SEMA3E REGULATES RESIDENT MACROPHAGES RESPONSE IN LIPOPOLYSACCHARIDE-INDUCED SEPSIS

by

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ABSTRACT

Sepsis is an overwhelming systemic inflammatory response to microbial infections. Macrophages are the key innate immune cells that provide the first line of defence against systemic infections during sepsis. Macrophages perform multiple functions during infections such as triggering inflammation, phagocytosis of microbes, and resolution of inflammation. So far, various molecules have been shown to be involved in the regulation of macrophages in inflammatory conditions. However, recently published studies suggest that Semaphorin3E (Sema3E) plays a pivotal role in the immune function of macrophages. The exact role of Sema3E associated with macrophages function in lipopolysaccharide (LPS) induced endotoxemia is unknown. To directly address the involvement of Sema3E in macrophages, we have used *Sema3e* gene deletion approaches in *in vivo* and cell-based setups. We found that *Sema3e*$^{-/-}$ mice displayed initial transient protection from LPS-induced hypothermia. *Sema3e*$^{-/-}$ mice showed lower inducible nitric oxide synthase (iNOS) expression in peritoneal macrophages without altering the integrity of TLR-4 after LPS injection. *Sema3e*$^{-/-}$ mice exhibit a lower level of tumour necrosis factor (TNF) and interleukin-6 (IL-6) in peritoneal lavage and serum as compared to wild type (WT) littermates. Bone marrow derived macrophages (BMDMs) from *Sema3e*$^{-/-}$ mice expressed low levels of pro-inflammatory cytokines and also exhibited significantly down-regulated phosphorylation of STAT3, ERK1/2, and NF-κB, upon LPS exposure. Overall, the current study provides direct evidence that the lack of Sema3E, makes macrophages to become less responsive to LPS by disturbing LPS-

initiated signaling transduction. These findings suggest that the inhibition of Sema3E might be a novel strategy to treat conditions triggered by the excessive production of inflammatory cytokines.

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LIST OF ABBRIVIATIONS

ACK : Ammonium-chloride-potassium

ANOVA : Analysis of variance

APC : Antigen presenting cells

BBB : Blood brain barrier

BMDMs : Bone marrow derived macrophages

CARS : Compensatory anti-inflammatory response syndrome

Cdk5 : Cyclin dependent kinase 5

CRMP-2 : Collapse response mediator protein-2

Cyt c : Cytochrome c

DAMPs : Damage-associated molecular patterns

DCs : Dendritic cells

EAE : Experimental autoimmune encephalomyelitis

ECM : Extracellular matrix

ELISA : Enzyme-linked immunosorbent assay

ET : Endotoxin tolerance

FADD : Fas-associated death domain

GPI : Glycosylphosphatidylinositol

GSK3 : Glycogen synthase kinase 3

HIV : Human immunodeficiency virus

HMGB-1 : High-mobility group protein 1

IFN-γ : Interferon- γ

IL-1 : Interleukin-1

IL-10 : Interleukin-10

IL-2 : Interleukin-2

IL-6 : Interleukin-6

IL-8 : Interleukin-8

JNK : C-jun n-terminal kinase

KS : Kallmann syndrome

LBP : LPS binding protein

LDL : Low-density lipoprotein

LIMK : Lim-kinase

LPS : Lipopolysaccharide

MAPK : Mitogen-activated protein kinase

MAPK : Mitogen activated protein kinase

MHC : Major histocompatibility complex

MICAL : Molecules interacting with casl

MIF : Migration inhibitory factor

MOG : Myelin oligodendrocyte glycoprotein

MyD88 : Myeloid differentiation primary response gene 88

NETs : Neutrophil extracellular traps

NF-kB : Nuclear factor kb

NK : Natural killer

NO : Nitric oxide

Nrp : Neuropilin

PAF : Platelet activating factor

Pak : P21-activated kinase

PAMPs : Pathogen-associated molecular patterns

PGE2 : Prostaglandine2

PGI2 : Prostaglandini2

PI3K : Phosphatidylinositide 3-kinases

PPRs : Pattern-recognition receptors

PSI : Plexin-semaphorin-integrin

ROCK : Rho-associated kinase

ROR- t : Retinoic acid receptor-related orphan receptor-γ t

ROS : Reactive oxygen species

Sema : Semaphorin

SIRS : Severe inflammatory response syndrome (sirs

SLPI : Secretory leukocyte protease inhibitor

SRAM : Sterile a and HEAT-Armadillo motifs-containing protein

SSH : Slingshot

TCR : T cell receptor

Td : T-dependent

TGF- β : Transforming growth factor-β

TIR : Toll IL-1 receptor

TIRAP : TIR domain-containing adaptor protein

TLR-4 : Toll-like receptor-4

TLR-9 : Toll-like receptor-9

TLRs : Toll-like receptors

TNF : Tumour necrosis factor

TRAM : TRIF-related adaptor molecule

TRIF : TIR domain-containing adaptor inducing IFN-β

VEGF : Vascular endothelial growth factor

WT : Wild type

1. INTRODUCTION

1.1 Sepsis

Sepsis is a serious medical condition caused by overwhelming systemic inflammatory response against microbial infection [1]. The immune system initiates a series of reactions including the storm of cytokines, inflammation, swelling and blood clotting. Consequently, impaired blood flow and deprivation of nutrient and oxygen to tissues / organs leads to multiple organ failure and death [2].

The sepsis mortality rate surpasses prostate cancer, breast cancer and HIV/AIDS combined [3]. Furthermore, pre-existing states such as human immunodeficiency virus (HIV), cancer, diabetes, cirrhosis, alcohol dependence, and bedsores contribute to disease severity in sepsis [4]. The occurrence of sepsis is increasing dramatically regardless of discoveries of modern medicines including antibiotics and vaccines.

1.1.1 Epidemiology

Approximately 20-30 million people are affected every year by sepsis worldwide and 50 patients die every hour. Globally, sepsis claims more than 6 million lives of neonates and children per year. Annually, more than 100,000 cases accounted of maternal sepsis worldwide [5]. In Canada, sepsis was the leading cause of death in 2011 with an overall death rate of 38.4 /100,000 persons. One in 18 deaths in Canada involves sepsis. Sepsis-

associated deaths increase with age and males have a higher risk of death than females [6]. Moreover, sepsis is the contributing factor for more than one-half of all deaths from infectious diseases and it has a significant economic burden to the health care system estimated at 17$ billion annually in the USA [7]. In Europe, a typical sepsis episode costs approximately 25,000€ on health care services [8]. The rate of hospitalization for sepsis has increased twice over the last ten years and surpasses the hospitalization rate of myocardial infarction in the USA [9-11].

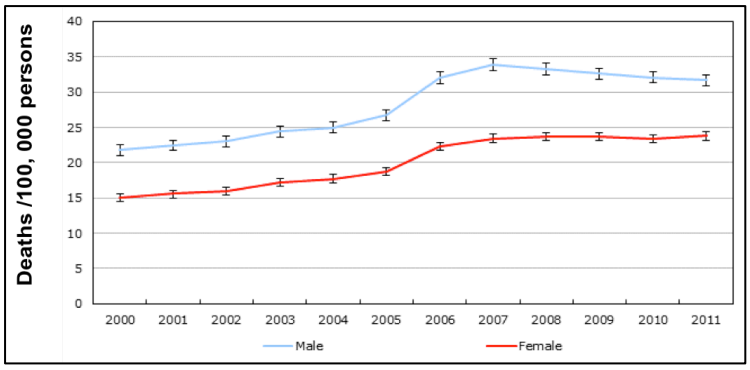

Figure 1. Data represent sepsis-associated mortalities (deaths per 100,000) in Canada between 200 - 2011.
Figure adapted from: *Statistics Canada,* Release date: January 21, 2016.

1.1.2 Pathophysiology of Sepsis:

Two opposite phenomena can be seen in sepsis. The first is a severe inflammatory response syndrome (SIRS) and the second a compensatory anti-inflammatory response syndrome (CARS). Both SIRS and CARS start immediately after infection. However, SIRS is predominant in the initial stage of sepsis while CARS appears during the later stage. During sepsis, recognition of pathogens by host immune cells initiate a pro-

inflammatory response against infection called SIRS [12]. An exaggerated inflammatory response during SIRS leads to tissue damage and organ failure. However, to protect the body from the negative SIRS effects, an anti-inflammatory feedback loop called CARS is induced [13, 14]. SIRS involves leukocyte activation, cytokine storm, systemic inflammation, and tissue injury and multiple organ failure, while CARS includes leukocyte deactivation, immunosuppression, endothelial/epithelial dysfunction leading to death due to secondary infections [15].

1.1.2.1 Systemic inflammatory response syndrome (SIRS)

SIRS is an overwhelming inflammatory response to injury, such as trauma, burns, hypoxia or infections. SIRS due to infection is called sepsis and this condition can be seen in the initial phase of infection where innate immune cells produce a cytokines storm, which in turn causes edema and multiple organ failure. During sepsis when microbes enter into the systemic circulation, resident cells detect the invaders and initiate immune responses. In the case of a limited number of microbes, the local immune response is sufficient to control infection. However, uncontrolled local microbial burden leads to systemic dissemination.

Immune cells initiate a host defense response after detection of microbes/microbial components through pattern-recognition receptors (PRRs) [16]. Microbial infection in the body is sensed by recognizing microbial products or conserved pathogen-associated molecular patterns (PAMPs), which are expressed by harmful microorganisms. However,

released components of damaged tissues (alarmins) are also recognized by the immune system. Alarmins and tissue components are referred to as damage-associated molecular patterns (DAMPs) [16]. In septic conditions, the release of high amounts of DAMPs from damaged tissues and components of microbes (PAMPs) leads to the intense immune response due to the overstimulation of immune cells. The result, an excessive release of cytokines (cytokines storm) from immune cells, is useful for fighting infections, but it causes tissue damage too [17].

Toll-like receptors (TLRs), which are a subfamily of PRRs, are key receptors for the recognition of PAMPs and DAMPs and initiate the inflammatory response. Gram-negative organisms are the main invading microbes in sepsis [18]. LPS is the membrane component of Gram-negative bacteria (PAMPs) and it is an important trigger of the inflammatory response in sepsis [19, 20]. LPS is recognized by TLR-4 receptors present on the surface of immune cells. CD14 and MD2 form a complex with TLR-4 and play an important role in the recognition of LPS [21, 22]. TLR-4 is a key molecule which interacts with its ligand (LPS) to initiates inflammatory signaling pathways, hence it is a potential therapeutic target for sepsis [23]. The exact role of TLR-4 is not well understood yet. In animal studies, inhibition of TLR-4 protects mice against polymicrobial sepsis [24]. LPS (TLR-4 agonist) is derived from outer membrane of Gram negative bacteria plays a critical role in human sepsis [25]. Blocking of TLR-4 has not shown any promising therapeutic benefit in human sepsis. Inadequate response upon TLR-4 blocking in human sepsis might be due to the increase in occurrence of non-Gram

negative bacteria and fungi infections. Thus, in the early phase of sepsis, a severe infection causes excessive activation of the host immune system along with an imbalance in tissue homeostasis, which eventually causes tissue damage and multiple organ failure [13, 14].

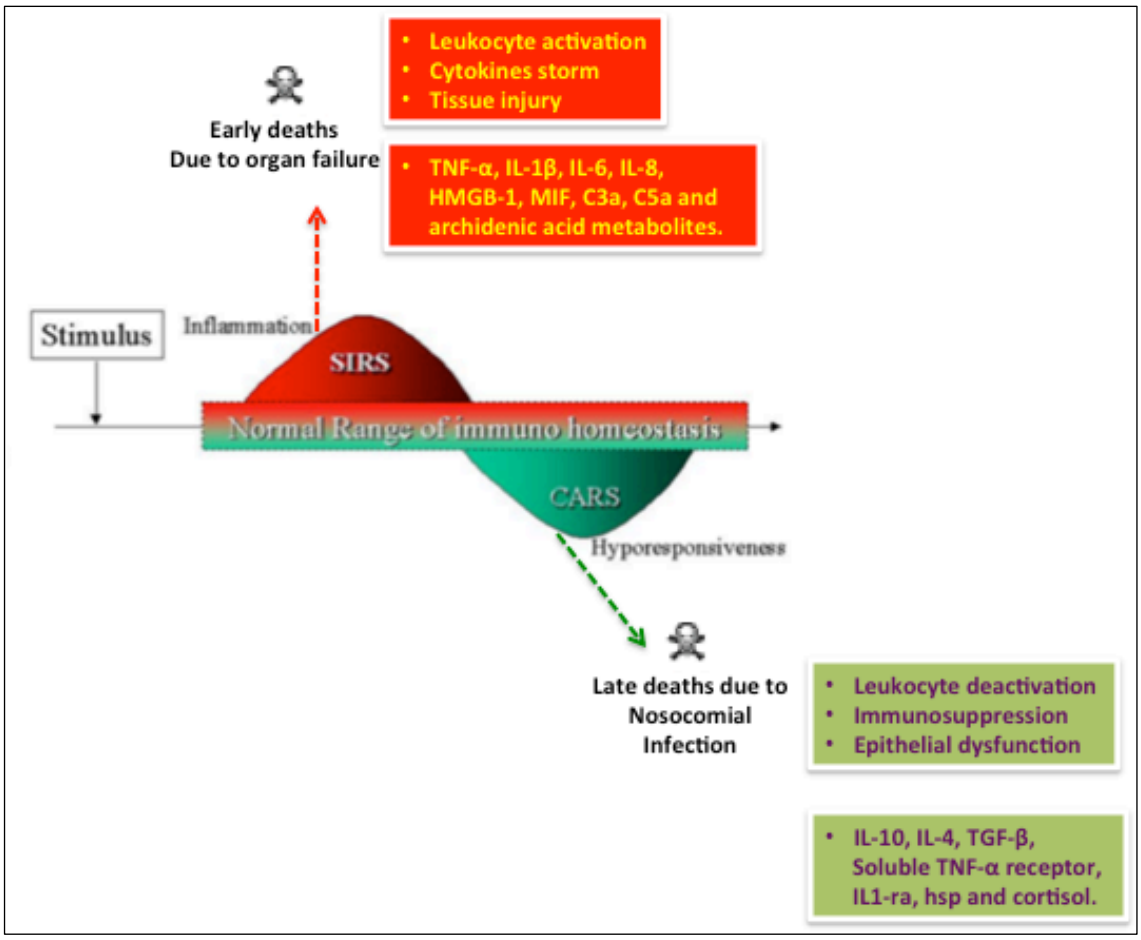

Figure 2. Immune response in sepsis: Two immunological characteristics can be seen in sepsis, these are SIRS and CARS. SIRS engages the pro-inflammatory response while CARS involves the anti-inflammatory or immunosuppressive response. SIRS commonly dominates immediately after infection, while CARS mostly appears in the later stage of sepsis. Leukocyte activation, cytokine storm, tissue injury and multiple organ failure are the common features of SIRS while nosocomial infections, immunosuppression, leukocyte deactivation, apoptosis of immune cells, epithelial/endothelial dysfunction and abundance of anti-inflammatory cytokines are the characteristics of CARS.

Adapted from: Intl art organ,25, 2002.

1.1.2.1.1 Pro-inflammatory mediators in SIRS:

During sepsis, pro-inflammatory mediators like tumor necrosis factor (TNF), interleukins (IL-6, IL-8/CXCL-8 and IL-1), nitric oxide (NO), high-mobility group protein 1 (HMGB-1), arachidonic acid derived mediators and complements help establishing inflammatory responses against infections [26]. TNF is a prime inflammatory mediator involved in the process of inflammation in severe sepsis. TNF correlates with mortality in severe sepsis patients [27]. TNF acts on the hypothalamus by crossing the blood brain barrier (BBB) and resets the temperature threshold to a lower level [28, 29]. IL-6 is mainly produced in macrophages, T cells, B cells and endothelial cells, and it is responsible for the production of acute phase proteins [30]. In sepsis, IL-8 is secreted by activated macrophages, monocytes, and Kupffer cells. It acts as a chemotactic agent for neutrophils [31]. Recently, HMGB-1 released from macrophages was recognized as a key cytokine in septic conditions. Levels of HMGB-1 elevate in the late phase of LPS stimulation and remain elevated in most patients with severe sepsis [32]. Macrophage migration inhibitory factor (MIF) is a pro-inflammatory mediator, which is released from macrophages and monocytes. MIF acts by promoting TNF expression during infection. Conversely, production of MIF is further increased by newly formed TNF [33].

Nitric oxide (NO) is a free radical that has cytotoxic and vasodilation properties. In inflammatory conditions, NO formation is induced by inducible nitric oxide synthase

(iNOS). iNOS is mainly synthesized in macrophages and the synthesis of iNOS is triggered by endotoxin, TNF, interleukin-1 (IL-1), or platelet activating factor (PAF). NO causes vasodilation and myocardial depression by reducing intracellular calcium [34]. In the early stage of sepsis, the complement system gets activated mainly via alternate pathway. Activation of the complement system generates a large amount of anaphylatoxin C5a. Complements such as C3a and C5a cause the release of histamine and act with kinin to enhance capillary permeability. C5a produces adverse effects on other systems including the coagulation cascade, TLR-4 response and cytokines release. Excessive complements levels are thought to be a contributing factor for multiple organ failure in sepsis [35].

Arachidonic acid metabolites are one of the major players in sepsis [37]. Arachidonic acid is converted into prostaglandins through cyclooxygenase enzyme [37]. The resultant major vasoactive metabolites are prostaglandinE2 (PGE2), prostacyclin (PGI1), and thromboxane A2 (TXA2). PGE2 and prostaglandinI2 (PGI2) exert a hypotensive effect [38]. PGI2 additionally exerts anti-inflammatory properties by suppression of TNF release in sepsis. However, TXA2 is a potent vasoconstrictor that plays an important role in the development of airway resistance and hypoxemia.

Table 1. Pro-inflammatory mediators during inflammatory stage of sepsis [36]

Pro-inflammatory mediators	Actions
TNF	Activation of PMN and endothelial cells
IL-6	Acute phase protein production, T and B cell proliferation.
IL-1	T cell and macrophage activation
IL-8	Chemoattractant for neutrophil and T cells
HMGB-1	Hypotension and shock
C3a-C5a	Histamine release, increase capillary permeability.
MIF	Increases TNF and TLR-4 expression
NO	Smooth muscle relaxation, activation of platelets and endothelial cells
PAF	Histamine release, vasodilation
PEG2, PGI2	Vasodilation
TXA2	Increased pulmonary resistance
LTC4, LTD4, LTE4	Increased pulmonary capillary permeability, bronchospasm

Leukotrienes are formed from arachidonic acid by the action of lipoxygenase. Leukotrienes such as LTC4, LTD4, and LTE4 (also called slow-reacting substances of anaphylaxis) may cause pulmonary capillary permeability and bronchospasm. Another leukotriene like factor, LTB4, is a potent chemotactic factor for polymorphonuclear leukocytes, eosinophils, and monocytes. Unlike other leukotrienes, LTB4 does not cause bronchospasm [39].

Synthesis of PAF takes place in endothelial cells, macrophages, and neutrophils. PAF enhances the expression of adhesion molecules and activates platelets to release adhesion molecules, histamine and serotonin. These mediators play a key role in the migration of

polymorphonuclear cells at the site of infections that ultimately leads to bacterial killing as well as tissue injury. It has been speculated that PAF might be the one among the other responsible elements for the acute pathogenesis of multiple organ dysfunction in sepsis[40].

The acute phase response is a series of secretion of proteins predominantly by the liver in response to inflammation or tissue injury. Interleukin-6 (IL-6) is an inducing factor of the acute-phase proteins. C-reactive protein is one of the acute phase proteins and acts as a protective agent against infection [41]. It binds to ribonucleoproteins, which helps in the clearance of nuclear material of injured and necrotic tissues. Another important acute phase protein is serum amyloid, which prevents the initiation of nuclear specific autoimmunity[42] and also causes disturbance of cholesterol metabolism.

Pentraxin 3 (PTX3) is a soluble PRR, and important component of innate immunity [43]. It is an acute phase protein, which helps in the removal of PAMPs and DAMPs during infections [43, 44]. Studies showed that the level of pentraxin3 in severe sepsis is a prediction of organ failure [45]. However, a recent study from Hamakubo et al. [46] demonstrated the protective role of pentraxin3 in sepsis. According to authors, pentraxin3 prevents sepsis-induced cytotoxicity by binding with extracellular histones.

Protease inhibitors α1-antitrypsin and α1-antichymotrypsin play a key role in neutralization of neutrophil associated proteases in sepsis. Inhibition of neutrophils proteases protects tissue matrix from digestion. Protease inhibitors also play an

immunomodulatory function such as reduction of the production of pro-inflammatory cytokines [47] and inhibition of neutrophil activation [48]. Overall, these protease inhibitors act as protective factors during inflammatory condition.

In sepsis, microbes/microbial components cause activation of coagulation. Initiation of the coagulation cascade is mainly mediated by tissue factor (TF) expressed on monocytes and macrophages [49]. In sepsis, coagulation is also activated by TNF and interleukins [50]. Pro-coagulant activity is further enhanced by inhibition of natural anti-thrombotic protein called protein Ca [51]. IL-6 suppresses the activity of anticoagulant factor protein Ca. Treatment with protein Ca in septic patients reduces TNF, IL-6, coagulation markers, and endothelial cell expression of adhesive molecules in a dose-dependent manner which may account for the high mortality rate in patients with lower protein Ca level [51].

1.1.2.1.2 Macrophages in SIRS

Macrophages are a primary source of inflammatory mediators in sepsis. These cells produce large amount of chemokines, cytokines, oxygen free radicals and lipid mediators [2]. Macrophages recognize not only PAMPs, but they also sense DAMPs through various receptors such as TLR, CD14, complements and Fc receptors [52, 53]. Apart from inflammatory mediators release, macrophages are also involved in phagocytosis and clearance of microorganisms.

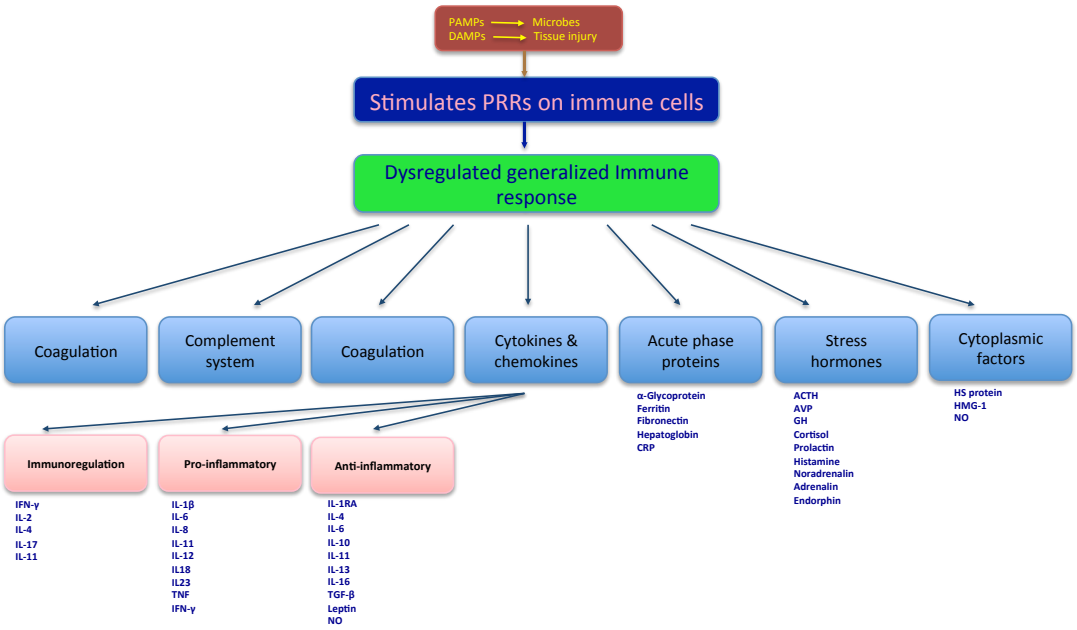

Figure 3. Pro-inflammatory and anti-inflammatory mediators in sepsis: During sepsis, microbial components (PAMPs) and injured tissues components (DAMPs) stimulate various patter recognition receptors (PRRs) on the immune cells, resulting initiation of intense immune response from immune cells network. Initiation of an immune response causes the release of pro-inflammatory and immunoregulatory factors belonging to the coagulation pathway, complement system, cytokines, chemokines, acute phase proteins, stress hormones and cytoplasmic factors. CRP= C reactive proteins, ACTH= Adrenocorticotropic hormone, AVP= Arginine vasopressin, GH=Growth hormone, HMG-1=High mobility group protein-1, HS protein=Heat shock protein, NO=Nitric oxide, IL-1RA=Interleukin-1 receptor antagonist.

In the later stage of sepsis, circulating monocytes undergo reprogramming to produce more anti-inflammatory mediators, which prevent an uncontrolled systemic immune response [54]. Thus, macrophages are the key immune cells responsible for initiation as well as regulation of innate immune response.

1.1.2.1.3 Neutrophils in SIRS

Neutrophils are key innate defense cells that are recruited immediately at the site of infection [55]. The release of interleukin (IL-8/CXCL-8) through macrophages pulls

neutrophils towards infection or an injured site [56]. After the arrival of neutrophils at the site of infection, they adopt various mechanisms to combat and neutralize infections. Neutrophils express TLRs, which sense microbes and microbial components as well as endogenous injured tissue debris during sepsis [57, 58]. Neutrophils also use other strategies such as phagocytosis of pathogens, releasing of proteases, generation of reactive oxygen species and extrusion of neutrophil extracellular traps (NETs) to limit microbial infection [59, 60]. NETs are complex fiber like structures composed of chromatin, bound to granular and selected cytoplasmic proteins. The process of trapping and killing of microbes through NETs is called NETosis [61]. Direct exposure of neutrophils to microbes or microbial products [62] in plasma of septic patients [61] leads to the formation of NETs. Trapping of microbes through NETosis facilitates the effective exposure of inflammatory mediators to microbes [63].

1.1.2.1.4 Lymphocytes in SIRS

B and T lymphocytes play a significant role in early phase of sepsis. Studies suggest that B cells increase early innate immune responses during sepsis. Specific B cell depleted mice exhibit reduced inflammatory cytokines expression and less survival [64]. Another report demonstrated that marginal zone (MZ) B cells initiate systemic inflammatory responses against infection via IL-6 and antibodies production in a mouse model of sepsis [65].

T cells are members of the adaptive immunity system and play an important role in various immune disorders. T cells are the key cells of adaptive immunity and receive

signaling from innate immune cells to initiate specific immune responses [66]. Antigens are phagocytized by APCs and chopped into small pieces. The processed antigen is then presented to T cells via major histocompatibility complex (MHC) class II or MHC class I. For the development of cell mediated and humoral adaptive immunity, T cells must be activated and the activation of T cells involves many signaling pathways. Cytokines released by T cells delineate the differentiation of the T cell subpopulations. The kind of T cells depends on the nature of the presented antigen, type of antigen presenting cells (APCs), and the microenvironment and cytokine milieu of the antigen presentation site. Various subsets of T cells include $CD4^+$ T helper (TH), $CD8^+$ cytolytic T cells and $CD4^+$ $CD25^+$ regulatory T cells (Treg) [67]. TH cells are subdivided into TH1, TH2, TH17 and Treg cells. TH1 lymphocytes produce cytokines like IFN-γ and IL-2 which augment cellular inflammation. TH2 lymphocytes release IL-4, IL-5, IL-6, IL-9, IL-10 and IL- 13 and boost humoral immunity [68]. TH17 kills extracellular microbes by producing IL-17 and IL-22. Treg cells produce TGF-β and IL-10, and their function is to dampen undesired immune activation. $CD8^+$ are cytotoxic cells, which identify and kill the cells infected with pathogens [69]. Very little information is available regarding lymphocytes in SIRS (severe sepsis).

A recent study from Jia Li et al, 2015 [70], described the role of T lymphocytes in severe sepsis. In this study, authors have observed that both TH1 (pro-inflammatory and TH2 (anti-inflammatory) activity was increased during severe sepsis. However, they found an uncontrolled balance between TH1 and TH2 immunity in severe sepsis. This balance was

found to be dysregulated in septic shock phase, where immunosuppression (TH2) was predominated. They also reported increased expression of t-bet and GATA3 in severe sepsis [70]. Collectively, like the innate immune system, the adaptive immune system also induces strong pro-inflammatory as well as anti-inflammatory activity simultaneously during infection. This kind of pro- and anti-inflammatory balance is required for effective eradication of microbes as well as controlling of exaggerated inflammation. Any disturbance in this equilibrium leads to either improper killing of microbes or tissue injury.

1.1.2.1.5 Regulatory T cells (Tregs) in SIRS:

It is well known fact that Tregs suppress immune responses and maintain homeostasis during inflammatory state [71]. Treg plays an important role in dampening of inflammatory response in sepsis. During sepsis, Tregs get stimulated either by microbes / microbial product or by other immune cells such as M2 macrophages [72]. Stimulation of Tregs increases in the production of anti-inflammatory cytokines while reduces the synthesis and release of of pro-inflammatory cytokines [73]. Recent studies demonstrated that adoptive transfer or expansion of Treg cells rescue mice from LPS as well as cecal ligation and puncture (CLP) induced sepsis [74, 75]. Collectively, Tregs increase survival of mice against experimental sepsis by diminishing inflammatory responses.

1.1.2.1.6 Natural killer (NK) cells in SIRS.

Natural killer (NK) cells are the key source of inflammatory cytokines against pathogens. They also recognize danger signaling from the pathogens with the help of other innate

immune cells. Interferons or macrophage-derived cytokines are responsible for activation of NK cells during sepsis. NK cells also produce cytotoxic action against own unhealthy cells with the help of perforin and granzyme B [76]. The exact role of NK cells in sepsis is contradictory in the literature. Some reports indicate that severe sepsis patients have a higher percentage of NK cells [77]. Other studies described that the NK cell numbers were reduced in patients with severe sepsis [78]. Higher NK counts reported to act as a protective factor against mortality [77]. While higher cytotoxicity of NK cells in sepsis patients is directly proportional to higher mortality and organ failure [79]. In case of animal studies, depletion of NK cells has been reported to provide protection against LPS as well as bacterial induced sepsis models [80, 81]. However, the exact role of NK cells in human sepsis is not clear.

1.1.2.2 Compensatory Anti-inflammatory Response Syndrome (CARS)

Any exaggerated inflammatory response makes the transition to a hypo-inflammatory state, CARS, to abrogate the harsh effects of persistent inflammation after clearing infections [82]. CARS can be seen in the later stages of sepsis and also in patients who have survived the initial phase of sepsis. CARS is described by the presence of leukocyte deactivation, immunosuppression, endothelial/epithelial dysfunction, and secondary infections. It is believed that CARS develops immediately after infection/injury but this condition is masked by SIRS predominance. However, CARS appears in the later stages of sepsis owing to a decline in SIRS dominancy [83]. Multiple mechanisms are thought to be involved in the transition from inflammatory to hypo-inflammatory state to abrogate

the uncontrolled cytokine responses that lead to organ dysfunction and shock.

The first possible mechanism is gene-specific epigenetic reprogramming and the second is to changes in the NADH/NA^+ ratio in immune cells. Gene-specific epigenetic reprogramming happens within a short time after TLR activation through NF-κB-p65 dependent silencing of pro-inflammatory genes and activation of anti-inflammatory pathways [84]. After TLRs activation, there is an energy burst, which causes changes in the NADH/NAD^+ ratio that result in an alteration in cellular metabolism leading to "cellular hibernation". These metabolic changes work with epigenetic reprogramming in the pro-inflammatory phase, which opens the door for a hypo-inflammatory phase of sepsis [85]. Immediately after the onset of the CARS phase, a stage of immune dormancy dominates. During this phase three main processes known for the immune dysfunction can be recognized. Those are:

- Immune cells anergy
- Shift to anti-inflammatory cytokines
- Apoptosis of immune cells

1.1.2.2.1 Immune cells anergy

It has been commonly observed that immune cells anergy is a major problem in sepsis. Abnormal responses to microbial stimuli are usually seen in cells of the innate and adaptive immune cells.

The major cause of immune dysfunction during sepsis is an improper activation of T cells and their diminished ability to release cytokines after exposure to antigen [86]. T-bet and GATA3 are the important transcription factors that regulate activation of TH1 and TH2 cell response respectively. In sepsis, expression of T-bet and GATA3 decreases which in turn impairs the function of TH1 and TH2 cells, respectively [87]. The TH17 response also decreases following diminished expression of retinoic acid receptor-related orphan receptor-γ t (RORγ t) in sepsis [88]. In contrast to apoptosis of other TH subsets, T cells called Tregs, expand efficiently with less apoptosis in sepsis, which enhances the suppression of immune responses against incoming infections [89].

Human studies indicated that severe sepsis patients exhibit increase in percentage of Treg while decrease in number of T effector cells. Increase in expression of anti-apoptotic factor (BCL-2) and also elevated level of Tregs inducers (heat shock proteins and histones) are responsible to increase in percentage of Tregs [90]. Tregs suppress immune system by variety of mechanisms. Tregs release IL-10 and TGF-β that suppress proliferation and release of TNF from TH1 cells [73]. Tregs also increase FasL expression that causes apoptosis of immune cells and also reduce release of pro-inflammatory cytokines[73]. Tregs interrupt in the most of the immune cells functions such as increase in Tregs causes disturbance in T effector cells proliferation and functions [91]. Tregs also suppress the function of neutrophils and monocytes [92]. Overall, Tregs are key players in severe sepsis associated immunoparalysis.

During CARS, macrophages/monocytes become tolerant to microbes by downregulating

HLA-DR and co-stimulatory molecules necessary for antigen presentation. The degree of decrease in CD14 and HLA-DR co-expression in macrophages/ monocytes correlates with severity of immunoparalysis in sepsis [93].

Neutrophils are important innate cells which control invading pathogens. Most of the neutrophils undergo apoptosis within one day after their release from bone marrow [94]. Neutrophils from animals with experimental sepsis exhibit impaired bacterial clearance, and reduction in ROS production and chemotaxis [95].

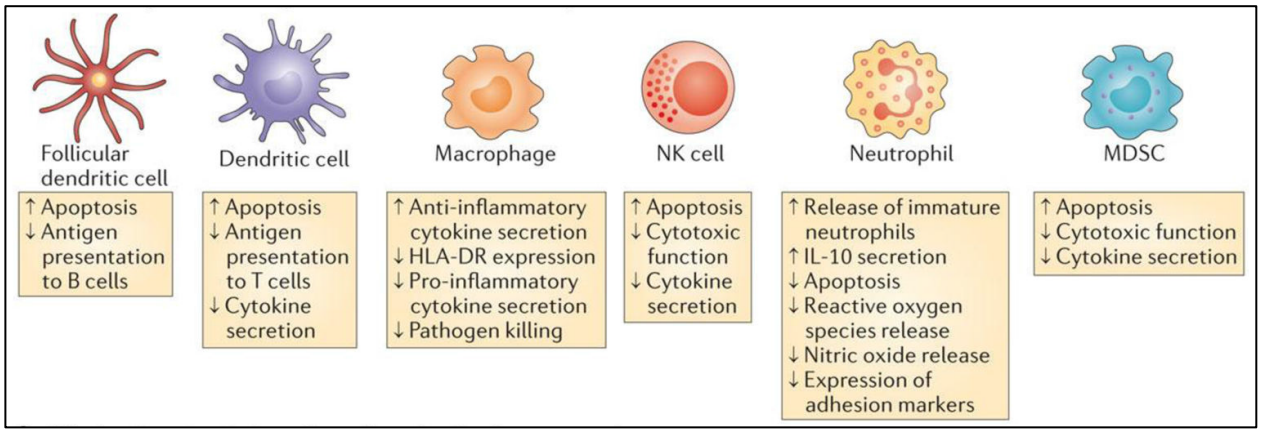

Figure 4 Effect of protracted sepsis on innate immune system: Persistent systemic infection in sepsis, shifts immune cells from pro-inflammatory to anti-inflammatory characteristics and also it initiates apoptotic pathways. An immunosuppressive phenomenon affects both innate and adaptive immune cells. Innate immune cells such as macrophages/monocytes, dendritic cells, NK cells and neutrophils become more prone to apoptosis in sepsis. The ability to produce pro-inflammatory cytokines, kill pathogens and express HLA-DR of macrophages/monocytes gets severely affected, while they produce an abundance of IL-10. Production of ROS, NO and expression of adhesion molecules in neutrophils also gets affected. Low expression of HLA-DR on APCs (macrophages/dendritic cells) decreases the antigen presentation ability to T cells, which in turn diminishes T cells activation.

Adapted from: *Nature Reviews Immunology 13, 862–874 (2013).*

Decreased expression of CXC-chemokine receptor-2 (CXCR2) may lead to loss of

chemotactic activity [96]. Some studies indicate that defects in TLR signaling might be the cause of improper immune responses in neutrophils [96]. During septis, neutrophils' ability to clear bacteria, production of oxygen species and effective TLR signaling gets diminished [95] which leads to nosocomial infections [97].

Dendritic cells (DCs) are the innate immune cells that act as APCs to activate T cells against the specific microbe. Similar to macrophages during sepsis, DCs also expresses low levels of HLA-DR and produce increased amounts of IL-10 57 [98]. DCs in septic patients are unable to initiate effective T cells activation, but induce Treg proliferation and T cell anergy [99]. Both splenic and lymph node dendritic cells displayed decreased ability for IL-2 synthesis [100].

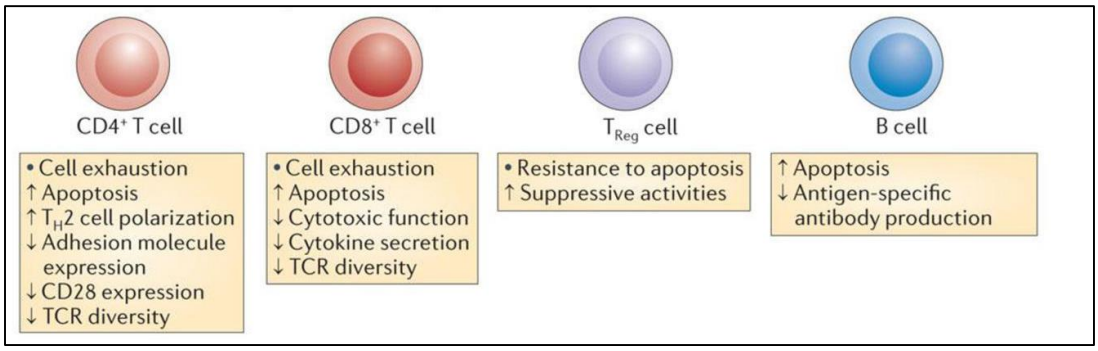

Figure 5. Effect of protracted sepsis on the adaptive immune system: In severe sepsis, lymphocytes (T and B cells) are vanished by apoptosis while Tregs are resilient to apoptosis that further accentuate the immunosuppressive effect of sepsis. Surviving CD4+ and CD8+ cells from apoptosis shifts from a pro-inflammatory to an anti-inflammatory state or an anergy state that eventually diminishes the immune response against invading pathogens.
Adapted from: *Nature Reviews Immunology 13, 862–874 (2013).*

NK cells are also key players of the innate immune system and play an important role in

the defense against intracellular microbes. They help to detect cells infected with microbes and kill immediately through perforin and granzyme B. NK cells also express TLR receptor and release cytokines upon binding to TLR agonists [76]. In polymicrobial sepsis, cytokine production by NK cells upon TLR agonist stimulation is impaired. This behavior of NK cells suggest that they become tolerant to TLR agonist and acquire similar features as endotoxin tolerance by macrophages/monocytes [101].

1.1.2.2.2 Shift to anti-inflammatory cytokines

Immediately after an insult, there is the initiation of the inflammatory process occurring by the release of various inflammatory mediators including pro-inflammatory cytokines and chemokines. During inflammation, an anti-inflammatory shift starts as a feedback mechanism against the pro-inflammatory environment by the production of immunosuppressive cytokines such as IL-10. A high level of TNF is responsible for stimulating monocytes/macrophages to produce IL-10 [102]. Evidence indicates that in macrophages, PD-1 receptor is responsible for the production of an immunosuppressive state by releasing IL-10 that subsequently impairs T cell proliferation [103, 104]. A number of other cytokines involved in the immunosuppressive response in sepsis-related immunoparalysis are mentioned in Table 2.

1.1.2.2.3 Apoptosis of immune cells

Apoptosis is the common phenomenon occurring in the later stages of sepsis and severely depletes innate and adaptive immune cells leading to immunosuppression [104]. Sepsis-induced apoptosis occurs by two pathways, first is the death receptor pathway, and

second is a mitochondrial-mediated pathway. Both pathways converge to activate common caspases. The death receptor pathway is an extrinsic pathway. This pathway is activated by death inducing factors (death receptor ligands) outside the cell. Binding of a death receptor ligand with a death receptor recruits an intracellular fas-associated death domain (FADD) that activates caspase-8. Activation of caspase-8 further activates caspase3/7, which in turn induce apoptosis. The mitochondrial-mediated pathway is also known as intrinsic apoptotic pathway. This pathway is induced by damage-associated intracellular components. Induction of the mitochondrial-mediated pathway increases mitochondrial permeability to release cytochrome c (Cyt c) from mitochondria.

Table 2. Anti-inflammatory mediators during the immunosuppressive stage of sepsis[36]

Anti-inflammatory mediators	Actions
IL-10	Suppression of IFN, IL-1, and macrophage functions
PGI2	Down-regulation of TNF
Soluble TNF receptor	Blockage of TNFα receptors
IL-1 receptor antagonist	Competitive binding to IL-1 receptors, blocking the action of IL-1
Heat shock proteins	Enhanced expression of IKK-β gene, negative feedback on NF-κB activation, inhibition of TNF and IL-1
Phosphatases	Dephosphorylation of cytoplasmic substrates, reduced NF-κB-dependent TNF production, deactivation of leukocytes and endothelial cells
Cortisol	Inhibition of NF-κB, reduction in production of TNF, IL-1, IL-6, eicosanoids, NO, liberation of heat shock proteins.

Releasing Cyt c triggers the activation of a series of caspases9/3/7 and leads to apoptosis [105, 106]. The Bcl-2 family is a conserved group of structurally closely related proteins. These proteins regulate cell death by induction or inhibition of apoptosis. Generally, Bcl-2 family molecules are divided into two groups: pro-apoptotic and anti-apoptotic. Anti-apoptotic family members are Bcl-2, Bcl-x¡, Bcl-w, myeloid cell leukemia-1 (Mcl-1) and Al. The pro-apoptotic Bcl-2 family is subdivided into two sub-groups: BH1-3 multidomain and BH3. Bcl-2-associated X protein (Bax) and Bcl-2 homologous antagonist/killer (Bak), are members of the BH1-3 multidomain sub-group and are important for induction of apoptosis [107]. Apoptosis-inducing factors activate the apoptotic pathway either by inducing pro-apoptotic Bcl-2 family proteins, inhibiting anti-apoptotic Bcl-2 proteins, or both [107].

1.1.2.2.4 Macrophages in sepsis

Macrophages perform a key role in immunity, tissue repair, maintaining homeostasis, and development [108, 109]. Macrophages perform multiple immunological functions such as triggering inflammation, phagocytosis, and microbicidal activity to fight an infection and resolution of inflammation.

Macrophages exhibit phenotypic and functional plasticity [110, 111]. Under normal physiological conditions, macrophages modulate themselves in terms of morphology and functions in the presence of various tissue microenvironments to become a distinct resident population, such as Kupffer cells in the liver, alveolar macrophages in lungs, lamina propria macrophages in the gut, and microglia in the brain. Under inflammatory

conditions, macrophages adopt two-district phenotypes according to type and duration of stimulation. Two district polarized states of macrophages known as "classical" M1 and "alternative" M2 can be distinguished. The polarization of macrophages depends on the kind of cytokine environment around, such as the presence of IFN-γ or IL-4/IL-13 causes polarization of M1 or M2 phenotype, respectively [108, 112, 113]. M1 macrophages release a large amount of pro-inflammatory cytokines (IL-1β, TNF, IL-6), and a meager quantity of IL-10. On the contrary, M2 macrophages express high levels of IL-10 and little pro-inflammatory cytokines [72]. Similar to inflammatory cytokines variability, macrophages (M1 and M2) display diverse chemokine production and chemokine receptor expression. M1 macrophages express CXCL9 and CXCL10 chemokines, which attracts Th1 lymphocytes, while M2 produces TH2 attracting chemokines such as CCL17, CCL22 and CCL24 [114, 115]. M1 macrophages activity is predominant in the inflammatory state while M2 macrophages play an important role during resolution of inflammation. The main functions of M1 macrophages are to promote the TH1 response, efficient antigen presentation, killing of intracellular pathogens, tumor destruction and tissue damage. M2 macrophages promote the TH2 response, encapsulate and clear parasites, tumor promotion, tissue remodeling and immunoregulation [72].

During persistent sepsis, apoptosis of T-, B- and dendritic cells occur in contrast to monocytes or macrophages [116, 117]. This indicates that macrophages are the key regulators of the immune response during sepsis and might be a potential therapeutic target for immune modulation. However, during sepsis "adaptation" or "re-programming"

processes of macrophages/monocytes lead to the development of immunosuppressive features (Figure 6). This immunosuppressive phenomenon can be well described by "Endotoxin Tolerance" (ET) [118].

Parental cells of macrophages (Monocytes) from sepsis patients fail to produce pro-inflammatory cytokines such as TNF upon endotoxin challenge and they display characteristics of endotoxin tolerance [119]. Macrophages from sepsis patients exhibit downregulation of MHC class II (HLA-DR), CD86, and CIITA [120]. They express more anti-inflammatory factors such as transforming growth factor-β (TGF- β), IL-1 receptor antagonist (IL-1RA), secretory leukocyte protease inhibitor (SLPI) and MIF [54, 83, 121].

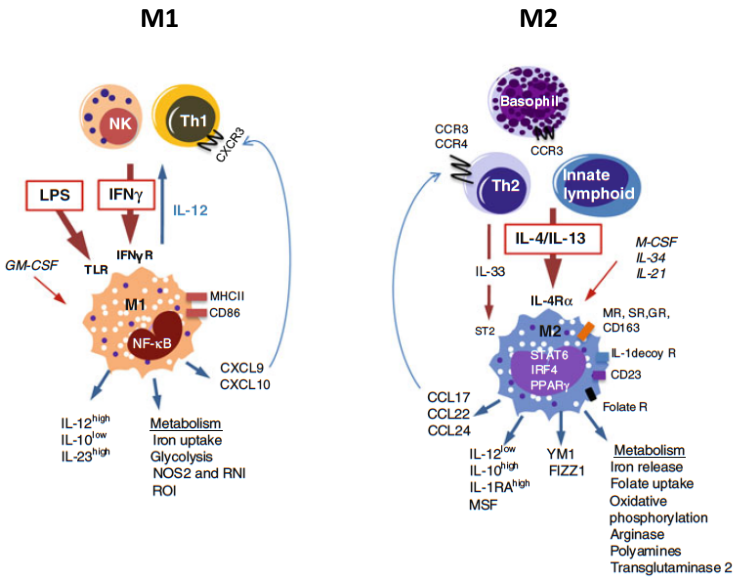

Figure 6. Polarization of macrophages. Upon exposure to a specific microenvironment, macrophages switch to either M1 or M2 state. Exposure of LPS, IFN-γ or GM-CSF gives rise to M1 macrophages while presence of Il-4/IL13, IL21, IL33 diverts macrophages towards the M2 state. Macrophages release

various factors that help to polarize TH cells such as M1 macrophages into TH1 and M2 macrophages into TH2.
Adapted from: *Macrophages biology and role in the pathology of diseases by S.K.Biswas, 2014.*

Overall, the down-regulation of inflammatory cytokines, upregulation of anti-inflammatory factors and increased phagocytic capacity indicates M2 like behavior of macrophages in sepsis [122]. However, it is difficult to assign the definite polarization state of macrophages due to overlapping of pro-inflammatory and immunosuppressive features. But certainly, presented data proves that macrophages have characteristics of functional and phenotypic plasticity during the course of sepsis.

Macrophages/monocytes from various organs such as lung, peritoneum, spleen, microglia macrophages, and Kupffer cells exhibit different characteristics in terms of their functions, gene expression and surface markers [123, 124]. Compared to blood monocytes, intestinal macrophages are unable to produce pro-inflammatory cytokines upon LPS stimulation [125]. The mechanism of TNF production upon bacterial stimuli is different in monocytes, peritoneal macrophages, and alveolar macrophages [126]. Alveolar macrophages are sensitive while blood monocytes and peritoneal macrophages develop tolerance upon repetitive exposure of endotoxin [127, 128]. Peritoneal macrophages express TLR-9 and also produce IL-10 in response to endotoxins, while alveolar macrophages do not express TLR-9 and do not produce IL-10 upon endotoxin stimulation [129, 130].

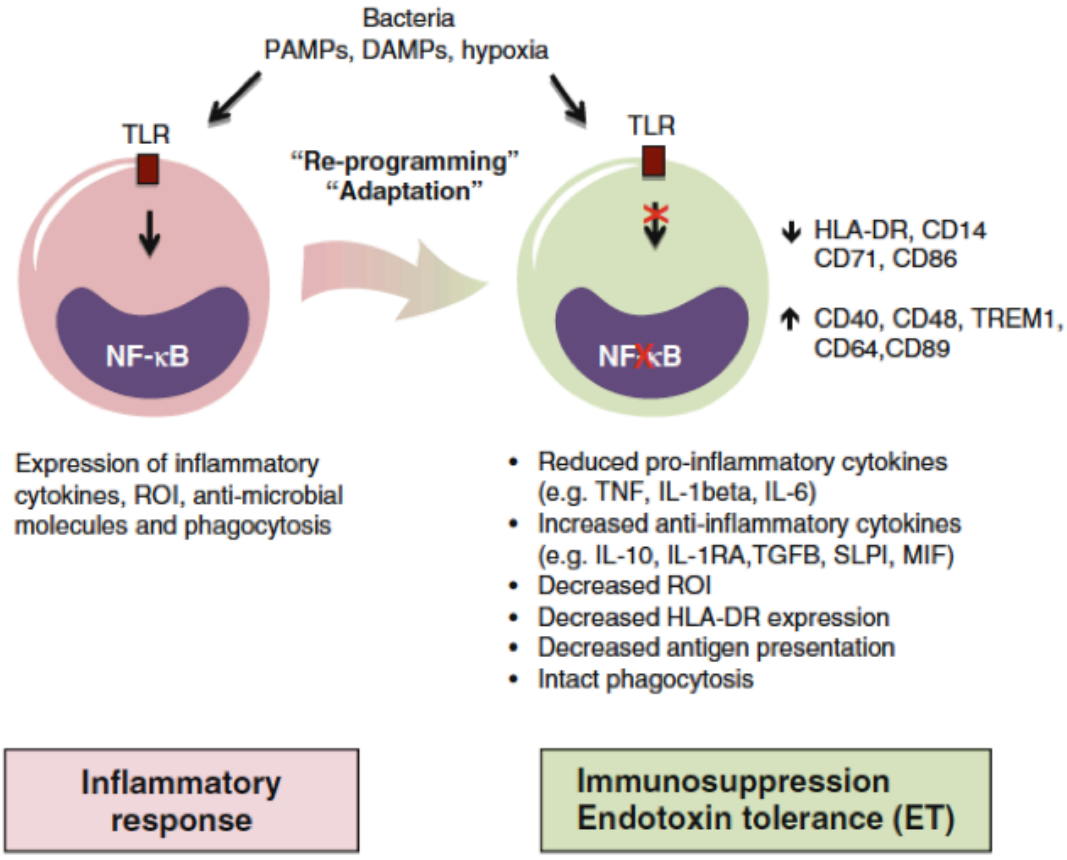

Figure 7. Functional re-programming of monocytes/macrophages in sepsis. In sepsis, monocytes/macrophages recognize PAMPs and DAMPs that initiate an immune response by releasing pro-inflammatory cytokines and generation of reactive oxygen species (ROS). Persistent systemic infection makes macrophages/monocytes to undergo gene re-programming that ultimately causes endotoxin tolerance. Gene-reprogrammed macrophages lose the ability to produce optimal pro-inflammatory cytokines, but they are able to express more anti-inflammatory cytokines upon a subsequent challenge of endotoxins.

Adapted from: *Macrophages biology and role in the pathology of diseases by S.K.Biswas, 2014.*

Recruitment of adaptor proteins activate various downstream signalling. MyD88 and TRIF signalling activates MAPK which in turn activate AP-1 transcription factor to

translocate in nucleus. In resting stage NFκ-B exist as making complex with IκB. Thus, IκB prevents translocation of NFκ-B to nucleus for gene transcription. Activation of TLR-4 signaling causes degradation of IκB which in turn make NFκ-B to translocate in to nucleus. Translocation of AP-1 and NFκ-B initiate expression of pro-inflammatory cytokines (IL-6, TNF) [134].

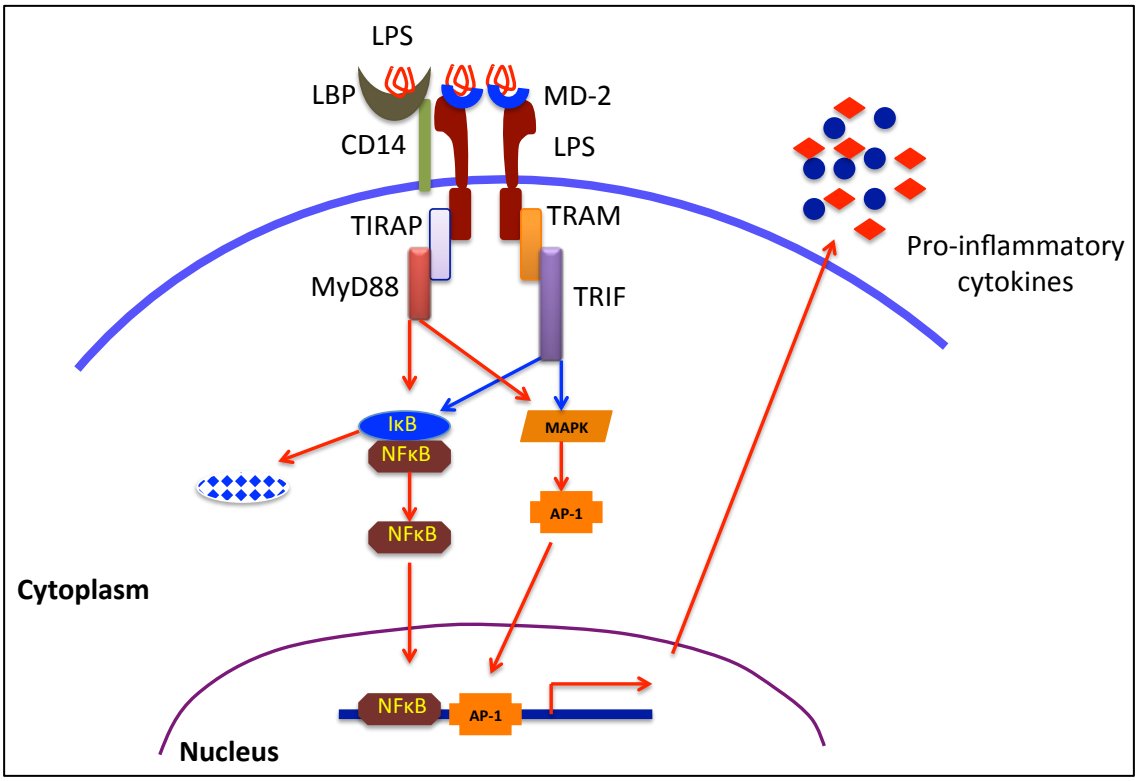

Figure 8 LPS/TLR-4 signaling: LPS is mainly recognized and transferred to TLR-4/MD-2 complex by LBP and CD14. Binding of LPS with TLR-4/MD-2 causes activation of TIR that recruits various adaptor proteins including MyD88. Recruitment of adaptor proteins activates transcription factors to translocate into the nucleus and to initiate pro-inflammatory cytokines gene transcription.

1.2 Semaphorins

Semaphorins are a versatile family of secreted and cell membrane bound glycoproteins. Secreted molecules act at a distance from the site of secretion while membrane-bound semaphorins are attached to the cell surface. Therefore, membrane-bound semaphorins act in a contact dependant fashion. More than 30 semaphorins have been identified and they are categorized into eight distinct classes on the basis of amino acid sequence and structural component similarity [136, 137]. Invertebrates express semaphorin class 1 and 2, virus encodes class 8 semaphorins, while vertebrates expresses class 3 to 7. Semaphorins class 2, 3, and 8 are secreted proteins while other members are glycosylphosphatidylinositol (GPI)-linked or transmembrane proteins (classes 1, 4, 5 and 6). Semaphorins range from 400-1000 amino acid in size. Each protein is designated by a letter code like Sema3E and Sema3A.

Semaphorins are made up of various domains. First there is the extracellular "Sema" domain, which consists of 400 amino acids and semaphorins activity is primarily regulated by this "Sema" domain [138, 139]. The second domain is a cysteine rich receptor binding domain PSI (plexin, semaphoring and integrins). An additional important structural feature of the semaphorins is the presence of a single immunoglobulin (Ig)-like domain, which consists of a set of thrombospondin type I repeats or a highly charged carboxy-terminal tail. Semaphorin class 2, 3, 4 and 7 have single Ig like domains whereas class 5 semaphorins have thrombospondin domains. Some semaphorins undergo proteolytic cleavage, and most cleaved fragments have been shown

to have modified potency compared to parent molecules. The process of proteolytic cleavage might be involved in functional regulation of semaphorins [140-142].

1.2.1 Semaphorin Receptors

Semaphorins are multifunctional proteins essential for various physiological and pathological processes. These molecules interact with receptors, leading to different kinds of intracellular signaling events. Two main families of semaphorin receptors have been identified: the plexins (Plxns) and neuropilins (Nrps) [143].

1.2.1.1 Plexins

Plexins are the main family of receptors and these are divided into four groups (A–D) based on overall homology. Plexins are subdivided into four types that are one A-type, three B-type, one C-type, and one D-type. Individual plexins are designated according to number codes like Plexin-D1 and Plexin-B1. Plexins were first identified for their cell adhesion properties. Structurally, plexins are similar to semaphorins. Apart from "Sema" domains they also consist of three IPT (Ig-like, plexins and transcription factors) domains and 2-3 PSI (a cysteine rich motif) domains towards the receptor binding site. The intracellular domain of plexins shares structural similarity with the GAP domain, which is highly conserved in different plexins.

1.2.1.2 Neuropilins

There are two types of neuropilins, namely NRP1 and 2 [140-142]. They are highly conserved transmembrane proteins and mainly act as a co-receptor with plexins [144]. Neuropilins have short cytoplasmic domains (≈ 40 amino acid residue) and were first recognized as a neuronal adhesion molecule with their distinct role in neuronal development [145, 146].

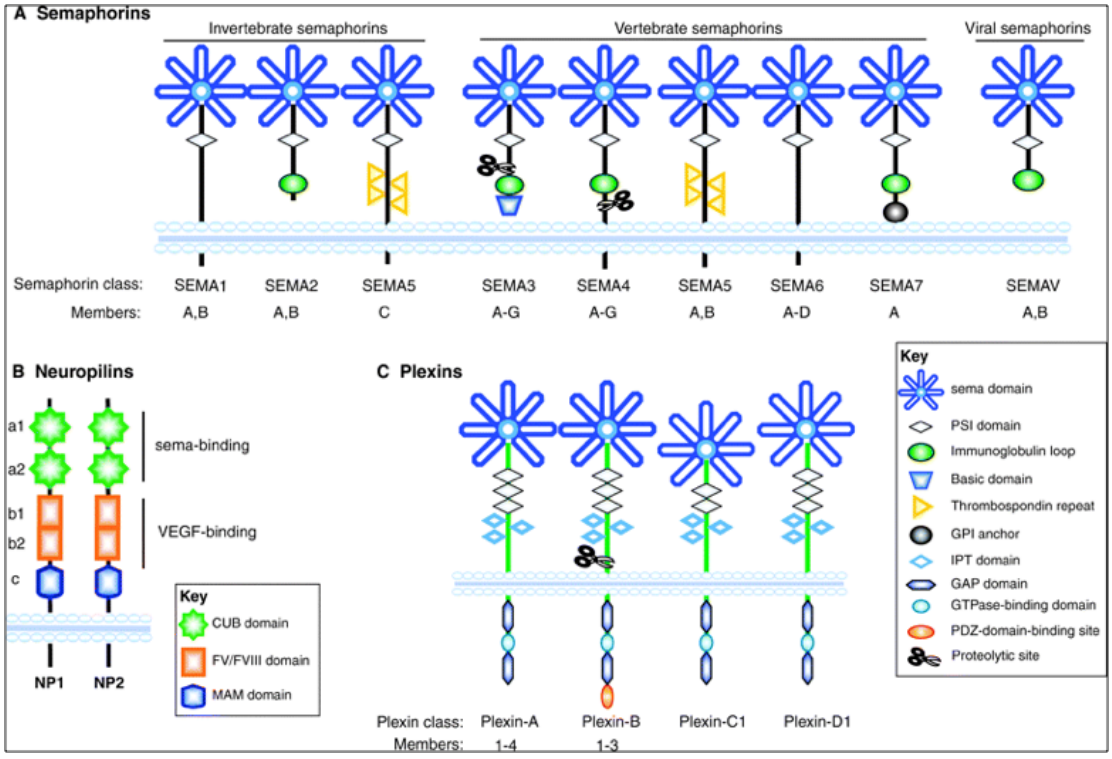

Figure 9. Semaphorins and their receptors: (A) Semaphorins mainly consist of a sema domain and a PSI domain. Additionally, semaphorin class 2, 3, 4, 7 and 8 have Ig (immunoglobulin) domains. Only class 5 comprises of thrombospondin repeats. Class 2, 3, and viral semaphorins are secreted proteins while class 1, 5, 4 and 6 are transmembrane molecules. Class 7 semaphorin is attached to the membrane via glycophosphatidylinositol (GPI). Some of the members of class 3 semaphorins and Sema4D undergo proteolytic cleavage by furin-convertases. Semaphrins bind with two kinds of receptors to initiate intracellular signaling. These receptors are neuropilins (Nrp) and plexins. (B) There are two types of

neuropilins Nrp1 and Nrp2. Neuropilins consist of two CUB domains (a1 and a2 domains) that mainly interact with semaphorins. A second domain helpful in the interaction with VEGF is FV/FVIII coagulation factor-like domains (b1 and b2 domains) and a third domain is the MAM domain (c domain). (B) Plexins are the main receptors of semaphorins. These receptors have structural similarity with semaphorins. The molecular structure of plexins includes one sema domain, 2-3 PSI domains and three IPT domains. The intracellular part of plexins mainly contains two GTPase-activating proteins (GAP) domains attached with one GTPase binding domain. Sub family-B of plexins additionally contain PDZ domain binding site. Similar to semaphorins, plexins also undergo proteolytic cleavage by furin-convertases.
Figure adapted from: *Journal of Cell Science*, 122, 2009.

NRPs also cooperate with other receptors like plexins, vascular endothelial growth factor (VEGF) and TGF-β that play a critical role in development, immunity and cancer [147, 148].

1.2.2 Semaphorins and intracellular signaling

In the absence of semaphorins, plexins get auto-inhibited by binding of their N-terminal domain with half of the extracellular domain. Binding of semaphorins to plexins leads to conformational changes that block the auto-inhibition and activates plexin receptors [149]. Upon activation of plexins, there is activation of several signaling pathways.

GTPase signaling pathway

Soluble and membrane bound semaphorins bind to the extracellular domain of plexins that activate GTPase-activating protein (GAP) in the cytoplasm, which in turn modulates cellular functions. Plexin GAP domain signaling activates downstream molecules such as GTPases, protein kinases and cytoskeleton-associated proteins. 150 small GTPases of the

Ras superfamily are known in mammalians, which are important for various cellular processes[150].

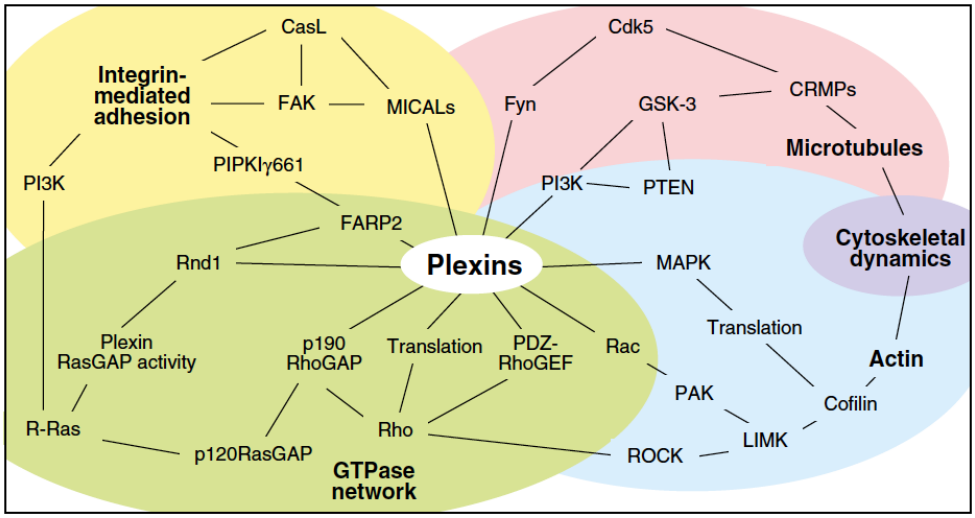

Figure 10. Plexins signaling network. Plexins are the main receptors of semaphorin proteins. Interaction of plexins with semaphorins initiates a network of signaling activities. Activation of these signaling pathways stimulates various cellular responses such as GTPase activity (green), actin dynamics (blue) and microtubule changes that eventually alter cytoskeleton dynamics. Moreover, plexins activation is also involved in integrins mediated cellular adhesion. (yellow). Figure adapted from: *Current biology, vol 19, Issue 13, 2009.*

Small GTPases are divided into five subfamilies: Ras, Rho, Arf, Rab, and Ran GTPases. Plexins are the receptors of the semaphorins, which are directly associated with several GTPases. The structure and dynamics of the cytoskeleton are mainly regulated by Rho GTPases [151]. Rho GTPases can be subdivided into several subgroups (Cdc42, Rho, Rac, Rndl, and RhoD). Rho and Rac regulate each other antagonistically; Rho activity causes retraction of neurite, while Rac produces the opposite effect [152]. GTPases signaling also inactivates integrins and causes its internalization by activation of Arf6.

Inhibition of integrins prevents endothelial cell adhesion to the extracellular matrix (ECM) that leads to filopodial retraction in endothelial tip cells [153].

MICAL(molecules interacting with CasL) signaling

MICAL consists of a flavoenzyme of the monooxygenase enzymes. In vertebrates three MICAL genes have been identified that are MICAL-1, MICAL-2 and MICAL-3 while invertebrates express only D-MICAL [154, 155]. These enzymes are oxidoreductases and they use FAD to insert one molecular oxygen into the substrate. MICALs are conserved signal transduction proteins and they have multiple domains which will interact with the cytoskeleton, cytoskeletal adaptor proteins and other signaling proteins. Apart from their binding ability to other proteins, it contains a large NADPH-dependent flavoprotein monooxygenase enzymatic domain. Sometimes MICAL generates reactive oxygen species (ROS) through oxygenase enzymes [156]. MICAL signaling in invertebrates such as *drosophila* involves sema1A/plexinA and sema2A/plexinB. Upon PlexinA/B activation, the MICAL family of redox enzymes can bind to the intracellular domain of plexins that causes generation of ROS followed by oxidation of actin filament subunits, which in turn induces F-actin disassembly, cytoskeleton remodeling and growth cone steering [157, 158]. In *Drosophila* Sema/lexins signaling in the context of MICAL is well studied. However, the involvement of MICAL in the vertebrate's sema/plexins axis is yet to be determined. Upon binding of sema3s to neuropilins/plexins, recruitment of two important classes of proteins (CRMPs and RanBPM) that bind to MICAL leads to the release of ROS and actin/tubulin cytoskeleton remodeling [154, 159].

PI3K (phosphatidylinositide 3-kinases)/Akt signaling

Plexin GAP domain signaling activates downstream molecules such as GTPases, protein kinases and cytoskeleton-associated proteins [160-162]. This signaling reduces the levels of active Ras and Rap GTPases [140, 163]. Activation of Ras decreases the activation of the PI3K/Akt signaling pathway, resulting in an increase in glycogen synthase kinase 3 (GSK3) phosphorylation leading to activation of collapse response mediator protein-2 (CRMP-2) and growth cone collapse [164].

1.2.3 Semaphorins in cellular responses

Semaphorins /plexins axis induces different cellular responses upon activation in various cell types. The cellular responses include actin and microtubule dynamics, axonal transport, protein translation and cell death responses.

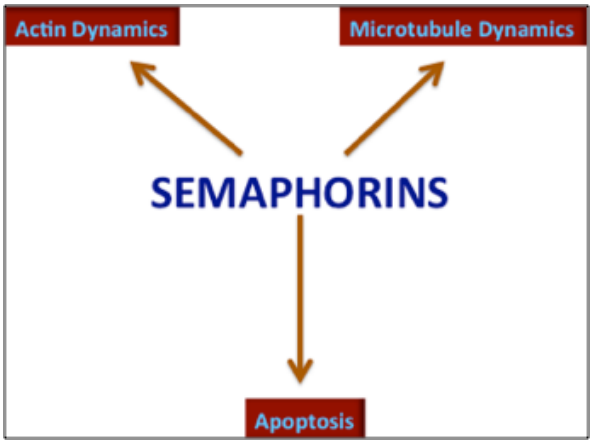

Figure 11. Effect of semaphorins on various cellular functions. Semaphorins interact with their receptors and activate various signaling pathways, which ultimately change cellular dynamics such as actin dynamics, microtubule dynamics and apoptosis.

1.2.3.1 Actin dynamics:

Semaphorins activate their common downstream target LIM-kinase (LIMK) via mainly two protein kinases called “Rho-associated kinase (ROCK)” and “p21-activated kinase (PAK)”. As a result, inactivation of ADF/cofilin that eventually causes dissociation of the actin filament [165, 166]. LIMK mediated inactivation of cofilin may be negatively regulated by signal transduction through PI3/Akt that causes activation of slingshot (SSH) family leads to blocking of inactivation of ADF/cofilin[167].

1.2.3.2 Microtubule dynamics

Cyclin dependent kinase 5 (Cdk5) and GSK3 are intracellular mediators of Sema3A [168, 169]. Cdk5 activation is regulated by upstream kinases called Fyn and Cdk5 that directly phosphorylate CRMP-2 resulting in decreased affinities between CRMP-2 and p-tubulin, resulting in an alteration in microtubule dynamics. R-Ras and PI3K and GSK3 are also involves in sema3A-induced changes in microtubule dynamics. Activation of Ras decreases the activation of the PI3K/Akt signaling pathway, resulting in an increase in GSK3 activation that causes phosphorylation of CRMP-2 followed by growth cone collapse [170].

1.2.3.3 Apoptosis

In addition to their other cellular activities, semaphorins can also cause cell death. Some of the class 3 semaphorins have been identified in the regulation of apoptosis in sensory neurons, small-cell lung cancer cell lines and DEV cells [171-173]. Sema3A-induced

apoptosis in sympathetic and cerebellar granular neurons upon prolonged exposure [174]. Sema4D from activated T cells also induces neuronal cell death [174, 175]. Furthermore, sema3A induces apoptosis in leukemic T cells, semaA3B in lung and breast cancer cells while semaA3F is capable to kill ovarian cancer cells. The role of semaphorins signaling in cell death is unknown. Sema3A might induce cell death via activation of c-Jun N-terminal kinase (JNK)/c-Jun signaling in neurons [176].

1.2.4 Semaphorins in Immune Functions

Semaphorins were originally known as axon guidance molecules in the neuronal system. Some of the semaphorins also play a critical role in various cells of innate and adaptive immune system called "immune semaphorins" [177]. These molecules regulate cytokine expression, immune cell migration, immune cell activation and immune cell interaction. The role of semaphorins in individual immune cells is discussed below.

1.2.4.1 T cells

Semaphorins play important roles in T cell activation and differentiation. Sema4D was the first semaphorin molecule identified in the immune system and is abundantly expressed by T cells and crucially involved in T cell activation, which requires DCs activation [178]. *Sema4d* gene deletion causes impairment of T-dependent (Td) antibody responses and priming of antigen specific T cells [179, 180]. *Sema4d* gene deletion in mice protects against experimental autoimmune encephalomyelitis (EAE) and this phenomenon might be due to impaired T cell function [179]. Sema4A is expressed

specifically in TH1 cells and requires TH1 cell differentiation via TIM-2. Absence of Sema4A causes impairment of Th1 responses and exaggerated TH2 responses in intestinal nematodes infections [181]. Recent studies demonstrated a novel function of Sema4A in the stability of Treg cells. Sema4A binds with Nrp-1 on Treg cells that enhances the Treg function and survival [182]. Sema7A is an important immune semaphorin expressed by activated T cells that stimulate monocytes/macrophages through α1β1 integrin, which in turn induces pro-inflammatory cytokine production [183].

1.2.4.2 B cells

B cells form the key pillar of the immune system. B cells produce an abundance of antibodies against infections and also enhances the innate immune response [65]. B cells homeostasis is disturbed in the absence of Sema4D. Sema4D helps to transduce BCR signaling through mitigation of CD72 inhibitory signals [180]. At baseline, Sema4D is expressed at low level in B cells while it is up-regulated after LPS or CD40 antibody [184]. *Sema4d*-deficient mice have altered antibody responses due to abnormal B cells. Sema4D signaling in B cells is mediated by plexins-B leading to increased B-cell proliferation and lifespan [185]. Sema4D derived from T cells may contribute in the interaction of T cells and germinal center B cells, which promotes survival of germinal center B cells [180].

1.2.4.3 DCs

Sema3A and sema4A are highly expressed by DCs and they play a critical role in T cell activation [186]. PlexinA1 is a receptor for Sema3A, and it mediates activation of DCs. Sema4A is an important immune semaphorin involved in the DCs mediated T cell activation [186]. *PlexinA1* deficient mice exhibit defective generation of antigen-specific T cells due to impaired function of DCs [187]. Sema3A/plexinA1 axis is also involved in the migration of DCs to lymph nodes during infection [188]. DCs from *Sema4a* -deficient mice produce poor stimulation of allogeneic T cells compared to wild-type DCs. However, there is no difference in stimulation of T cells derived from *Sema4a* -deficient mice and T cells from their wild type littermates cultured with DCs derived from wild type mice [178]. Sema4D released from T cells is also a key player in DCs activation by increasing surface expression of CD80, CD86, and major histocompatibility complex (MHC) class II molecules on DCs [179]. Similar to Sema4D, another semaphorin sema6D also derived from T cells and acts by binding to plexin-A1–TREM2–DAP12 (on DCs) can promote DC activation and maturation [186].

1.2.4.4 Basophils

Basophils play a key role in humoral memory response and TH2 polarization [189]. Sema4B promotes T cell-basophil interaction and negatively regulates basophils [190]. *Sema4b* deficient mice exhibit TH2 skewing by producing more IL-4 and IgE [190]. Thus, the presence of Sema4B suppresses an exaggerated TH2 response by inhibiting IL4

and IgE production. It is speculated that sema4B regulates basophils functions via ITIM.

Table 3. Expression and actions of immune semaphorins

Semaphorins/receptors	Expression	Binding partner	Activities
Semaphorins			
Sema3A	T cells		Inhibition of monocyte migration
	Tumour cells	Plexin-A proteins	Inhibition of T-cell activation
	Endothelial cells		Inhibition of tumor angiogenesis
Sema4A	Dendritic cells	Plexin-B proteins	T-cell activation
	Activated T cells	Plexin-D1	Promotion of Th1 differentiation
	Th1 cells	TIM-2	
Sema4D	T cells	Plexin-B1	B-cell activation
	Activated B cells	CD72	DC activation
	Dendritic cells		Microglial activation
Sema6D	T cells	Plexin-A1	DC activation
	B cells		Production of type I interferon
	NK cells		Differentiation of osteoclast
Sema7A	Activated T cells	Plexin-C1	Monocyte/macrophage

Semaphorins/receptors	Expression	Binding partner	Activities
		Integrin a1b1	activation
Receptors			
Neuropilin-1	Treg cells Tumor cells Endothelial cells	VEGF	Tumor angiogenesis
Plexin-A1	Dendritic cells Plasmacytoid DCs (osteoclast)	Class VI semaphorins	DC activation Production of type I interferon Differentiation of osteoclast
Plexin-A4	T cells Dendritic cells Macrophages	Class VI semaphorins	Inhibition of T-cell activation
Plexin-B1	Microglia Oligodendrocytes	Class IV semaphorins	Microglial activation Injury of oligodendrocytes
TIM-2	Activated T cells	Sema4A	T-cell activation

Semaphorins/receptors	Expression	Binding partner	Activities
	Th2 cells		
CD72	B cells (Dendritic cells)	Sema4D	B-cell activation DC activation
Integrin a1b1	Monocytes Macrophages	Sema7A	Monocyte/macrophage activation

Adapted from: *Cellular & Molecular Immunology* (2010) 7, 83–88

1.2.5 Therapeutic effects of semaphorins.

The semaphorins/plexins axis has a wide range of functions in homeostasis. They also play important roles in different pathological conditions. Recently, semaphorins emerged as immune mediators with potential clinical relevance in the field of immunopathological diseases. Administration of exogenous semaphorins or inhibitors of semaphorins or plexins modulate immune responses. Exogenous recombinant Sema4D and Sema3A enhance antigen-specific antibody production and antigen-specific T cell generation [191]. Exogenous recombinant Sema4D and Sema3A along with conventional immunization boost the immune responses against infections via stimulation of CD72 or Tim-2. Thus, the combination of immune semaphorin stimulation with vaccination or antibiotics might be beneficial against infections. In contrast to exogenous semaphorins, blocking of semaphorins might also be beneficial in some immune diseases, such as blocking of sema3A attenuates the severity of EAE. *Sema4d*-deficient mice have impaired T cell generation in EAE induced by MOG-derived peptides in mice [192]. Collectively, animal studies and cell-based assays reveal that the blocking or promoting of semaphorin/plexins signaling can modulate disease conditions. No drugs have made it into clinical practice till now. Thus, semaphorins/plexins might be a potential therapeutic target for drug development to target immune oriented diseases conditions.

Table 4. Therapeutic effects of semaphorins

Targets	Indications	Validation
Semaphorins		
SEMA3A	Ischaemic or diabetic retinopathy	*Sema3a* knockdown protects microvessel in hyperoxia-induced retinopathy in rats [193].
		Knockdown or neutralization of Sema3A attenuates diabetes-induced retinal damage by reduction in vascular leakage in mice [194].
	Neurotrophic corneal disease	Inhibition of Sema3A using small molecules helps in regeneration of nerves in corneal transplantation model in mice [195].
	Cancer	Sema3A effectively suppresses tumor growth in head and neck squamous cell carcinoma [196].
	Sepsis	Anti-Sema3A neutralization monoclonal antibody attenuates endotoxin-induced shock in mice [197].
	Cardiac arrhythmia and myocardial injury	Sema3A attenuates ventricular arrhythmias and myocardial injury in rodents [198, 199].
	Diabetic nephropathy	Sema3A aggravates advanced diabetic nephropathy [200].
	Pain	Sema3A might be a potential therapeutic target in low back pain [201].
	Spinal cord injury	Sema3A antagonist improves regeneration of nerves in spinal cord injury model in rats [202].
SEMA3E	Cancer	Knockdown of *Sema3e* in cancer cells reduces metastasis in xenograft as well as allograft models [203].
		Exogenous administration or expression of a soluble form of plexinD1 suppresses tumor and metastasis of tumor cells in an allograft model in mice [204].

Targets	Indications	Validation
	Kallmann syndrome	Disturbance in Sema3E signaling causes degeneration of gonadotropin-releasing hormone neuron in Kallmann syndrome [205].
	Type 2 diabetes mellitus	*Sema3e* -knockout and blockade of Sema3E binding reduce adipose tissue inflammation that improves insulin resistance in mice [206].
	Liver regeneration	Sema3E secreted by damaged hepatocytes regulates regeneration of liver during liver injury [207].
	Peripheral vascular disease	*Sema3e* -knockout mice show effective recovery by reperfusion in a hind limb ischemia model in mice [208].
SEMA4A	Multiple sclerosis	Anti-Sema4A antibody inhibits induction of EAE in mice [178].
	Myocarditis	*Sema4d*-knockout mice exhibit reduced severity of autoimmune myocarditis [209].
SEMA4D	Arterial thrombosis, atherosclerosis	*Sema4d* gene deletion attenuates arterial thrombus formation and atherosclerosis [210].
	Cancer	Knockdown *Sema4d* in cancer cells or Anti-Sema4D antibody treatment reduces tumor angiogenesis and growth in xenograft models in mice [211].
		Sema4d-knockout mice exhibit reduced angiogenesis and tumor size in an allografts model [212].
		Anti-Sema4D antibody inhibits tumor angiogenesis and tumor growth in a xenograft model in mice [213].
	Multiple sclerosis	*Sema4d*-knockout in mice or Anti-Sema4D antibody treatment efficiently inhibits the development of EAE [192].
	Glomerulonephritis	*Sema4d* gene deletion reduces the levels of immune-complex in a glomerulonephritis model in mice [214].

Targets	Indications	Validation
	Osteoporosis	*Sema4d* gene deletion or Anti-Sema4D antibody increases bone density in mice [215].
	Asthma	*Sema4d*-knockout mice are protected from airway inflammation in ovalbumin-induced mouse model of asthma. [216].
SEMA6A	Cancer	Subcutaneous tumor allografts grown in *Sema6a*-knockout mice exhibit reduced angiogenesis and tumor growth [217].
SEMA6B	Cancer	*Sema6b*- knockdown cancer cells reduce tumor growth in a xenograft model in mice [218].
SEMA7A	Cancer	*Sema7a*- knockdown cancer cells reduces tumor growth and angiogenesis in an allograft tumor model [219].
	Contact hypersensitivity	*Sema7a* -knockout mice get protected from contact hypersensitivity [220].
	Multiple sclerosis	*Sema7a* -knockout mice are resistance to development of EAE [220].
	Lung and liver fibrosis	*Sema7A*-knockout mice protected from TGFβ1-induced lung and liver fibrosis [221, 222].
	Airway inflammation	Sema7A regulates IgE-mediated airway inflammation in mice [223].
Plexins		
Plexin A1	Multiple sclerosis	*Plxna1*-knockout mice are resistance to the development of EAE [224].
	Osteoporosis	*Plxna1*-knockout mice have increased bone density in mice [224].
Plexin A2	Spinal cord injury	*Plxna2*-knockout mice exhibit improved regeneration of nerves in pyramidotomy [225].
Plexin A4	Cancer	Knockdown of *plxna4* cancer cells reduces tumor growth in a xenograft model in mice [218].

Targets	Indications	Validation
	Sepsis	*Plxna4*-knockout mice are protected from cecal ligation and puncture (CLP) and LPS induced septic shock in mice [226].
Plexin B1	Cancer	*Plxnb1*-knockout mice exhibit less severe metastasis in an ERBB2-positive breast cancer model [227].
	Multiple sclerosis	*Plxnb1*-knockout mice show resistance to development of EAE [192].
	Osteoporosis	*Plxnb1*-knockout mice have increased bone density [215].
	Hepatic Injury	Inhibition of Plexin C1 protects mice against hepatic Injury [228].
Plexin C1	Acute lung injury	*Plxnc1*-knockout mice and mice treated with anti-plexin-C1 antibodies or a peptide binding to the plexin-C1–SEMA7A binding site show protection against high pressure ventilation- induced lung injury model in mice [229].
Plexin D1	Cancer	Knockdown of *Plxnd1* in cancer cells reduces metastasis in a xenograft model in mice [203].
		Uncleaved Sema3E interferes with binding of Sema3E–p61 to plexin D1, which inhibits metastasis of cancer cells, angiogenesis and tumor growth in xenograft models [230].

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1.3 Semaphorin3E

Sema3E is a secreted protein originally known as a regulator of axonal growth of neurons [231]. Sema3E is biosynthesized as a 85 – 90 kDa protein [232]. Sema3E undergoes photolytic cleavage by furin convertase that gives rise to P61 fragment. Similar to other Sema3s, Sema3E comprises of Sema, PSI (plexin-semaphorin-integrin), Ig (immunoglobulin) domains and a basic C terminus tail. Sema3E act as a repulsive cue in axon guidance while cleaved fragment (P61) of the Sema3E serves as an attractant [233]. Sema3E only interacts with plexinD1 and acts as a repellent, but Sema3E behaves like an attractant when plexinD1is co-expressed with neuropilin-1 in the system [234]. The functional variation of Sema3E depends on its structural alteration and the availability of its receptors.

Plexin D1 is the only receptor from which Sema3E interacts. Sema3E/PlexinD1 axis plays a critical role in normal physiology such as vascular development [235], neuronal development [236] and hormonal control [205] as well as various pathological conditions such as metastasis in cancer [233], immune cell migration [237], insulin resistance in obesity, release of pro-inflammatory cytokines [206] and proliferation [238]. Vascular patterning is a key activity for the physiological and pathological process. Full-length Sema3E and P61 fragments have different functions such as P61 fragment of Sema3E has a pro-metastatic role in malignant cancer but the whole Sema3E acts against metastasis by obstructing P61 activity. The Sema3E/PlexinD1 axis dynamically contributes to vascular patterning via feedback regulation of VEGF.

Immune cell migration is a hallmark of the inflammatory process. Sema3E also modulates migration of thymic cells and macrophages by regulating small GTPase and Rho activity [239]. Sema3E restore GnRH release by protecting GnRH neuron degeneration in Kallmann syndrome (KS) [205]. In obesity, releasing of pro-inflammatory cytokines in fat tissues may cause insulin resistance. Sema3E is a key molecule, which enhances insulin resistance through releasing of pro-inflammatory cytokines in fat tissues. Overall, Sema3E is a key regulatory molecule, which mediates regulation of various physiological and pathological processes.

1.3.1 Sema3E in immune system

Sema3E is a secreted protein originally known as a regulator of axonal growth of neurons [231]. Sema3E exerts a significant role in the immune system such as immune responses [206], cell migration [237] and proliferation [238]. Recently published studies demonstrated that Sema3E has a significant impact on macrophages and thymocytes.

In the thymus, Sema3E is mainly expressed in the medullar region. Sema3E interacts with plexinD1 receptors of CD69+ cells in the thymic medulla that causes repression of CCL25 induced signaling. Suppression of CCL25 signaling inhibits the migration of CD69+ thymocytes towards thymic cortex. In contrast, CD69+ cells migrate towards the thymic medulla in response to CCL19/21. Thus, Sema3E plays a key role in thymocytes development by aiding migration of $CD69^{+}$ to thymic medulla [240]. Sema3E/plexinD1 axis is also involved in the process of inflammation and migration of macrophages.

Sema3E is upregulated in macrophages when stimulated with oxidized low-density lipoprotein (LDL), LPS and hypoxia. These stimulations make macrophages to polarize towards M1, which exert predominantly a pro-inflammatory action. However, *Sema3e* expression does not change in M2 macrophages, which are known to have immunosuppression activity [241]. Recent studies showed that Sema3E attracts monocytes via induction of p53 into adipose tissue, which eventually becomes pro-inflammatory macrophages [206]. In contrast, Sema3E also causes retention of M1 macrophages in atherosclerotic plaque by blocking CCL19 and CCL21 [241].

2 RATIONAL

Sepsis is an overwhelming systemic inflammatory response to bacterial infection. Innate immune cells are the first line of defense, which play an important role in mounting the immune response against microbes. Among innate immune cells, macrophages are the primary responders to infection. During acute sepsis, resident macrophages act by engulfing microbes and releasing pro-inflammatory mediators. The immune efficiency of macrophages precedes the intensity of further inflammatory responses.

Sema3E is a secreted protein originally known as a regulator of axonal growth of neurons [231]. Sema3E exerts various physiological and pathological roles in immune response, cell migration [237], proliferation [238] and angiogenesis [235]. Recently published studies demonstrated that the Sema3E has a significant impact on macrophages in terms of their inflammatory and migratory functions. Sema3E attracts monocytes via induction of p53 into adipose tissue, which eventually becomes pro-inflammatory macrophages. Sema3E also causes retention of M1 macrophages in the atherosclerotic plaque by blocking CCL19 and CCL21 [241]. This evidences suggests that Sema3E plays a pivotal role in the regulation of macrophages. However, the exact role of Sema3E in macrophages behavior in LPS induced endotoxemia is unknown. Thus, we aimed to study the role of Sema3E deficiency in modulating macrophage function in the context of LPS induced endotoxemia .

3 HYPOTHESIS

Sema3E regulates resident macrophages response in lipopolysaccharide-induced endotoxemia.

4 AIMS

- To examine the functional impact of *Sema3e* gene deletion in a mouse model of LPS-induced endotoxemia.
- Effect of *Sema3e* gene deletion on macrophages functions upon LPS exposure.

5 MATERIALS AND METHODS

5.1 Animal Housing and Breeding

The 129 P2 *Sema3e*$^{-/-}$ mice were obtained from an in-house breeding colony maintained by the Central Animal Care Services, University of Manitoba. Parent breeders of these animals were gifted by Dr. F. Mann, Université de la Méditerranée, Marseille, France. 129 P2 mice were used as wild type littermate control. All animals were housed in individualized ventilated cages (IVC) with wooden chip bedding in an environment-enriched condition in the animal care facility of the University of Manitoba. A maximum of four animals were housed in each cage. Animals were maintained in a 12-hour dark-light cycle, and supplied with standard chow and water *ad libitum*. Animal usage was strictly according to instructed guidelines by the Canadian Council for Animal Care.

5.2 LPS-induced Endotoxemia

Animals (*Sema3e*$^{+/+}$ and *Sema3e*$^{-/-}$) were treated with LPS (5-30mg/kg, i.p) or saline. Animals were observed for clinical score (Table. 5.) and rectal temperature. For higher doses of LPS (20 or 30mg/kg, i.p), animals were observed for clinical score (Table.5.) and rectal temperature up to 72 hours every three hours during the day and every 5 hrs during night time. For sub-lethal dose LPS (5mg/kg, i.p), animals were sacrificed at various times (8, 12 and 24 hrs) after LPS treatment. Peritoneal lavages (PL) and tissues/organs were collected for further processing.

Table 5. Scoring system of clinical signs

	Clinical signs	Clinical score
Appearance	Normal	0
	Ruffled hair	1
	Hunched posture	2
Eye condition	Sunken eyes	1
	Closed eyes	2
Movement	Normal	0
	Moderate	1
	Slow	2
	Unable	3
Response to tactile stimuli	Normal	0
	Moderate response	1
	Mild response	2
	No response	3

For blood collection, animals were anesthetized and blood was collected by cardiac puncture. PL was collected after injecting 5mL of RPMI media in the abdominal cavity. The animal's abdomen was massaged for 30 seconds and the lavage was collected (we observe 85% of recovery of injected solution) through a sterile syringe. After collecting peritoneal lavage, the abdomen was cut open and mesenteric lymph nodes were collected from the center of the omentum. Lungs were collected and subjected to digestion in lung digestion buffer (collagenase type IV at 1mg/ml in RPMI) for about 45 min at 37^0C on a shaker. Lymph nodes were crushed and passed through a cell restraint (40μm, Corning® cell restrainer, USA) to obtain a single cell suspension. PL was processed to centrifugation at 1200rpm for 5 min at 4 ^{0}C. The supernatant was collected and stored at – 80 ^{0}C for cytokines estimation. Cell pellets were processed for flow cytometry.

5.2.1 Processing of cells

Cell suspensions from peritoneal lavage, blood, lung and mesenteric lymph nodes were subjected to ammonium-chloride-potassium (ACK) lysis buffer treatment for 5 minutes to lyse red blood cells. ACK buffer was neutralized by adding flow buffer (PBS containing 0.1% newborn calf serum and 0.1% sodium azide) and centrifuged at 1200rpm for 5 min at 4 ^{0}C. Cell pellets were resuspended in flow buffer.

5.2.2 Depletion of peritoneal macrophages

Mice were intraperitoneally injected with 0.2ml of clodronate liposomes 24 hrs before LPS injection. A control group received the same volume of control liposomes. This method primarily eliminates peritoneal macrophages, which undergo apoptosis after phagocytosis of clodronate liposomes [242]. Depletion of macrophages in peritoneal lavage, lung and spleen was confirmed by flow cytometry.

5.2.3 Surface staining of cells with fluorochrome-conjugated antibody

Above processed cell suspensions (0.5 x 10^{6} cells /tube) were added into flow cytometry tubes (Falcon, BD Biosciences, San Diego, CA). The cells were treated with Fc blocker for 5 min, and then washed with 1 mL flow buffer by centrifuging at 1200rpm for 5 min at 4 ^{0}C. Supernatant was discarded and the excess fluid was blotted out using a paper towel. Cells were then incubated with 20 µl (containing 0.1 to 0.25µg) of fluorochrome-labeled antibodies against the given surface or intracellular molecules: CD11b (APC or

PE.cy.7), F480 (PE or PE.cy.7), iNOS2 (APC), TLR4 (PE.cy.7), Gr-1(PE or FITC), Ly6C (FITC), CD3e (eflour450), CD4 (APC) and CD69 (PE). After 25-30 min of incubation at 4 ^{0}C, cells were washed and resuspended in 300µl flow buffer. For intracellular staining (iNOS), cells were permeabilized with saponin (0.1% in 1xPBS) for 15 min before fluorochrome-labeled antibodies treatment.

5.2.4 Flow cytometry analysis

Processed cells were acquired by using BD FACSCanto II (BD Biosciences, USA) by adopting standard instrumental protocol. Briefly, stained cells were acquired (at least 50,000 events) and the gating strategies for the cells and markers are indicated in respective figures. For making accurate gating, we have used fluorescence minus one (FMO) control of flow antibody conjugated with respective fluorochrome for each type of biomarker. FlowJo key was used for analyses of flow data.

5.2.5 Sandwich enzyme-linked immunosorbent assays (ELISA)

The level of TNF-a, IL-6 and MCP-1 in serum and PL was quantified by ELISA as per the manufacturer's instructions (Biolegends, San Diego, CA). Briefly, ELISA plates (Immulon VWR, Mississauga, ON) were coated with primary antibody (100µL/well) and incubated overnight at 4^{0}C. The next day, non-specific bindings were blocked by adding 200µL /well diluent buffer. After 1 hour of blocking, plates were washed 4 - 5 times with washing buffer (1X PBS + 0.05% Tween-20). Standards and samples were diluted in assay diluent according to their respective concentration range, and they are added to

each well in 100µL volume. After 2 hours of incubation at room temperature with shaking, plates were washed 4-5 times with washing buffer. Detection antibody was added (100 µL/well) and incubated for 1 hour at room temperature with shaking. Plates were washed 4-5 times with washing buffer and 100 µL of diluted Avidin-HRP solution was added to each well. Plates were incubated at RT for 30 minutes with shaking. Plates were washed 4-5 times with washing buffer. TMB Substrate Solution (100 µL/well) was added and kept in dark for 15 minutes then the reaction was stopped by adding stop solution (100 µL/well). Plates were read at 450 nm (Spectra Max) after the appropriate color development.

5.3 Generation of bone marrow derived macrophages (BMDMs)

Bone marrow cells obtained from *Sema3e*$^{+/+}$ and *Sema3e*$^{-/-}$ mice were treated with ACK buffer to lyse RBCs. The resultant cell suspension was plated on a petriplate (100 x 15mm) in the concentration of $5X10^5$ cells/plate, suspended in 5 mL of BMDMs medium (30% of L929 conditioned medium +20% fetal bovine serum+1%penicillin-streptomycin in RPMI 1640 medium). Cells were incubated at 37°C in a 5% CO_2 atmosphere and grown for 6 days. Media (BMDMs medium) was changed every alternate day. On day 6, adhered cells (BMDMs) were washed twice with sterile 1X PBS and detached by gently scraping using sterile scraper. Cells are then centrifuged at 1200rpm for 5 min at 4 ^{0}C and the cell resultant pellet was resuspended in complete media.

5.4 LPS Stimulation of BMDMs

The scraped BMDMs on day 6 (as described above) from *Sema3e*$^{+/+}$ and *Sema3e*$^{-/-}$ mice, were seeded in the concentration of 2x10^5 /well in 12 well plates. Cells were left for 4 hours for adherence and deprived them from serum for another 6 hours (provided 1%FBS in RMPI). After deprivation period cells were washed and replaced with complete RPMI media and stimulated with LPS (100ng/mL in complete RPMI) for various time points.

5.4.1 Real-time quantitative PCR

At the end of the stimulation period, cells were lysed by using lysis buffer and processed for RNA isolation as per manufacturer's kit (RNeasy mini kit, Qiagen, Mississauga, ON, Canada). The RNA concentration was measured using BioPhotometer (Eppendorf AG, Hamburg, Germany). Single stranded cDNA was generated as per the manufacturer instruction using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA, Cat #: 4368814). Briefly, RNA (1000ng/reaction) in 10µL, dTNP, reverse transcriptase, RNase inhibitor, RT buffer were mixed to get the total volume of 20 µL/reaction. The samples were incubated in a thermocycler instrument (Eppendorf AG, Hamburg, Germany) for specified time (indicated in figure. 16.) to generate cDNA. The resultant cDNA was stored at -20 ^{0}C unless it was used for real time-PCR. For real-time PCR, cDNA of each sample (35ng/reaction) and sequence specific to IL-6, TNF, *Sema3e* and GAPDH primers (10 □M) were mixed with SYBR Select Master Mix (Applied Biosystems, USA, Cat #: 4472908) to get a total volume of 20µL/well in 96 well PCR

plates (VWR cat# 82006-650). The PCR reaction was run using the BioRad CFX with protocol, initial 1 cycle denaturation step:10 min at 95°C, 40 cycles of PCR (95°C for 15 s, 60 °C for 35 s and 72°C for 35 s), 1 cycle of melting and 1 cooling cycle. Specificity of the product was determined by observing melting curve analysis and examining the quality of amplification curves. Target gene amplification was calculated by normalizing against the amplification of GAPDH.

Table 6. Real-Time PCR primers (forward and reverse) used in analysis of gene expression

Gene	**Forward (5′- 3′)**	**Reverse (5′- 3′)**
TNF	5'-ATGAGCACAGAAAGCATGATC-3'	5'-TACAGGCTTGTCACTCGAAT-3'
IL-6	5'-GAGGATACCACTCCCAACAGACC-3'	5'-AAGTGCATCATCGTTGTTCATACA-3'
GAPDH	5′-AACTTTGGCATTGTGGAAGG-3′	5′-ACACATTGGGGGTAGGAACA-3′

5.5 Stimulation of BMDMs for Western blotting

Cells (BMDMs) from *Sema3e*$^{+/+}$ and *Sema3e*$^{-/-}$ mice were deprived from serum for 6 hours (provided 1%FBS in RMPI 1640 media). After the deprivation period, cells were washed and replaced with complete RPMI media. Cells were stimulated with LPS (*E.coli* 0111:B4, cat# L2630, Sigma-Aldrich, USA) in 100ng/mL. At predetermined time points (0, 1, 5, 15, 30, 60 and 120 min) after LPS stimulation, media was removed and the signaling reaction was stopped by adding ice cold 1XPBS. After that, PBS was removed

and cells were lysed with lysis buffer containing M-PER (Thermo scientific, USA, cat # 78501) and protease inhibitor cocktail (Abcam, Cat # ab65621, UK). Cell lysates were subjected to centrifugation at 10000rpm for 10 min at 4 ^{0}C. The supernatant was collected and stored at -80^{0}C western blot.

5.5.1 Determination of protein concentration

Protein concentration in the cell lysate was determined by using the bicinchoninic acid assay (BCA) protein assay kit. The assay procedure was performed according to the manufacturer's instructions (San Diego, CA, USA, Cat #: 71285-3). After protein estimation, supernatants were stored at -80 ^{0}C for Western blot.

5.5.2 Determination of signaling molecules from Western blot

5.5.2.1 Preparation of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE gel was prepared by making two solutions; one is for staking the gel and another for separating the gel. The contents were added in the following order. Distilled water, 40% Bisacrylamide, Tris buffer, 10% SDS, Ammonium persulfate and TMT (composition is given in the table. 6. The solution for the separating gel was poured first into the gap between two gel making plates up to 80% level.

The remaining space was filled with alcohol to get a uniform level of the separating gel. Plates were kept aside for 30 minutes. After that, the alcohol was removed by inverting the plates and the remaining space was filled with stacking solution. Plates were again

kept aside for 30 minutes for gel solidification and then stored at 4^0C by wrapping it in wet paper towel until used.

Table 7. Composition of SDS-PAGE gel for western blot analysis

Reagents	10% separating solution	4% stacking solution
ddH_20	4.8mL	6.3mL
40% Bisacrylamide	2.5mL	1mL
Tris-HCl (1.5M)	2.5mL	-
Tris-HCl (0.5M)	-	2.5mL
10% SDS	100µL	100µL
10% ammonium persulfate	100µL	100µL
TMT	5µL	6µL

5.5.2.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for signaling proteins

Frozen cell lysates supernatant from BMDMs of *Sema3e*$^{+/+}$ and *Sema3e*$^{-/-}$ mice were thawed and the required amounts of each sample were mixed with 4X loading dye and lysis buffer. The tube with loading mixture was centrifuged at 2000rpm for 1 minute. The total protein of 20µg/ in 15µL volume was loaded in each well. Standard size protein marker (Precision Plus Protein Standard Dual Color, BioRad laboratories Inc., ON, Canada) was also loaded to track the molecular weight of our desired protein. Standard

size protein marker is an indicator of molecular weight ranging from 10 – 250kDa. Running buffer (Table 1) is used to run electrophoresis. Initial voltage was 90 volts for 20 minutes followed by 120 volts for about 2 hours.

After completion of electrophoresis, separated proteins were transferred using semi-drying transfer method. The transfer was performed from gel to polyvinylidenediflouride (PVDF) membrane (BioRad) at 20 volts for 50 minutes using transfer buffer. Prior to transfer, PVDF membrane was activated in pure methanol for 10 minutes. After completion of the transfer, membrane was blocked in 5% of skimmed milk in Tris buffer saline with tween 20 (TBST) for 1 hr at room temperature (RT).

Table 8. Composition of stock solution used in Western blot.

Composition	**Stock solutions**			
	10x running buffer	1.5 M Tris, pH 6.8	1.5 M Tris, pH 8.8	10X transfer buffer
SDS	200mL of 20%	-	-	-
Tris base	121.1g	181.65 g	181.65 g	121.1 g
Glycin	576g			576 g
H_2O	4000mL	1000mL	1000mL	4000mL
PH	-	Adj with HCL	Adj with HCL	-

Table 9. Composition of working buffers used in Western blot

Composition	Working solutions		
	Running buffer	Transfer buffer	Stripping buffer
10% SDS	10mL	-	10mL
10 X Running buffer	100mL	100mL	-
Methanol	-	200mL	-
1 M Glycin	-	-	200mL
Tween 20	-	-	10mL
H_2O	900mL	700mL	780mL

Thereafter, the membrane was divided into desired pieces according to required molecular weight of phosphor-proteins. Each piece of membrane (required for detection of the specific molecule) was incubated overnight at 4^0C, with respective rabbit anti-mouse primary antibodies (Cell Signaling Technology, USA) targeting NFκ-B, STAT-3 and ERK1/2 molecules at a dilution of 1: 1000 in 1% skimmed milk in TBST. The next day, membranes were washed (5 min X4 times) with TBST. After that, membranes were incubated with goat anti-rabbit secondary antibody (1:5000 dilution in 1% skimmed milk in TBST) for 2 hours at RT, then membranes were washed (10 min X 5 times) with TBST and developed using ECL kit (GE Healthcare). The multiple images of emitted signal were captured at different time points using the ChemiDoc™ MP System (Bio-Rad). After detection of phosphorylated proteins, membranes were stripped using stripping buffer (Table 7). After that, membranes were again washed and re-probed with respective total protein rabbit anti-mouse primary antibodies to detect total proteins.

5.5.3 Statistical analysis

All results are expressed as mean ± SEM. A potential significant differences between the groups in clinical score, rectal temperature and cytokines expression from BMDMs were determined through Two-way analysis of variance (ANOVA) and multiple comparisons between the groups were made by Tukey's post hoc test. The significance of other sets of data was determined by using one-way ANOVA followed by Bonferroni post hoc test. Data were analyzed by using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA) and alpha was set at 0.05.

6 RESULTS

6.1 *Sema3e*$^{-/-}$ mice showed initial transient protection from LPS induced clinical score and hypothermia

Before the start of experimental work, we asked the following question: what is the outcome of *Sema3e* deletion on LPS induced alteration of physiological and clinical parameters in mice. Animals were injected with LPS (5 and 20 mg/kg) and observed for clinical score and rectal temperature. Animals exhibited significant hypothermia after LPS compared to baseline temperature. However, *Sema3e*$^{-/-}$ mice were less hypothermic as compared to *Sema3e*$^{+/+}$ mice for the transient period just after LPS (after 4 hrs) but not later (Figure 11A and 11C). Similarly, *Sema3e*$^{-/-}$ mice showed less clinical score compared to their WT littermates for the brief initial period after LPS but not later (Figure 11B and 11D).

Table 10. Raw data: Clinical score after LPS treatment (5mg/kg, i.p.)

Mice Groups	Animal ID	Appearance	Movement	Eye condition	Response to tactile stimuli	Total
Clinical score 0 hr after LPS						
***Sema3e*$^{+/+}$ 5mg/kg**	1	0	0	0	0	0
	2	0	0	0	0	0
	3	0	0	0	0	0
	4	0	0	0	0	0
	5	0	0	0	0	0
	6	0	0	0	0	0
***Sema3e*$^{-/-}$ 5mg/kg**	1	0	0	0	0	0
	2	0	0	0	0	0
	3	0	0	0	0	0
	4	0	0	0	0	0
	5	0	0	0	0	0
	6	0	0	0	0	0
Clinical score 2 hr after LPS						
***Sema3e*$^{+/+}$ 5mg/kg**	1	1	1	1	0	3
	2	1	1	1	0	3
	3	2	1	1	1	5
	4	2	1	0	0	3
	5	1	1	1	0	3
	6	1	1	1	0	3
***Sema3e*$^{-/-}$ 5mg/kg**	1	1	0	0	0	1
	2	1	0	0	0	1
	3	1	0	0	0	1
	4	1	0	0	0	1
	5	1	0	0	0	1
	6	1	0	0	0	1
Clinical score 4 hr after LPS						
***Sema3e*$^{+/+}$ 5mg/kg**	1	1	1	1	0	3
	2	2	1	0	0	3
	3	2	1	0	0	3
	4	2	1	0	0	3
	5	1	1	1	0	3
	6	1	1	1	0	3
***Sema3e*$^{-/-}$ 5mg/kg**	1	1	1	0	0	2
	2	1	1	1	0	3
	3	1	1	0	0	2
	4	1	1	0	0	2
	5	1	1	0	0	2
	6	2	1	0	0	3
Clinical score 8 hr after LPS						
***Sema3e*$^{+/+}$ 5mg/kg**	1	2	1	1	0	4
	2	2	1	1	0	4

Mice Groups	Animal ID	Appearance	Movement	Eye condition	Response to tactile stimuli	Total
	3	2	1	0	0	3
	4	2	2	1	0	5
	5	2	1	0	0	3
	6	2	1	1	0	4
***Sema3e*$^{-/-}$ 5mg/kg**	1	1	1	1	0	3
	2	2	1	1	0	4
	3	2	1	0	0	3
	4	2	1	1	1	5
	5	1	1	1	0	3
	6	2	1	0	0	3
Clinical score 12 hr after LPS						
***Sema3e*$^{+/+}$ 5mg/kg**	1	1	1	0	0	2
	2	1	1	0	0	2
	3	1	1	1	0	3
	4	1	1	0	0	2
	5	1	1	0	0	2
	6	2	1	1	0	4
***Sema3e*$^{-/-}$ 5mg/kg**	1	1	1	0	0	2
	2	1	1	1	0	3
	3	1	1	0	0	2
	4	1	1	1	0	3
	5	2	1	0	0	3
	6	1	1	1	0	3

Table 11. Raw data: Clinical score after LPS treatment (20mg/kg, i.p.)

Mice Groups	Animal ID	Appearance	Movement	Eye condition	Response to tactile stimuli	Total
Clinical score 0 hr after LPS						
***Sema3e*$^{+/+}$ 20mg/kg**	1	0	0	0	0	0
	2	0	0	0	0	0
	3	0	0	0	0	0
	4	0	0	0	0	0
***Sema3e*$^{-/-}$ 20mg/kg**	1	0	0	0	0	0
	2	0	0	0	0	0
	3	0	0	0	0	0
	4	0	0	0	0	0
Clinical score 2 hr after LPS						
***Sema3e*$^{+/+}$ 20mg/kg**	1	1	1	1	0	3
	2	1	1	1	0	3
	3	1	1	1	0	3
	4	1	1	1	0	3
***Sema3e*$^{-/-}$ 20mg/kg**	1	1	0	0	0	1
	2	1	1	0	0	2
	3	1	0	0	0	1
	4	1	0	0	0	1
Clinical score 4 hr after LPS						
***Sema3e*$^{+/+}$ 20mg/kg**	1	2	1	2	0	5
	2	2	1	1	1	5
	3	1	1	1	0	3
	4	2	1	0	0	3
***Sema3e*$^{-/-}$ 20mg/kg**	1	1	1	1	0	3
	2	2	1	1	1	5
	3	1	1	0	1	3
	4	1	1	1	0	3
Clinical score 8 hr after LPS						
***Sema3e*$^{+/+}$ 20mg/kg**	1	2	1	1	1	5
	2	2	1	2	0	5
	3	2	1	1	1	5
	4	2	1	1	1	5
***Sema3e*$^{-/-}$**	1	2	2	1	1	6

Mice Groups	Animal ID	Appearance	Movement	Eye condition	Response to tactile stimuli	Total
20mg/kg	2	2	1	0	1	4
	3	2	2	1	1	6
	4	2	1	1	1	5
Clinical score 12 hr after LPS						
***Sema3e*$^{+/+}$ 20mg/kg**	1	2	2	1	1	6
	2	2	1	1	1	5
	3	2	1	1	1	5
	4	2	1	1	1	5
***Sema3e*$^{-/-}$ 20mg/kg**	1	2	2	2	0	6
	2	2	1	1	1	5
	3	2	1	1	0	4
	4	2	2	1	1	6
Clinical score 24 hr after LPS						
***Sema3e*$^{+/+}$ 20mg/kg**	1	2	2	1	1	6
	2	2	1	1	1	5
	3	2	1	1	1	5
	4	2	2	1	1	6
***Sema3e*$^{-/-}$ 20mg/kg**	1	2	2	1	1	6
	2	2	2	2	0	6
	3	2	2	1	1	6
	4	2	2	2	0	6

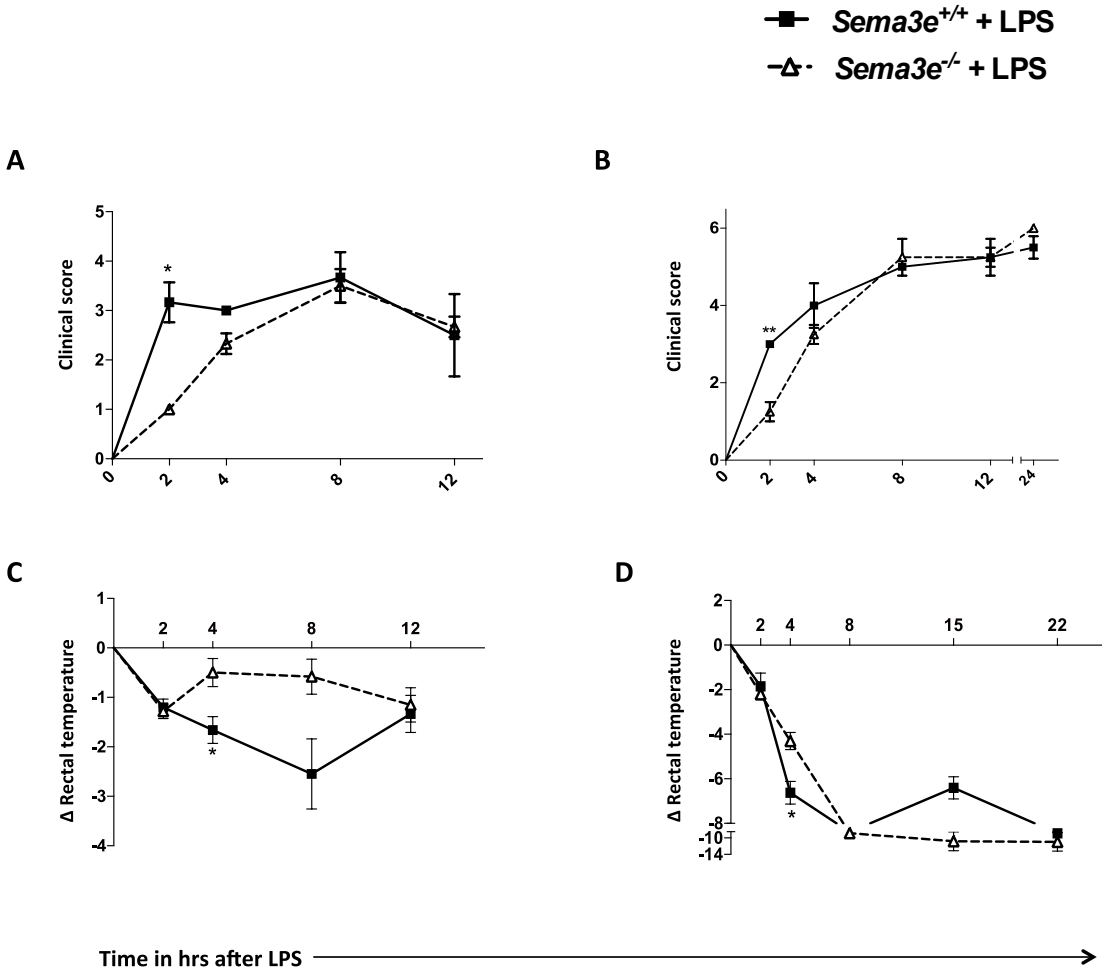


Figure 12. *Sema3e*$^{-/-}$ mice showed initial transient protection from LPS induced hypothermia. LPS treated mice were observed for clinical score A & B at 5mg/kg and 20mg/kg respectively, and rectal temperature C & D at 5mg/kg and 20mg/kg respectively. Both the doses of LPS were given intraperitoneally. Results are expressed in Mean ± SEM (n=6), Two-way ANOVA with Tukey's post hoc test was performed to determine the significance difference between the groups at various time points, **p<0.05, **p<0.01*.

6.2 *Sema3e*$^{-/-}$ mice release less pro-inflammatory cytokines upon LPS injection

In the light of clinical score and rectal temperature results, we hypothesized that protection might be due to a difference in cytokines release between *Sema3*$^{+/+}$ and *Sema3e*$^{-/-}$ mice. In LPS-induced systemic inflammation, primarily macrophages release TNF and IL-6 upon LPS exposure. Released TNF and IL-6 are transported to hypothalamus where they modulate the temperature threshold that eventually leads to hypothermia [243]. To elucidate the effect of cytokines on rectal temperature, we have quantified pro-inflammatory cytokines in peritoneal lavage and serum to assess the extent of inflammation after a sub-lethal dose of LPS using the sandwich ELISA method. TNF (Figure 12A) and IL-6 (Figure 12B) are significantly lower in serum of *Sema3e*$^{-/-}$ mice compared to *Sema3e*$^{+/+}$ upon LPS treatment. Moreover, *Sema3e*$^{-/-}$ mice exhibit significantly lower levels of IL-6 (Figure 12D) in PL. Furthermore, we found lower levels of monocyte chemotactic factor-1 (MCP-1) in serum (Figure 12C) and PL (Figure 12E) in *Sema3e*$^{-/-}$ mice.

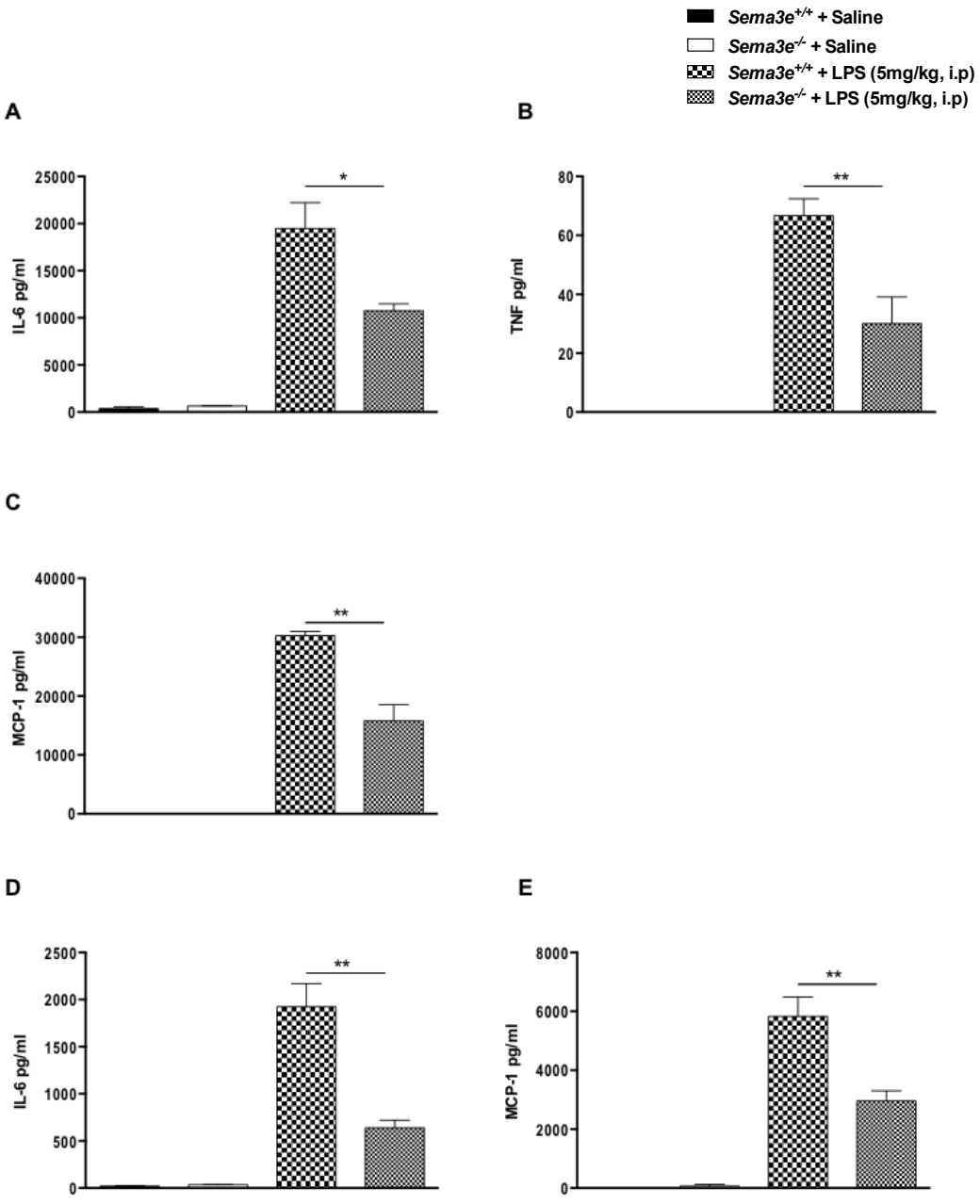
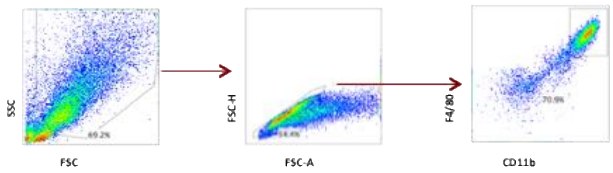


Figure 13. ***Sema3e*$^{-/-}$ mice release less pro-inflammatory cytokines.** Mice were injected with a sub-lethal dose of LPS (5mg/kg, i.p.), and sacrificed after 8 hrs. Pro-inflammatory cytokines in serum (A, B & C) and in peritoneal lavage (D & E) were analyzed by ELISA. Results are expressed in Mean ± SEM, One-way ANOVA with post-hoc Bonferroni analysis was performed to determine the significance difference between the groups, *$p<0.05$, **$p<0.01$.

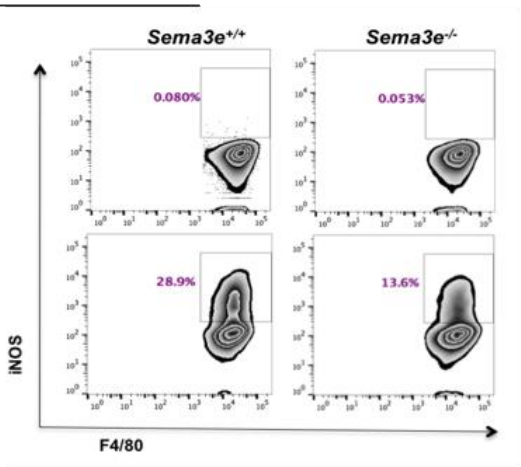
6.3 Peritoneal macrophages from *Sema3e*$^{-/-}$ mice display reduced iNOS expression upon LPS stimulation in vivo

Macrophages are the primary responders among innate cells against LPS [244]. They are key players to release pro-inflammatory cytokines such as TNF and IL-6 [243]. The releasing of TNF and IL-6 from macrophages is depends on the degree of activation of macrophages. iNOS is a well-known marker of macrophages activation upon LPS stimulation [245]. The expression of iNOS in macrophages is directly proportional to the extent of macrophage activation during acute inflammation [246]. To know the activation status of peritoneal macrophages, we investigated iNOS expression after LPS injection using flow cytometry. We found low iNOS expression in peritoneal macrophages of *Sema3e*$^{-/-}$ mice compared to WT (Figure 13B & C). This result suggests that macrophages from *Sema3e*$^{-/-}$ mice are less responsive to LPS.

A. Gating strategy



B. Gated on F4/80+ CD11b+ cells



C.

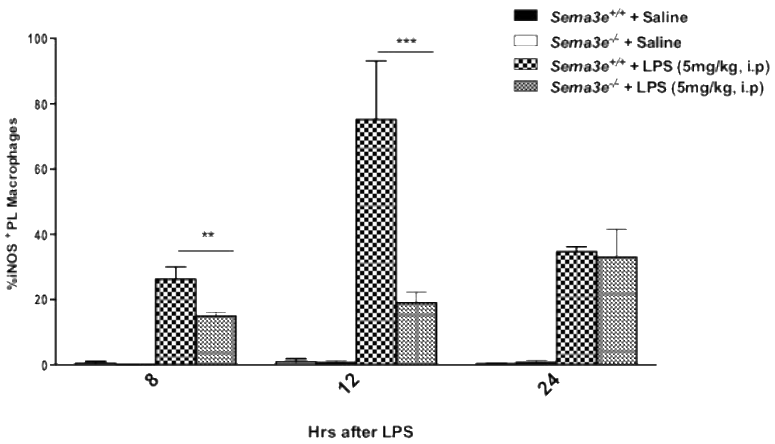


Figure 14. Peritoneal macrophages from *Sema3e*$^{-/-}$ mice display reduced iNOS expression upon LPS stimulation *in vivo*. Mice were injected with a sub-lethal dose of LPS (5mg/kg, i.p.) and sacrificed at the indicated time point. iNOS in PL macrophages was analyzed by flow cytometry. (A) Gating strategy, (B) Flow graphs and (C) Statistical representation. Results are expressed in Mean ± SEM (n=6), One-way ANOVA with post-hoc Bonferroni analysis was performed to determine significances difference between the groups,**p<0.01, ***p<0.001*.

6.4 *Sema3e*$^{-/-}$ mice displayed reduced influx of pro-inflammatory monocytes in blood upon LPS injection.

Intraperitoneal LPS expands pro-inflammatory monocytes in the blood where they recognize infections and release pro-inflammatory cytokines, which causes progression of inflammation [247]. Our cytokines data showed that pro-inflammatory cytokines were significantly lower in serum and PL of *Sema3e*$^{-/-}$ mice. Therefore, we were interested to evaluate the status of monocytes in blood. We found significantly less pro-inflammatory blood monocytes in *Sema3e*$^{-/-}$ mice compared to WT littermates after a sub-lethal dose of LPS injection *in vivo* (Figure 14 B & C.).

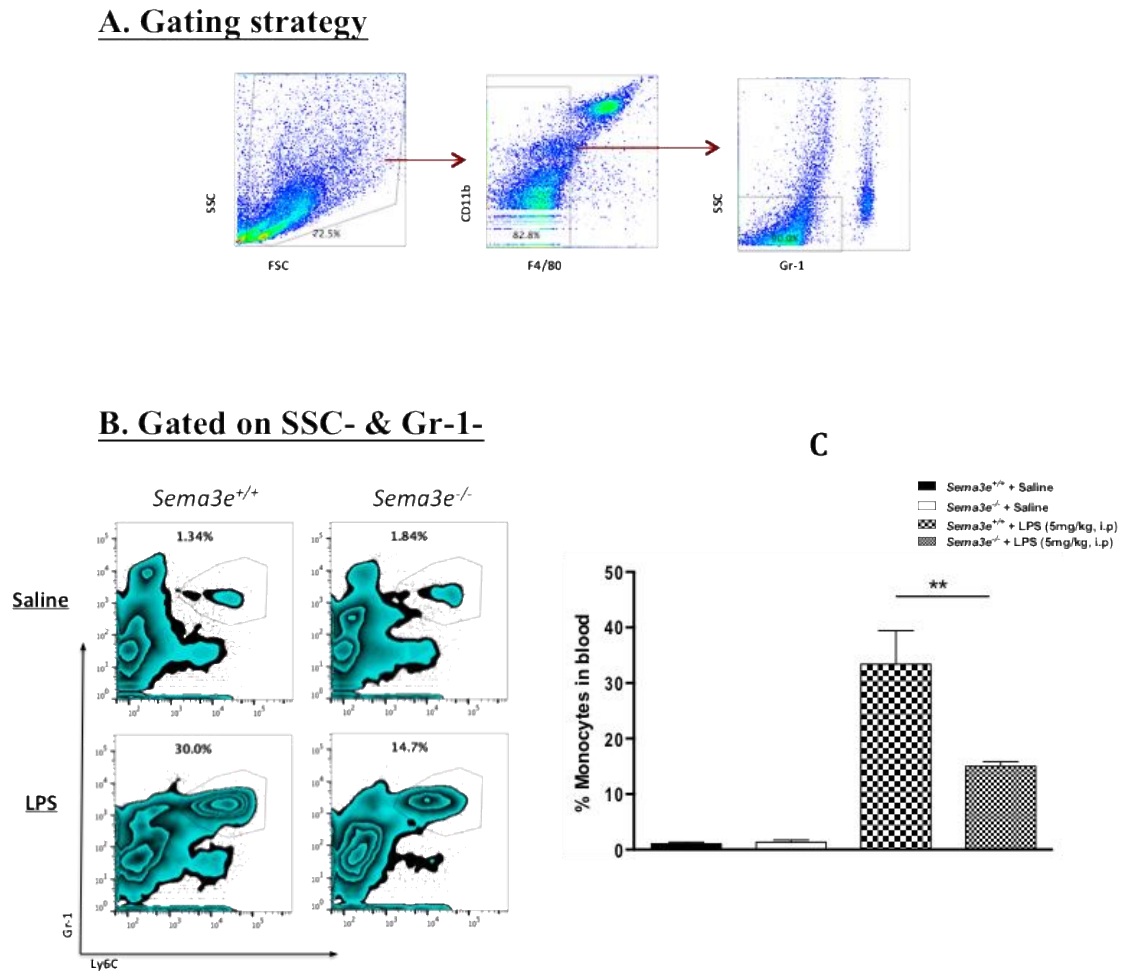


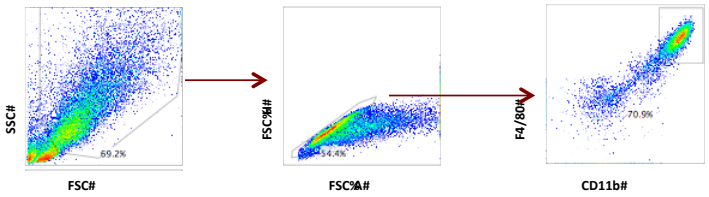
Figure 15. ***Sema3e*$^{-/-}$ mice displayed a reduced influx of pro-inflammatory monocytes in blood upon LPS injection.** Mice were injected with a sub-lethal dose of LPS (5mg/kg, i.p.) and sacrificed after 8 hrs of LPS. Blood Monocytes were analyzed by flow cytometry. (A) Gating strategy, (B) Flow graphs and (C) Statistical representation. Results are expressed in Mean ± SEM (n=7), One-way ANOVA with post-hoc Bonferroni analysis was performed to determine significance difference between the groups,**p<0.01*.

6.5 No difference in surface TLR-4 on peritoneal macrophages after LPS injection

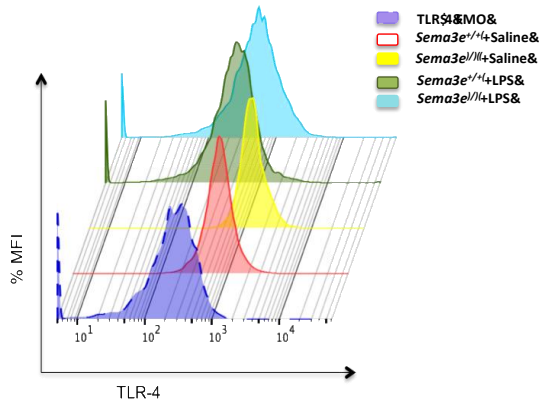
Based on iNOS and pro-inflammatory cytokines data, macrophages from *Sema3e*$^{-/-}$ mice seem to be a less active. Thus, we anticipated that hyporesponsiveness of Mφ in *Sema3e*$^{-/-}$ mice might be the result of defective TLR-4 expression or internalization. TLR-4 binds to its agonist (LPS) and gets internalized into the cell cytoplasm to activate pro-inflammatory signaling pathways.

Here we have measured the extent of internalization of TLR-4 receptors after LPS injection in peritoneal Mφ. TLR-4 receptors at the cell surface were significantly decreased after LPS injection compared to saline control (figure. 15C). However, we did not find a significant difference between *Sema3e*$^{-/-}$ and *Sema3e*$^{+/+}$ mice in terms of TLR-4 internalization in peritoneal macrophages after LPS (Figure 15B & C). Interestingly, at the baseline peritoneal Mφ from *Sema3e*$^{-/-}$ express higher TLR4 compared to WT littermates (Figure 15B & C).

A. Gating strategy



B. Gated on F4/80+ CD11b+ cells



C.

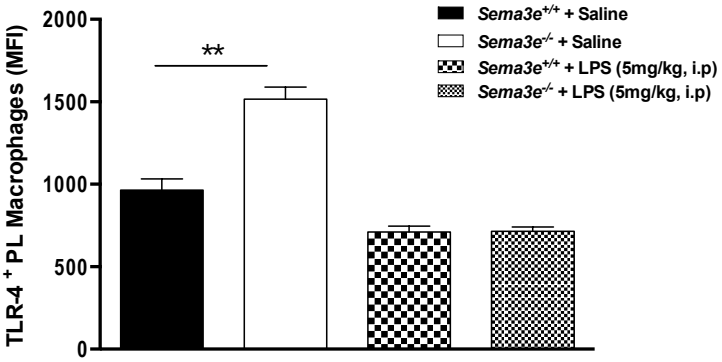


Figure 16. No difference in surface TLR-4 on peritoneal macrophages after LPS injection. Mice were injected with a sub-lethal dose of LPS (5mg/kg, i.p.) and sacrificed after 8 hours of LPS. TLR-4 on PL macrophages was analyzed by flow cytometry. (A) Gating strategy, (B) Flow graph, (C) Statistical representation. Results are expressed in Mean ± SEM (n=6). One-way ANOVA with post-hoc Bonferroni analysis was performed to determine significance difference between the groups,***p<0.01*.

6.6 *Sema3e*$^{-/-}$ BMDMs expressed a low level of pro-inflammatory cytokines after LPS stimulation

In vivo data suggested that macrophages from *Sema3e*$^{-/-}$ are hyporesponsive to LPS. Peritoneal lavage and serum from *Sema3e*$^{-/-}$ animals exhibits lower levels of pro-inflammatory cytokines (TNF and IL-6). It is well known that resident macrophages (from specific tissue) exhibit animal-to-animal biological variations. These variations are depending upon genetic makeup, environmental factors and microbiota of the individual animal. To get macrophages with more uniform functions with less biological variations, we grew BMDMs from a single animal (*Sema3e*$^{-/-}$ or *Sema3e*$^{+/+}$) to extensively study the functions of macrophages. BMDMs were stimulated with LPS (100ng/ml) and mRNA and protein were quantified by RT-qPCR and ELISA, respectively. BMDMs from the *Sema3e*$^{-/-}$ showed less mRNA expression of IL-6 (figure. 16A) and TNF (Figure 16C) compared to *Sema3e*$^{+/+}$ at predetermined time points after LPS stimulation. Supernatant from *Sema3e*$^{-/-}$ BMDMs also expressed less IL-6 (Figure 16B) and TNF (figure. 16D) compared to *Sema3e*$^{+/+}$ BMDMs.

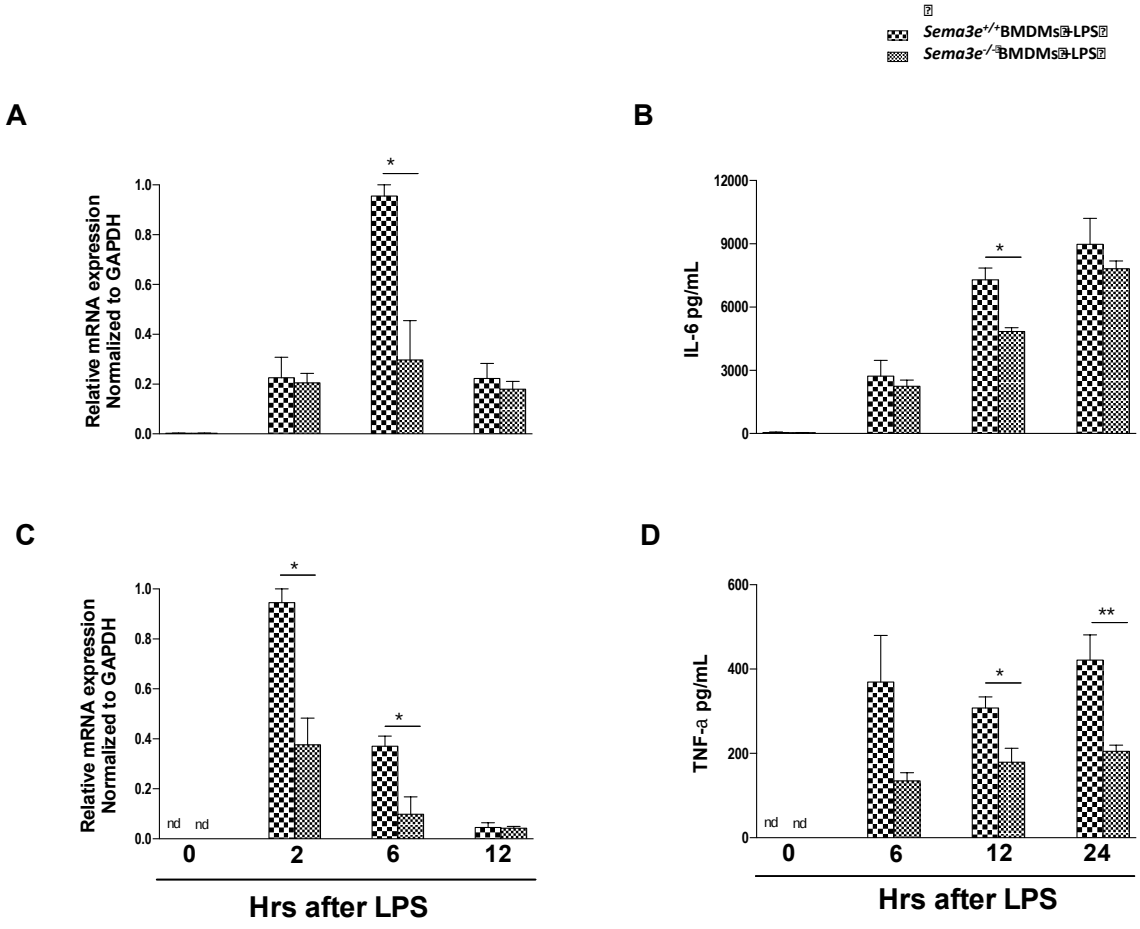


Figure 17. *Sema3e*$^{-/-}$ BMDMs expressed a low level of pro-inflammatory cytokines after LPS stimulation. BMDMs from *Sema3e*$^{+/+}$ and *Sema3e*$^{-/-}$ mice were stimulated with LPS (100ng/ml). mRNA expression of IL-6 (A) and TNF(B) were quantified by RT-qPCR. Protein expression of IL-6 (B) and TNF (D) were measured by using ELISA. The results are from three independent experiments and are expressed as mean±SEM (n=4). Two-way ANOVA with Tukey's post hoc test was performed to determine the significances between the groups at various time points *$p<0.05$, **$p<0.01$.

6.7 Decreased activity of pro-inflammatory signaling molecules in *Sema3e*$^{-/-}$ BMDMs upon LPS stimulation

Intracellular signaling proteins (MAPKs and STATs) regulate the production of pro-inflammatory cytokines in macrophages. Therefore, we determined the impact of a lack of *Sema3e* in BMDMs on signaling proteins upon LPS stimulation. We have stimulated BMDMs with LPS and cell lysates were collected at 0, 1, 5, 15, 30, 60 and 120 min. Total and phosphorylated proteins of signaling molecules (STAT3, ERK1/2, and NF-κB) were quantified by Western blot. Results indicated that, the absence of *Sema3e,* impaired phosphorylation of STAT3 (Figure 17A), ERK1/2 (Figure 17B), NF-κB (Figure 17C) in BMDMs following LPS stimulation.

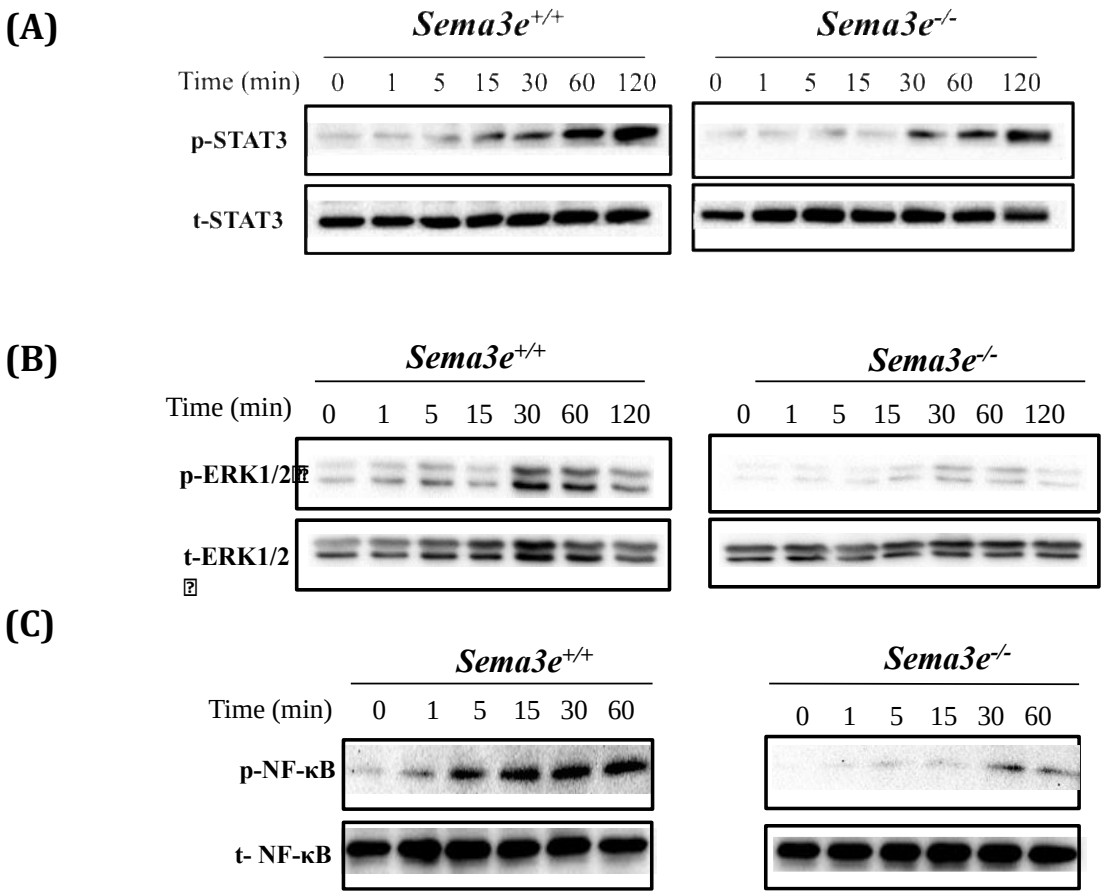


Figure 18. *Sema3e*$^{-/-}$ BMDMs have decreased activity of pro-inflammatory signaling molecules upon LPS stimulation. BMDMs from *Sema3e*$^{+/+}$ and *Sema3e*$^{-/-}$ mice were stimulated with LPS (100ng/ml) and cell lysates were collected at the indicated time points. Total and phosphorylated signaling STAT3 (A), Erk1/2 (B) and NF-κB (C) were quantified by Western blotting.

6.8 Impact of peritoneal macrophages depletion on the initial phase of LPS induced clinical score

We found an initial brief protection in hypothermia and clinical score in *Sema3e*$^{-/-}$ mice after LPS exposure. We hypothesized that this brief protection might be due to hyporesponsive macrophages. To see the exact role of macrophages during this initial brief protection, we depleted peritoneal macrophages by injecting clodronate liposomes (figure. 18B). As per previous publications, depletion of macrophages reduces the severity of LPS induced endotoxemia [248, 249]. Similar to previous reports, depletion of peritoneal macrophages reduces the severity of clinical score and hypothermia in both *Sema3e*$^{-/-}$ and *Sema3e*$^{+/+}$ mice. However, in the absence of peritoneal macrophages, the brief initial protection disappeared in *Sema3e*$^{-/-}$ mice, (Figure 18C & D.). This suggests that initial protection from LPS in *Sema3e*$^{-/-}$ mice is due to hyporesponsiveness of macrophages and this was confirmed in earlier experiments.

Table 12. Clinical score in macrophages depleted and macrophages sufficient animals after LPS treatment

Mice Group	Animal ID	Appearance	Movement	Eye condition	Response to tactile stimuli	Total
Clinical score 0 hr after LPS						
***Sema*$^{+/+}$ + CLD Lip + LPS**	1	0	0	0	0	0
	2	0	0	0	0	0
	3	0	0	0	0	0
***Sema*$^{-/-}$ + CLD Lip + LPS**	1	0	0	0	0	0
	2	0	0	0	0	0
	3	0	0	0	0	0
***Sema*$^{+/+}$ + Ctrl Lip + LPS**	1	0	0	0	0	0
	2	0	0	0	0	0
	3	0	0	0	0	0
***Sema*$^{-/-}$ + Ctrl Lip + LPS**	1	0	0	0	0	0
	2	0	0	0	0	0
	3	0	0	0	0	0
Clinical score 2 hr after LPS						
***Sema*$^{+/+}$ + CLD Lip + LPS**	1	0	0	0	0	0
	2	0	0	0	0	0
	3	1	0	0	0	1
***Sema*$^{-/-}$ + CLD Lip + LPS**	1	0	0	0	0	0
	2	1	0	0	0	1
	3	1	0	0	0	1
***Sema*$^{+/+}$ + Ctrl Lip + LPS**	1	1	1	0	0	2
	2	1	1	0	0	2
	3	1	1	0	0	2
***Sema*$^{-/-}$ + Ctrl Lip + LPS**	1	0	0	0	0	0
	2		0	0	0	0
	3	1	1	0	0	2
Clinical score 4 hr after LPS						
***Sema*$^{+/+}$ + CLD Lip + LPS**	1	1	1	0	0	2
	2	1	0	0	0	1
	3	0	0	0	0	0
***Sema*$^{-/-}$ + CLD Lip + LPS**	1	1	1	0	0	2
	2	0	0	0	0	0
	3	1	0	0	0	1
***Sema*$^{+/+}$ + Ctrl Lip**	1	2	1	1		4

Mice Group	Animal ID	Appearance	Movement	Eye condition	Response to tactile stimuli	Total
+ LPS	2	2	1	0	0	3
	3	2	1	0	0	3
***Sema*$^{-/-}$ + Ctrl Lip + LPS**	1	1	0	0	0	1
	2	1	1	0	0	2
	3	1	1	0	0	2
Clinical score 8 hr after LPS						
***Sema*$^{+/+}$ + CLD Lip + LPS**	1	1	1	0	0	2
	2	1	1	0	0	2
	3	2	1	0	0	3
***Sema*$^{-/-}$ + CLD Lip + LPS**	1	1	1	0	0	2
	2	2	1	0	0	3
	3	2	1	0	0	3
***Sema*$^{+/+}$ + Ctrl Lip + LPS**	1	2	2	1	1	6
	2	2	2	1	1	6
	3	2	2	1	1	6
***Sema*$^{-/-}$ + Ctrl Lip + LPS**	1	2	2	1	1	6
	2	2	2	1	1	6
	3	2	1	1	1	5
Clinical score 12 hr after LPS						
***Sema*$^{+/+}$ + CLD Lip + LPS**	1	2	1	1	1	5
	2	1	1	1	1	4
	3	1	1	1	1	4
***Sema*$^{-/-}$ + CLD Lip + LPS**	1	2	1	1	1	5
	2	2	2	1	1	6
	3	1	1	1	1	4
***Sema*$^{+/+}$ + Ctrl Lip + LPS**	1	2	2	1	1	6
	2	1	2	2	1	6
	3	2	2	1	1	6
***Sema*$^{-/-}$ + Ctrl Lip + LPS**	1	1	2	2	1	6
	2	1	2	2	1	6
	3	2	2	1	1	6
Clinical score 24 hr after LPS						
***Sema*$^{+/+}$ + CLD Lip + LPS**	1	2	1	1	1	5
	2	2	1	1	1	5
	3	1	1	2	1	5
***Sema*$^{-/-}$ + CLD Lip + LPS**	1	2	1	1	1	5
	2	2	2	2	1	7
	3	2	2	2	1	7

Mice Group	Animal ID	Appearance	Movement	Eye condition	Response to tactile stimuli	Total
***Sema*$^{+/+}$ + Ctrl Lip + LPS**	1	2	2	1	1	6
	2	2	1	2	1	6
	3	2	2	2	1	7
***Sema*$^{-/-}$ + Ctrl Lip + LPS**	1	2	2	2	1	7
	2	2	2	2	1	7
	3	2	2	2	1	7

Ctrl Lip = Control liposomes

CLD Lip = Clodronate liposomes

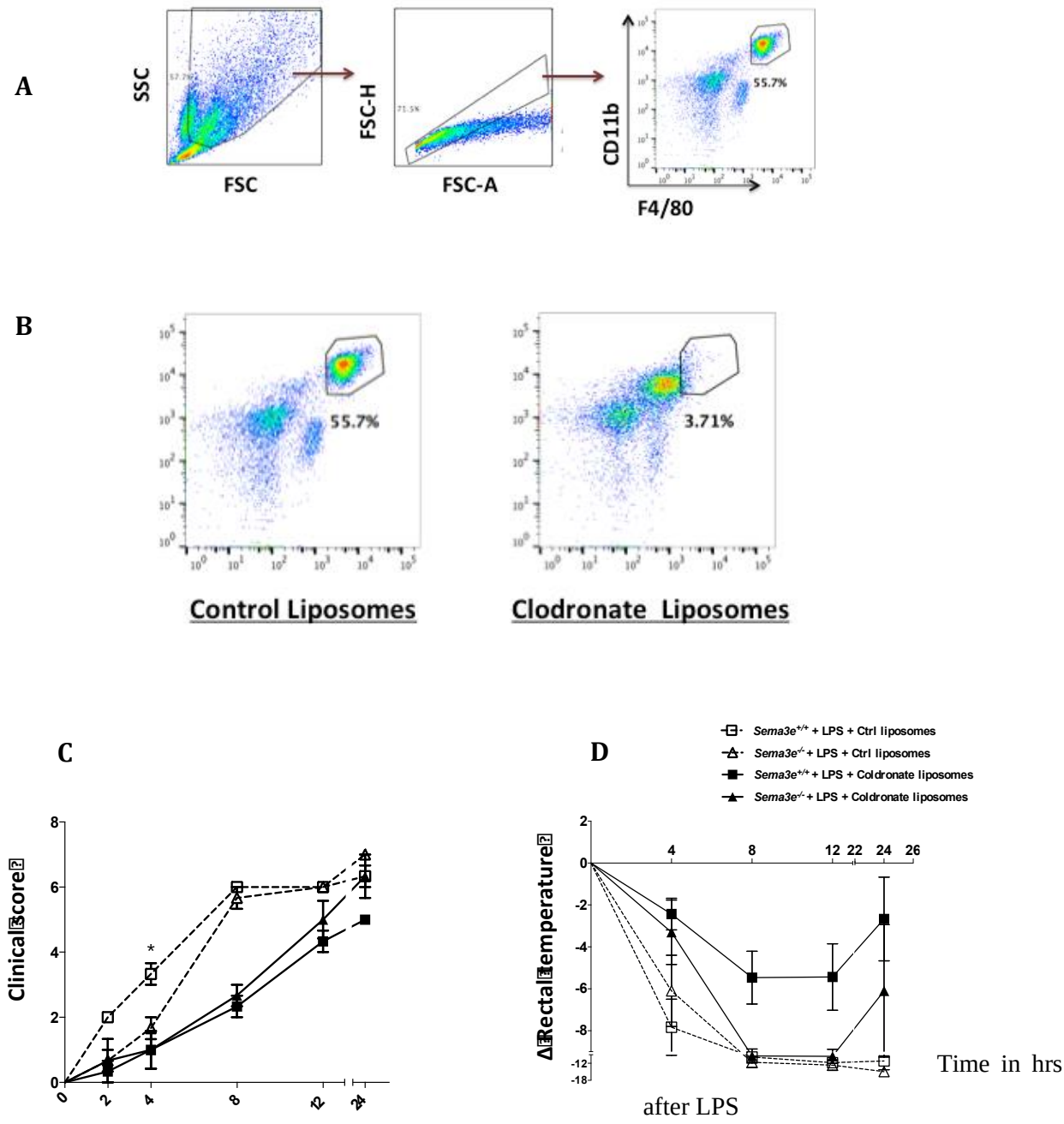


Figure 19. Depletion of peritoneal macrophages eliminates the differences in initial transient hypothermia between *Sema3e*$^{-/-}$ and *Sema3e*$^{+/+}$ mice after LPS. Mice were depleted with peritoneal macrophages by injecting clodronate liposomes intraperitoneally. After 24 of clodronate liposomes injection, animals were treated with LPS (30mg/kg, i.p). Gating strategy (A), Representative macrophage depletion flow graph (B), Clinical score (C) & rectal temperature (D). Results are expressed in Mean ± SEM (n=4), Two-way ANOVA with Tukey's post hoc test was performed to determine the significances between the groups at various time points *$p<0.05$.

6.9 T cells from *Sema3e*$^{-/-}$ mice are less activated upon LPS exposure

Apart from DCs, macrophages also contribute to activation of T cells [108]. Macrophages stimulate T cells via direct interaction through MHC II or by releasing IL-12 [108]. Based on the literature, we were interested to explore the influence of macrophages on the activation status of CD+ T cells in peripheral tissues, such as spleen, mesenteric lymph nodes (MLN) and lungs. We have used CD69 as an activation marker of T cells since CD69 is a well characterized indicator of T cell activation [250]. Interestingly, we found less expression of CD69 in CD+ cells of the spleen (Figure 20A & 21A), MLN (Figure 20B & 21B) and lungs (Figure 20C & 21C) in *Sema3e*$^{-/-}$ mice compared to WT littermates upon LPS treatment.

Gating strategy

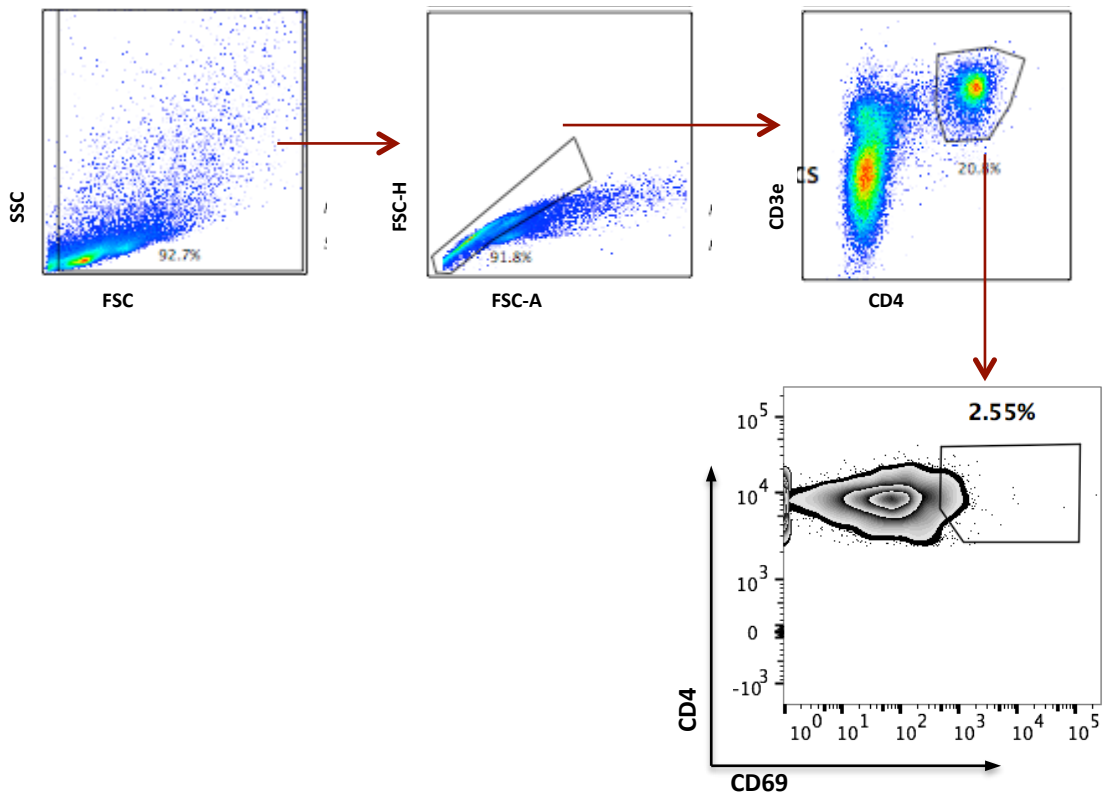


Figure 20. Gating strategy for T cells activation. Gating strategy for T cells activation in spleen, mesenteric lymph node (MLN) and lung. The above representative flow graphs are from spleen.

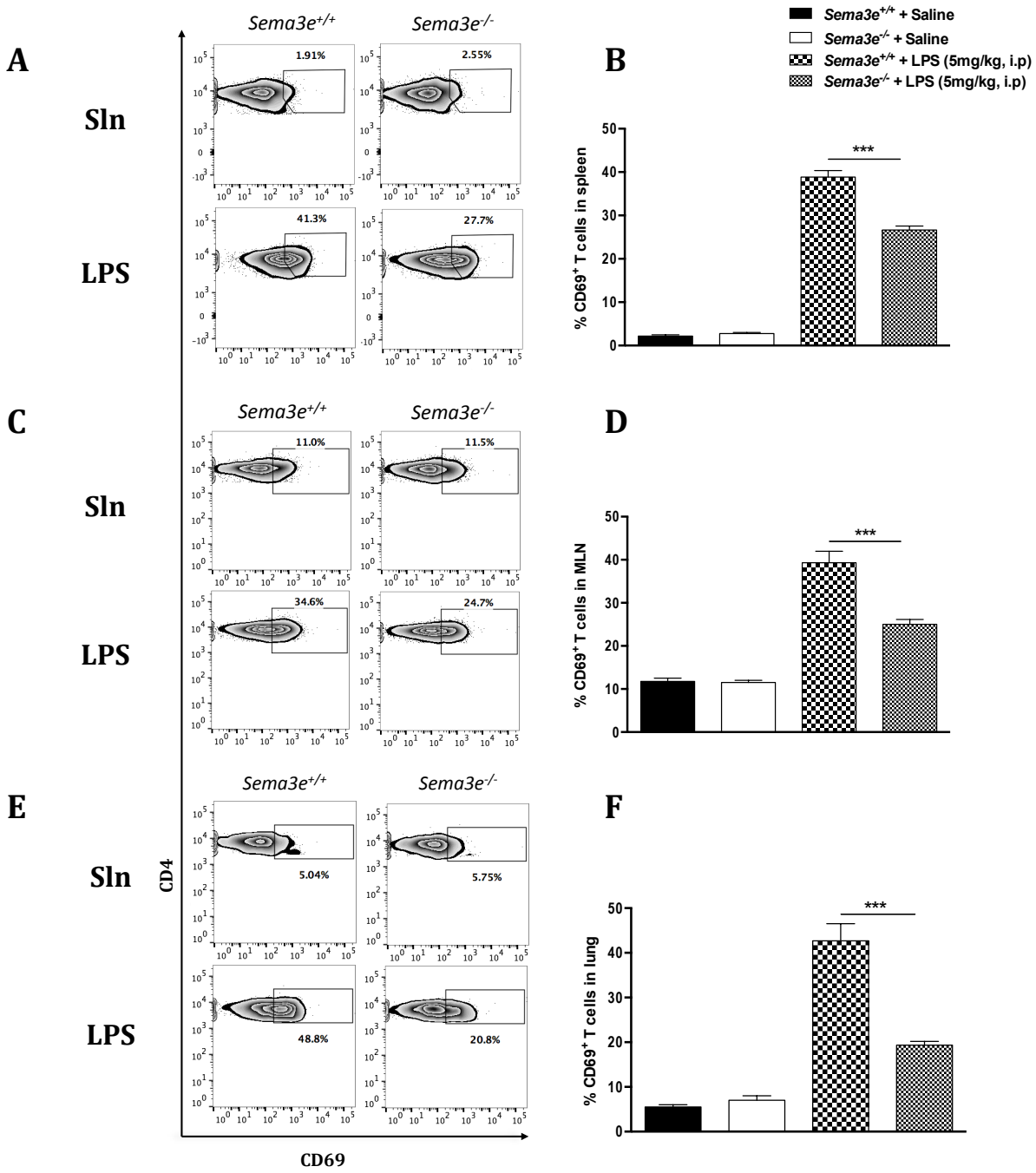


Figure 21. T cells from *Sema3e*$^{-/-}$ mice are less activated upon LPS exposure. Mice were injected with a sub-lethal dose of LPS (5mg/kg, i.p) and sacrificed after 8 hrs of LPS. CD69+ T cells were analyzed by flow cytometry. Flow graph of CD69+: Spleen (A), MLN (C) and Lung (E). Statistical representation of % CD69+ T cells: Spleen (B), MLN (D) and Lung (F). Results are expressed in Mean ± SEM (n=6).

One-way ANOVA with post-hoc Bonferroni analysis was performed to determine significances difference between the groups ***$p<0.001$. Sln= Saline.

Gated on CD3e+ CD4+ cells

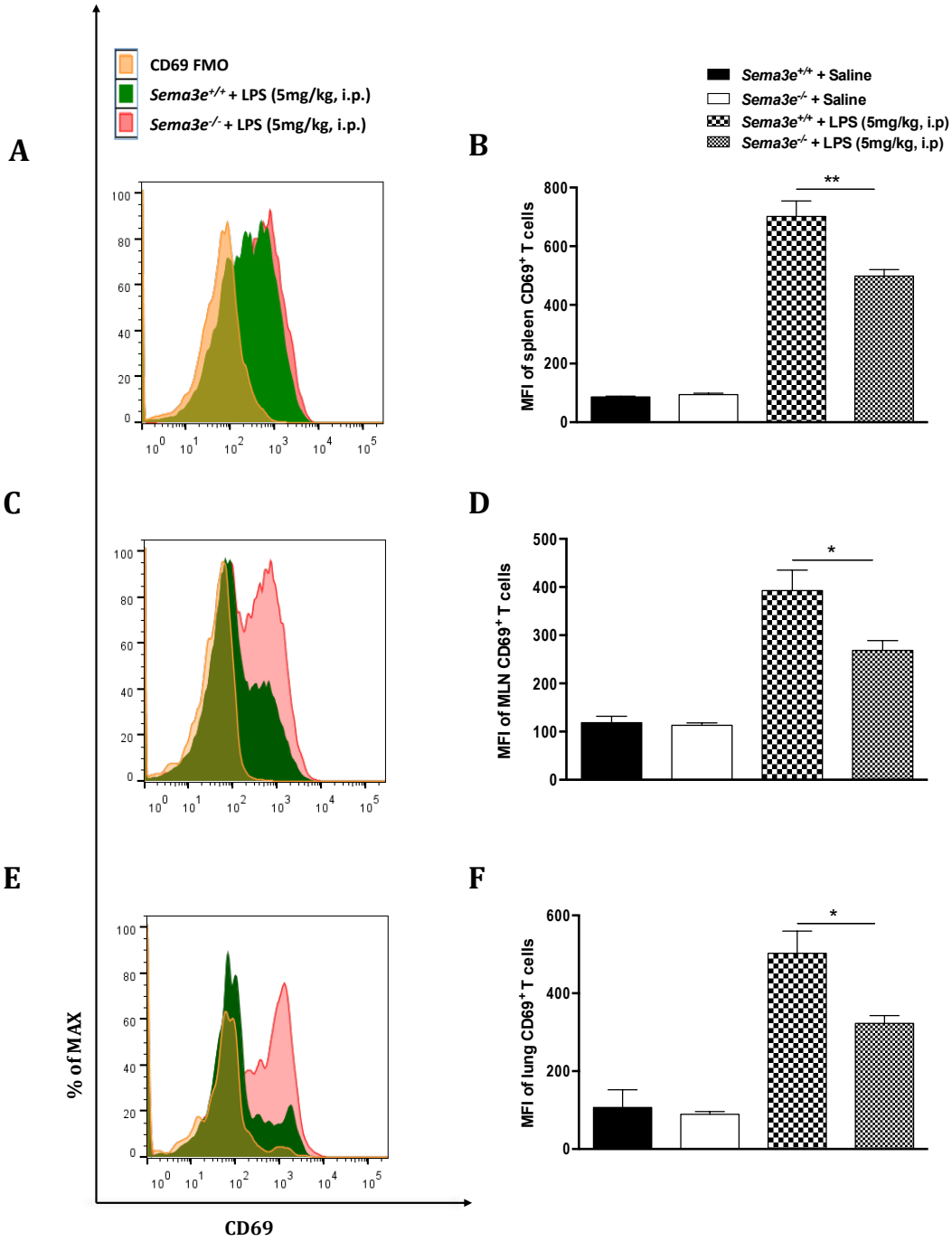


Figure 22. T cells from *Sema3e*$^{-/-}$ mice are less activated upon LPS exposure. Mice were injected with a sub-lethal dose of LPS (5mg/kg, i.p) and sacrificed after 8 hrs of LPS. CD69+ T cells were analyzed by flow cytometry. Flow graph of CD69+: Spleen (A), MLN (C) and Lung (E). Statistical representation of MFI of CD69+ T cells: Spleen (B), MLN (D) and Lung (F). Results are expressed in Mean ± SEM (n=6).

One-way ANOVA with post-hoc Bonferroni analysis was performed to determine significances difference between the groups $*p<0.05$, $**p<0.01$.

7 SUMMARY OF RESULTS

1. *Sema3e*$^{-/-}$ mice are protected from LPS-induced hypothermia transiently at an early time point

2. LPS induced serum and PL pro-inflammatory cytokines are significantly suppressed in *Sema3e*$^{-/-}$ mice compared to WT littermates.

3. PL macrophages from *Sema3e*$^{-/-}$ exhibited reduced expression of iNOS upon LPS injection.

4. *Sema3e*$^{-/-}$ mice showed less expansion of blood monocytes after LPS treatment.

5. Expression of pro-inflammatory cytokines was significantly less in *Sema3e*$^{-/-}$ BMDMs.

6. Deficiency in Sema3E impairs phosphorylation of STAT3, ERK1/2 and NF-κB in BMDMs upon LPS stimulation in vitro.

7. Depletion of macrophages from *Sema3e*$^{-/-}$ and *Sema3e*$^{+/+}$ mice abolished the difference in LPS-induced hypothermia and clinical score.

8. *Sema3e* gene deletion impairs T cell activation *in vivo*.

8 DISCUSSION

Macrophages are essential to innate immunity. These innate cells limit infections through their ability of phagocytosis and the release of inflammatory mediators [251]. Macrophages play an essential role in initiation and progression of inflammation [252], so that the response of macrophages against infections will determine the severity of inflammation [253]. Presence of hyporesponsive macrophages or total absence of macrophages fails to initiate a strong immune response against LPS [83, 248]. In this dissertation, we have demonstrated that the deletion of the *Sema3e* gene makes resident macrophages hyporesponsive to LPS. We found less iNOS expression in PL macrophages of *Sema3e*$^{-/-}$ mice upon LPS injection. BMDMs from *Sema3e*$^{-/-}$ mice produce lesser TNF and IL-6 upon LPS exposure. Our data also suggest that decreased macrophage activation against LPS is due to a defect in TLR-4 signaling. Finally, the *Sema3e* gene deletion causes impairment in T cell activation *in vivo*. Collectively, these results indicate that Sema3E is essential for optimal activation of macrophages.

Most of the semaphorins are expressed by immune cells and they act through either paracrine or autocrine manner. Among semaphorins, Sema3E is an important protein that takes part in various physiological and pathological conditions. A recent study demonstrated that Sema3E attracts pro-inflammatory monocytes/macrophages in adipose tissue, where these cells produce pro-inflammatory cytokines (IL-6 and TNF) [206]. However, this study did not show a direct involvement of Sema3E on macrophages activation. In another published report the stimulation of macrophages with LPS caused

an increase in the expression of Sema3E [241], though the authors did not address whether higher expression of Sema3E actually dampens macrophage activation or facilitates this phenomenon. Recently published studies reveal that one of the members of class 3 semaphorins such as Sema3A is crucial for the activation of macrophages [226, 254]. Direct stimulation of macrophage parental cells (monocytes) with Sema7A induces pro-inflammatory factors [255]. Our results indicate that *Sema3e* gene deletion in mice causes tissue macrophages to express less iNOS and pro-inflammatory cytokines against LPS. We also found BMDMs from *Sema3e*$^{-/-}$ release reduced levels of pro-inflammatory cytokines (IL-6 & TNF) upon LPS exposure. These findings indicate that the absence of Sema3E renders macrophages to become hyporesponsive to LPS exposure.

It is well known that LPS binds with TLR-4 receptors and causes activation of macrophages. Abnormality in activation of the macrophages could be the result of a defect in TLR-4 signaling. Activation of TLR-4 leads to downstream activation of ERK1/2 and NF-κB that eventually leads to production of pro-inflammatory cytokines and iNOS expression [134, 256]. Some of the semaphorins have been reported as potential modulators of TLRs signaling molecules. One study revealed that the absence of the sema3A-plexins axis reduces activation of NF-κB on the other hand this axis did not alter ERK1/2 phosphorylation in peritoneal macrophages [226]. Another paper showed the importance of Sema3A in ERK1/2 and NF-κB activation in microglial cells [254]. According to this study, Sema3A presence is necessary for appropriate activation of NF-κB and Erk1/2 through TLR-4 signaling pathway [254]. Our results show that the

Sema3e gene deletion disturbs the TLR-4 signaling pathway. Absence of Sema3E impairs activation of NF-κB, ERK1/2 and STAT3. Inhibition of ERK1/2 and NF-κB in *Sema3e*$^{-/-}$ BMDMs seems to be due to a disturbance in TLR-4 signaling. We also observed less activation of STAT3 in BMDMs upon *Sema3e* deletion. Literature showed that the STAT3 activation is mainly mediated by IL-6R [257, 258]. Interestingly, we found less IL-6 release from BMDMs upon LPS exposure. This indicates that diminished activation of STAT3 in *Sema3e*$^{-/-}$ macrophages in our study might be the result of reduced expression of IL-6. Overall, our study revealed that the *Sema3e* gene is an important element for activation of key pro-inflammatory signaling molecules in macrophages upon LPS exposure. Our results are restricted to activation of key TLR-4 signaling molecules (ERK1/2 and NF-κB), but involvement of other TLR-4 associated downstream signaling pathways in context of Sema3E is yet to be clearly known. Impact of Sema3E/TLR-4 signaling pathways in macrophages can be studied at different levels by using specific inhibitors for each signaling molecules.

In blood, monocytes encounter with systemic infections and release a plethora of pro-inflammatory cytokines [247]. These monocytes will extravasate into tissue spaces where they become activated macrophages [259]. During inflammation, the absolute number of blood monocytes rises dramatically due to an increase in the rate of migration from bone marrow [259]. In our study, we found less pro-inflammatory monocytes in *Sema3e*$^{-/-}$ mice blood compared to WT mice. A reduced level of pro-inflammatory cytokines in the serum of *Sema3e*$^{-/-}$ mice might be the reason for less expansion of blood monocytes. However, these observations can be strengthened by performing *in vitro*

stimulation of monocytes and also distribution of monocytes between tblood and bone marrow at the specified time points after LPS administration. *In vitro* activation explains the extent of monocytes capability to release pro-inflammatory cytokines. While a distribution study will elucidate the migration pattern of monocytes from bone marrow to blood. An *in vitro* monocytes migration assay might also be helpful to know the impact of Sema3E on migration.

Previous reports demonstrated that TNF and IL-6 are key cytokines to induce hypothermia upon LPS treatment [29]. In our study, we found less TNF and IL-6 level in the serum of *Sema3e*$^{-/-}$ mice upon LPS injection. This could be explained as follows: *Sema3e*$^{-/-}$ mice are protected from hypothermia for a transient time since TNF and IL-6 appear in the blood for a brief period immediately after LPS. Peritoneal macrophages are key innate cells responsible for initiation and progression of the immune response upon intraperitoneal injection of LPS. However, we have seen impaired activation of peritoneal macrophages in *Sema3e*$^{-/-}$mice, which could be the reason we observed less cytokines in the serum which in turn protected animals from hypothermia. To explain the relation between macrophages and hypothermia, we depleted peritoneal macrophages using clodronate liposomes from *Sema3e*$^{-/-}$ as well as *Sema3e*$^{+/+}$ mice. After macrophages depletion, the difference in rectal temperature at initial time points (up to 4 hours of post LPS) between *Sema3e*$^{-/-}$ and WT mice was abolished. This indicates that the hyporesponsive macrophages in *Sema3e*$^{-/-}$ mice are responsible for the initial transient hypothermia.

It is well known that macrophages play a significant role in the activation of T cells. Macrophages release factors that commit T cells to become specific TH cells [260]. The output of mediators from macrophages depends upon the nature of stimuli from which macrophages have been encountered [111]. Upon LPS exposure, macrophages polarize towards a classical M1 subset (more iNOS expression). These M1 macrophages activate CD4+ cells to become a TH1 subset via direct interaction through MHCII or by releasing IL-12 [108]. Moreover, chemokines (CXCL 9 and CXCL 10) released from activated M1 macrophages attracts TH1 cells in the vicinity[261]. Collectively, M1 macrophages are the key players in boosting TH1 immunity. Previous reports indicate that other members of semaphorins such as sema4D and Sema3A are important for T cell activation [179] [181]. We found that *Sema3e*$^{-/-}$ mice show less activated CD4+ cells upon LPS exposure. This phenomenon might be due to hyporesponsive M1 macrophages (iNOS+) in *Sema3e*$^{-/-}$ mice. This could be explained by measuring MHCII surface expression on macrophages in various tissues after LPS injection [108]. Furthermore, direct *in vitro* interaction of macrophages and CD4+ cells in LPS environment will also provide valuable information about the influence of macrophages on T cells activation in context of Sema3E.

In conclusion, our results demonstrate that Sema3E is involved in the immune response and activation of signaling molecules associated with inflammatory cytokine production in macrophages. Deletion of Sema3e makes macrophages less responsive to inflammatory stimuli. Thus, targeting Sema3E could be a novel strategy to control

macrophage-mediated inflammatory diseases such as sepsis, rheumatoid arthritis, chronic obstructive pulmonary disease and inflammatory bowel disease.

9 REFERENCES

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