytes that enter the plaque. In the context of angiogenesis, Sema-3A recruits a specific NRP-1 positive myeloid cells that contribute to arterial formation and tumor vessel normalization through paracrine effects [136, 15]. Further experiments are needed to investigate the role of this specific cell population on plaque formation.

Activated macrophages are routinely classified into M1 and M2 macrophages. The classically activated M1 macrophages have an acute inflammatory phenotype. The alternatively activated, anti-inflammatory M2 macrophages have various functions, including regulation of immunity, maintenance of tolerance and tissue repair [80]. Cells of the monocyte/macrophage lineage exhibit plasticity in response to different stimuli. M2-polarized macrophages can convert to the M1-activated status under certain conditions and vice versa. M1 and M2 macrophages are both present in the plaque, M1 macrophages are known to be proinflammatory and are associated with plaque instability. However M2 macrophages have been shown to resolve inflammation by promoting efferocytosis and by clearing apoptotic macrophages [73, 120].

We next investigated the function of bone marrow derived-macrophages. Interestingly, basal and MCP-1-induced migration of only M2 type "patrolling" and not M1 "inflammatory" macrophages was increased when Sema-3A was added at the bottom chamber (figure 2–5A), suggesting that Sema-3A is a chemotactic agent for these cells. This result was in accordance with a higher level of NRP-1 receptor in M2

as compared to M1 macrophages, suggesting that M2 macrophages are more reactive to the chemo-attractant function of Sema-3A. Recently, Casazza *et al.* reported that Sema-3A/NRP-1 signaling tightly controls the localization of tumor-associated macrophages into vascular areas, with Sema-3A acting as an attractant [18].

The next question was if Sema-3A would cause polarization of recruited cells to the M2 phenotype. We showed *in vitro* that Sema-3A does not influence macrophage polarization in short term or long term. Furthermore, increased M2 macrophage motility by Sema-3A was not associated with increased efferocytosis of these cells (figure 2–12).

To determine what differences in M1 and M2 macrophages underlie their different responses in terms of motility, to Sema-3A; we investigated integrin expression. β integrins have different functions in migration: β_1 and β_3 integrins drive migration [1, 60, 82], whereas β_2 integrins tend to promote firm binding and rather serve as a brake [133]. Interestingly, integrin β_3 expression was strongest in M2 macrophages, whereas M1 cells rather expressed abundant β_2 integrin (figure 2–13). In further support of a potential role of integrins in macrophage motility, we found that the active form of β_1 integrin was increased by Sema-3A in human PBMCs (figure 2–5B) without any regulation of the mRNA levels in murine macrophages (figure 2–13). In endothelial cells, endogenous and exogenous Sema-3A has been shown to regulate integrin-mediated cell migration towards extracellular matrix [101]. Sema-3A regulates also tumor cell migration through integrins [86]. An inhibitory effect of Sema-3A on endothelial cell migration as well as aortic ring sprouting was reported in tumor angiogenesis [17].

Increased migration of M2 macrophages in response to Sema-3A correlated with increased phosphorylation of FAK (figure 2–5C) that is typically activated through the formation of integrin-dependent focal adhesions [124, 76]. It has been reported that FAK functions downstream of Sema-3A signaling during axonal remodeling [21]. Furthermore, Sema-3A promotes the extension of hippocampal dendrites by a pathway that requires FAK. The stimulation of dendrite growth and FAK phosphorylation by Sema3A depend on integrin engagement [99]. We believe that in M2 cells, Sema-3A might stimulate β_1-dependent engagement at the leading edge, facilitating motility through FAK-dependent actin cytoskeleton remodeling. Further experiments are needed to determine the exact molecular mechanism involved in Sema-3A-induced M2 macrophage migration.

These novel data suggest that Sema-3A, by stimulating mainly M2 macrophage migration, possibly through β_1 integrin and FAK, decreases plaque progression in an atherosclerotic mouse model. These data provide an attractive conceptual approach to curb atherosclerosis.

Source of funding This study was supported by Canada Foundation for Innovation, Canada Research Chairs and Canadian Institutes of Health Research.

Disclosures: None.

Supplemental Figures

2.6 Supplemental Table & Figures

Target	mRNA sense primers	mRNA anti-sense primer
Arg1	5'-CTCCAAGCCAAAGTCCTTAGAG-3'	5'-GGAGCTGTCATTAGGGACATCA-3'
Fizz1	5'-TCCAGCTAACTATCCCTCCACTGT-3'	5'-GGCCCATCTGTTCATAGTCTTGA-3'
Ym1	5'-GGGCATACCTTTATCCTGAG-3'	5'-CCACTGAAGTCATCCATGTC-3'
NOS2	5'-TTCACCCAGTTGTGCATCGACCTA-3'	5'-TCCATGGTCACCTCCAACACAAGA-3'
TNF-α	5'-ATCCGCGACGTGGAACTG-3'	5'-ACCGCCTGGAGTTCTGGAA-3'
IL6	5'-TAGTCCTTCCTACCCCAATTTCC-3'	5'-TTGGTCCTTAGCCACTCCTTC-3'
Rps16	5'-ATCTCAAAGGCCCTGGTAGC-3'	5'-ACAAAGGTAAACCCCGATCC-3'

Table 2–1: Oligonucleotide sequences

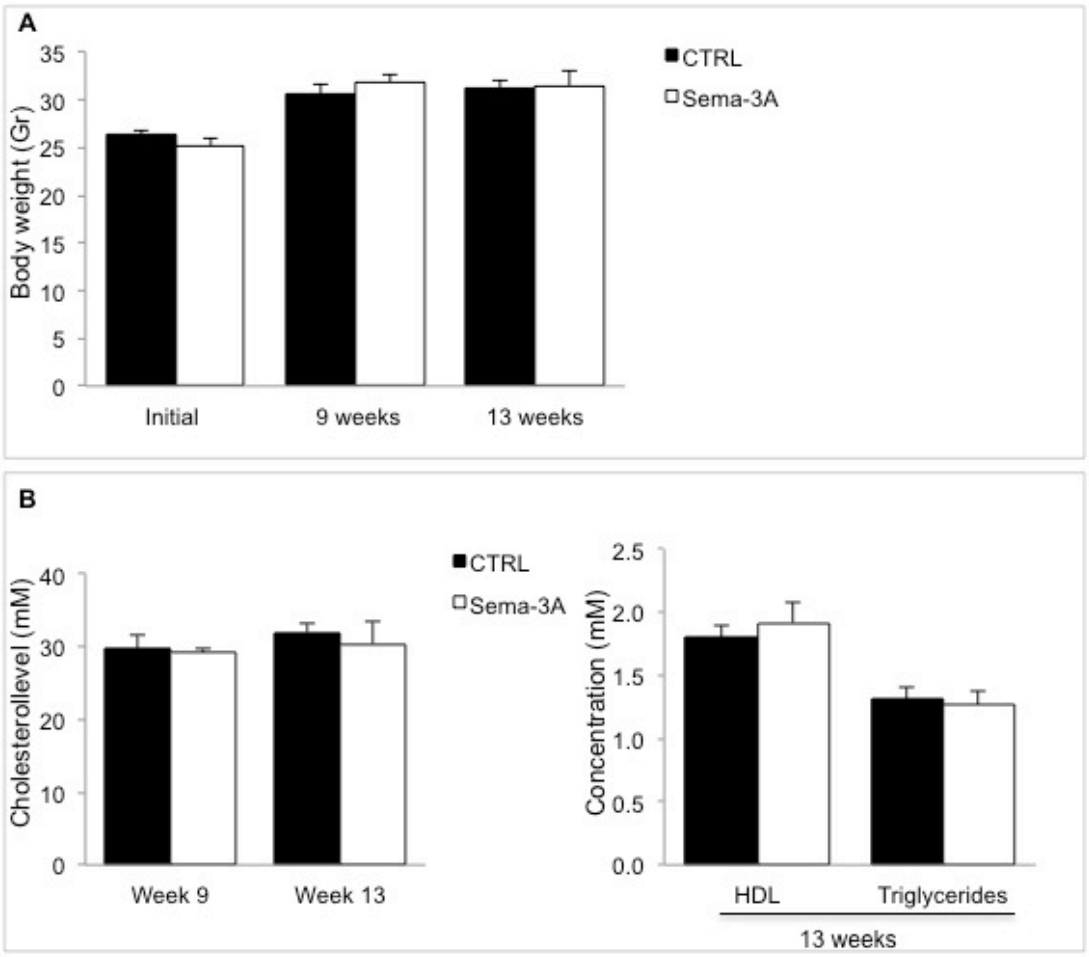


Figure 2–6: No differences in body weight and plasma lipid levels in Sema-3A overexpressing and control mice. A: Body weight of Sema-3A overexpressing and control (CTRL) mice was measured before and after 9 and 13 weeks of high fat diet Data are mean±SEM of n=5-8. B: Plasma cholesterol, HDL, and triglyceride levels were measured after 9 and /or 13 weeks of high fat diet. Data are mean±SEM of n=5.

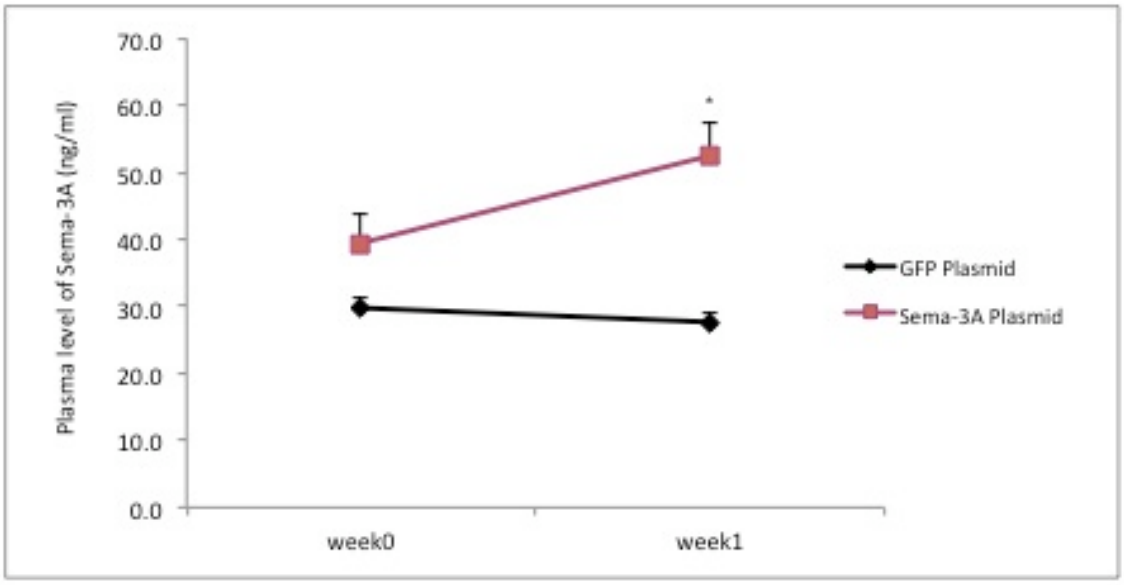


Figure 2–7: Sema-3A level in the blood serum of Sema-3A overexpressing mice vs. control GFP plasmid. Serum levels of Sema-3A were verified by Elisa, 80% elevation was observed one week after intramuscular administration of the Sema-3A plasmid (52.4 $\pm$ 5 ng/ml with Sema-3A vs 28 $\pm$ 1.5 ng/ml with control plasmid).

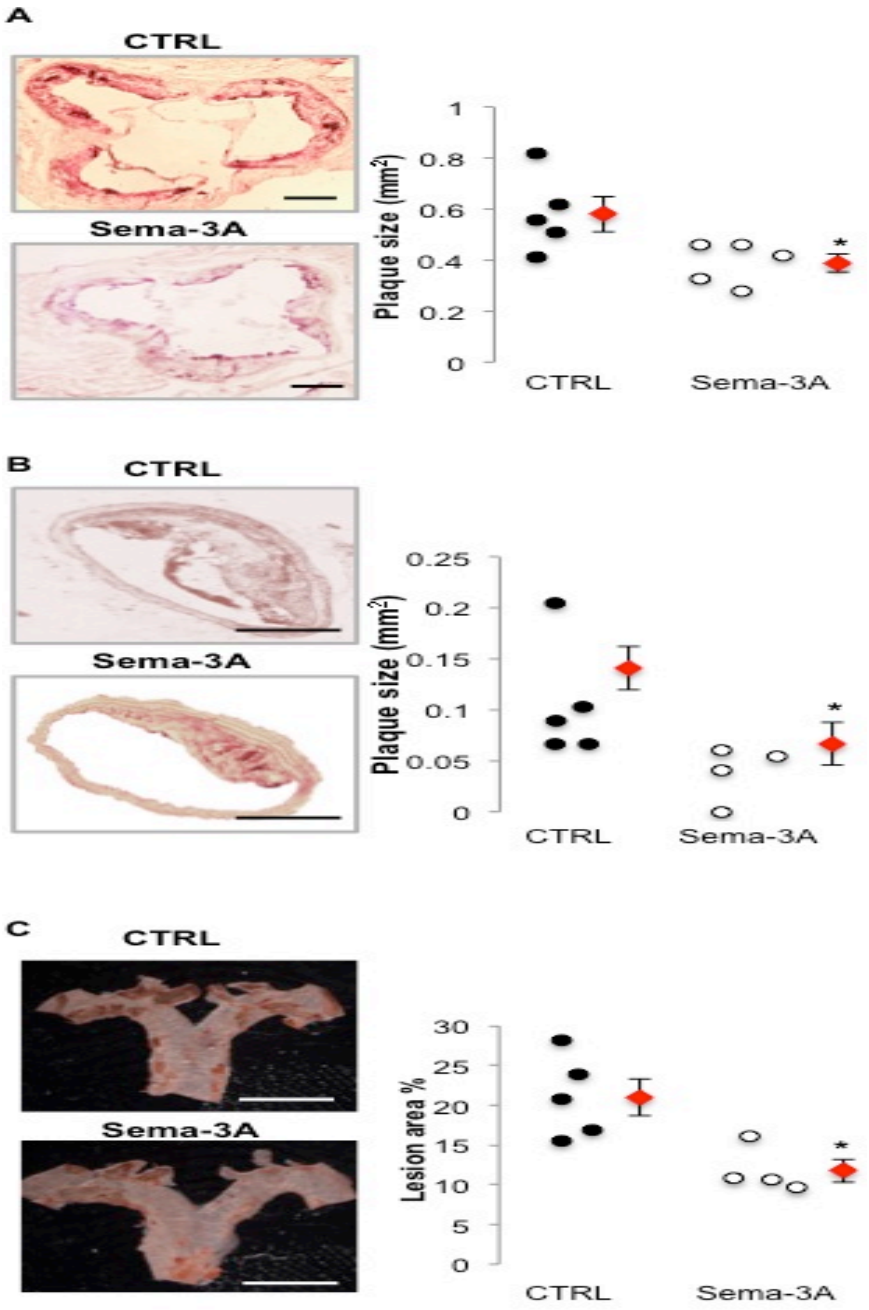


Figure 2–8: Sema-3A overexpression reduces atherosclerotic plaque formation in ApoE$^{-/-}$ mice. ApoE$^{-/-}$ overexpressing Sema-3A protein and control (CTRL) mice were placed on a high fat diet for 13 weeks. A,B,C. Lesion size was assessed with oil red O staining in the aortic sinus (A), the brachiocephalic artery (B), and the aorta (C).Data are presented as mean/pmSEM of n=5. $*p<0.05$ vs.CTRL, $**p<0.01$ vs. CTRL. Scale bars = 50 μm (A, B, C), 25 μm (D).

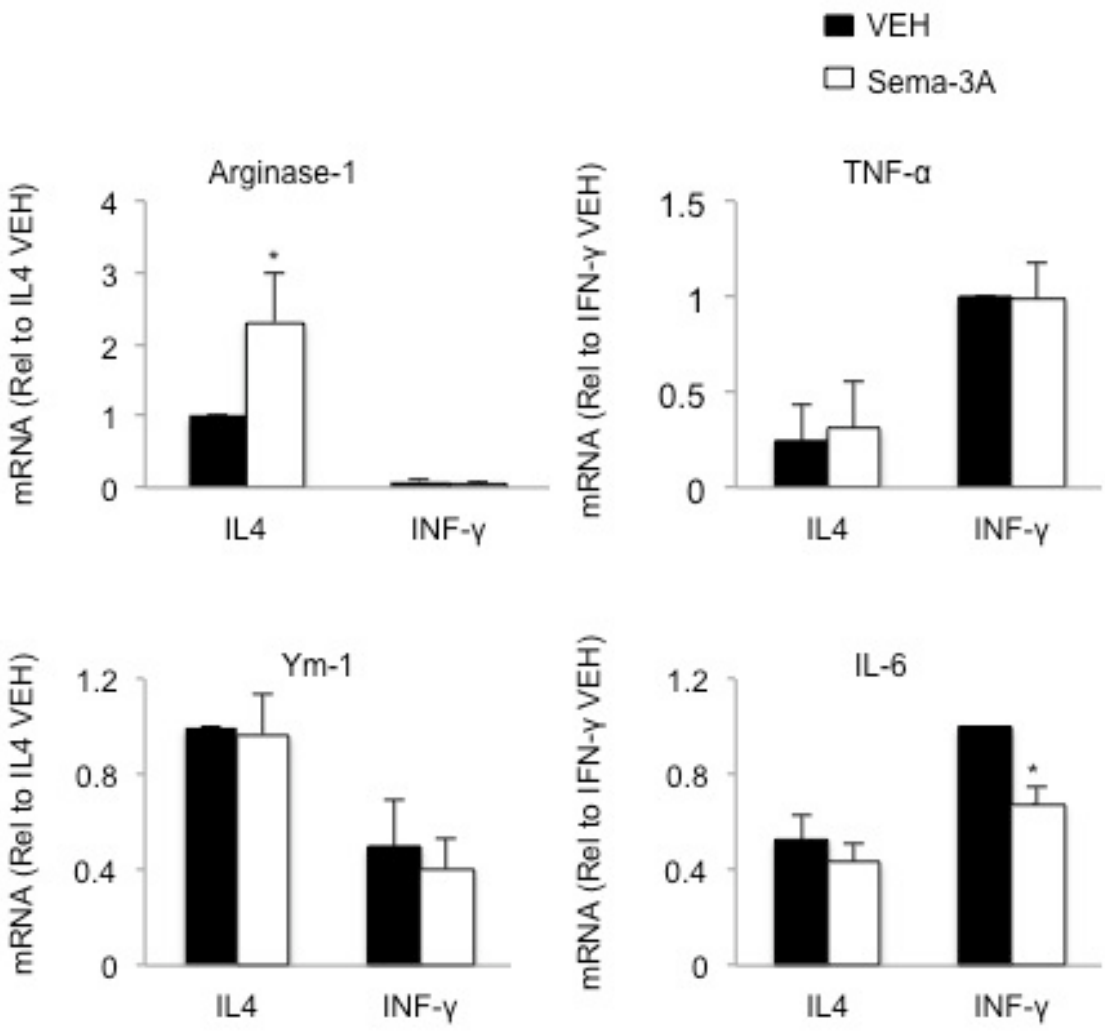


Figure 2–9: Sema-3A influences on Arg-1 and IL-6 expression in favor of M2 phenotype. Bone marrow cells were extracted from C57BL/6 mice, differentiated into M2 or M1 macrophages with interleukine-4 (IL-4) or interferon-γ (INF-γ) respectively and stimulated for 12 hours with Sema-3A or vehicle (VEH). RT-qPCR was performed to detect the mRNA levels of M1 and M2 macrophage markers. Although Sema-3A increased Arginase-1 expression in IL-4-polarized macrophages and decreased IL-6 expression in INF-γ-polarized macrophages, the other M1 and M2 markers are not influenced. Data are mean$\pm$SEM of n=8. $*p<0.05$ vs. IL4VEH (for arginase-1) and INF-γVEH (for IL-6).

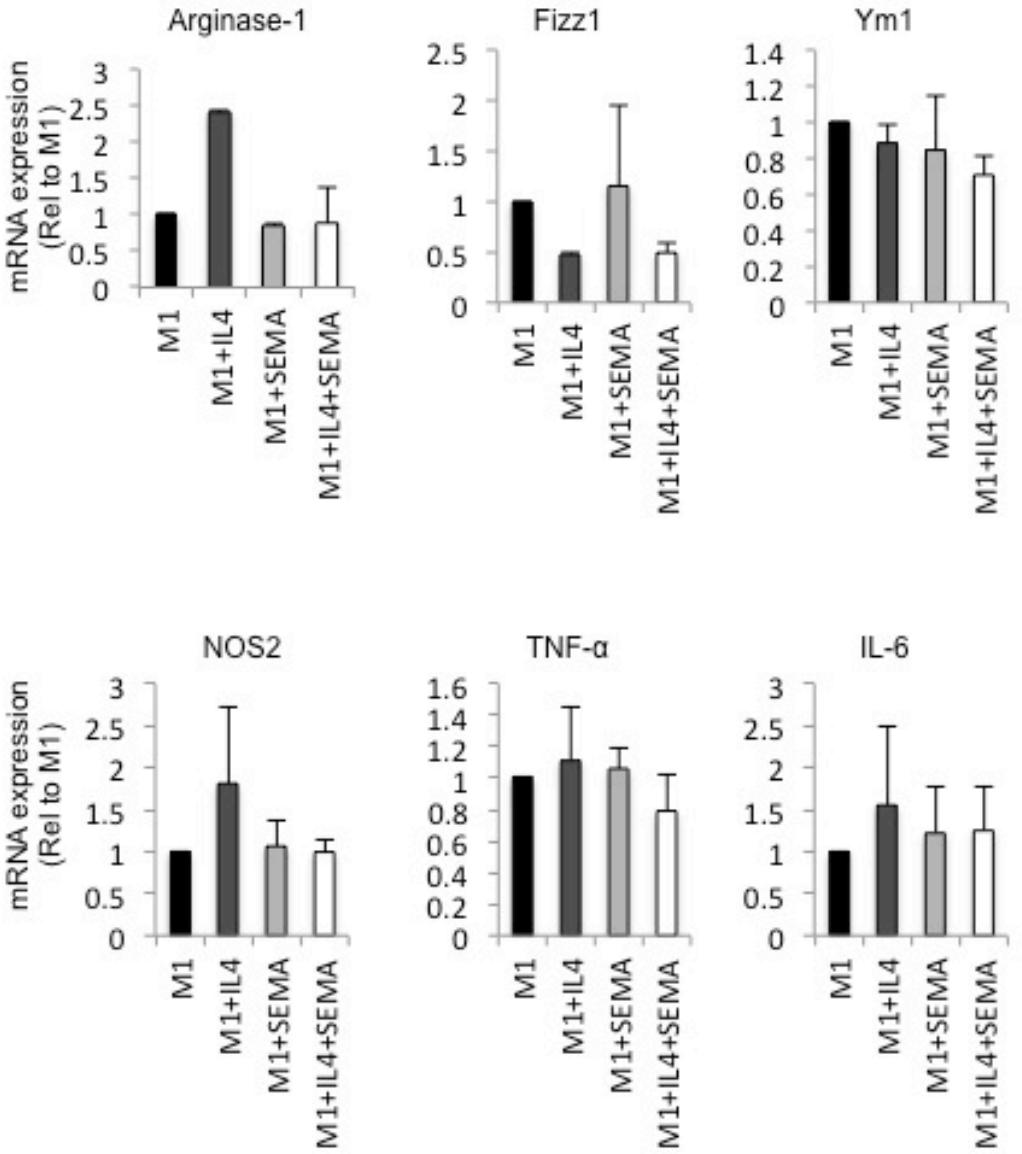


Figure 2–10: Sema-3A does not influence bone marrow-derived M1 macrophage phenotype switch to M2. Bone marrow cells were extracted from C57BL/6 mice, differentiated into M1 with interferon-γ (INF-γ) and stimulated for 24 hours with Sema-3A, IL-4, or both. RT-qPCR was performed to detect the mRNA levels of M1 and M2 macrophage markers. Data are mean$\pm$SEM of n=5. $*p<0.05$ vs. M1.

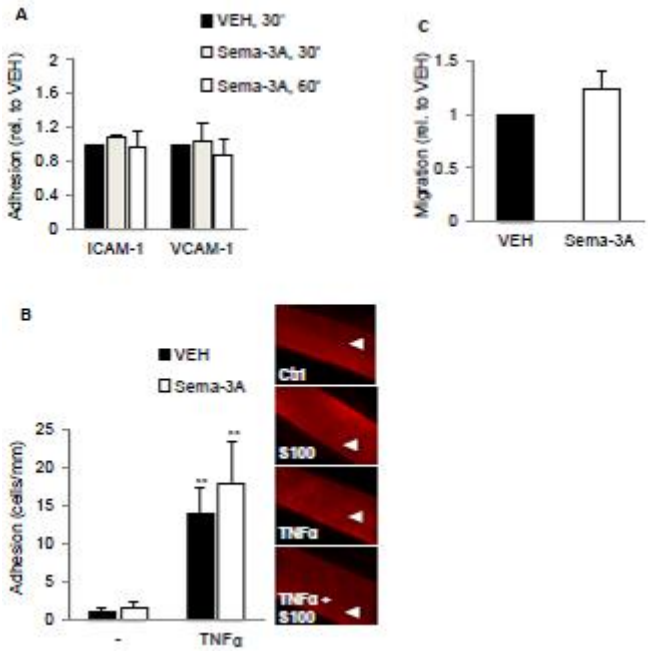


Figure 2–11: Sema-3A has little impact on monocyte function. A) Mouse monocytes were exposed to 20 (S25) or 100 (S100) ng/mL of Sema-3A and allowed to adhere to recombinant ICAM-1 or VCAM-1 for 30 or 60 min. Attached cells were counted after washout. B) Monocyte migration was assessed using Boyden chamber assay. Monocytes were placed in the upper chamber, and Sema-3A was added to the lower chamber. Migrated cells were counted after 18h. C) Mouse carotid arteries were mounted in an *ex vivo* perfusion system and incubated for 6 hours with cell culture medium alone, or cell medium containing Sema-3A (100ng/ml), TNFα (10ng/ml), or Sema-3A+TNFα. After 6 hours, fluorescently labelled monocytes were injected in the intraluminal compartment of the vessels and allowed to adhere for 30 min before washout. Cells attached to the vessels were visualized by microscopy (right panels, arrowheads) and counted. Results are representative of n=3-4. $**p<0.01$ vs. no TNFα.

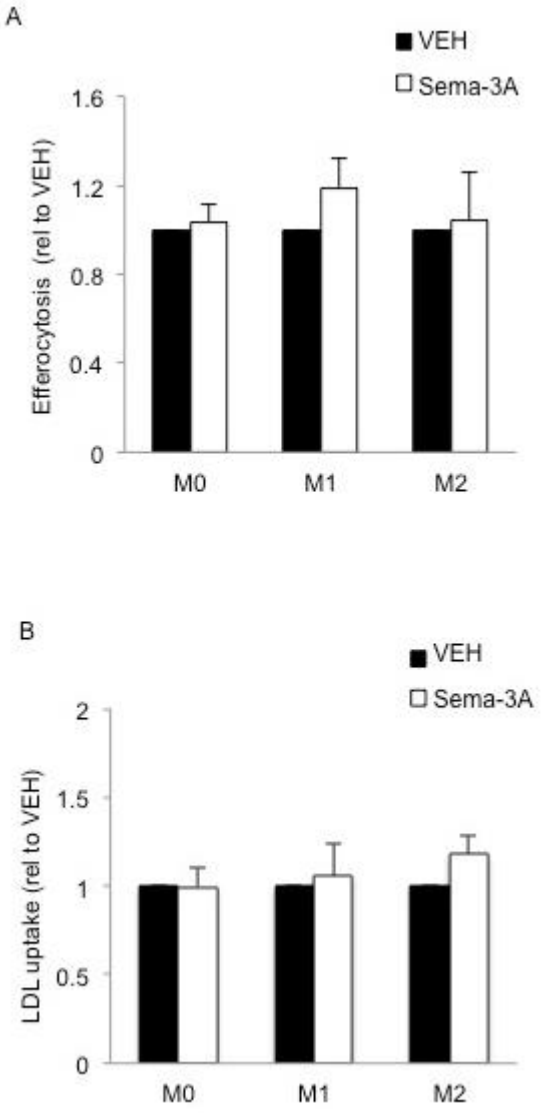


Figure 2–12: Sema-3A has no effect on efferocytosis and foam cell formation.A. M0, M1, and M2 cells were stimulated with vehicle or 100ng/ml Sema-3A for 30 minutes then incubated 1 hour with labeled apoptotic cells. Efferocytosis was measured by counting the number of phagocytic cells. B. Cells were incubated for 4hours in the presence of labeled actylated LDL. LDL uptake was quantified by fluorescence intensity as a function of cell density. M0, M1, and M2 bone marrow-derived macrophages incubated with vehicle or 100 ng/mL Sema-3A 30 minutes prior to Ac-LDL uptake, N=4-5.

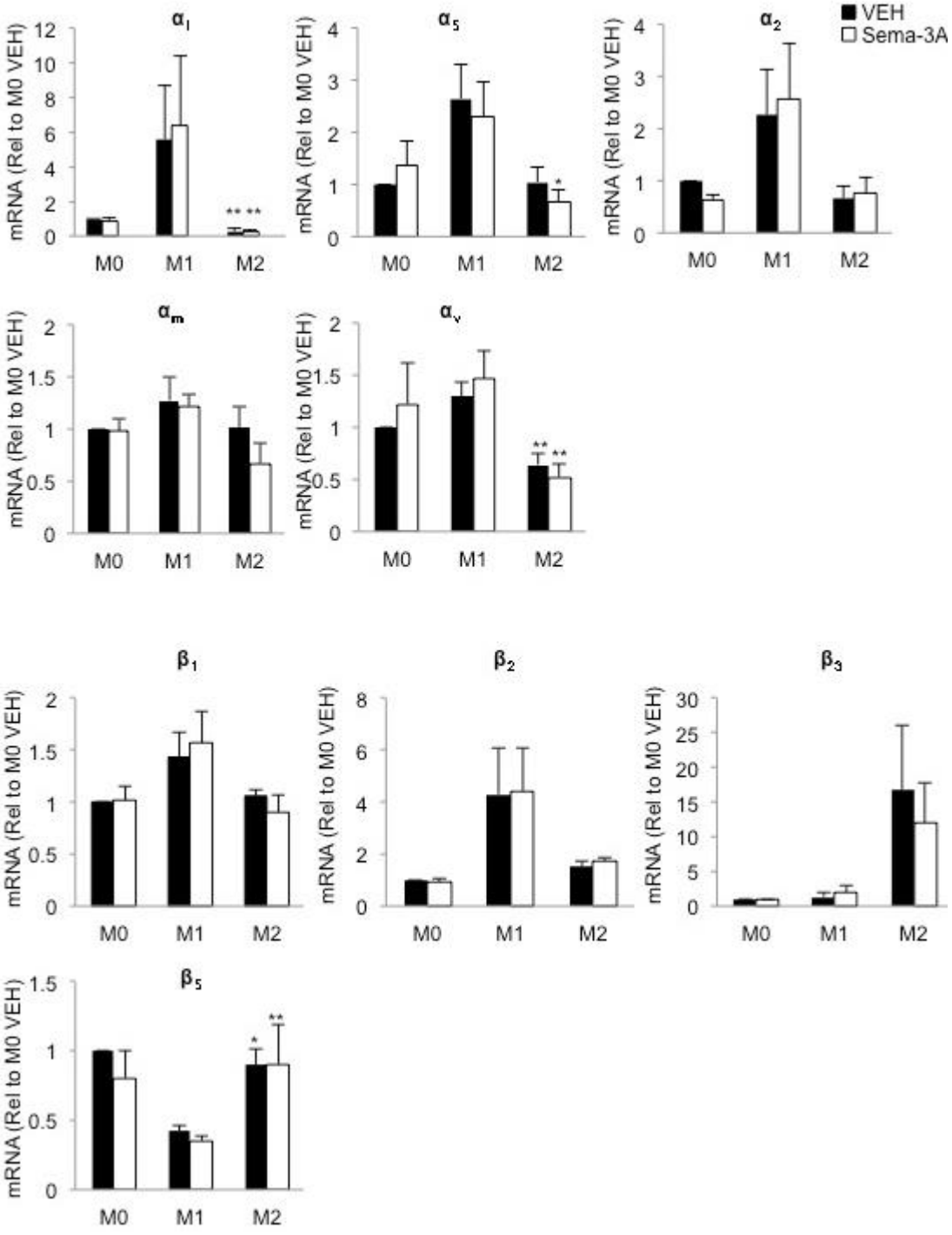


Figure 2–13: (Sema-3A does not influence integrin levels in macrophages. Bone marrow cells were extracted from C57BL/6 mice, differentiated into M0, M1, or M2 macrophages and stimulated for 12 hours with Sema-3A or vehicle (VEH). RT-qPCR was performed to detect the mRNA levels of α and β integrin subtypes. Data are mean±SEM of n=8, $*p<0.05$, $**p<0.01$ vs M0VEH.

Chapter III: Sema-3A and acute Inflammation

CHAPTER 3
Semaphorin-3A and acute inflammation

Introduction, Hypothesis, and Objectives

Inflammation is a process in which the immune system of the body responds to the injury. It is classified in two different, but related categories, chronic inflammation and acute inflammation. Leukocytes and monocyte/macrophages are responsible to resolve acute inflammation. However, these cells also contribute to activate lymphocytes in response to chronic inflammation.

In the previous chapter we focused on the effect of Sema-3A on atherosclerosis, a chronic inflammatory disease, and we discussed extensively in the first chapter that the monocytes and macrophages are the main players to initiate plaque formation. Although there is relatively good knowledge about the immuno-regulatory role of Sema-3A on chronic inflammation and cancer, it has not been previosly investigated in terms of acute inflammation yet. So in this chapter we try to take the initial steps to identify the effect of Sema-3A on regulation of immune cells in an acute model of inflammation to see how Sema-3A regulates immune cells involved in innate immunity. *We hypothesize* that Sema-3A reduces acute inflammation by regulating macrophages and T cells.

Specific objective:

- To determine the effect of Sema-3A on acute, local inflammation *in vivo* in a mouse model of acute inflammation

3.1 Materila & Methods

3.1.1 Animal Housing & LPS injection

C57BL/6 male mice were obtained from Jackson laboratory (Bar Harbor, ME) and mated in house. The McGill University animal use committee approved the experimental protocols and animals were handled in accordance with institutional guidelines. One hour before electroporation hyaluronidase was injected in each thigh muscle of animals (30±lμl/thigh) to increase electroporation efficiency by 50% (1±lμl in 70±lμl of 0.9% NaCl) (Calbiochem) under isofluorane anesthesia. After one hour, Sema-3A or GFP plasmid was injected into each thigh (50μg/thigh dissolved in 50 ±lμl of 0.9% NaCl) under anesthesia. Thigh muscles were then lightly massaged and then immediately electroporated using a previously described protocol [26]. Briefly, eight 10 millisecond electrical pulses at 200 V/cm with a frequency of 1 Hz were applied. The pulses were generated using an ECM 830 Square Wave Electroporator and administered using electrodes (BTX Harvard Apparatus). Then five days later the mice again went under isofluorane anesthesia and either LPS (1 mg/ml, Sigma) or PBS was injected into the skin of mouse ears under the disecting microscope. The mice were kept in the house for 2 days and then sacrificed to study the inflammation.

3.1.2 Physiological parameter measurement

Blood was removed via a syringe from the heart at the time of sacrifice and was collected in heparin-coated tubes (Sarstedt). Blood cell enumeration (white blood cells, lymphocytes, monocytes, granulocytes, red blood cells, % hematocrit, hemoglobin and platelets) was performed with a hematology analyzer (scil vet abc

animal blood counter, Vet Novations). The blood was centrifuged to collect plasma and Sema-3A level was determined by ELISA.

3.1.3 Inflammation measurement and characterization

At sacrifice the ears were removed and placed in 4% paraformaldehyde for 3 days at 4 °C shaker and then rinsed with 1X PBS and changed to 30% sucrose (in PBS) overnight at 4 °C on a shaker, and again rinsed with 1X PBS. The hair of ears were shaved by blade and embedded horizontally in Tissue-tek (Sakura), and serial 10μm thick sections were cut using a cryostat (Leica CM 3050 S, Leica Microsystems) and collected on gelatin-coated slides. To measure the thickness of the ear sections H+E staining was done and slide were mounted using Eukitt mounting medium (Electron Microscopy Sciences). Bright field pictures were acquired using a Leica microscope. The mean thickness in mm^2 for ears were calculated using ImageJ software (National Institute of Health). Macrophage and T cell content of the ears were evaluated by immunohistochemistry staining for monocytes/macrophages (10% goat serum block, 1/50 polyclonal MOMA-2 antibody, abcam) and T cells (10% goat serum block, 1/100 polyclonal rabbit anti-human CD3, Dako) respectively for 1 hour, followed by 1 hour incubation by secondary antibody and mounted using mounting media with dapi (Vector). Immunofluorescent pictures were taken using Leica microscope. Percentage of ear staining was calculated using ImageJ.

3.1.4 ELISA

The blood plasma level of Sema-3A was determined using a mouse Sema-3A ELISA kit following the instruction protocol (MyBioSource Inc.)

3.2 Results:

3.2.1 Sema-3A reduces LPS-induced acute, local inflammation in mouse ear.

To determine if Sema-3A can exert a protective effect in acute inflammation, C57BL/6 mice were injected with Sema-3A expression plasmid under control of the cytomegalovirus promoter or control plasmid. As discussed earlier, in this model, Sema-3A is overexpressed in muscle cells and secreted by these cells. Five days after Sema-3A injection, LPS or PBS was injected into the skin of ears and after 48 hours inflammation in the external ear was investigated. Our results show that inflammatory signs like redness (figure 3–1A) and ear thickening (figure 3–1B were significantly lower in Sema-3A overexpressing mice compared to the control group (0.28 ± 0.03mm vs. 0.5 ± 0.06mm)

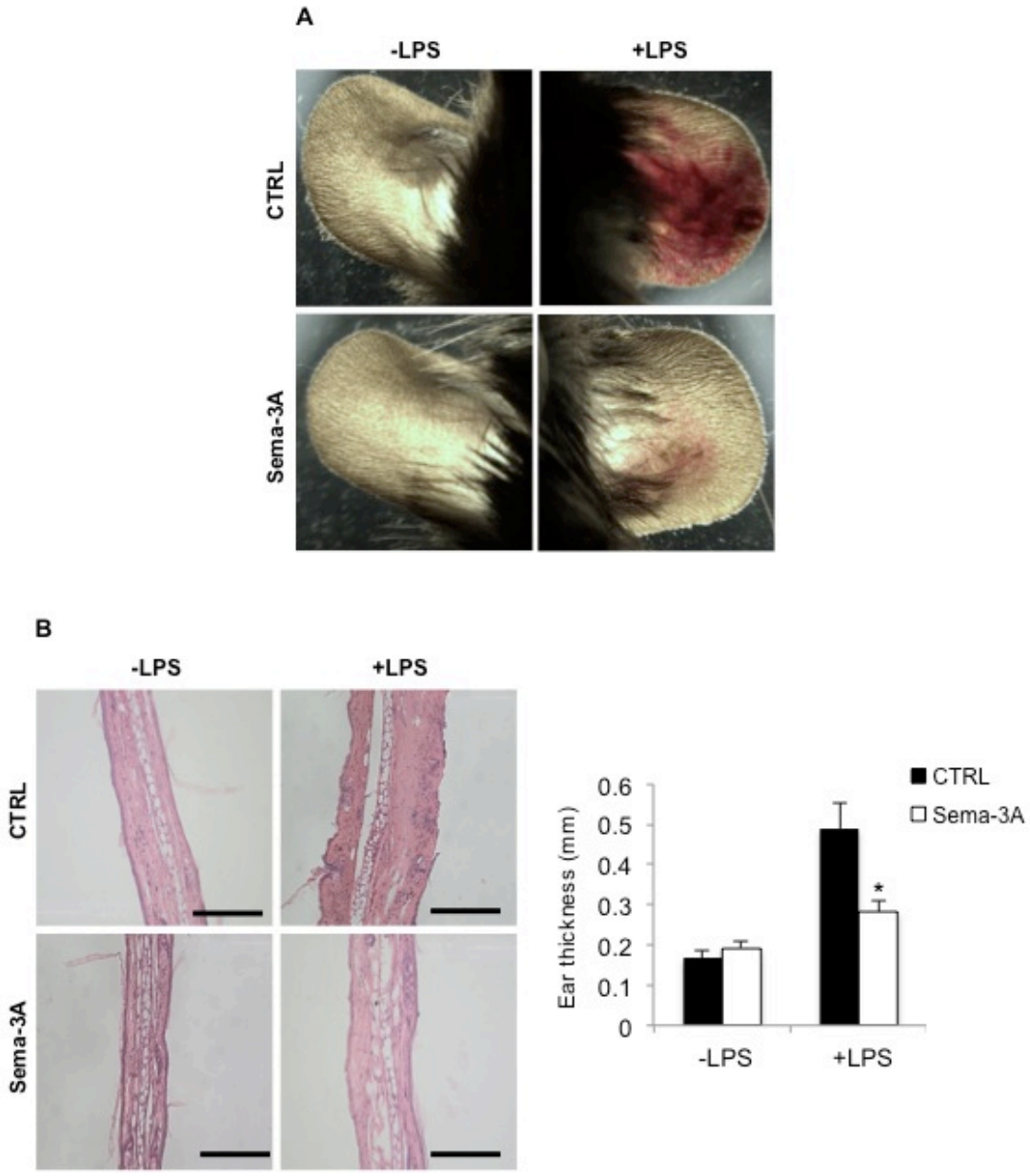


Figure 3–1: Sema-3A reduces LPS-induced inflammation. LPS or PBS were injected into the skin of ear of C57BL/6 overexpressing Sema-3A protein and control (CTRL) mice. A. As it is shown, the redness is significantly more in LPS-injected ear of control mice compared to the Sema-3A overexpressing mice. B. H+E staining of ear sections. Data are presented as mean ± SEM of n=5-6. $*p<0.05$ vs. CTRL, Scale bars = 25 μm

3.2.2 Reduced acute inflammation in the ears was associated with lower macrophage and T cell content in Sema-3A overexpressing mice.

To understand the mechanism underlying decreased acute, local inflammation by Sema-3A, we have started the characterization by examining macrophages and T cells. According to our previous findings discussed earlier that Sema-3A regulates macrophage function, we expected to see fewer macrophages in Sema-3A overexpressing mice. We characterized ear macrophage content by MOMA-2 immunostaining and found that it was significantly reduced in Sema-3A overexpressing vs. control mice (3.7 ± 0.28 vs. 5.7 ± 0.38) (figure3–2A). Interestingly the T cell content was also remarkably lower in this group as measured by CD3 immunostaining (2.5 ± 0.8 vs. 8.2 ± 1.8) (figure3–2B).

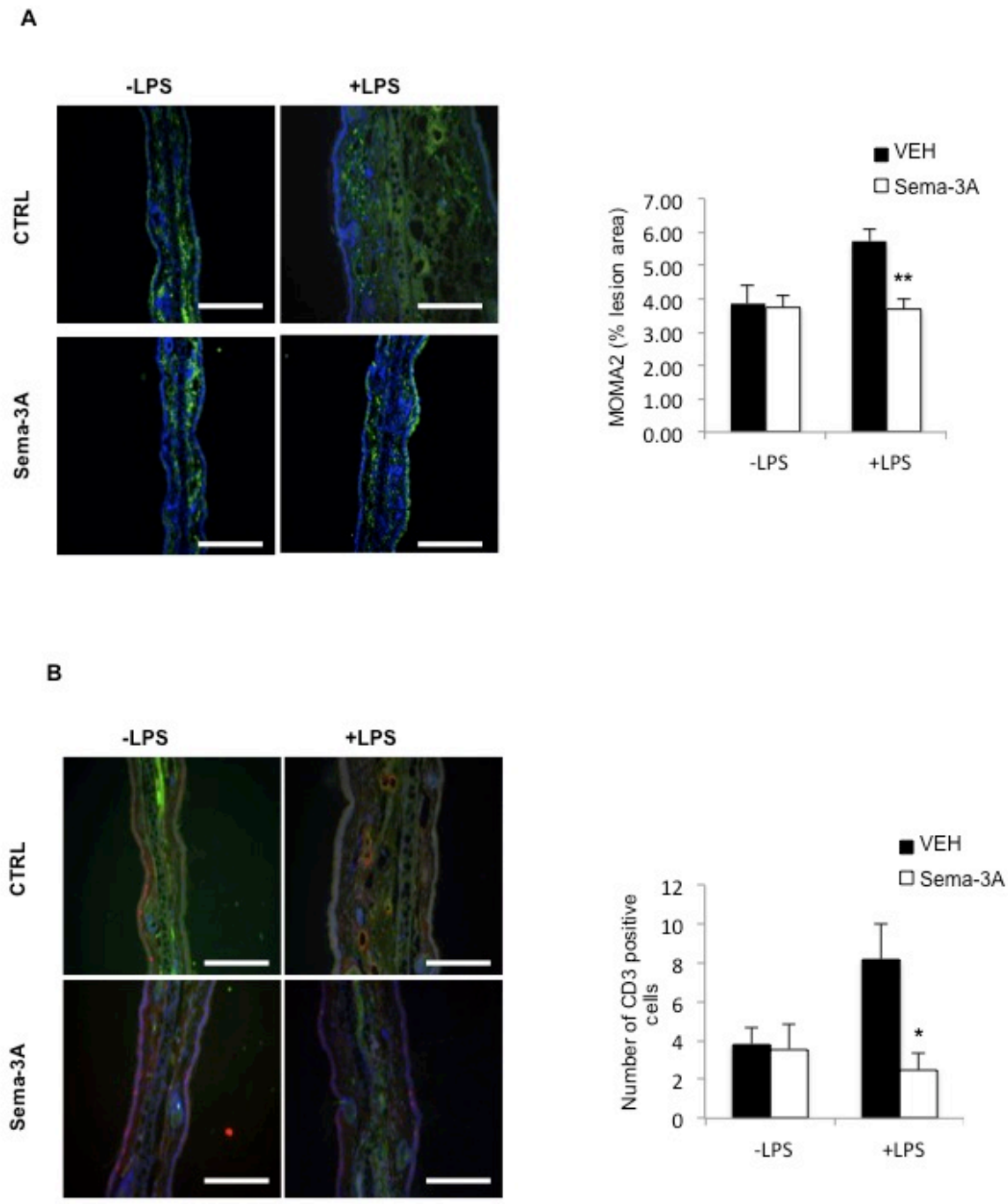


Figure 3–2: Decreased inflammation is associated with decreased macrophage and T cell content of the ears. After 48 hours of LPS injection, lesion macrophage and T cell content were assessed by MOMA-2 (A) and CD3 (B) immunostaining. Data are presented as mean±SEM of n=5-6, $*p<0.05$ and $**p<0.01$ vs. CTRL, Scale bars = 25 μm

3.3 Discussion

Innate immunity is the first line of defence against pathogens and is the first immune reaction activated upon injury. Leukocytes, monocytes, macrophages, DCs and NK cells are the cells responsible to respond to the pathogen entry. Moreover, most of them have a role as antigen presenting cells to activate the lymphocytes.

Sema-3A and its receptor expression were found in most of the cells involved in immune reactions [46, 15, 53]. Many investigators have focused on the effect of Sema-3A in auto-immune diseases and cancer [19, 20, 121, 83]. However, no systematic studies have been done on the impact of Sema-3A on acute inflammation and innate immunity.

To find a link between Sema-3A and acute inflammation, we used an LPS-induced local inflammation mouse model. We showed that Sema-3A significantly reduces inflammation in these mice. Furthermore we provided evidence that this immunoprotective effect of Sema-3A could be due to regulation of macrophages and T cells. Sema-3A remarkably reduced LPS-induced ear thickening (figure 3–1B), which supports our hypothesis that Sema-3A reduces acute inflammation. It has been shown that Plx-A4$^{-/-}$ cells have impaired NF-κB activation [126]. However, PlX-A4 is not the main receptor for Sema-3A and more investigation is needed to be done in terms of Nrp-1 deficiency and LPS induced signaling. Interestingly our immunofluorescent studies showed that the protective effect of Sema-3A is associated with lower macrophage and T cell content in Sema-3A overexpressing mice. So the next steps in this study could be to determine if there is any difference in terms of different sub-populations of macrophages recruited into the ears, and to study the effect of

Sema-3A on downstream signaling of LPS/TLR4 in these mice. All together our findings so far show that Sema-3A is a very interesting candidate to be considered as a suppressor molecule of acute inflammation.

Chapter IV: Conclusion & Discussion

CHAPTER 4
Conclusion & Discussion

In the present study we have tried to identify the role of Sema-3A and its receptors in two different type of inflammatory diseases: chronic inflammation and acute inflammation. First, in the second chapter we have discussed atherosclerosis as an example of chronic inflammation. In the third chapter we focused on LPS-induced technique as a model of local, acute inflammation.

Atherosclerosis is a chronic inflammatory disease which develops in the regions of the vasculature where the laminar blood flow is disturbed i.e. in arterial branches and bifurcations. Although clinical studies have determined many risk factors associated with this disease including age, gender, life style, diabetes, smoking, and socio-economical conditions, most recent data show that the process of atherosclerotic plaque formation is under the control of the immune system.

Sema-3A is one of the members of semaphorin family of proteins, first found in nervous system as an axon guidance protein. However, recent studies have shown it also regulates immune cell function [107]. This protein and its receptors Nrp-1 and Plx-A1/A4 are expressed by both leukocytes and lymphocytes indicating the possibility that Sema-3A could play role in immune-related diseases.

Although many immune cells like macrophages, DCs, and T cells are detected in atherosclerotic plaques, and it has been shown that Sema-3A and its receptors are expressed by all of these cells [20, 62, 126], no link had been made between this

protein and its receptors and atherosclerosis. In this study for the first time we have demonstrated that Sema-3A reduces plaque development in the ApoE$^{-/-}$ mouse model of atherosclerosis. Moreover, we have provided evidence that this atheroprotective effect of Sema-3A could be at least in part due to decreased circulating monocytes and increased mobility of M2 "reparative" macrophages, possibly through regulation of β_1 integrin activity in these cells.

It has been shown that Sema-3A and its receptors are expressed in monocytes and macrophages of both humans and mice [135, 129]. Here we have shown that all three receptors of Sema-3A are expressed by cells in both healthy vessels and the atherosclerotic plaque. Our confocal microscopy data showed that these receptors are expressed by ECs of healthy BCA and those covering the plaques. In addition, we saw a colocalization between Nrp-1 and macrophage staining within the plaque indicating that these cells could be responsive to Sema-3A (figure 2–1A). So we used one of the classic models of atherosclerosis to study the effect of this protein on plaque formation. Sema-3A or control plasmid were injected intramuscularly into the ApoE$^{-/-}$ mice and then electroporated and kept on high fat diet for short and long term studies (9 weeks and 13 weeks, respectively). The well established electroporation technique successfully increases serum level of secreted proteins like Sema-3A [26, 127]. We confirmed the increased serum level of Sema-3A by ELISA in Sema-3A treated ApoE$^{-/-}$ mice compared to control group (figure 2–7). At both 9 weeks and 13 weeks, we found that Sema-3A overexpressing ApoE$^{-/-}$ mice had smaller atherosclerotic plaques in BCA, aortic sinus, and aortic arch compared to the control group (figure 2–1B-D), while the cholesterol, triglyceride, and HDL level

were comparable in both groups (figure 2–6). These findings support our hypothesis that Sema-3A has an atheroprotective effects. Our results are consistent with the paper by Ait-Oufella and his colleague showing that when ApoE$^{-/-}$ mice on high fat diet were treated with measles virus, which is known to induce secretion of Sema-3A [119], the atherosclerotic plaques were smaller [2].

Regarding plaque stability, our histological studies did not show any differences in collagen and smooth muscle cell content between two groups (figure 2–2), indicating Sema-3A does not either increase or decrease plaque stability. Interestingly the flow cytometery analysis of blood from Sema-3A overexpressing ApoE$^{-/-}$ mice showed decreased circulating monocyte population compared to the control group, which was associated with less macrophage content within the plaque of this group (figure 2–3). To investigate the possibility that decreased monocyte recruitment would lead to fewer macrophages within the plaque, we determined the effect of Sema-3A on monocyte adhesion in normal and inflammatory conditions, as well as monocyte migration in response to chemoattractant *in vitro*. We found that Sema-3A has no effect on either monocyte adhesion or migration, which confirms a study done by Ji *et al.* showing Sema-3A has no effect on human monocyte migration in response to MCP-1 [46]. However more experiments are necessary to find the link between Sema-3A and monocyte recruitment into the plaque *in vivo*. One experiment that could be proposed is the intravenous injection of fluorescently labeled monocytes into the apoE$^{-/-}$ under high fat diet in Sema-3A overexpressing and control mice to determine the effect of Sema-3A on the number of monocytes present within the plaques of both groups in different stages on plaque progression.

To address the reason for fewer macrophages within the plaque of Sema-3A over-expressing ApoE$^{-/-}$ mice, we focused on the effect of Sema-3A on macrophage function in both M1 (proinflammatory) and M2 (reparative or healing) macrophages. Interestingly we found increased M2 macrophage migration in response to Sema-3A *in vitro* (figure 2–5A). It has been shown that when monocytes were differentiated to M2 macrophages, expression of Nrp-1 and Plx-A1 were significantly increased [46]. We also showed that Nrp-1 is abundantly expressed in M2 macrophages (figure2–4). However, we saw that its expression was decreased with Sema-3A. So it is possible that higher reaction of M2 macrophages to chemoattractant function of Sema-3A is due to higher expression of Sema-3A in these cells. Although Sema-3A is primarily a chemorepulsive protein in the nervous system, it has been shown that the chemorepulsive or chemoattractant response elicited by semaphorins depends on both cell type and the receptor complex that neuropilin and plexin forms in response to the microenvironment. There is a study showing that Sema-3A plays a role as an attractant to control the localization of tumor macrophages into the vascular areas [18]. Further experiments are needed to show that Sema-3A increases macrophage egress from the plaque *in vivo*. Previously it has been shown that macrophage emigration out of the plaque to adventitial lymphatics is dependant on CCR7 [66]. Therefore to confirm the effect of Sema-3A on macrophage egress *in vivo*, ApoE$^{-/-}$Ccr7$^{-/-}$ double-knockout mice [91] could be tested in our setting. Our anticipation is that the atherosclerotic plaque size would be comparable in ApoE$^{-/-}$Ccr7$^{-/-}$ overexpressing Sema-3A or control plasmid due to ablolished macrophage egress.

The atherosclerotic plaque is a complex environment within which macrophages encounter different and even opposite stimuli. Some signals are proinflammatory and some are anti-inflammatory. The balance between these stimuli determines the phenotype and function of macrophages [80]. Since both M1 and M2 phenotypes are present in the plaque, we tried to determine if Sema-3A would shift the polarization of recruited macrophages toward M2 phenotype. It was shown that M2 macrophages resolve inflammation by increasing efferocytosis and clearance of apoptotic bodies [73, 120]. Our data showed that in longer polarization time (12 hours) Sema-3A increased expression of Arg-1 in macrophages polarized with IL-4 (M2), while decreasing IL-6 expression in cells polarized with IFN-γ (M1), but did not have any impact on other markers (figure 2–9). Such an increase or decrease of one or two markers is not a perfect indication of macrophage M2 phenotype switch *in vivo*, but it does suggest that Sema-3A tends to push macrophages towards a less inflammatory, more reparative program. Therefore it would be interesting to study the effect of Sema-3A on phenotype of plaque macrophages by flow cytometery of cells present in the plaque. Also Sema-3A did not influence on phenotype switching from M1 to M2 (figure 2–10).

At the early stages of plaque formation efferocytosis is very effective in eliminating debris. However, in advanced plaques where efferocytosis is impaired, increased apoptosis leads to release of cholesterol and inflammatory contents of apoptotic cells which creates secondary necrotic core [132]. There is almost no data in the literature regarding Sema-3A and phagocytosis and efferocytosis. Interestingly it has been shown that constitutively activated R-Ras activates $\alpha_M\beta_2$ in macrophages which

enhances macrophage phagocytosis [14]. In addition, increased migratory capacity of cells is essential for phagocytosis, and there is some evidence indicating that the Sema-3A/Plxn-A1 complex increases migration of some types of cancer cells via activation of R-Ras [56]. But further investigations are necessary to find a clear link between Sema-3A and phagocytosis/efferocytosis in macrophages *in vivo*. To investigate such effect, we could suggest an experiment in which fluorescent particles could be injected intravenously then fluorescent intensity could be compared in macrophages derived from Sema-3A overexpressing mice with control [117]. In a study Ji *et al.* showed that Sema-3A had no effect on phagocytosis of opsonized human erythrocytes by macrophages [46]. Similar to this finding we also determined that Sema-3A did not have any effect on cholesterol uptake and foam cell formation as well as efferocytosis *in vitro* (figure 2–12).

To address what differences in M1 and M2 macrophages underlies their different response to chemoattractant function of Sema-3A, we investigated integrin expression as a major molecules involve in cell migration. β integrins have different functions in terms of migration: β_1 and β_3 integrins promote migration [1, 60, 82], while β_2 integrin serves as a brake by making firm binding to the ECM [133]. Keeping this in mind, we tried to define the role of Sema-3A on expression of both α and β subunites of integrin known to be involved in migration of macrophages, at the mRNA level. We saw β_3 integrin was strongly expressed in M2 macrophages, whereas expression of β_2 was strongest in M1 macrophages (figure 2–13). However, we did not see any impact of Sema-3A on these differences or on mRNA expression of any integrin. Interestingly when we looked at the active forms of the integrins we found that β_1 integrin was

highly activated by Sema-3A in human blood-derived macrophages (figure 2–5B). The link between Sema-3A and integrins was previously shown by several studies. For example, both endogenous and exogenous Sema-3A have been shown to regulate integrin-mediated cell migration towards ECM in ECs [101]. Also, in tumor cells, Sema-3A regulates migration through integrins [86]. It would be great to investigate the effect of Sema-3A on activated forms of other integrins if the antibodies would be commercially available.

Focal adhesion complexes are common structures regulated by integrins in the context of migration [76]. In the investigation of mechanisms involved in increased M2 macrophage migration, we found that FAK is highly phosphorylated in response to Sema-3A in these cells (figure 2–5C). Regulation of FAK by Sema-3A was extensively studied in neural cells. Chacon *et al.* has reported that FAK functions downstream of Sema-3A signaling in axonal remodeling [21]. Moreover Sema-3A increased phosphorylation of FAK and stimulation of dendrite growth through integrins [99]. So we believe that Sema-3A might increase M2 macrophage migration at least in part through activation of β_1 integrin at the leading edge, facilitating cell motility through FAK-dependent actin cytoskeleton remodeling. Another mechanism that could be proposed is that at the rear of migrating cells binding of Sema-3A could promote the intrinsic GTPase activity of R-Ras. The increase in GDP-bound (inactive) R-Ras leads to a decrease in integrin-mediated attachment to the ECM. As a result M2 macrophages are more detached from the matrix and have more mobility (figure 4–1). Obviously more experiments are needed to determine the exact molecular mechanism involved in this process. For instance to show in our settings if

phosphorylation of FAK is due to interaction of PLX-A1 with focal adhesion proteins we could undertake immunoprecipitation experiments.

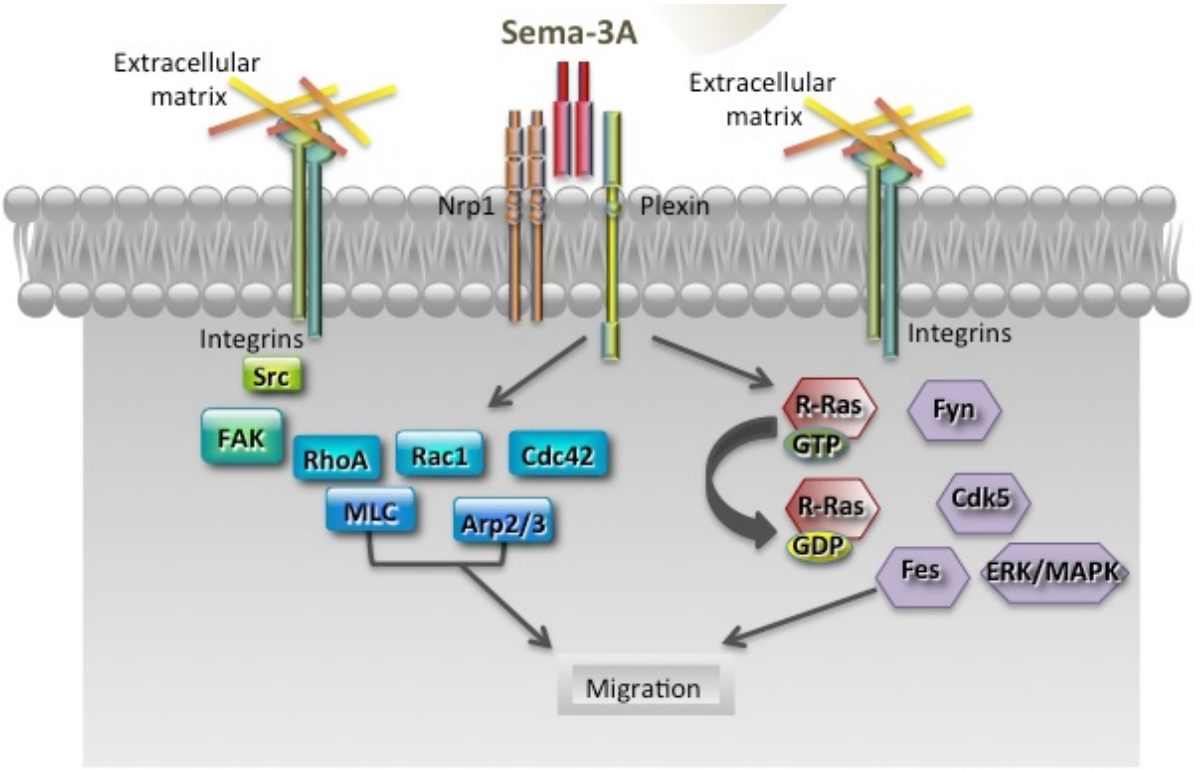


Figure 4–1: Schematic illustration on how Sema-3A may regulate M2 macrophage migration. Sema-3A enhances migration of M2 macrophages at least in part by activating $\beta 1$ integrins and increasing phosphorylation of FAK. Therefore our hypothesis is that it may increase M2 macrophage migration via two possible mechanisms: 1)By activating the small GTPases RhoA, Rac1, and Cdc42 and their downstream targets myosin light chain (MLC) and Arp2/3. 2)By deactivating GTP-bound R-Ras and increase activation of Fyn and Cdk5, or by acivation of ERK/MAPK and their downstream molecules.

The ongoing research part of our studies is to show the direct effect of Sema-3A on more local acute inflammation which triggers the response of innate immunity.

Innate immunity is not only responsible to respond to pathogens induced inflammation, but also it is responsible to activate adaptive immunity in regards to chronic inflammation, so it would be beneficial to know how Sema-3A may regulate innate immune cell function and cytokine secretion.

Our preliminary data show that inflammation signs including increased redness and thickness are significantly less in Sema-3A overexpressing C57BL/6 mice compared to control (figures 3–1A and B respectively). Intrestingly we have found that Sema-3A significantly reduced both macrophage and T cell content of the LPS-injected ears (figure 3–2).

So far there is not much known about Sema-3A and acute inflammatory diseases. There are a few papers showing that Sema-3A positively promotes the innate immune response. For example it has been shown that in PLx-A4$^{-/-}$ cells, the impaired TLR-induced Rac1 activation results in impaired JNK and NF-κB activation and inflammatory cytokine production is defective [126]. Ranganathan *et al.* found that when Sema-3A was inactivated, mice were protected from ischemia-reperfusion-induced acute kidney injury (AKI), and these mice showed less epithelial cell apoptosis, reduced neutrophil infiltration, and increased cytokine and chemokine excretion in urine. Also *in vitro*, Sema-3A enhanced TLR4-mediated inflammation in epithelial cells, macrophages, and dendritic cells [93]. Although yet we don't have enough data to be able to conclude whether Sema-3A has an anti-inflammatory role in innate immunity, according to our observations and data, Sema-3A seems to have a protective effect and this makes it an interesting molecule to be followed in the

context of innate immunity.

limitations and future perspective

Like all research projects, we have confronted these questions with some limitations in this research. Sema-3A protein in the serum of animals was very unstable, and we had to process the serum immediately after blood was drawn from the mice otherwise it was not detectable in the plasma. We would like to also study the effect of Sema-3A on the activated form of β_3 integrin, one of the potent integrins involved in cell migration. However there was no antibody available for the activated form of this integrin. Besides, the other antibodies that we have used in this research for activated forms of integrins were for use with human cells, since there is no antibody for activated β integrins in mouse cells. We used FACS analysis to measure the activated integrins, the only approach available for this aim. Although FACS is one of the most precise quantitative methods to sort the cells into different populations, due to lack of diversity in antibodies for activated integrins, we could not verify the accuracy of the low percentages that we have obtained for the activated integrins. However there are other publications that used the same antibody and had low percentages regarding their flow cytometery analysis [131].

Regardless of all these limitations, our findings and those of others [19, 134] shows that Sema-3A is possibly one of the major immunoregulatory players in the immune system. This involvement encouraged the investigation of its possible therapeutic application in many immune-mediated disorders. A challenge for the future will be to link the increasing knowledge of semaphorin/plexin signalling pathways to

specific cell events in immune function. These efforts will probably focus on further works on the regulation of integrins and Rho kinases especially R-Ras family in immune cells. In addition since all patients having clinical symptoms already have established plaques, it would be very interesting to verify the protective effect of the Sema-3A on atherosclerotic plaque regression as well. The other interesting point that deserve further investigations is that spleens of Sema-3A overexpressing $ApoE^{-/-}$ group were always significantly bigger than control group. We studied spleenocyte T cell populations in these both groups. However, we didn't see any impact of Sema-3A on spleenocyte T cells. Since B cells are the other major cell populations in spleens, it would be interesting to assess the effect of Sema-3A on this population as well.

References

REFERENCES

[1] Michelle Y Abshire, Keena S Thomas, Katherine A Owen, and Amy H Bouton. Macrophage motility requires distinct $\alpha 5\beta 1$/fak and $\alpha 4\beta 1$/paxillin signaling events. *Journal of leukocyte biology*, 89(2):251–257, 2011.

[2] Hafid Ait-Oufella, Branka Horvat, Yann Kerdiles, Olivier Herbin, Pierre Gourdy, Jamila Khallou-Laschet, Régine Merval, Bruno Esposito, Alain Tedgui, and Ziad Mallat. Measles virus nucleoprotein induces a regulatory immune response and reduces atherosclerosis in mice. *Circulation*, 116(15):1707–1713, 2007.

[3] Hafid Ait-Oufella, Benoît L Salomon, Stéphane Potteaux, Anna-Karin L Robertson, Pierre Gourdy, Joffrey Zoll, Régine Merval, Bruno Esposito, José L Cohen, Sylvain Fisson, et al. Natural regulatory t cells control the development of atherosclerosis in mice. *Nature medicine*, 12(2):178–180, 2006.

[4] Shizuo Akira and Kiyoshi Takeda. Toll-like receptor signalling. *Nature reviews immunology*, 4(7):499–511, 2004.

[5] Shizuo Akira, Satoshi Uematsu, and Osamu Takeuchi. Pathogen recognition and innate immunity. *Cell*, 124(4):783–801, 2006.

[6] Guangyu An, Huan Wang, Rong Tang, Tadayuki Yago, J Michael McDaniel, Samuel McGee, Yuqing Huo, and Lijun Xia. P-selectin glycoprotein ligand-1 is highly expressed on ly-6chi monocytes and a major determinant for ly-6chi monocyte recruitment to sites of atherosclerosis in mice. *Circulation*, 117(25):3227–3237, 2008.

[7] Manuela Battaglia, Angela Stabilini, Barbara Migliavacca, Jutta Horejs-Hoeck, Thomas Kaupper, and Maria-Grazia Roncarolo. Rapamycin promotes expansion of functional cd4+ cd25+ foxp3+ regulatory t cells of both healthy subjects and type 1 diabetic patients. *The Journal of Immunology*, 177(12):8338–8347, 2006.

[8] Oded Behar, Jeffrey A Golden, Hiroshi Mashimo, Frederick J Schoen, and Mark C Fishman. Semaphorin iii is needed for normal patterning and growth of nerves, bones and heart. *Nature*, 383:525–528, 1996.

[9] Peer Bork, Tobias Doerks, Timothy A Springer, and Berend Snel. Domains in plexins: links to integrins and transcription factors. *Trends in biochemical sciences*, 24(7):261–263, 1999.

[10] JJ Boyle. Macrophage activation in atherosclerosis: pathogenesis and pharmacology of plaque rupture. *Current vascular pharmacology*, 3(1):63–68, 2005.

[11] Chiara Buono, Christoph J Binder, George Stavrakis, Joseph L Witztum, Laurie H Glimcher, and Andrew H Lichtman. T-bet deficiency reduces atherosclerosis and alters plaque antigen-specific immune responses. *Proceedings of the National Academy of Sciences of the United States of America*, 102(5):1596–1601, 2005.

[12] Chiara Buono, Carolyn E Come, George Stavrakis, Graham F Maguire, Philip W Connelly, and Andrew H Lichtman. Influence of interferon-γ on the extent and phenotype of diet-induced atherosclerosis in the ldlr-deficient mouse. *Arteriosclerosis, thrombosis, and vascular biology*, 23(3):454–460, 2003.

[13] Larissa Cardilo-Reis, Sabrina Gruber, Sabine M Schreier, Maik Drechsler, Nikolina Papac-Milicevic, Christian Weber, Oswald Wagner, Herbert Stangl, Oliver Soehnlein, and Christoph J Binder. Interleukin-13 protects from atherosclerosis and modulates plaque composition by skewing the macrophage phenotype. *EMBO molecular medicine*, 4(10):1072–1086, 2012.

[14] Emmanuelle Caron, Annette J Self, and Alan Hall. The gtpase rap1 controls functional activation of macrophage integrin αmβ2 by lps and other inflammatory mediators. *Current Biology*, 10(16):974–978, 2000.

[15] Alessandro Carrer, Silvia Moimas, Serena Zacchigna, Lucia Pattarini, Lorena Zentilin, Giulia Ruozi, Miguel Mano, Milena Sinigaglia, Federica Maione, Guido Serini, et al. Neuropilin-1 identifies a subset of bone marrow gr1- monocytes that can induce tumor vessel normalization and inhibit tumor growth. *Cancer research*, 72(24):6371–6381, 2012.

[16] Leslie A Cary, James F Chang, and Jun-Lin Guan. Stimulation of cell migration by overexpression of focal adhesion kinase and its association with src and fyn. *Journal of cell science*, 109(7):1787–1794, 1996.

[17] Andrea Casazza, Xi Fu, Irja Johansson, Lorena Capparuccia, Fredrik Andersson, Alice Giustacchini, Mario Leonardo Squadrito, Mary Anna Venneri, Massimiliano Mazzone, Erik Larsson, et al. Systemic and targeted delivery of semaphorin 3a inhibits tumor angiogenesis and progression in mouse tumor models. *Arteriosclerosis, thrombosis, and vascular biology*, 31(4):741–749, 2011.

[18] Andrea Casazza, Damya Laoui, Mathias Wenes, Sabrina Rizzolio, Nicklas Bassani, Marco Mambretti, Sofie Deschoemaeker, Jo A Van Ginderachter, Luca Tamagnone, and Massimiliano Mazzone. Impeding macrophage entry into hypoxic tumor areas by sema3a/nrp1 signaling blockade inhibits angiogenesis and restores antitumor immunity. *Cancer Cell*, 24(6):695–709, 2013.

[19] Alfonso Catalano. The neuroimmune semaphorin-3a reduces inflammation and progression of experimental autoimmune arthritis. *The Journal of Immunology*, 185(10):6373–6383, 2010.

[20] Alfonso Catalano, Paola Caprari, Simona Moretti, Monica Faronato, Luca Tamagnone, and Antonio Procopio. Semaphorin-3a is expressed by tumor cells and alters t-cell signal transduction and function. *Blood*, 107(8):3321–3329, 2006.

[21] Mariola R Chacón, Gloria Fernández, and Beatriz Rico. Focal adhesion kinase functions downstream of sema3a signaling during axonal remodeling. *Molecular and Cellular Neuroscience*, 44(1):30–42, 2010.

[22] Hang Chen, Alain Chédotal, Zhigang He, Corey S Goodman, and Marc Tessier-Lavigne. Neuropilin-2, a novel member of the neuropilin family, is a high affinity receptor for the semaphorins sema e and sema iv but not sema iii. *Neuron*, 19(3):547–559, 1997.

[23] Jose C Crispin, Araceli Martınez, and Jorge Alcocer-Varela. Quantification of regulatory t cells in patients with systemic lupus erythematosus. *Journal of autoimmunity*, 21(3):273–276, 2003.

[24] Onno J de Boer, Jelger J van der Meer, Peter Teeling, Chris M van der Loos, and Allard C van der Wal. Low numbers of foxp3 positive regulatory t cells are present in all developmental stages of human atherosclerotic lesions. *PloS one*, 2(8):e779–e779, 2007.

[25] B Ebbell. The papyrus ebers, the greatest egyptian medical document. levin & munksgaard/h. milford, 1937.

[26] Daniel Eefting, Margreet R de Vries, Jos M Grimbergen, Jacco C Karper, J Hajo van Bockel, and Paul HA Quax. In vivo suppression of vein graft disease by nonviral, electroporation-mediated, gene transfer of tissue inhibitor of metalloproteinase-1 linked to the amino terminal fragment of urokinase (timp-1. atf), a cell-surface directed matrix metalloproteinase inhibitor. *Journal of vascular surgery*, 51(2):429–437, 2010.

[27] Michael R Ehrenstein, Jamie G Evans, Animesh Singh, Samantha Moore, Gary Warnes, David A Isenberg, and Claudia Mauri. Compromised function of regulatory t cells in rheumatoid arthritis and reversal by anti-tnfα therapy. *The Journal of experimental medicine*, 200(3):277–285, 2004.

[28] Britta J Eickholt, Sarah L Mackenzie, Anthony Graham, Frank S Walsh, and Patrick Doherty. Evidence for collapsin-1 functioning in the control of neural crest migration in both trunk and hindbrain regions. *Development*, 126(10):2181–2189, 1999.

[29] Amanda C Foks, Andrew H Lichtman, and Johan Kuiper. Treating atherosclerosis with regulatory t cells. *Arteriosclerosis, thrombosis, and vascular biology*, 35(2):280–287, 2015.

[30] Mélanie Franco and Luca Tamagnone. Tyrosine phosphorylation in semaphorin signalling: shifting into overdrive. *EMBO reports*, 9(9):865–871, 2008.

[31] Peter Friedl and Bettina Weigelin. Interstitial leukocyte migration and immune function. *Nature immunology*, 9(9):960–969, 2008.

[32] Elena Galkina and Klaus Ley. Immune and inflammatory mechanisms of atherosclerosis. *Annual review of immunology*, 27:165, 2009.

[33] Flavia Garcia, Yves Lepelletier, Salete Smaniotto, Réda Hadj-Slimane, Mireille Dardenne, Olivier Hermine, and Wilson Savino. Inhibitory effect of semaphorin-3a, a known axon guidance molecule, in the human thymocyte migration induced by cxcl12. *Journal of leukocyte biology*, 91(1):7–13, 2012.

[34] Teresa Gerhardt and Klaus Ley. Monocyte trafficking across the vessel wall. *Cardiovascular research*, page cvv147, 2015.

[35] Godfrey S Getz and Catherine A Reardon. Animal models of atherosclerosis. *Arteriosclerosis, thrombosis, and vascular biology*, 32(5):1104–1115, 2012.

[36] Yelena Glinka and Gérald J Prud'homme. Neuropilin-1 is a receptor for transforming growth factor β-1, activates its latent form, and promotes regulatory t cell activity. *Journal of leukocyte biology*, 84(1):302–310, 2008.

[37] Siamon Gordon. Alternative activation of macrophages. *Nature reviews immunology*, 3(1):23–35, 2003.

[38] David J Grainger, Jill Reckless, and Elaine McKilligin. Apolipoprotein e modulates clearance of apoptotic bodies in vitro and in vivo, resulting in a systemic proinflammatory state in apolipoprotein e-deficient mice. *The Journal of Immunology*, 173(10):6366–6375, 2004.

[39] Chenghua Gu, E Rene Rodriguez, Dorothy V Reimert, Tianzhi Shu, Bernd Fritzsch, Linda J Richards, Alex L Kolodkin, and David D Ginty. Neuropilin-1 conveys semaphorin and vegf signaling during neural and cardiovascular development. *Developmental cell*, 5(1):45–57, 2003.

[40] Mausumee Guha and Nigel Mackman. Lps induction of gene expression in human monocytes. *Cellular signalling*, 13(2):85–94, 2001.

[41] Sanjay Gupta, Anne Marie Pablo, X c Jiang, Nan Wang, Alan R Tall, and Christian Schindler. Ifn-gamma potentiates atherosclerosis in apoe knock-out mice. *Journal of Clinical Investigation*, 99(11):2752, 1997.

[42] Bruno Halioua and Bernard Ziskind. *Medicine in the Days of the Pharaohs*. Harvard University Press, 2005.

[43] Zhigang He and Marc Tessier-Lavigne. Neuropilin is a receptor for the axonal chemorepellent semaphorin iii. *Cell*, 90(4):739–751, 1997.

[44] Takuji Ito, Tokiko Morita, Kenji Yoshida, Takayuki Negishi, and Kazunori Yukawa. Semaphorin 3a-plexin-a1 signaling through erk activation is crucial for toll-like receptor-induced no production in bv-2 microglial cells. *International journal of molecular medicine*, 33(6):1635–1642, 2014.

[45] Akiko Iwasaki and Ruslan Medzhitov. Toll-like receptor control of the adaptive immune responses. *Nature immunology*, 5(10):987–995, 2004.

[46] Jong-Dae Ji, Kyung-Hyun Park-Min, and Lionel B Ivashkiv. Expression and function of semaphorin 3a and its receptors in human monocyte-derived macrophages. *Human immunology*, 70(4):211–217, 2009.

[47] H Jin and J Varner. Integrins: roles in cancer development and as treatment targets. *British journal of cancer*, 90(3):561–565, 2004.

[48] Zhao Jin and Stephen M Strittmatter. Rac1 mediates collapsin-1-induced growth cone collapse. *The Journal of neuroscience*, 17(16):6256–6263, 1997.

[49] Jenny Jongstra-Bilen, Mehran Haidari, Su-Ning Zhu, Mian Chen, Daipayan Guha, and Myron I Cybulsky. Low-grade chronic inflammation in regions of the normal mouse arterial intima predisposed to atherosclerosis. *The Journal of experimental medicine*, 203(9):2073–2083, 2006.

[50] Taro Kawai and Shizuo Akira. Pathogen recognition with toll-like receptors. *Current opinion in immunology*, 17(4):338–344, 2005.

[51] Jamila Khallou-Laschet, Aditi Varthaman, Giulia Fornasa, Caroline Compain, Anh-Thu Gaston, Marc Clement, Michaël Dussiot, Olivier Levillain, Stéphanie Graff-Dubois, Antonino Nicoletti, et al. Macrophage plasticity in experimental atherosclerosis. *PloS one*, 5(1):e8852, 2010.

[52] Adil A Khan, Christian Bose, Lung S Yam, Mark J Soloski, and Fabio Rupp. Physiological regulation of the immunological synapse by agrin. *Science*, 292(5522):1681–1686, 2001.

[53] Hitoshi Kikutani and Atsushi Kumanogoh. Semaphorins in interactions between t cells and antigen-presenting cells. *Nature Reviews Immunology*, 3(2):159–167, 2003.

[54] Andreas Klostermann, Marion Lohrum, Ralf H Adams, and Andreas W Püschel. The chemorepulsive activity of the axonal guidance signal semaphorin d requires dimerization. *Journal of Biological Chemistry*, 273(13):7326–7331, 1998.

[55] Alex L Kolodkin, David J Matthes, and Corey S Goodman. The semaphorin genes encode a family of transmembrane and secreted growth cone guidance molecules. *Cell*, 75(7):1389–1399, 1993.

[56] Robert P Kruger, Jennifer Aurandt, and Kun-Liang Guan. Semaphorins command cells to move. *Nature Reviews Molecular Cell Biology*, 6(10):789–800, 2005.

[57] Anjli Kukreja, Giulia Cost, John Marker, Chenhui Zhang, Zhong Sun, Karen Lin-Su, Svetlana Ten, Maureen Sanz, Mark Exley, Brian Wilson, et al. Multiple immuno-regulatory defects in type-1 diabetes. *The Journal of clinical investigation*, 109(109 (1)):131–140, 2002.

[58] Atsushi Kumanogoh, Satoko Marukawa, Kazuhiro Suzuki, Noriko Takegahara, Chie Watanabe, EweSeng Ch'ng, Isao Ishida, Harutoshi Fujimura, Saburo Sakoda, Kanji Yoshida, et al. Class iv semaphorin sema4a enhances t-cell activation and interacts with tim-2. *Nature*, 419(6907):629–633, 2002.

[59] Carlene MM Lawes, Stephen Vander Hoorn, Anthony Rodgers, et al. Global burden of blood-pressure-related disease, 2001. *The Lancet*, 371(9623):1513–1518, 2008.

[60] Chun-Yi Lee, Mei-Ju Su, Chun-Yin Huang, Meng-Yi Chen, Horng-Chaung Hsu, Ching-Yuang Lin, and Chih-Hsin Tang. Macrophage migration inhibitory factor increases cell motility and up-regulates $\alpha v\beta 3$ integrin in human chondrosarcoma cells. *Journal of cellular biochemistry*, 113(5):1590–1598, 2012.

[61] Sik Lee, Sarah Huen, Hitoshi Nishio, Saori Nishio, Heung Kyu Lee, Bum-Soon Choi, Christiana Ruhrberg, and Lloyd G Cantley. Distinct macrophage phenotypes contribute to kidney injury and repair. *Journal of the American Society of Nephrology*, 22(2):317–326, 2011.

[62] Yves Lepelletier, Ivan Cruz Moura, Réda Hadj-Slimane, Amédée Renand, Susana Fiorentino, Cédric Baude, Anat Shirvan, Ari Barzilai, and Olivier Hermine. Immunosuppressive role of semaphorin-3a on t cell proliferation is mediated by inhibition of actin cytoskeleton reorganization. *European journal of immunology*, 36(7):1782–1793, 2006.

[63] Andrew C Li and Christopher K Glass. The macrophage foam cell as a target for therapeutic intervention. *Nature medicine*, 8(11):1235–1242, 2002.

[64] Xudong Liao, Nikunj Sharma, Fehmida Kapadia, Guangjin Zhou, Yuan Lu, Hong Hong, Kaavya Paruchuri, Ganapati H Mahabeleshwar, Elise Dalmas, Nicolas Venteclef, et al. Krüppel-like factor 4 regulates macrophage polarization. *The Journal of clinical investigation*, 121(7):2736, 2011.

[65] Peter Libby. Inflammation in atherosclerosis. *Nature*, 420(6917):868–874, 2002.

[66] Jaime Llodrá, Véronique Angeli, Jianhua Liu, Eugene Trogan, Edward A Fisher, and Gwendalyn J Randolph. Emigration of monocyte-derived cells from atherosclerotic lesions characterizes regressive, but not progressive, plaques. *Proceedings of the National Academy of Sciences of the United States of America*, 101(32):11779–11784, 2004.

[67] Donald Lloyd-Jones, Robert J Adams, Todd M Brown, Mercedes Carnethon, Shifan Dai, Giovanni De Simone, T Bruce Ferguson, Earl Ford, Karen Furie, Cathleen Gillespie, et al. Heart disease and stroke statistics2010 update a report from the american heart association. *Circulation*, 121(7):e46–e215, 2010.

[68] Yong-Chen Lu, Wen-Chen Yeh, and Pamela S Ohashi. Lps/tlr4 signal transduction pathway. *Cytokine*, 42(2):145–151, 2008.

[69] Yuling Luo, David Raible, and Jonathan A Raper. Collapsin: a protein in brain that induces the collapse and paralysis of neuronal growth cones. *Cell*, 75(2):217–227, 1993.

[70] Aldons J Lusis. Atherosclerosis. *Nature*, 407(6801):233–241, 2000.

[71] Henry H Majed, Siddharthan Chandran, Simone P Niclou, Richard S Nicholas, Alastair Wilkins, Mark G Wing, Kate E Rhodes, Maria Grazia Spillantini, and Alastair Compston. A novel role for sema3a in neuroprotection from injury mediated by activated microglia. *The Journal of neuroscience*, 26(6):1730–1738, 2006.

[72] Alberto Mantovani, Antonio Sica, and Massimo Locati. Macrophage polarization comes of age. *Immunity*, 23(4):344–346, 2005.

[73] Fernando O Martinez, Laura Helming, and Siamon Gordon. Alternative activation of macrophages: an immunologic functional perspective. *Annual review of immunology*, 27:451–483, 2009.

[74] Christina Mayerl, Melanie Lukasser, Roland Sedivy, Harald Niederegger, Ruediger Seiler, and Georg Wick. Atherosclerosis research from past to presenton the track of two pathologists with opposing views, carl von rokitansky and rudolf virchow. *Virchows Archiv*, 449(1):96–103, 2006.

[75] Ashley M Miller, Damo Xu, Darren L Asquith, Laura Denby, Yubin Li, Naveed Sattar, Andrew H Baker, Iain B McInnes, and Foo Y Liew. Il-33 reduces

the development of atherosclerosis. *The Journal of experimental medicine*, 205(2):339–346, 2008.

[76] Satyajit K Mitra, Daniel A Hanson, and David D Schlaepfer. Focal adhesion kinase: in command and control of cell motility. *Nature reviews Molecular cell biology*, 6(1):56–68, 2005.

[77] Kathryn J Moore, Frederick J Sheedy, and Edward A Fisher. Macrophages in atherosclerosis: a dynamic balance. *Nature Reviews Immunology*, 13(10):709–721, 2013.

[78] Kathryn J Moore and Ira Tabas. Macrophages in the pathogenesis of atherosclerosis. *Cell*, 145(3):341–355, 2011.

[79] Adi Mor, David Planer, Galia Luboshits, Arnon Afek, Shula Metzger, Tova Chajek-Shaul, Gad Keren, and Jacob George. Role of naturally occurring cd4+ cd25+ regulatory t cells in experimental atherosclerosis. *Arteriosclerosis, thrombosis, and vascular biology*, 27(4):893–900, 2007.

[80] David M Mosser and Justin P Edwards. Exploring the full spectrum of macrophage activation. *Nature reviews immunology*, 8(12):958–969, 2008.

[81] Fumio Nakamura, Masaki Tanaka, Takuya Takahashi, Robert G Kalb, and Stephen M Strittmatter. Neuropilin-1 extracellular domains mediate semaphorin d/iii-induced growth cone collapse. *Neuron*, 21(5):1093–1100, 1998.

[82] Ichiro Nakamura, Mary F Pilkington, Päivi T Lakkakorpi, Lorraine Lipfert, Stephen M Sims, S Jeffrey Dixon, Gideon A Rodan, and Le T Duong. Role of alpha (v) beta (3) integrin in osteoclast migration and formation of the sealing zone. *Journal of cell science*, 112(22):3985–3993, 1999.

[83] Gera Neufeld and Ofra Kessler. The semaphorins: versatile regulators of tumour progression and tumour angiogenesis. *Nature Reviews Cancer*, 8(8):632–645, 2008.

[84] World Health Organization et al. Global atlas on cardiovascular disease prevention and control. mendis s, puska p, norrving b editors. *Geneva. Rehabilitation Therapies in the Elderly Stroke Population*, 177, 2011.

[85] Mireille Ouimet, Hasini N Ediriweera, U Mahesh Gundra, Frederick J Sheedy, Bhama Ramkhelawon, Susan B Hutchison, Kaitlyn Rinehold, Coen van Solingen, Morgan D Fullerton, Katharine Cecchini, et al. Microrna-33–dependent

regulation of macrophage metabolism directs immune cell polarization in atherosclerosis. *The Journal of clinical investigation*, 125(12):0–0, 2015.

[86] Hongjie Pan, Luke S Wanami, Thusitha R Dissanayake, and Robin E Bachelder. Autocrine semaphorin3a stimulates alpha2 beta1 integrin expression/function in breast tumor cells. *Breast cancer research and treatment*, 118(1):197–205, 2009.

[87] J Thomas Parsons. Focal adhesion kinase: the first ten years. *Journal of cell science*, 116(8):1409–1416, 2003.

[88] Kim E Paulson, Su-Ning Zhu, Mian Chen, Sabrina Nurmohamed, Jenny Jongstra-Bilen, and Myron I Cybulsky. Resident intimal dendritic cells accumulate lipid and contribute to the initiation of atherosclerosis. *Circulation research*, 106(2):383–390, 2010.

[89] Andrew S Plump, Jonathan D Smith, Tony Hayek, Katriina Aalto-Setälä, Annemarie Walsh, Judy G Verstuyft, Edward M Rubin, and Jan L Breslow. Severe hypercholesterolemia and atherosclerosis in apolipoprotein e-deficient mice created by homologous recombination in es cells. *Cell*, 71(2):343–353, 1992.

[90] Franck Polleux, Theresa Morrow, and Anirvan Ghosh. Semaphorin 3a is a chemoattractant for cortical apical dendrites. *Nature*, 404(6778):567–573, 2000.

[91] Stephane Potteaux, Emmanuel L Gautier, Susan B Hutchison, Nico van Rooijen, Daniel J Rader, Michael J Thomas, Mary G Sorci-Thomas, and Gwendalyn J Randolph. Suppressed monocyte recruitment drives macrophage removal from atherosclerotic plaques of apoe–/–mice during disease regression. *The Journal of clinical investigation*, 121(5):2025–2036, 2011.

[92] Myrto Raftopoulou and Alan Hall. Cell migration: Rho gtpases lead the way. *Developmental biology*, 265(1):23–32, 2004.

[93] Punithavathi Ranganathan, Calpurnia Jayakumar, Riyaz Mohamed, Neal L Weintraub, and Ganesan Ramesh. Semaphorin 3a inactivation suppresses ischemia-reperfusion-induced inflammation and acute kidney injury. *American Journal of Physiology-Renal Physiology*, 307(2):F183–F194, 2014.

[94] Anna-Karin L Robertson and Göran K Hansson. T cells in atherogenesis for better or for worse? *Arteriosclerosis, thrombosis, and vascular biology*, 26(11):2421–2432, 2006.

[95] Atsuko Sakurai, Colleen Doci, and J Silvio Gutkind. Semaphorin signaling in angiogenesis, lymphangiogenesis and cancer. *Cell research*, 22(1):23–32, 2012.

[96] Gennaro Sardella, Leonardo De Luca, Vittorio Francavilla, Daniele Accapezzato, Massimo Mancone, Maria Isabella Sirinian, Francesco Fedele, and Marino Paroli. Frequency of naturally-occurring regulatory t cells is reduced in patients with st-segment elevation myocardial infarction. *Thrombosis research*, 120(4):631–634, 2007.

[97] Milka Sarris, Kristian G Andersen, Felix Randow, Luzia Mayr, and Alexander G Betz. Neuropilin-1 expression on regulatory t cells enhances their interactions with dendritic cells during antigen recognition. *Immunity*, 28(3):402–413, 2008.

[98] Haruna Sawaki, Fumio Nakamura, Michiko Aihara, Yoji Nagashima, Junko Komori-Yamaguchi, Naoya Yamashita, Masatoshi Nakazawa, Yoshio Goshima, and Zenro Ikezawa. Intranasal administration of semaphorin-3a alleviates sneezing and nasal rubbing in a murine model of allergic rhinitis. *Journal of pharmacological sciences*, 117(1):34–44, 2011.

[99] Uwe Schlomann, Jens C Schwamborn, Myriam Müller, Reinhard Fässler, and Andreas W Püschel. The stimulation of dendrite growth by sema3a requires integrin engagement and focal adhesion kinase. *Journal of cell science*, 122(12):2034–2042, 2009.

[100] Dorien M Schrijvers, Guido RY De Meyer, Arnold G Herman, and Wim Martinet. Phagocytosis in atherosclerosis: Molecular mechanisms and implications for plaque progression and stability. *Cardiovascular research*, 73(3):470–480, 2007.

[101] Guido Serini, Donatella Valdembri, Sara Zanivan, Giulia Morterra, Constanze Burkhardt, Francesca Caccavari, Luca Zammataro, Luca Primo, Luca Tamagnone, Malcolm Logan, et al. Class 3 semaphorins control vascular morphogenesis by inhibiting integrin function. *Nature*, 424(6947):391–397, 2003.

[102] J Shim, RH Al-Mashhadi, CB Sørensen, and JF Bentzon. Large animal models of atherosclerosis–new tools for persistent problems in cardiovascular medicine. *The Journal of pathology*, 2015.

[103] Nathalie Spassky, Fernando de Castro, Barbara Le Bras, Katharina Heydon, Françoise Quéraud-LeSaux, Evelyne Bloch-Gallego, Alain Chédotal, Bernard

Zalc, and Jean-Léon Thomas. Directional guidance of oligodendroglial migration by class 3 semaphorins and netrin-1. *The Journal of neuroscience*, 22(14):5992–6004, 2002.

[104] Kazuhiro Suzuki, Atsushi Kumanogoh, and Hitoshi Kikutani. Semaphorins and their receptors in immune cell interactions. *Nature immunology*, 9(1):17–23, 2008.

[105] Ira Tabas. Macrophage death and defective inflammation resolution in atherosclerosis. *Nature Reviews Immunology*, 10(1):36–46, 2010.

[106] Frank Tacke, David Alvarez, Theodore J Kaplan, Claudia Jakubzick, Rainer Spanbroek, Jaime Llodra, Alexandre Garin, Jianhua Liu, Matthias Mack, Nico van Rooijen, et al. Monocyte subsets differentially employ ccr2, ccr5, and cx3cr1 to accumulate within atherosclerotic plaques. *The Journal of clinical investigation*, 117(1):185–194, 2007.

[107] Hyota Takamatsu and Atsushi Kumanogoh. Diverse roles for semaphorin-plexin signaling in the immune system. *Trends in immunology*, 33(3):127–135, 2012.

[108] Hyota Takamatsu, Tatsusada Okuno, and Atsushi Kumanogoh. Regulation of immune cell responses by semaphorins and their receptors. *Cellular & molecular immunology*, 7(2):83–88, 2010.

[109] Hyota Takamatsu, Noriko Takegahara, Yukinobu Nakagawa, Michio Tomura, Masahiko Taniguchi, Roland H Friedel, Helen Rayburn, Marc Tessier-Lavigne, Yutaka Yoshida, Tatsusada Okuno, et al. Semaphorins guide the entry of dendritic cells into the lymphatics by activating myosin ii. *Nature immunology*, 11(7):594–600, 2010.

[110] Kiyoshi Takeda, Tsuneyasu Kaisho, and Shizuo Akira. Toll-like receptors. *Annual review of immunology*, 21(1):335–376, 2003.

[111] Noriko Takegahara, Hyota Takamatsu, Toshihiko Toyofuku, Tohru Tsujimura, Tatsusada Okuno, Kazunori Yukawa, Masayuki Mizui, Midori Yamamoto, Durbaka VR Prasad, Kazuhiro Suzuki, et al. Plexin-a1 and its interaction with dap12 in immune responses and bone homeostasis. *Nature cell biology*, 8(6):615–622, 2006.

[112] Osamu Takeuchi and Shizuo Akira. Pattern recognition receptors and inflammation. *Cell*, 140(6):805–820, 2010.

[113] Luca Tamagnone, Stefania Artigiani, Hang Chen, Zhigang He, Guo-li Ming, Hong-jun Song, Alain Chedotal, Margaret L Winberg, Corey S Goodman, Muming Poo, et al. Plexins are a large family of receptors for transmembrane, secreted, and gpi-anchored semaphorins in vertebrates. *Cell*, 99(1):71–80, 1999.

[114] Luca Tamagnone and Paolo M Comoglio. Signalling by semaphorin receptors: cell guidance and beyond. *Trends in cell biology*, 10(9):377–383, 2000.

[115] Luca Tamagnone and Paolo M Comoglio. To move or not to move? *EMBO reports*, 5(4):356–361, 2004.

[116] Luca Tamagnone and Silvia Giordano. Semaphorin pathways orchestrate osteogenesis. *Nature cell biology*, 8(6):545–547, 2006.

[117] Karrie Tartaro, Maria VanVolkenburg, Dean Wilkie, Timothy M Coskran, John M Kreeger, Thomas T Kawabata, and Sandra Casinghino. Development of a fluorescence-based in vivo phagocytosis assay to measure mononuclear phagocyte system function in the rat. *Journal of immunotoxicology*, 12(3):239–246, 2015.

[118] Rafaèle Tordjman, Yves Lepelletier, Valérie Lemarchandel, Marie Cambot, Philippe Gaulard, Olivier Hermine, and Paul-Henri Roméo. A neuronal receptor, neuropilin-1, is essential for the initiation of the primary immune response. *Nature immunology*, 3(5):477–482, 2002.

[119] Hieu Tran-Van, Elita Avota, Charlene Börtlein, Nora Mueller, and Sibylle Schneider-Schaulies. Measles virus modulates dendritic cell/t-cell communication at the level of plexina1/neuropilin-1 recruitment and activity. *European journal of immunology*, 41(1):151–163, 2011.

[120] Eugene Trogan, Jonathan E Feig, Snjezana Dogan, George H Rothblat, Véronique Angeli, Frank Tacke, Gwendalyn J Randolph, and Edward A Fisher. Gene expression changes in foam cells and the role of chemokine receptor ccr7 during atherosclerosis regression in apoe-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America*, 103(10):3781–3786, 2006.

[121] Zahava Vadasz, Tharwat Haj, Katalin Halasz, Itzhak Rosner, Gleb Slobodin, Dina Attias, Aharon Kessel, Ofra Kessler, Gera Neufeld, and Elias Toubi. Semaphorin 3a is a marker for disease activity and a potential immunoregulator in systemic lupus erythematosus. *Arthritis Res Ther*, 14(3):R146, 2012.

[122] Zahava Vadasz, Tharwat Haj, Aharon Kessel, and Elias Toubi. B-regulatory cells in autoimmunity and immune mediated inflammation. *FEBS letters*, 587(13):2074–2078, 2013.

[123] Janine M van Gils, Bhama Ramkhelawon, Luciana Fernandes, Merran C Stewart, Liang Guo, Tara Seibert, Gustavo B Menezes, Denise C Cara, Camille Chow, T Bernard Kinane, et al. Endothelial expression of guidance cues in vessel wall homeostasis dysregulation under proatherosclerotic conditions. *Arteriosclerosis, thrombosis, and vascular biology*, 33(5):911–919, 2013.

[124] Jeeva Varadarajulu, Martin Laser, Markus Hupp, Rongxue Wu, and Christof R Hauck. Targeting of α v integrins interferes with fak activation and smooth muscle cell migration and invasion. *Biochemical and biophysical research communications*, 331(2):404–412, 2005.

[125] Vissia Viglietta, Clare Baecher-Allan, Howard L Weiner, and David A Hafler. Loss of functional suppression by cd4+ cd25+ regulatory t cells in patients with multiple sclerosis. *The Journal of experimental medicine*, 199(7):971–979, 2004.

[126] Haitao Wen, Yu Lei, So-Young Eun, and Jenny P-Y Ting. Plexin-a4–semaphorin 3a signaling is required for toll-like receptor–and sepsis-induced cytokine storm. *The Journal of experimental medicine*, 207(13):2943–2957, 2010.

[127] Jon A Wolff, Robert W Malone, Phillip Williams, Wang Chong, Gyula Acsadi, Agnes Jani, and Philip L Felgner. Direct gene transfer into mouse muscle in vivo. *Science*, 247(4949):1465–1468, 1990.

[128] Ine MJ Wolfs, MMPC Donners, Menno PJ de Winther, et al. Differentiation factors and cytokines in the atherosclerotic plaque micro-environment as a trigger for macrophage polarisation. *Thrombosis and haemostasis*, 106(5):763, 2011.

[129] Athena W Wong, W June Brickey, Debra J Taxman, Hendrick W van Deventer, William Reed, Jian Xin Gao, Pan Zheng, Yang Liu, Ping Li, Janice S Blum,

et al. Ciita-regulated plexin-a1 affects t-cell–dendritic cell interactions. *Nature immunology*, 4(9):891–898, 2003.

[130] Kevin J Woollard and Frederic Geissmann. Monocytes in atherosclerosis: subsets and functions. *Nature Reviews Cardiology*, 7(2):77–86, 2010.

[131] Bo Wu, Yi Wang, Xiang-Min Yang, Bao-Qing Xu, Fei Feng, Bin Wang, Qiang Liang, Yu Li, Yang Zhou, Jian-Li Jiang, et al. Basigin-mediated redistribution of cd98 promotes cell spreading and tumorigenicity in hepatocellular carcinoma. *Journal of Experimental & Clinical Cancer Research*, 34(1):1, 2015.

[132] Wei Xu, Anja Roos, Nicole Schlagwein, Andrea M Woltman, Mohamed R Daha, and Cees van Kooten. Il-10–producing macrophages preferentially clear early apoptotic cells. *Blood*, 107(12):4930–4937, 2006.

[133] Valentin P Yakubenko, Nataly Belevych, Daria Mishchuk, Aleksey Schurin, Stephen C-T Lam, and Tatiana P Ugarova. The role of integrin α d β 2 (cd11d/cd18) in monocyte/macrophage migration. *Experimental cell research*, 314(14):2569–2578, 2008.

[134] Junko Yamaguchi, Fumio Nakamura, Michiko Aihara, Naoya Yamashita, Hiroshi Usui, Tomonobu Hida, Kohtaro Takei, Yoji Nagashima, Zenro Ikezawa, and Yoshio Goshima. Semaphorin3a alleviates skin lesions and scratching behavior in nc/nga mice, an atopic dermatitis model. *Journal of Investigative Dermatology*, 128(12):2842–2849, 2008.

[135] Midori Yamamoto, Kazuhiro Suzuki, Tatsusada Okuno, Takehiro Ogata, Noriko Takegahara, Hyota Takamatsu, Masayuki Mizui, Masahiko Taniguchi, Alain Chédotal, Fumikazu Suto, et al. Plexin-a4 negatively regulates t lymphocyte responses. *International immunology*, 20(3):413–420, 2008.

[136] Serena Zacchigna, Lucia Pattarini, Lorena Zentilin, Silvia Moimas, Alessandro Carrer, Milena Sinigaglia, Nikola Arsic, Sabrina Tafuro, Gianfranco Sinagra, and Mauro Giacca. Bone marrow cells recruited through the neuropilin-1 receptor promote arterial formation at the sites of adult neoangiogenesis in mice. *The Journal of clinical investigation*, 118(6):2062, 2008.

[137] Yeping Zhou, Rou-Afza F Gunput, and R Jeroen Pasterkamp. Semaphorin signaling: progress made and promises ahead. *Trends in biochemical sciences*, 33(4):161–170, 2008.