Sex-Dependent Immune Modulation: The Impact of Sema3E/PlexinD1 Deficiency on Macrophages

by

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ABSTRACT

The Sema3E/PlexinD1 axis has been shown to regulate immune responses, especially in allergic inflammation and macrophage cytokine production. Previous studies have shown that this axis is required for immune homeostasis and its disruption can worsen inflammatory responses, as seen in lung interstitial macrophages (IMs). Sex differences in innate immunity, including macrophages, are well established, with males being more pro-inflammatory and females being more anti-inflammatory. This sex difference can impact how immune cells, regulate cytokine production, including IL-10, a key anti-inflammatory cytokine. My thesis focuses on the role of Sema3E/PlexinD1 in modulating IL-10 and TNF in macrophages, with an emphasis on sex differences and receptor dynamics, particularly TLR4 and CD14.

The main hypothesis of this work is that deletion of Sema3E/PlexinD1 changes IL-10 production in response to LPS in a sex-dependent manner, leading to different inflammatory outcomes in males and females. To test this, the project used an HDM model of allergic asthma *in vivo* and measured cytokines in bone marrow-derived macrophages (BMDMs) *in vitro*, following TLR4 activation. BMDMs were challenged with 100 ng/ml of LPS and cytokine protein levels were measured by ELISA. Flow cytometry was used to analyze TLR4 and CD14 internalization dynamics. Sema3E-Fc was added before LPS stimulation to see if cytokine production and receptor dynamics could be restored.

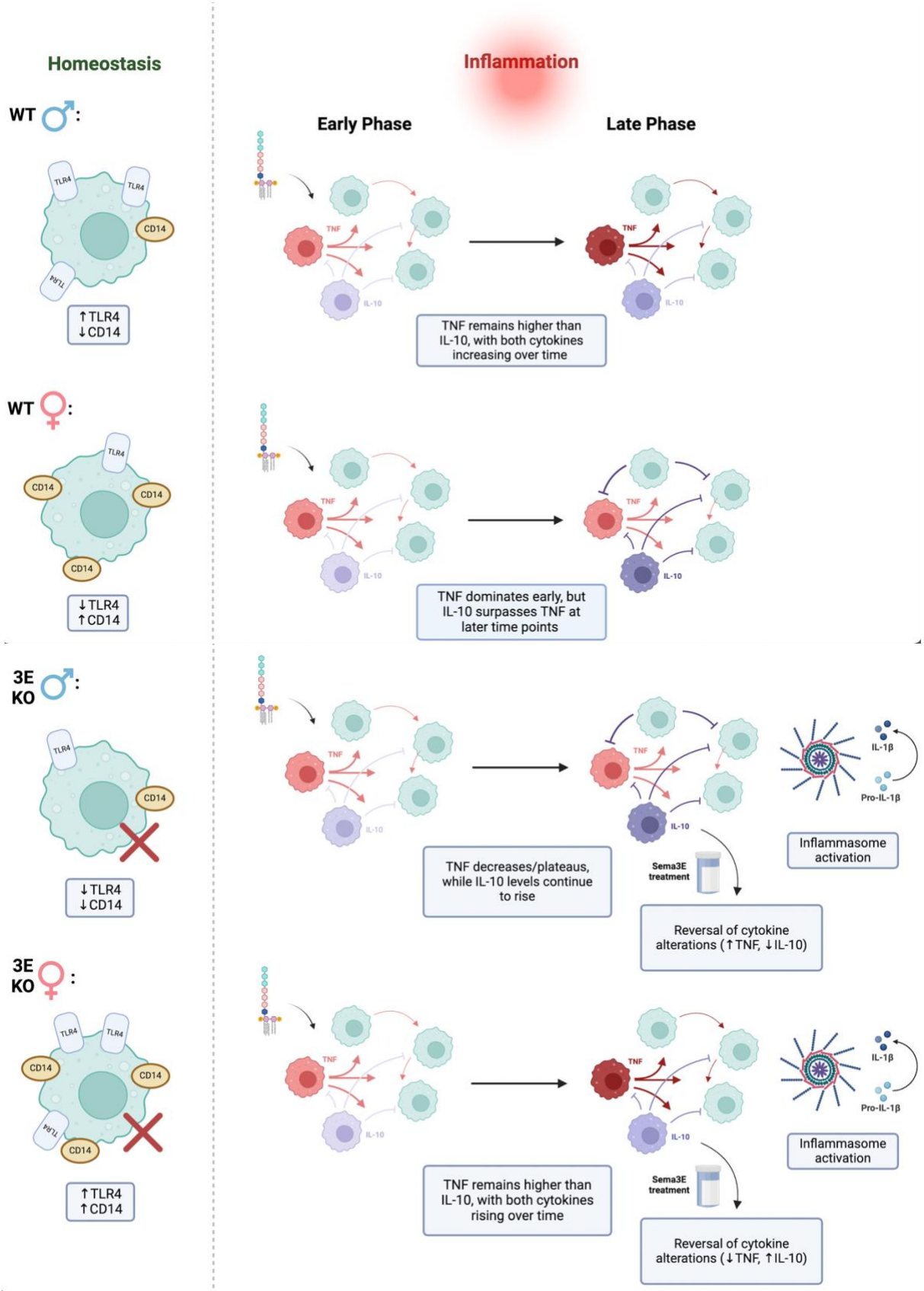
The results showed sex-specific cytokine production in Sema3E KO BMDMs. Males produced more IL-10, while females produced more TNF. PlexinD1 KO BMDMs displayed an early increase in TNF production shortly after LPS stimulation, indicative of an immediate pro-inflammatory response. This was followed by a delayed increase in IL-10 levels, a key anti-inflammatory cytokine, at later times. This sequential pattern suggests a counter-regulatory mechanism where the initial pro-

inflammatory TNF response triggers a subsequent rise in IL-10 to limit inflammation and promote resolution of the immune response.

Sema3E-Fc restored cytokine profiles in a sex-dependent manner. In female BMDMs, Sema3E-Fc treatment decreased TNF and increased IL-10 levels, whereas in male BMDMs, it increased TNF and decreased IL-10 levels. This highlights the importance of Sema3E's role in modulating inflammation differently between sexes, further highlighting its regulatory function in immune responses. Sema3E KO disrupted TLR4 and CD14 receptor dynamics, with an inverse correlation in WT BMDMs and a positive correlation in Sema3E KO BMDMs. Sema3E KO and PlexinD1 KO BMDMs secreted IL-1β after LPS stimulation, suggesting NLRP3 inflammasome activation. This was sex-dependent, with Sema3E enhancing IL-1β secretion in males and decreasing it in females.

Overall, this demonstrates the complex role of Sema3E in macrophage polarization, cytokine production, and inflammasome activation, and that there are sex differences in immune responses.

GRAPHICAL ABSTRACT



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To my lab, thank you for being a constant source of support, always being there to lend a helping hand, and becoming like family to me. Your encouragement and love made the many long hours in the lab not only manageable but enjoyable.

I also owe a huge debt of gratitude to my family. Thank you for your unwavering belief in me and for supporting me throughout my many years of education. I especially want to thank you for helping to take care of my two sweet boys while I spent long hours in the lab. Your love and support have been invaluable.

Finally, to my amazing husband, Sam, thank you for being my rock throughout this journey. You've been by my side through all the ups and downs, always reminding me to laugh when things got tough. You've been my partner in everything, and I couldn't have done this without you—thank you for your endless love, support, and for making me laugh when I needed it most.

DEDICATION

To my two incredible boys, Jack and Will,

Thank you for not burning the house down while I spent long hours in the lab, pipetting away instead of watching *Sesame Street* with you. Nothing is better than when you both run into my arms after a long day. My hope is that one day, when you are older, you'll look back and know that all this hard work was for you. May it inspire you to chase your own dreams (but maybe with fewer late nights and less coffee). You two are my greatest motivation and best buddies, and I hope I've made you proud.

Love,

Mom

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACK: Ammonium-chloride-potassium

ACPAs: Anti-citrullinated protein antibodies

AKT: Protein kinase B (PKT)

AMs: Alveolar macrophages

ANOVA: Analysis of variance

APCs: Antigen-presenting cells

AP1: Activator protein 1

ARDS: Acute respiratory distress syndrome

AREs: Adenosine-uridine-rich regions

ARF6: ADP-ribosylation factor 6

ATP: Adenosine triphosphate

BMDMs: Bone marrow-derived macrophages

β-ME: β-mercaptoethanol

CARS: Compensatory anti-inflammatory response syndrome

CCL2: Monocyte chemoattractant protein 1

CCL11: Eotaxin-1

CD14: Cluster of differentiation 14

C/EBPβ: CCAAT/enhancer binding protein-β

COPD: Chronic obstructive pulmonary disease

DAMPs: Damage-associated molecular patterns

DCs: Dendritic cells

DNA: deoxyribonucleic acid

DUSP1: Dual-specificity protein phosphatase 1

ECP: Eosinophil cationic protein

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme-linked immunosorbent assay

EMT: epithelial-to-mesenchymal transition

EPO: Eosinophil peroxidase

ER: Estrogen receptor

ERK: Extra-cellular signal regulated kinases

FMO: Fluorescence minus one

FOXP3: Forkhead box p3

GAP: GTPase-activating protein

GM-CSF: Granulocyte/macrophage colony-stimulating factor

GnRH: Gonadotropin-hormone releasing hormone

GPI: Glycosylphosphatidylinositol

GSK3: Glycogen synthase kinase 3

HUVECs: Human umbilical vein endothelial cells

IBD: Inflammatory bowel disease

ICs: Immune complexes

IFNs: Type I interferons

IFN-β: Interferon-β

IFN-γ: Interferon- γ

IKK: IκB kinase

IKKε: IκB kinase epsilon

IL: Interleukin

ILCs: Innate lymphoid cells

IMCs: Immature myeloid cells

IMs: Interstitial macrophages

iNOS: Nitric oxide synthase

IPF: Idiopathic pulmonary fibrosis

IRAK: IL-1 receptor-associated kinase

IRF3: Interferon regulatory factor 3

JAK: Janus kinase

JNK: C-jun n-terminal kinase

KO: Knock-out

LBP: LPS binding protein

LDL: Low-density lipoprotein

LPS: Lipopolysaccharide

MAPK: Mitogen-activated protein kinase

MBP: Major basic protein

M-CSF: Macrophage colony-stimulating factor

MDA5: Melanoma differentiation-associated gene 5

MDSCs: Myeloid-derived suppressor cells

MD-2: Myeloid differentiation factor 2

MHC: Major histocompatibility complex

miRNAs: MicroRNAs

MMPs: Matrix metalloproteinases

MODS: Multiple organ dysfunction syndrome

MyD88: Myeloid differentiation primary response gene 88

NEMO: NF-κB essential modulator

NETs: Neutrophil extracellular traps

NF-kβ: Nuclear factor kb

NK: Natural killer

NLRs: Nucleotide-binding oligomerization domain-like receptors

NO: Nitric oxide

NOD: Nucleotide-binding oligomerization domain

OXPHOS: Oxidative phosphorylation

PAMPs: Pathogen-associated molecular patterns

PBMCs: Peripheral blood mononuclear cells

PGE2: Prostaglandin E2

PIP5K: Phosphatidylinositol-4-phosphate 5-kinases

PI3K: Phosphatidylinositide 3-kinases

PRRs: Pattern-recognition receptors

PSI: Plexin-semaphorin-integrin

RA: Rheumatoid arthritis

RBCs: Red blood cells

RBD: Rho-GTPase-binding domain

RF: Rheumatoid factor

RhoJ: Ras homolog family member J

RIG-I: Retinoic acid-inducible gene I

RLRs: Retinoic acid-inducible gene I-like receptors

RNA: Ribonucleic acid

RNS: Reactive nitrogen species

ROS: Reactive oxygen species

RPMI: Roswell Park Memorial Institute

RT-PCR: Real-time reverse transcription-polymerase chain reaction

SCF: Stem cell factor

Sema: Semaphorin

Sema3E: Semaphorin3E

SIRS: Severe inflammatory response syndrome

SLE: Systemic lupus erythematosus

STAT: Signal transducer and activator of transcription

TAK1: Transforming growth factor-β-Activated Kinase 1

TAM: Tamoxifen

TBK1: TANK-binding kinase 1

TGF- β: Transforming growth factor-β

TICAM1: TIR-domain-containing adaptor molecule 1

TIR: Toll/IL-1 receptor

TIRAP: TIR domain-containing adaptor protein

TLR4: Toll-like receptor 4

TLRs: Toll-like receptors

TNF: Tumour necrosis factor

TPL2: Tumour progression locus 2

TRAF6: TNF receptor-associated factor 6

TRAM: TRIF-related adaptor molecule

Tregs: Regulatory T cells

TRIF: TIR domain-containing adaptor inducing interferon-β

TTP: Tristetraprolin

UC: Ulcerative colitis

VEGF: Vascular endothelial growth factor

WT: Wild-type

1. INTRODUCTION

1.1 Inflammation

1.1.1 What is Inflammation?

When the body encounters something harmful like a virus, damaged cells or irritants, inflammation is part of the immune response [1, 2]. The five signs of inflammation are heat, pain, inflammation, swelling and loss of function [3-6]. The body uses inflammation as a key player in its innate immune system, the first line of defence. This system works independently of the specific attributes of the invading pathogen [7]. The adaptive immune system is specific, targeting each pathogen individually.

By immune cells, blood vessels and chemical mediators working together, inflammation serves a protective function. The main purposes are to eliminate the source of harm, remove dead or damaged cells, and start the healing of tissues [8]. Modulation of this process is critical because too little inflammation can lead to tissue damage from toxic chemicals and can be life-threatening. Too much/prolonged inflammation can lead to chronic inflammation, which is linked to autoimmune disorders, cardiovascular diseases and metabolic syndromes [9].

Historically, inflammation was seen as a reaction to microbial invasion or tissue damage. However, new research has shown that metabolic abnormalities like those seen in diabetes and obesity can trigger obvious and hidden inflammation. These metabolic disorders are now known to cause chronic low-grade inflammation, sometimes referred to as "meta-inflammation" [10], which greatly impacts the development of chronic diseases.

Inflammation is a complex process involving many immune cells, signalling molecules and regulatory pathways. Activated immune cells, like macrophages, produce important pro-inflammatory cytokines like tumour necrosis factor-alpha (TNF), interleukin (IL)-1β and IL-6 [11]. These cytokines are key to the inflammatory response. TNF, for example, triggers the production of more pro-inflammatory cytokines, attracts immune cells to the site of infection or injury, and induces fever and apoptosis [12,13]. TNF is important in fighting infections and tumour growth. However, when not regulated properly, it can contribute to chronic inflammatory conditions like rheumatoid arthritis [12] and inflammatory bowel disease [14-16]. Sepsis, an exaggerated inflammatory response which causes inflammation all over the body, blood vessel leakage, and failure of many organs, can result from too much TNF production [17, 18].

Conversely, anti-inflammatory cytokines like IL-10, reduce inflammation and restore tissue balance [19-21]. When there is inflammation, various immune cells produce IL-10. Its main function is to be a negative regulator by stopping the production of pro-inflammatory cytokines and preventing dendritic cells (DCs) and macrophages from presenting antigens [19-21]. It is crucial to maintain immunological homeostasis by balancing pro-inflammatory and anti-inflammatory cytokines. For example, TNF and other pro-inflammatory cytokines attract immune cells during bacterial infections. At the same time, IL-10 regulates inflammation to prevent excessive persistence and protect against tissue damage once the infection is under control.

Cytokine balance is important in viral infections as cytokines like TNF and interferons trigger antiviral response, and IL-10 regulates this response to prevent immune system damage. Too much IL-10 can lead to immunosuppression, which impairs the body's ability to get rid of

infections. This is seen in compensatory anti-inflammatory response syndrome (CARS) during sepsis [22, 23]. Patients in this state of immunosuppression are more prone to secondary infections, which can lead to higher mortality.

Chronic inflammation contributes to the development and progression of cancer by creating a tumour-promoting environment [24-26]. Pro-inflammatory cytokines like TNF can stimulate tumour growth, survival and spread [25-27]. Anti-inflammatory cytokines like IL-10 can help tumours evade the immune system [28-30].

In the end, inflammation serves a dual purpose: to boost the immune system, repair tissue, and handle infections and injuries (Figure 1). However, in the case of metabolic disorders, if inflammation persists over time, it can lead to continuous tissue damage and disease progression. Therapies that target pro-inflammatory cytokines, like TNF inhibitors, have been shown to reduce inflammation [31-36]. However, we need to be careful in regulating the levels of anti-inflammatory cytokines, like IL-10, to prevent immune suppression.

In summary, inflammation is both a defence mechanism and a cause of damage depending on its control and persistence. The body counteracts damaging stimuli and repairs by maintaining a balance between pro and anti-inflammatory responses and limiting the negative effects of prolonged or uncontrolled inflammation [37].

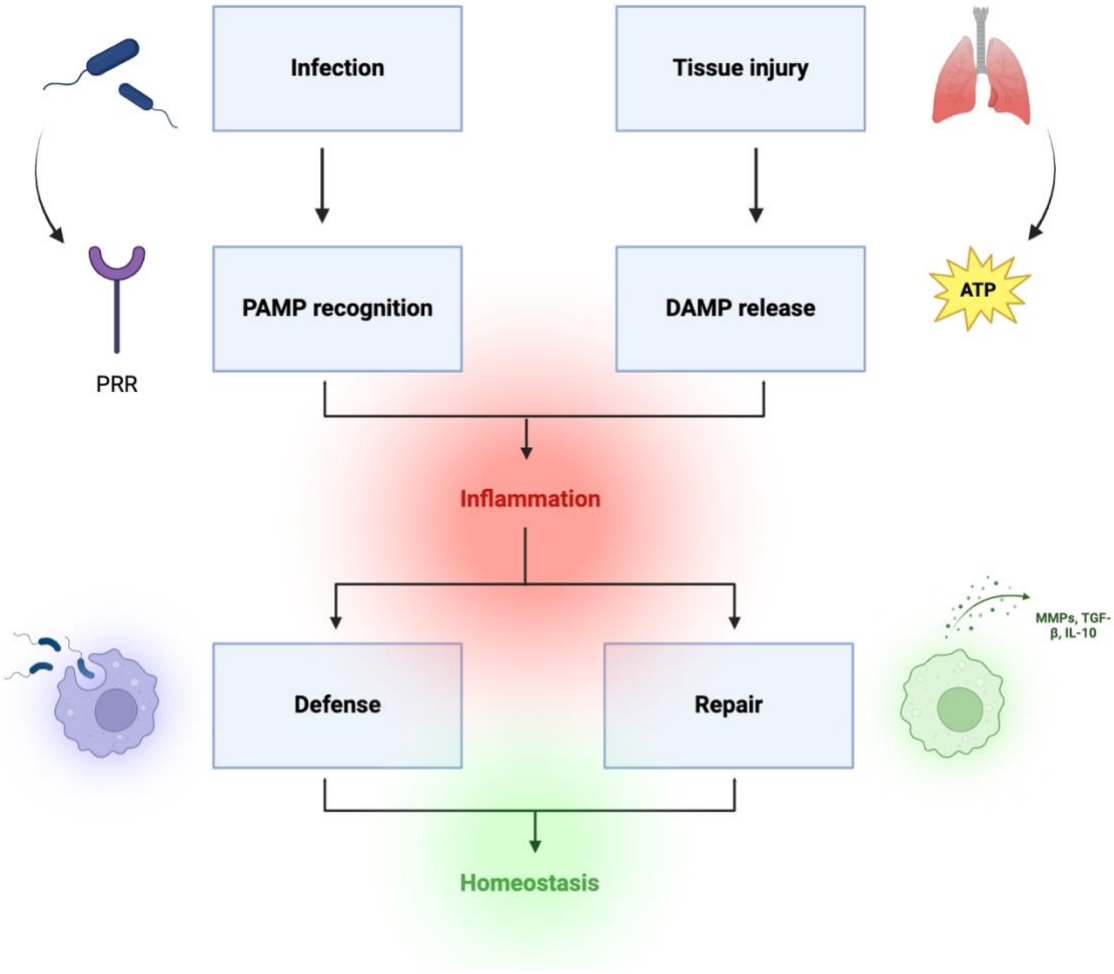

Figure 1. Inflammatory response in amplifying innate immunity and tissue repair

Inflammation is a biological response to harmful stimuli such as infection or tissue injury. This process is triggered by specific molecular signals such as pathogen-associated molecular patterns (PAMPs) from infections and damage-associated molecular patterns (DAMPs) like adenosine triphosphate (ATP) from injured tissue. These signals activate the inflammatory response, which directs two key processes: defence and repair. Defence involves immune cell activation, such as macrophages engulfing pathogens. Repair focuses on tissue healing, supported by anti-inflammatory mediators released by immune cells. Together, these processes restore homeostasis when properly regulated, preventing the transition to chronic inflammation.

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Adapted from: Robbins, S., Cotran, R., Kumar, V., Abbas, A., & Aster, J. (2020). *Pathologic basis of disease* (10th ed.). Philadelphia, PA: Saunders Elsevier.

1.1.2 Innate immunity and inflammation

The innate immune system is the first line of defence against microbial infections and is necessary to trigger adaptive immune responses and initiate tissue repair. It consists of two main parts: the cellular and humoral arms [38]. The cellular arm has pattern recognition receptors (PRRs) located in different cellular compartments such as the plasma membrane, endosomes, and cytoplasm, which recognize microbial invaders and tissue injury. These receptors such as toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), inflammasomes and scavenger receptors trigger the production of cytokines, adhesion molecules and antimicrobial agents and eliminate pathogens through phagocytosis [39-40].

Conversely, the humoral arm has chemicals such as pentraxins, collectins and ficolins. These molecules are precursors to antibodies by triggering the complement system, making pathogens more susceptible to engulfment by immune cells, and counteracting the effects of microorganisms [41]. These molecules are important in regulating inflammation and immune responses.

The innate immune system relies on molecular receptors and various immune cells to recognize and respond to infections. Important players are macrophages, DCs, neutrophils, natural killer (NK) cells, eosinophils and basophils, which respond quickly to infections and tissue injury. These cells have the receptors to recognize and eliminate danger and orchestrate adaptive immune responses [42].

Cells of the innate immune system

Macrophages are immune cells located in various tissues of the body [43]. Their main role is to constantly survey the tissues for signs of infection or injury (Figure 2). When macrophages encounter pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) they become active and initiate an inflammatory response [44]. This activation leads to the production of important pro-inflammatory cytokines, such as TNF, which increases the permeability of blood vessels to allow entry of immune cells; IL-1β which causes fever and induces expression of adhesion molecules on endothelial cells; and IL-6 which triggers the acute-phase response and synthesis of acute-phase proteins by the liver [42, 45]. Macrophages also secrete chemokines such as CXCL8 (IL-8), which attracts neutrophils to the site of infection or injury and intensifies the inflammatory response [42].

Beyond their inflammatory roles, macrophages carry out a wide range of critical functions. One of their key tasks is phagocytosis, where they engulf and digest pathogens, dead cells, and debris [46]. This process helps clear infections and maintain tissue health by removing harmful substances and damaged cells, preventing further injury or chronic inflammation. Macrophages can also activate the NLRP3 inflammasome in response to infections or cellular stress [47]. This activation leads to the production of IL-1β, a potent pro-inflammatory cytokine, and triggers pyroptosis, a type of cell death that eliminates intracellular pathogens while amplifying the immune response. Additionally, macrophages produce reactive oxygen species (ROS), which not only help kill bacteria and viruses but also serve as signalling molecules that regulate inflammation and attract other immune cells to the site of infection or injury [48].

Macrophages also play important roles beyond pathogen defence. They use autophagy to break down intracellular pathogens and damaged cellular components, a process that limits pathogen survival and prevents excessive immune activation by clearing danger signals [49]. In certain situations, they can induce apoptosis in infected or damaged cells, helping to remove potential threats and maintain tissue homeostasis. As antigen-presenting cells, macrophages process and display antigens to T cells, linking the innate and adaptive arms of the immune system and helping tailor responses to specific pathogens [50].

In addition to their defensive roles, macrophages contribute to tissue repair and remodelling. They release growth factors like TGF-β and VEGF and matrix metalloproteinases (MMPs) to promote healing and restore normal tissue structure after injury [51]. They also produce anti-inflammatory cytokines like IL-10 [52], which help resolve inflammation and support recovery. Together, these diverse functions highlight the vital role macrophages play in both immunity and maintaining the overall health of tissues.

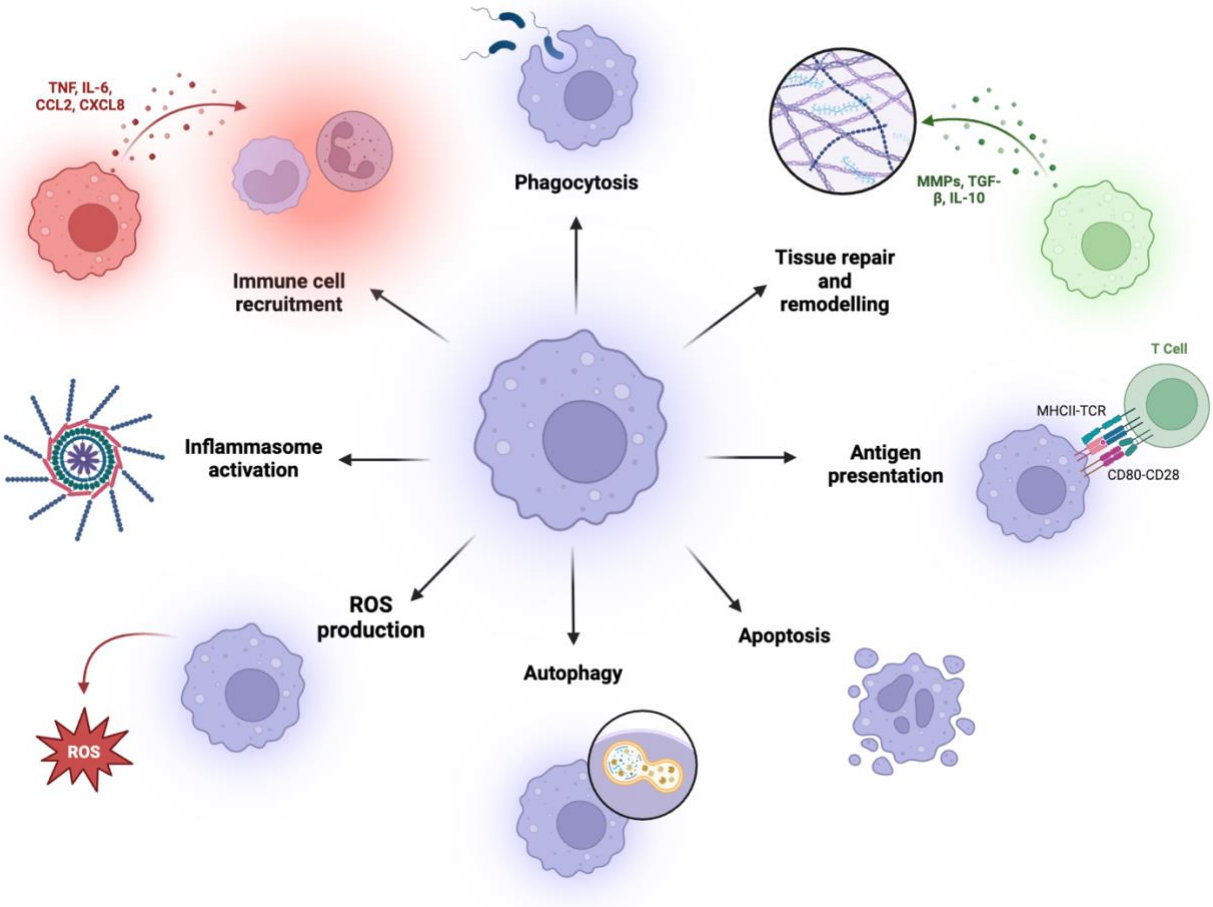

Figure 2. Key functions of macrophages in immunity and tissue homeostasis

Macrophages are versatile immune cells that play central roles in inflammation and tissue repair. Upon activation by PAMPs or DAMPs, macrophages produce pro-inflammatory cytokines (e.g., TNF, IL-1β, IL-6) and chemokines (e.g., CXCL8) to recruit immune cells and amplify the inflammatory response. Beyond their inflammatory functions, macrophages perform phagocytosis to clear pathogens and debris, activate the NLRP3 inflammasome, produce ROS, and engage in autophagy and apoptosis to maintain tissue health. Additionally, they act as antigen-presenting cells to link innate and adaptive immunity and promote tissue repair by releasing growth factors and anti-inflammatory cytokines like IL-10.

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DCs bridge the innate and adaptive immune responses. Like macrophages, they have PRRs that recognize PAMPs and DAMPs [53]. When activated DCs undergo maturation, which is characterized by an increase in co-stimulatory molecules, this increase allows them to present antigens to T cells [54]. Mature DCs then go to lymphoid organs where they present processed antigens to naive T lymphocytes. The presentation of antigens, plus the addition of stimulatory signals and cytokines, is required for T cell activation, differentiation and initiation of adaptive immune response [53].

Neutrophils are the most abundant white blood cells and are the first immune cells to arrive at the site of infection or tissue injury [55]. Chemokines such as CXCL8 recruit them [42]. Activated macrophages and other cells in the inflamed area produce these chemokines. Upon arrival at the site of inflammation, neutrophils perform various functions, including phagocytosis, which is engulfing and digesting microorganisms [56-58]. Neutrophils have antimicrobial peptides and enzymes stored in granules. These granules are released into the phagosome to eliminate and break down ingested bacteria [59]. They also produce reactive oxygen species (ROS) through the respiratory burst, which results in the production of highly reactive chemicals that damage microbial membranes and proteins [60]. Moreover, neutrophils secrete neutrophil extracellular traps (NETs), which are complex structures composed of deoxyribonucleic acid (DNA), histones and antimicrobial proteins [61, 62]. These NETs ensnare and inactivate microorganisms. Neutrophilia is a type of neutrophil cell death that helps eliminate pathogens.

NK cells are a subset of lymphocytes that play a role in the initial immune response against virus-infected cells and malignant cells [63, 64]. Unlike T and B cells, NK cells do not need prior

sensitization to recognize and eliminate their targets. Instead, NK cells rely on a balance of activating and inhibitory receptors to distinguish between normal and diseased cells [65]. NK cells release cytotoxic granules containing perforin and granzymes when they detect an abnormal cell. Perforin forms pores in the membrane of the target cell, allowing granzymes to enter and induce apoptosis [65]. Besides killing cells, NK cells also secrete cytokines like interferon-gamma (IFN-γ) [66, 67, 68]. This cytokine activates macrophages and promotes Th1 cells, a type of T helper cell involved in the immune response to intracellular infections [68].

Eosinophils are recruited to the site of inflammation in response to chemokines such as eotaxin-1 (CCL11), and cytokines such as IL-5, which promotes their proliferation and activation [69]. These cells have granules that are packed with toxic proteins and enzymes such as major basic protein (MBP), eosinophil cationic protein (ECP), and eosinophil peroxidase (EPO) [70]. When eosinophils are activated, they release these granules, making them effective against infections [70-72]. They target and destroy important pathogens like helminths that are resistant to phagocytosis.

Eosinophils play a role in allergic diseases like asthma, where they release inflammatory mediators that cause tissue damage and inflammation in the airways [69-73]. Eosinophils also secrete several cytokines and chemokines that affect other immune cells, regulate the immune response, and in some cases, facilitate tissue healing during the resolution phase of inflammation [69].

Basophils play a role in inflammatory responses, especially allergic reactions and parasite infections [74]. Basophils have granules that are rich in histamine [75]. These granules are released when the basophils are activated, causing increased vascular permeability, vasodilation

and the characteristic swelling and redness of inflammation. Like eosinophils, basophils have a role in type I hypersensitivity reactions such as allergic asthma, hay fever, and anaphylaxis by releasing histamine and other pro-inflammatory mediators [76]. Basophils play a role in protecting against parasitic infections by recruiting and activating other immune cells, especially eosinophils [76, 77]. Basophils also secrete cytokines such as IL-4 and IL-13 which are important in stimulating Th2 immune response, antibody production, and overall defence against allergies and parasites [78-82].

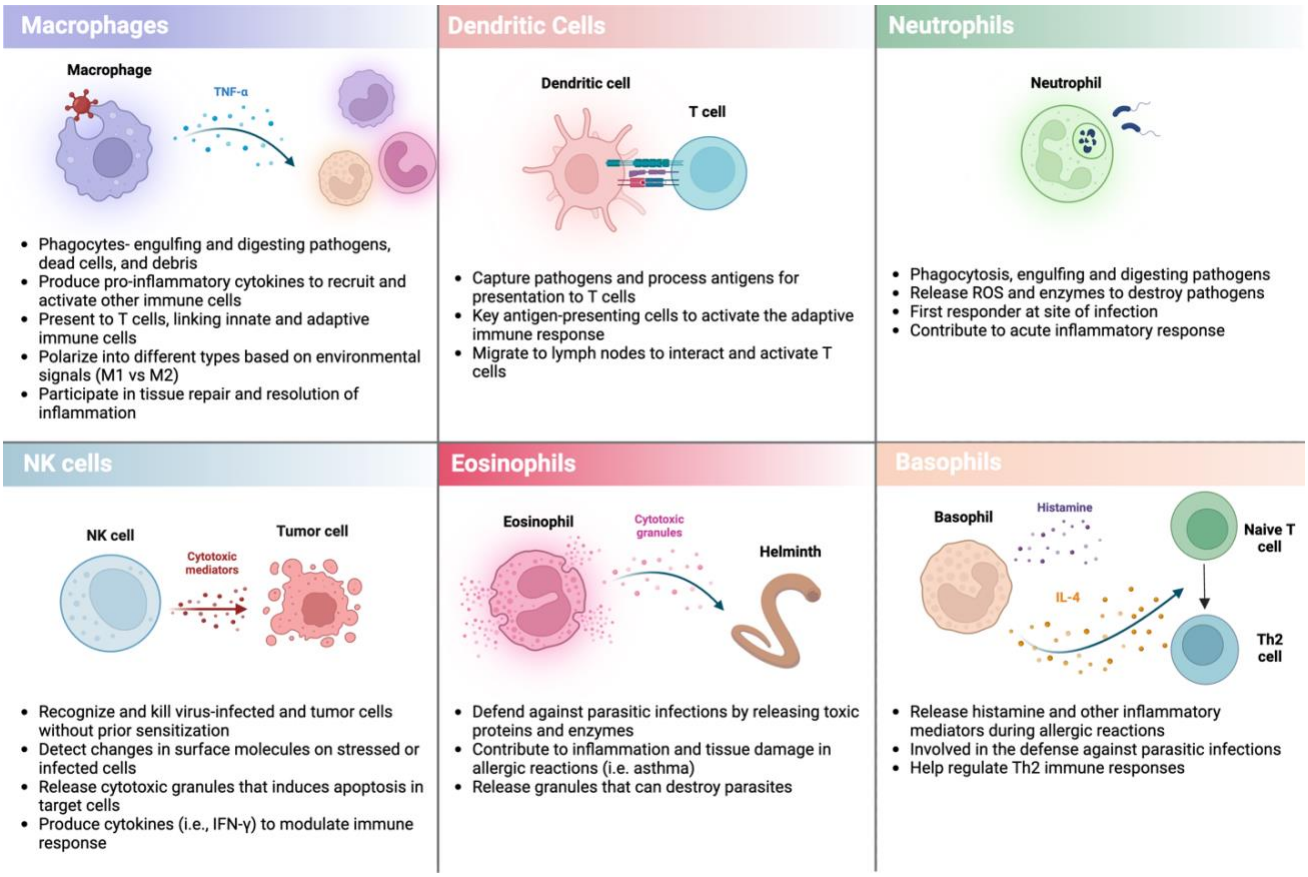

Figure 3. Primary functions of innate immune cells

Macrophages are phagocytic cells that produce pro-inflammatory cytokines, present antigens to T cells and repair tissue. DCs capture and present antigens to T cells, bridge innate and adaptive immunity. Neutrophils are first responders, phagocytose and release ROS to kill pathogens. NK cells recognize and kill virus infected and tumour cells, release cytotoxic granules and cytokines like IFN-γ. Eosinophils defend against parasitic infections and contribute to allergic

inflammation by releasing toxic proteins and enzymes. Basophils release histamine and other mediators during allergic reactions and regulate Th2 immune response.

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Innate molecular receptors

TLRs are a type of PRR that recognizes microbial infections and triggers innate immune responses [83, 84]. These receptors are found either on the surface of immune cells or inside endosomes [85]. They can recognize various PAMPs such as bacterial lipopolysaccharides (LPS), flagellin, and viral ribonucleic acid (RNA). When these microbial components are detected, TLRs initiate intracellular signalling pathways that produce pro-inflammatory cytokines and type I interferons (IFNs)[86-88]. This amplifies the immune response and activates adaptive immunity. TLRs are present in different types of immune cells, such as macrophages, dendritic cells and neutrophils. Hence, TLRs are important for recognizing pathogens and regulating the immune response.

NLRs are cytoplasmic PRRs that recognize PAMPs and DAMPs inside the cells caused by infections, cellular stress or tissue injury [89]. NLRs, such as NOD1 and NOD2, can recognize bacterial peptidoglycans [90, 91]. NLRP3 and similar receptors recognize signs of cellular damage or stress [92]. When activated, NLRs can initiate signalling pathways that produce pro-inflammatory cytokines or form inflammasomes [93]. NLRs are important for triggering immune responses against bacteria inside cells and maintaining immune homeostasis within tissues.

RLRs are a family of intracellular PRRs that recognize viral RNA inside infected cells. They are important for recognizing and fighting viral infections [94, 95]. Two well-known RLRs are RIG-I and melanoma differentiation-associated gene 5 (MDA5). These receptors recognize viral RNA

by distinguishing it from host RNA based on molecular characteristics, such as uncapped or double-stranded RNA [96]. When activated, RLRs initiate signalling cascades that produce IFNs and other antiviral cytokines, leading to strong antiviral response [97]. They are important for protecting the host from RNA viruses, including influenza and hepatitis C [98-100].

Inflammasomes are multi-protein complexes formed in response to microbial infections, cellular injury, or metabolic stress [101]. These complexes are typically triggered by PRRs such as NLRs, including NLRP3, which respond to diverse stimuli like bacterial toxins, crystalline compounds, and adenosine triphosphate (ATP) [102, 103]. Inflammasomes play a critical role in the immune system by activating caspase-1, which processes and promotes the secretion of pro-inflammatory cytokines IL-1β and IL-18 [102-105].

Lipopolysaccharide (LPS), a component of Gram-negative bacterial cell walls, is a key activator of inflammasome pathways. In the *canonical inflammasome pathway* (Figure 4), PRRs such as NLRP3 are directly activated by signals like ATP or potassium efflux following initial LPS priming through TLR4. This leads to the assembly of the inflammasome complex, activation of caspase-1, and secretion of IL-1β and IL-18 [102-105]. In contrast, the *non-canonical inflammasome pathway* involves direct recognition of intracellular LPS by caspase-11 (in mice) or caspase-4/5 (in humans) [106]. Activation of these caspases leads to gasdermin D-mediated pyroptosis, a pro-inflammatory form of cell death, and indirect activation of NLRP3 and cytokine release .

Pyroptosis, an inflammatory form of cell death that helps remove infected or damaged cells, can enhance immune responses [107]. However, dysregulation of inflammasome activity can contribute to the pathology of inflammatory diseases such as gout, atherosclerosis, and sepsis [101, 102].

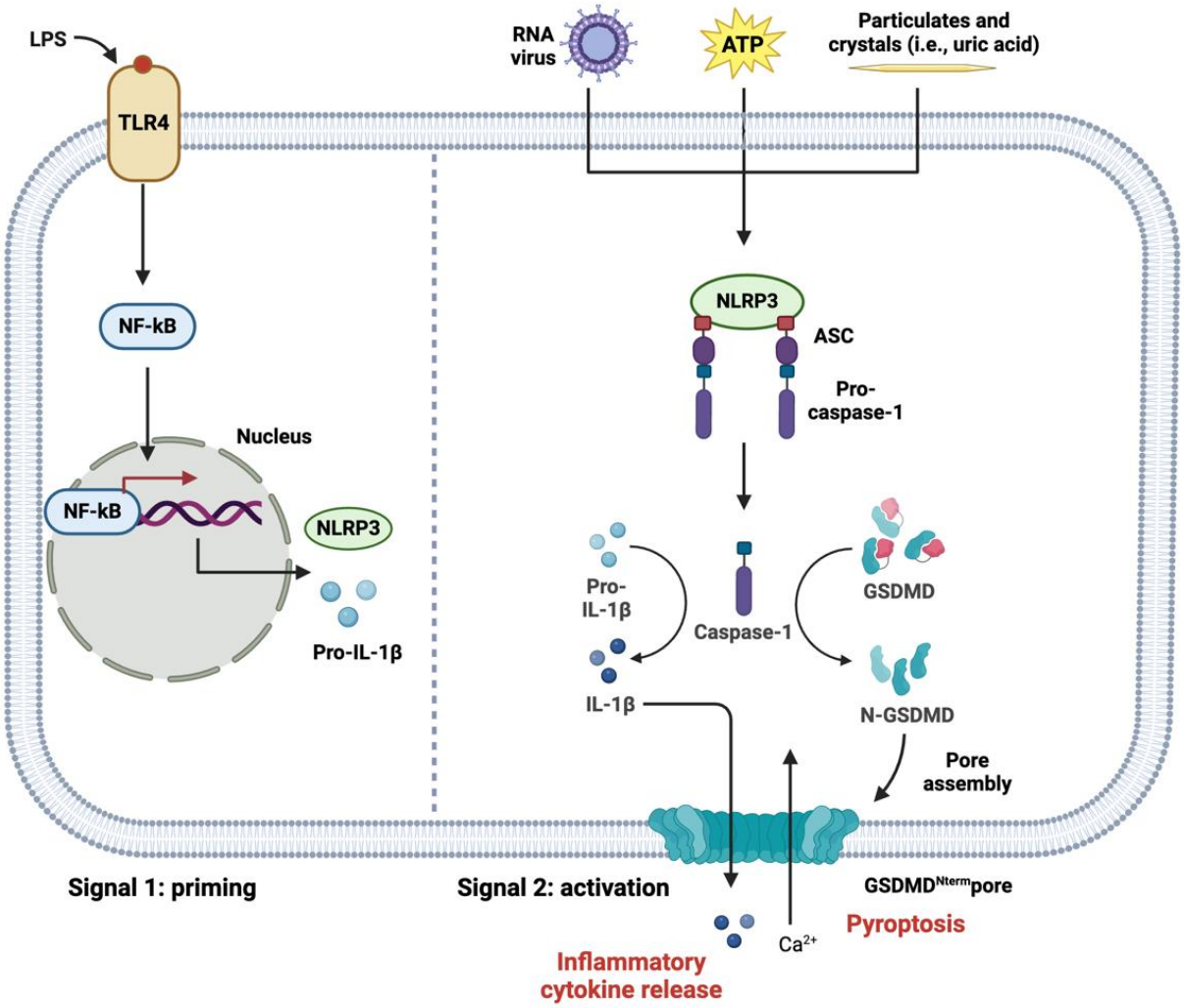

Figure 4. The two-step activation of NLRP3 inflammasome in macrophages

Step 1: Priming involves the recognition of PAMPs or DAMPs by PRRs such as TLR4, which activates NF-κB signalling. This leads to transcriptional upregulation of pro-IL-1β, pro-IL-18 and NLRP3, preparing the cell for subsequent activation.

Step 2: Activation occurs when additional stimuli such as potassium efflux, mitochondrial dysfunction or the presence of bacterial toxins are detected. These stimuli trigger NLRP3 oligomerization and inflammasome assembly, which activates caspase-1 that cleaves pro-IL-1β and pro-IL-18 into their mature active forms and release the pro-inflammatory cytokines.

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Adapted from: Swanson, K. V., Deng, M., & Ting, J. P. Y. (2019). The NLRP3 inflammasome: Molecular activation and regulation to therapeutics. *Nature Reviews Immunology*, *19*(8), 477–489.

Scavenger receptors are a family of receptors on the surface of cells. Their main function is to recognize and remove a wide range of substances, such as modified low-density lipoproteins (LDLs), dying cells and components of microorganisms [108]. These receptors are important for host protection by acting as PRRs that enhance the phagocytosis of pathogens and waste materials [109-111]. Scavenger receptors are found on several types of cells, such as macrophages and dendritic cells. They regulate the immune response by removing toxins from the circulation. Scavenger receptors are involved in innate immunity, tissue homeostasis, and anti-inflammatory response.

TLR4 in macrophages

The innate immune system relies on PRRs, like TLR4, to recognize microbial invaders and trigger inflammatory response. TLR4 is important in macrophages to detect LPS from Gram-negative bacteria [112, 113]. This detection triggers a series of signalling events that leads to production of pro-inflammatory cytokines.

The TLR4 signalling pathway is complex and involves many steps and components. Before LPS binds to TLR4, the macrophages express the receptor on their surface along with many co-receptors and accessory molecules such as myeloid differentiation factor 2 (MD-2), cluster of differentiation 14 (CD14), and LPS-binding protein (LBP) [114, 115]. MD-2 is a small protein that forms a bridge between TLR4 on the outer surface of the cell and is essential for LPS binding [116, 117]. CD14 is a protein attached to the cell membrane by a glycosylphosphatidylinositol (GPI) linkage [118]. It is a co-receptor for LPS, which can be found in both membrane bound and soluble form. CD14 helps in transferring LPS to the TLR4-MD-2 complex However, some immune cells, such as specific B and dendritic cells, can still respond to

LPS without CD14 [119-121]. This CD14-independent pathway enables these cells to activate TLR4 signalling and initiate an immune response, although it may occur with differing sensitivity or through alternative co-receptors or accessory molecules. LBP is a blood protein that binds to LPS and brings it to CD14, hence increasing the efficiency of LPS detection by TLR4.

TLR4 activation starts when LPS binds to the TLR4-MD-2 complex (Figure 5). LPS, usually in clusters, is recognized by LBP, which brings individual LPS to CD14. CD14 helps in transferring LPS to the TLR4-MD-2 complex and TLR4 changes its shape, resulting in the formation of TLR4-MD-2 complex [122-125]. Dimerization is crucial in initiating downstream signalling. When TLR4 forms dimers, it triggers two main signalling pathways: the myeloid differentiation primary response 88 (MyD88)-dependent pathway and the TIR domain-containing adaptor inducing interferon-β (TRIF)-dependent pathway. The MyD88-dependent pathway is responsible for the initial stage of TLR4 activation and leads to the production of pro-inflammatory cytokines. The adaptor protein MyD88 is brought to the TLR4 complex through its Toll/IL-1 receptor (TIR) domain [126, 127]. MyD88 binds and activates IL-1 receptor-associated kinase (IRAK) 4, which leads to the phosphorylation and activation of IRAK1 and IRAK2 [127-129]. Activated IRAKs interact with TNF receptor-associated factor 6 (TRAF6), resulting in the activation of TRAF6. TRAF6, together with Transforming growth factor-β-Activated Kinase 1 (TAK1), activates the IκB kinase (IKK) complex, which consists of IKKα, IKKβ and NF-κB essential modulator (NEMO). The IKK complex phosphorylates IκB proteins, causing them to be ubiquitinated and degraded. This leads to the release of NF-κB, which then moves into the nucleus and triggers the transcription of pro-inflammatory genes.

The TRIF-dependent pathway is responsible for the late stage of TLR4 signalling and leads to the production of IFNs and other inflammatory mediators [130-134]. TLR4 can signal through TRIF in a MyD88-independent manner. This signalling occurs through TIR-domain-containing adaptor molecule 1 (TICAM1), also known as TRIF. The adaptor protein TRIF-related adaptor molecule (TRAM) connects TRIF to TLR4 [130, 131]. TRIF activates TANK-binding kinase 1 (TBK1) and IκB kinase epsilon (IKKε), which, in turn, phosphorylates and activates interferon regulatory factor 3 (IRF3). Activated IRF3 moves to the nucleus and triggers the transcription of genes that produce type I interferons, such as IFN-β [132, 134].

Activation of these pathways leads to production and release of various cytokines and chemokines [135]. Pro-inflammatory cytokines such as TNF, IL-1β and IL-6 are produced and released, causing inflammation and attracting more immune cells to the site of infection. Chemokines such as CXCL8 attract neutrophils to the site of infection. IFN-α and IFN-β are type I interferons, have antiviral properties, and regulate the immune response.

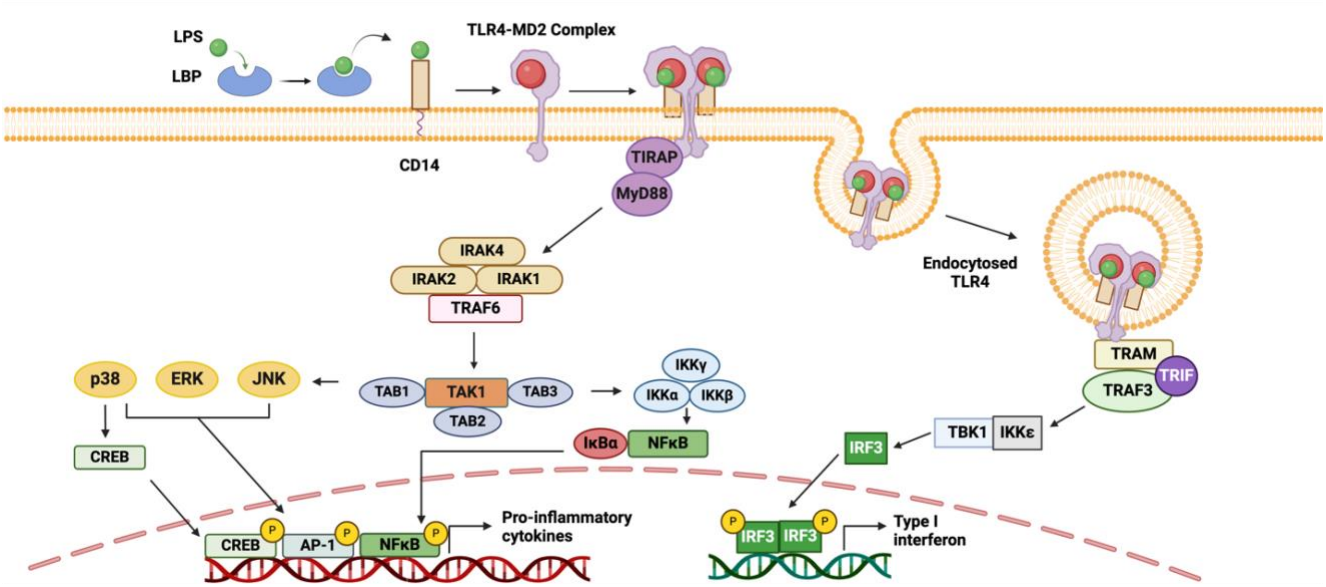

Figure 5. TLR4 signalling pathway

LBP binds to LPS and facilitates its transfer to CD14 or MD-2, essential accessory proteins for ligand recognition, TLR4 dimerization and endocytosis. Upon dimerization, TLR4 can signal through two different pathways: the MyD88-dependent and the MyD88-independent (TRIF-dependent) pathway. The MyD88-dependent pathway activates IRAKs and TRAF6 which leads to phosphorylation and nuclear translocation of transcription factors like NF-κB and AP-1 which drive the transcription of pro-inflammatory cytokine genes. The MyD88-independent pathway involves TRAF3 and leads to the activation of IRF-3, which promotes the production of type I interferons such as IFN-α and IFN-β.

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Adapted from: Ciesielska, A., Matyjek, M., & Kwiatkowska, K. (2021). TLR4 and CD14 trafficking and its influence on LPS-induced pro-inflammatory signaling. *Cellular and Molecular Life Sciences, 78*(4), 1233–1261.

Innate Immune Response

The innate immune system acts as the first line of defence against invading pathogens, a rapid and non-specific response to infection. The immune response is triggered when pathogens, such as bacteria, enter the body's protective barriers, which include the skin, mucous membranes and epithelial linings of the respiratory, gastrointestinal and genital tracts [136] (Figure 6). These

barriers act as physical and chemical barriers to prevent the entry of harmful microbes. However, if these barriers are breached, viruses can enter the underlying tissues and trigger the innate immune response.

A typical scenario is when bacteria enter the gut lumen and then pass through the epithelial barrier, which is a key defence mechanism in the gastrointestinal tract [136]. When bacteria, especially Gram-negative bacteria, breach this barrier, they secrete components such as LPS, which is a major component of their outer membrane. LPS is a very potent endotoxin and plays a key role in activating the innate immune system. When LPS enters the underlying tissues, such as the lamina propria, it is detected by PRRs expressed on resident immune cells such as macrophages [136]. Out of these PRRs, TLR4 is specifically designed to recognize LPS, which then initiates a series of intracellular signalling pathways previously described.

When TLR4 recognizes LPS, it triggers a series of intracellular signalling cascades in macrophages. These signalling events lead to the activation of NF-κB and other transcription factors, which induce the production of pro-inflammatory cytokines such as TNF, IL-1β and IL-6 [135]. These cytokines play a key role in coordinating the local inflammatory response by recruiting more immune cells to the site of infection. At the same time, chemokines such as monocyte chemoattractant protein-1 (CCL2) and CXCL8 are secreted, which attract monocytes and neutrophils from the circulation to the site of infection.

Once recruited to the site of infection, these immune cells actively participate in defence against the invading pathogens. Neutrophils, being the first responders, reach the site and participate in phagocytosis, which involves engulfing and breaking down bacteria and other harmful microbes [56-58]. Macrophages, which can be resident in the tissue or produced from

recruited monocytes, also participate in phagocytosis, and secrete more cytokines to boost the immune response [44, 46]. This coordinated attack helps to eliminate the invading bacteria and prevent the spread of infection.

However, it is important to tightly control the inflammatory response to prevent tissue damage. During the process of the immune system clearing the infection, anti-inflammatory cytokines such as IL-10 become important. IL-10 is produced by several immune cells such as macrophages and regulatory T cells [137]. It inhibits the production of pro-inflammatory cytokines, reduces the expression of co-stimulatory molecules, and prevents dendritic cells and macrophages from presenting antigens [137-139]. This modulation helps in resolution of inflammation and restoring tissue homeostasis.

When the innate response is not sufficient to clear the pathogen, antigen presenting cells (APCs) such as dendritic cells and macrophages take up and present antigens from the pathogen on major histocompatibility complex (MHC) molecules to T cells [50]. This presentation triggers the adaptive immune response which is a more specific and longer lasting immune response. The adaptive response involves activation of specific T and B cells and provides long term immunity and memory to the pathogen upon subsequent encounters.

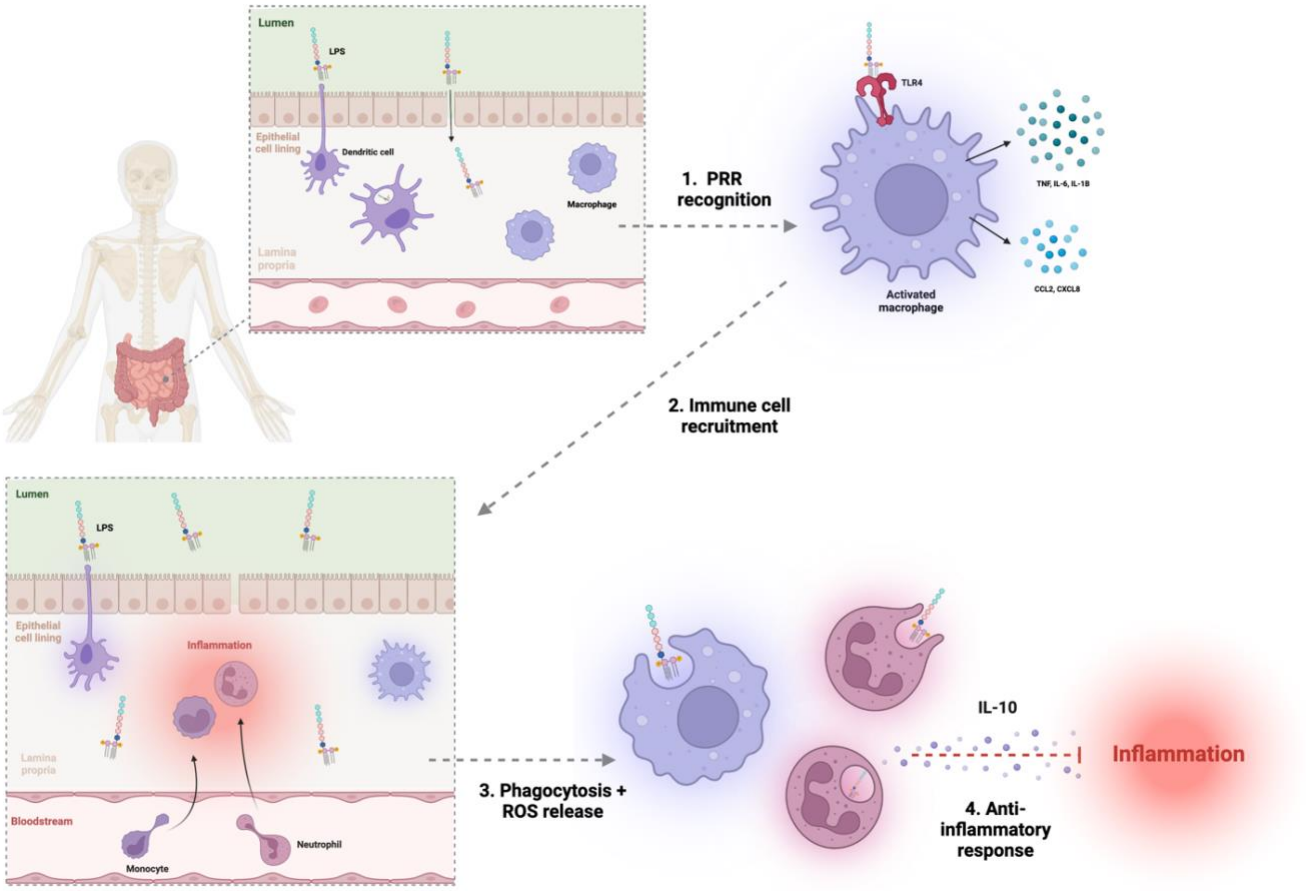

Figure 6. Innate immune response to LPS

The innate immune response starts when pathogens like bacteria breach the body's barrier. For example, when bacteria enter the gut lumen and cross the epithelial barrier, their components, such as LPS from Gram-negative bacteria, can reach the underlying tissue, such as the lamina propria. **(1)** In these tissues, LPS is recognized by PRRs like TLR4 on resident macrophages. This recognition triggers intracellular signalling cascades in the macrophages producing pro-inflammatory cytokines (e.g. TNF, IL-1β, IL-6) and chemokines (e.g. CCL2, CXCL8). **(2)** These cytokines initiate and amplify local inflammation, while the chemokines attract more immune cells such as monocytes and neutrophils from the circulation to the site of infection. **(3)** Once recruited, these immune cells participate in defence by phagocytosis to eliminate the invading pathogens. **(4)** To prevent tissue damage, anti-inflammatory cytokines like IL-10 are released to resolve the inflammation. IL-10 exerts anti-inflammatory effects by binding to its receptor on macrophages and other immune cells, activating the transcription factor STAT3. Activated STAT3 then suppresses the transcription of pro-inflammatory genes (e.g., TNF, IL-1β) and promotes the expression of anti-inflammatory molecules, dampening the immune response and helping to restore tissue homeostasis.

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The innate immune response is a multi-cellular and multi-signalling pathway process. Macrophages and dendritic cells are the primary sensors of infection and damage and produce cytokines and chemokines that recruit and activate other immune cells [44-46, 54]. Neutrophils provide rapid antimicrobial defence through phagocytosis, ROS production and NETosis [56-62]. This integrated response provides broad defence against various pathogens and allows transition to adaptive immunity. The interplay between innate and adaptive immune response is key to resolving infections and establishing long term immunity. However, dysregulation of these processes can lead to chronic inflammation and tissue damage, hence the importance of tightly controlled inflammatory responses in health.

1.1.3 Adaptive immune response and inflammation

Although this thesis focuses on innate immunity, it is important to acknowledge the role of adaptive immunity in the broader context of inflammation. Adaptive immunity which includes T and B cells, provides a more specific and longer lasting immune response [140]. The interplay between innate and adaptive immunity is key to a complete immune response where cytokines from innate immune cells can influence the activation and specialization of adaptive immune cells and vice versa.

Macrophages and dendritic cells play a crucial role in linking the innate and adaptive immune response. When these cells encounter harmful microorganisms, they release a range of cytokines and chemokines that affect the adaptive immune response. When activated by PRRs, dendritic cells process and present antigens to T cells in the lymphoid organs. DCs produce

cytokines such as IL-12, IL-6 and TNF, which can influence the development of naive T cells into different types of effector T cells such as Th1, Th2, Th17 and regulatory T cells (Tregs) [141]. IL-12 drives the differentiation of undifferentiated T cells into Th1 cells, which are important in fighting against intracellular pathogens such as viruses and some bacteria [142]. In addition to TGF-β, IL-6 drives the development of Th17 cells, which are important in fighting against bacteria and fungus outside of cells and contribute to autoimmune inflammation [143]. TNF stimulates and prolongs T cells and can influence the inflammatory conditions to facilitate adaptive immunological responses [143, 144].

Macrophages can also influence adaptive immunity through cytokine production and cell-to-cell interaction. IL-10 secreted by macrophages and dendritic cells helps to resolve inflammation. IL-10 is also involved in the creation and activity of Tregs, which are important in maintaining immunological tolerance and preventing autoimmune responses [145].

Conversely, adaptive immune cells can also influence innate immunity. Activated T cells release cytokines such as IFN-γ, which stimulate macrophages, and make them more effective in killing microbes [50, 146]. This leads to a stronger and more efficient innate immune response. B cells can enhance the recognition and removal of infections by phagocytes by producing antibodies that bind to the pathogens, in a process called opsonization [147]. This reciprocal interaction ensures that the immune system can respond to different infections while maintaining regulatory control over inflammatory responses to prevent tissue damage.

1.1.4 Chronic inflammation and innate immune dysregulation

Chronic inflammation occurs when your body's response to inflammation persists due to ongoing infections, autoimmune responses or prolonged exposure to irritants [147, 148]. Unlike acute inflammation, which resolves once the trigger is removed, chronic inflammation lingers even after the initial cause is gone. It is characterized by the presence of immune cells, specifically macrophages and lymphocytes, which release cytokines and other substances that maintain and spread the inflammatory response [150, 151].

Chronic inflammation often arises from persistent infections that the immune system cannot fully eliminate. For example, bacteria, such as *Mycobacterium tuberculosis*, which causes tuberculosis, can evade the immune system and remain inside host cells, specifically macrophages [152, 153]. The continuous battle to manage the infection results in the formation of granulomas, which are collections of immune cells that try to isolate the pathogen. Activated macrophages, epithelial cells, and multinucleated giant cells release pro-inflammatory cytokines within these granulomas, leading to chronic inflammation and potential tissue damage and fibrosis. Another example is LPS, a potent stimulator of inflammation due to its interaction with TLR4 on immune cells. Prolonged exposure to LPS can lead to persistent inflammatory responses that contribute to chronic inflammation [154]. Experimental models have shown that repeated or prolonged stimulation with LPS can lead to chronic inflammation [155, 156]. This state is characterized by the continuous production of pro-inflammatory cytokines such as TNF, IL-1β and IL-6. Research has shown that prolonged exposure to LPS can result in symptoms that resemble chronic inflammatory diseases. For example, continuous exposure to LPS in animal models can replicate certain features of chronic diseases such as chronic obstructive pulmonary disease (COPD)[157]. The models show long-lasting lung inflammation, airway remodeling and

fibrosis due to the continuous production of pro-inflammatory substances and attraction of immune cells. The continuous activation of TLR4 signalling due to prolonged exposure to LPS is one way by which chronic inflammation can persist and cause tissue damage. However, TLR4 signalling can also become desensitized over time, a process known as endotoxin tolerance. This desensitization often occurs through mechanisms like TLR4 internalization, which removes TLR4 from the cell surface and decreases its responsiveness to LPS, potentially protecting tissues from excessive inflammation [158, 159]. Interestingly, IFN-γ can counteract this desensitization by maintaining TLR4 surface expression and enhancing its responsiveness, which may contribute to sustained inflammation in chronic conditions [160-162]. These dynamics highlight the complexity of TLR4-mediated inflammation and its role in chronic inflammatory diseases.

Autoimmune reactions refer to the immune system's mistake of attacking the body's tissues, resulting in persistent inflammation, a hallmark of autoimmune diseases. In diseases such as rheumatoid arthritis (RA) autoantibodies such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPAs) attack joint tissues [163]. These autoantibodies form immunological complexes that accumulate in the joints and trigger the activation of complement and attraction of immune cells. T lymphocytes and macrophages infiltrate the synovium and secrete pro-inflammatory cytokines such as TNF, IL-1β and IL-6, which stimulate inflammation and damage to the joints. Systemic lupus erythematosus (SLE) is characterized by the production of autoantibodies that form immunological complexes resulting to persistent inflammation and organ damage [164].

Chronic inflammation can occur when we are exposed to irritants for a long time such as tobacco smoke, asbestos and environmental pollutants. In COPD, prolonged exposure to tobacco smoke leads to chronic inflammation in the airways. Macrophages, neutrophils and T lymphocytes infiltrate the lung tissue and produce pro-inflammatory cytokines, proteases and ROS that damage the alveoli and small airways [165, 166]. This chronic inflammatory response results in structural changes in the airways, scar tissue formation and decline in lung function.

Macrophages have a dual function in chronic inflammation. M1 macrophages, or classically activated macrophages, produce pro-inflammatory cytokines such as TNF, IL-1β and IL-6 and reactive nitrogen species (RNS) that worsen tissue damage [167] (Figure 7). These cells are induced by microbial products and Th1 cytokines such as IFN-γ. In chronic inflammatory diseases, the continuous activation of M1 macrophages is responsible for tissue destruction and inflammation. M2 macrophages, or alternatively activated macrophages, are stimulated by Th2 cytokines such as IL-4 and IL-13. The cells secrete anti-inflammatory cytokines such as IL-10 and TGF-β and growth factors that promote tissue repair and remodeling. However, uncontrolled M2 macrophage activity in chronic inflammation can result in over repair and fibrosis, which are features of diseases like liver cirrhosis and pulmonary fibrosis [51, 168-169]. Additional subtypes of M2 macrophages will be explored in detail in a later to illustrate their specific roles in chronic inflammation and tissue repair.

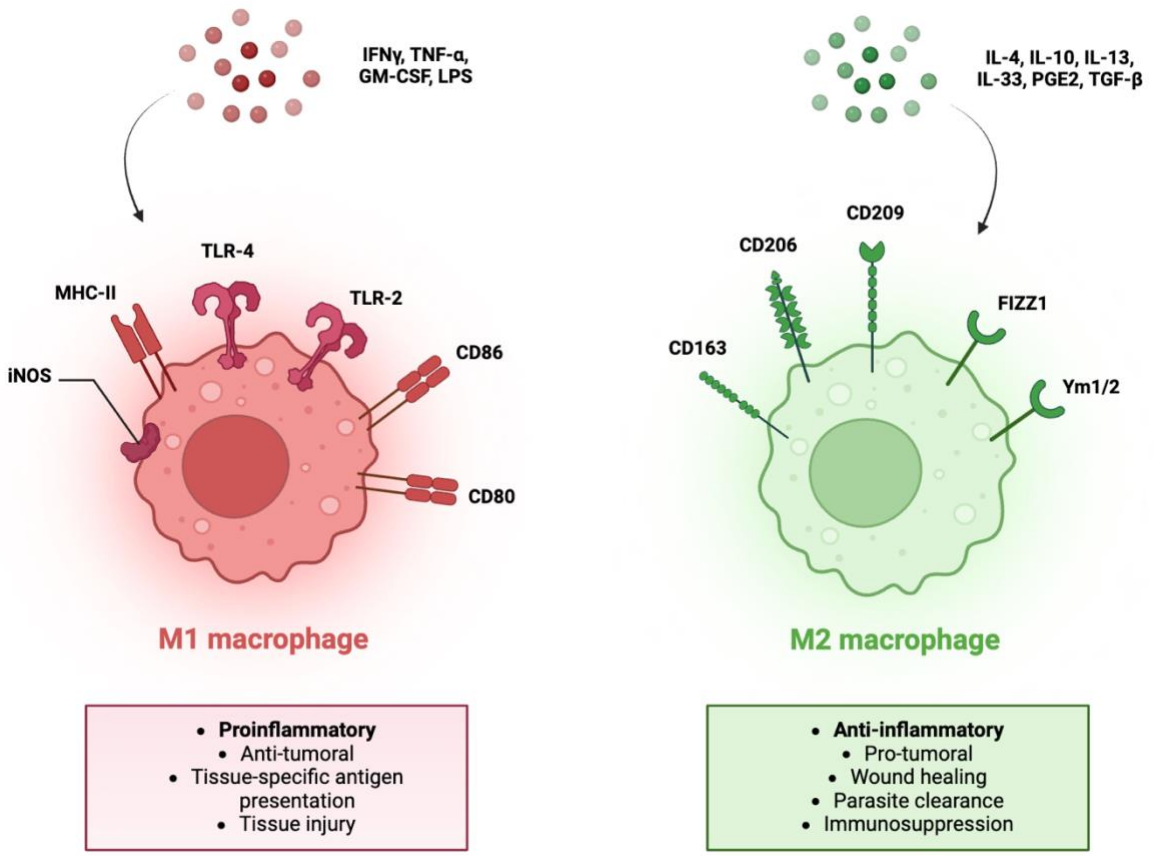

Figure 7. Macrophage polarization

Macrophages polarize into two distinct phenotypes, M1 and M2, in response to various environmental stimuli. M1 macrophages are induced by cytokines such as IFN-γ and PRRs such as TLRs and produce pro-inflammatory cytokines (e.g. IL-1β, IL-6 and TNF) and mediators like ROS and NO. These macrophages enhance antigen presentation and are associated with pro-inflammatory and antimicrobial functions. M2 macrophages are driven by Th2 cytokines such as IL-4, IL-10 and IL-13, and factors such as TGF-β and prostaglandin E2 (PGE2). M2 macrophages are involved in anti-inflammatory processes, tissue repair, wound healing, and immunosuppression, and secrete cytokines like IL-10 and TGF-β to modulate immune responses and promote tissue remodeling.

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Adapted from: Sica, A., & Mantovani, A. (2012). Macrophage plasticity and polarization: In vivo veritas. *Journal of Clinical Investigation, 122*(3), 787–795.

Chronic inflammation is a complex interplay of pro-inflammatory and anti-inflammatory signals. Pro-inflammatory cytokines such as TNF, IL-1β and IL-6 stimulate and enhance the

inflammatory response by attracting and activating immune cells such as macrophages, neutrophils and lymphocytes. These cytokines perpetuate the inflammatory process, resulting in tissue injury and fibrosis. Anti-inflammatory cytokines, such as IL-10 and TGF-β, control and resolve inflammation by suppressing pro-inflammatory cells and stimulating tissue healing. Inadequate production or communication of these anti-inflammatory cytokines can lead to uncontrolled inflammation and tissue damage.

Chronic inflammation and sepsis

Pro-inflammatory cytokines play a major role in chronic inflammation. For example, these cytokines have a crucial function in the body's response to severe infection in sepsis. TNF, mainly secreted by activated macrophages, amplifies the inflammatory response by inducing expression of adhesion molecules on endothelial cells, increasing vascular permeability and triggering the production of other cytokines and chemokines [170, 171]. In sepsis, high levels of TNF leads to extensive inflammation, increased permeability of blood vessels, fluid accumulation in tissues known as edema, and attraction of immune cells to different parts of the body. This can ultimately lead to organ dysfunction and failure. IL-1β is a key pro-inflammatory cytokine in sepsis, stimulating various immune cells, and produces MMPs that break down the extracellular matrix and cause tissue damage. IL-1β plays a major role in the sequence of inflammatory events that occur in sepsis. It causes fever, attracts white blood cells to the site of infection and intensifies the inflammatory response. This can worsen tissue damage and organ dysfunction [172, 173]. IL-6 is a versatile cytokine that affects immunological responses, inflammation and hematopoiesis. IL-6 is produced by several types of cells such as macrophages, T cells and endothelial cells in response to infections in sepsis [174, 175]. It

converts B cells into plasma cells that produce antibodies and activates T cells, which is important in the acute phase response. Higher levels of IL-6 is associated with severity of sepsis and leads to widespread inflammation and severe clinical outcomes. Sepsis is characterized by the excessive release of pro-inflammatory cytokines such as TNF, IL-1β and IL-6, resulting in a phenomenon called a cytokine storm [176]. This excessive inflammatory state can cause significant tissue damage, organ failure and increased mortality.

IL-10 and TGF-β are anti-inflammatory cytokines that play a major role in maintaining a balanced immune system and resolving inflammation. These cytokines play a crucial role in toning down the strong inflammatory response in sepsis to prevent excessive tissue damage and facilitate healing. IL-10 produced by macrophages, Tregs and DCs hinders the production of pro-inflammatory cytokines like TNF, IL-1β and IL-6. IL-10 minimizes the excessive inflammatory response in sepsis by suppressing immune cells and decreasing MHC class II and co-stimulatory molecules on APCs [21, 177]. This suppression is important to prevent over stimulation of the immune system, which can lead to widespread inflammation and organ damage. Studies show that sepsis patients with high levels of IL-10 have better outcomes. This is because IL-10 regulates the excessive inflammation and promotes resolution of immunological responses. TGF-β is an important anti-inflammatory cytokine produced by macrophages, Tregs and fibroblasts. TGF-β suppresses T cell and macrophage activation and proliferation in sepsis and attenuates the inflammatory response [178]. TGF-β also specializes Tregs, increases its anti-inflammatory property through tolerance and prevention of autoimmunity. Moreover, TGF-β induces the production of extracellular matrix components by fibroblasts and facilitates tissue repair and recovery from the septic injury [178-180]. However, dysregulation of TGF-β signalling can

worsen pathological fibrosis and impede recovery in disorders such as systemic sclerosis and idiopathic pulmonary fibrosis (IPF).

The continuous activation of immune cells and production of pro-inflammatory mediators in sepsis creates a vicious cycle of inflammation and tissue damage (Figure 8). In sepsis, pro-inflammatory cytokines like TNF, IL-1β and IL-6 attracts and stimulates more immune cells and produces more pro-inflammatory mediators [181, 182]. This cycle can lead to persistent tissue damage and continuation of the inflammatory response. In sepsis, macrophages and other innate immune cells produce high levels of TNF, IL-1β and IL-6 in response to bacterial endotoxins. These cytokines amplify the inflammatory response by increasing blood vessel permeability, allowing white blood cells to infiltrate and producing more cytokines. This state of excessive inflammation can cause extensive tissue damage and organ dysfunction.

The activation of these pro-inflammatory pathways leads to the development of conditions such as acute respiratory distress syndrome (ARDS) and multiple organ dysfunction syndrome (MODS) [183, 184]. ARDS is characterized by an overabundance of neutrophils and macrophages in the lungs, which produces proteases and reactive oxygen species. This causes damage to the alveoli, poor gas exchange and ultimately respiratory failure. In the same way, in MODS, systemic inflammation and cytokine storm causes damage to the endothelial cells, capillary leak and reduced blood flow to organs and ultimately organ failure.

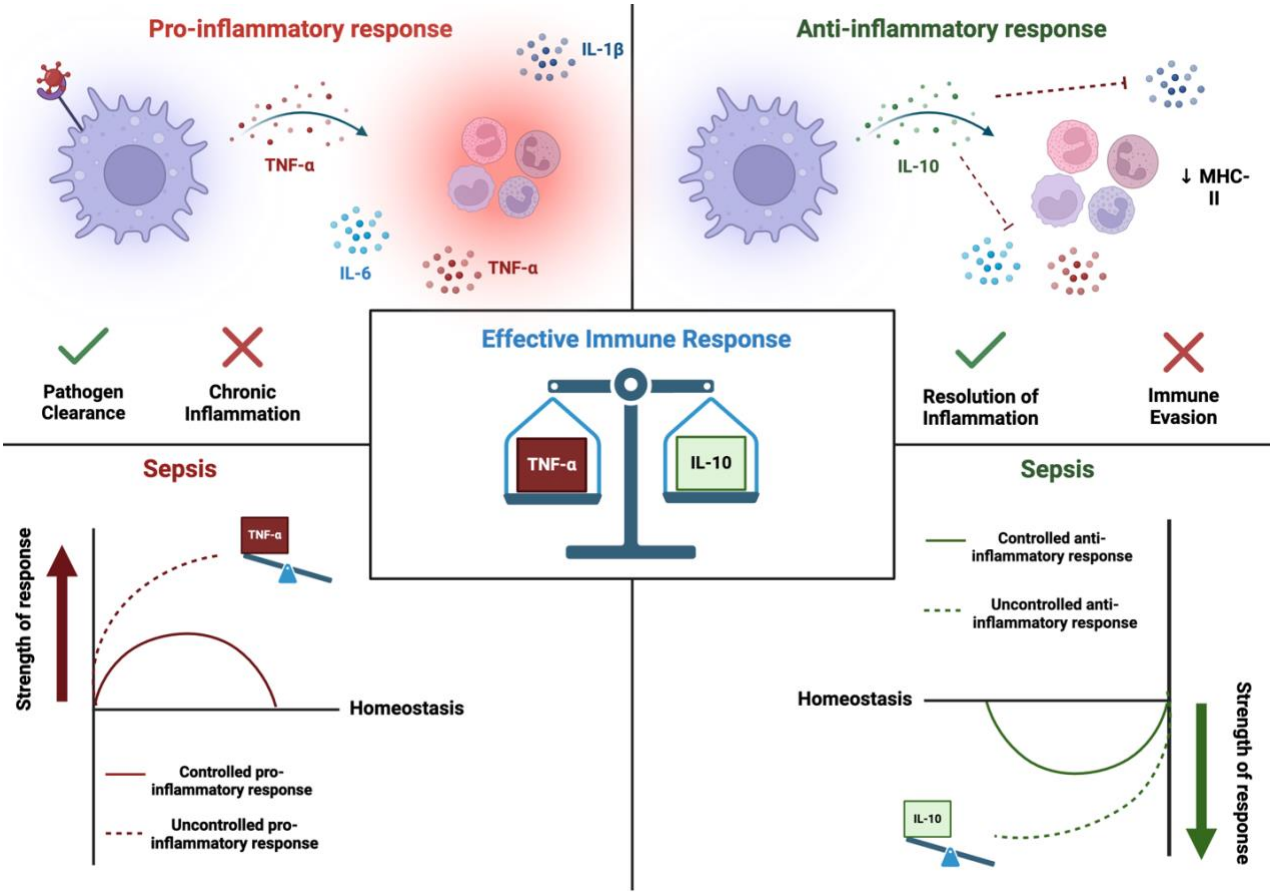

Figure 8. Balance of pro-inflammatory and anti-inflammatory cytokines in immune response

The balance between pro-inflammatory cytokines (e.g. TNF) and anti-inflammatory cytokines (e.g. IL-10) is key to regulating the immune response. TNF amplifies inflammation by recruiting immune cells and producing cytokines, which is important for infection control but can lead to chronic inflammatory diseases if uncontrolled (top left). IL-10 suppresses inflammation by inhibiting cytokine production and antigen presentation which aids in resolution of inflammation but can lead to immune evasion (top right). Sepsis can trigger an uncontrolled pro-inflammatory response often referred to as a 'cytokine storm'. This massive release of pro-inflammatory cytokines like TNF, IL-1β and IL-6 leads to widespread inflammation, vascular leakage and organ failure. The intense immune response can cause septic shock, MODS and ultimately early death if not controlled rapidly (bottom left). After the initial hyper-inflammatory phase, some patients develop CARS which can lead to immunosuppression. During this phase, the immune system becomes less responsive and IL-10 production is increased. This immunosuppressive state can make patients vulnerable to secondary infections, reactivation of latent infections, and unable to clear ongoing infections, leading to late death due to unresolved sepsis or new infections (bottom right).

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1.1.5 Inflammation and Innate Immune Cell Regulation

Inflammation regulation involves multiple mechanisms to avoid harm to the body's tissues. The complex network of immune cells and their interactions ensures the inflammatory response is regulated and stops once the harmful agent is removed. Key players in this regulation network are macrophages, myeloid-derived suppressor cells (MDSCs) and DCs.

Macrophages have a crucial role in both initiation and resolution of inflammation. As mentioned before, these cells can change and can be pro-inflammatory (M1) or anti-inflammatory (M2), depending on the signals they receive from their environment. As previously stated, M1 macrophages, when stimulated by signals like IFN-γ and LPS, produce pro-inflammatory cytokines (e.g. TNF, IL-1β and IL-6) and mediators like ROS and NO [48, 50]. These molecules are important for pathogen elimination and inflammatory response. However, persistent stimulation of M1 macrophages can cause tissue damage and continue inflammatory conditions. Conversely, M2 macrophages, when stimulated by IL-4 and IL-13, produce anti-inflammatory cytokines like IL-10 and TGF-β, which aid in tissue healing and resolution of inflammation [151, 168-169]. M2 macrophages have a significant role in wound healing and fibrosis by promoting the synthesis of extracellular matrix components and transforming fibroblasts into myofibroblasts. An imbalance in macrophage polarization can lead to chronic inflammatory diseases, where excessive M1 activity can lead to persistent inflammation and tissue damage and excessive M2 activity can lead to pathological fibrosis.

MDSCs play a key role in inflammation. MDSCs are derived from myeloid progenitor cells and immature myeloid cells (IMCs) in the bone marrow [185]. Under normal physiological conditions, IMCs differentiate rapidly into fully developed granulocytes, macrophages or DCs.

However, in pathological conditions such as cancer, viral diseases or autoimmune disorders, the normal differentiation process is blocked, and IMCs increase in number and have strong immune-suppressive ability [186, 187]. As a result, these cells become MDSCs. Multiple factors contribute to the activation of MDSCs. Tumour derived factors such as granulocyte/macrophage colony-stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF) and stem cell factor (SCF) are important in promoting the proliferation of MDSCs and preventing the differentiation of IMCs [188, 189]. Also, MDSCs activation relies heavily on cytokines such as IFN-γ, IL-4, IL-13 and TGF-β, produced by activated T cells and tumour stromal cells [189, 190]. This activation involves multiple signalling pathways including the Janus kinase (JAK) and signal transducer and activator of transcription (STAT) pathways with STAT3 and STAT6 playing a key role [191].

When MDSCs are active, they control innate immune cells by inhibiting T-cell responses. Their inhibitory effects are by producing high amounts of ROS, NO, and enzymes like arginase 1[192]. These chemicals inhibit T-cell growth and activity by blocking T-cell receptor signalling, inducing T-cell apoptosis and reducing L-arginine availability in the microenvironment [192]. MDSCs also interact with other innate immune system components like macrophages, modulating their cytokine production and creating an inhibitory and anti-inflammatory environment [193]. MDSCs have a key role in suppressing immune responses, especially in cancer and chronic diseases.

DCs bridge the innate and adaptive immune responses [194]. During inflammation, DCs capture antigens and then go to lymphoid organs to deliver these antigens to T cells. Activated DCs secrete cytokines, including IL-12, which induces naive T cells to become Th1 cells [195].

Th1 cells are important for an effective immune response against intracellular infections. DCs also produce IL-10, which helps to suppress inflammation and resolve the inflammatory process [196].

Inflammation is a balance between pro-inflammatory and anti-inflammatory signals mediated by multiple innate immune cells. Macrophages, MDSCs and DCs play a crucial role in maintaining this balance so that the immune response is both effective and regulated to avoid tissue damage.

1.2 Macrophages

1.2.1 Macrophages: An Overview

Macrophages are derived from blood monocytes that exit the circulation and differentiate in various tissues [197, 198]. They are highly diverse depending on the tissue they reside in [199]. The diversity of macrophages is due to their ability to specialize to adapt to the different conditions in each tissue. Their diversity is seen in their morphology, the pathogens they recognize, and the amount inflammatory cytokines, ROS, and NO they produce [48, 50]. The heterogeneity of these cells is due to their differentiation and can also be influenced by traits inherited from their monocyte precursors [200].

Macrophages in all tissues can adapt to different tissue environments. The flexibility of these cells is important for homeostasis and responding to infections or injuries. Their specialization results in macrophage populations that have different functions based on the stimuli they encounter in different tissues.

Macrophages are specialized phagocytes that are important for recognizing and eliminating infections and other foreign substances, and they play a key role in innate immunity. These cells can transform into multiple types, M1 and M2 macrophages, meaning they promote inflammation and reduce inflammation, respectively. Recent research has emphasized the fluid and dynamic nature of macrophage polarization instead of a rigid M1/M2 division [201-203]. Macrophages can respond to many different stimuli, which enables them to play a crucial role in tissue homeostasis and in responding to infection and injury.

Macrophages are involved in the initiation and maintenance of inflammation by producing many cytokines and chemokines that coordinate the recruitment and activation of other immune cells. The polarization of macrophages can determine the type of inflammation that occurs. For example, M1 macrophages are associated with Th1 type of inflammation. They help in eliminating pathogens and promoting inflammatory responses [48][50], while M2 macrophages are associated with Th2 type of inflammation and help in tissue repair and resolution of inflammation [151][168, 169]. The dual function of macrophages in inflammation highlights the complexity of their role, as they can either enhance or reduce inflammatory responses depending on the signals they receive from their surroundings.

Over time, many theories have merged to suggest that tissue macrophages come from two distinct sources. During development, organs are initially populated with primitive macrophages from the fetal liver and yolk sac, which are the resident macrophage population [204, 205]. During maturity, macrophages are also produced from hematopoietic stem cells in the bone marrow. Monocytes in the blood are the intermediate cells in this process. Macrophages can adapt to their environment, and this is seen in the M1 vs M2 spectrum of macrophage

polarization [206]. M1 macrophages have a pro-inflammatory phenotype with high phagocytic and cytotoxic activity. The identity of these cells is determined by their expression of MHC II, CD14, CD80/CD86, CD38 and iNOS [207]. M1 macrophages secrete pro-inflammatory cytokines such as IL-6, IL-12, IL-1β and TNF and chemokines such as CCL2 and CCL5. This means M1 macrophages can attract and activate other immune cells such as T cells and B cells at the site of infection.

Conversely, M2 macrophages have a role in reducing inflammation and promoting tissue healing. They have cell surface markers such as CD36, CD206 and CD163 [196]. M2 macrophages have functional diversity compared to M1 macrophages, with different subtypes (M2a, M2b, M2c, M2d) expressing different combinations of cytokines, chemokines and growth factors [208] (Figure 9). M2a macrophages, which are associated with Th2 polarized allergic inflammation in the lungs, are stimulated by IL-4 and IL-13 and express high levels of IL-10, TGF-β and inflammatory chemokines such as CCL17, CCL18, CCL22 and CCL24 [209-211]. M2b macrophages are activated by immune complexes (ICs) and require two activation signals: ICs combined with either TLR ligands or IL-1 receptor agonists. They have an important role in Th2 immune responses by producing TNF, IL-1β, IL-6, IL-10, and CCL1 [212-214]. An environment rich in IL-10 and PGE2 leads to the formation of M2c macrophages. These macrophages persistently produce IL-10 and TGF-β and play a crucial role in inflammation resolution and tissue repair [215-217]. M2d macrophages are generated by activation of TLR and adenosine A2A receptor ligands and the presence of IL-6. These macrophages have been shown to promote angiogenesis by producing VEGF and IL-10 [199]. It is important to note that macrophage polarization exists on a spectrum, as these cells can take on intermediate phenotypes with different physiological functions.

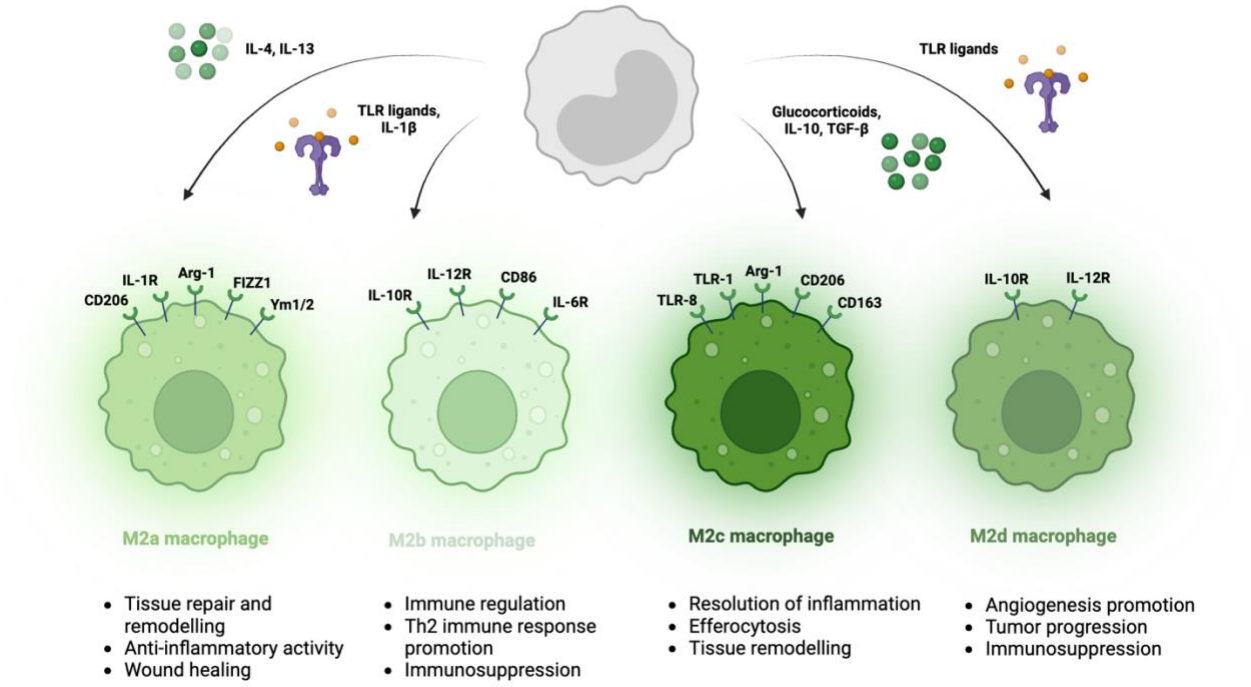

Figure 9. M2 macrophage subtypes

Key functions of M2 macrophage subtypes and their activating stimuli. M2a macrophages, driven by IL-4 and IL-13, play crucial roles in tissue repair, wound healing, and reducing inflammation. M2b macrophages, activated by immune complexes, IL-1 receptor agonists, and TLR ligands, help regulate immune responses by balancing pro- and anti-inflammatory signals, fostering Th2 polarization through IL-10 and IL-6 production, and suppressing Th1 and Th17 responses. M2c macrophages, stimulated by IL-10, glucocorticoids, and TGF-β, are key players in resolving inflammation, clearing apoptotic cells, and promoting tissue remodelling. M2d macrophages, influenced by IL-6, adenosine, and TLR ligands in tumour environments, contribute to angiogenesis, tumour progression, and the suppression of inflammation while supporting tissue growth.

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Adapted from: Li, P., Ma, C., Li, J., You, S., Dang, L., Wu, J., Hao, Z., Li, J., Zhi, Y., Chen, L., & Sun, S. (2022). Proteomic characterization of four subtypes of M2 macrophages derived from human THP-1 cells. *Journal of Zhejiang University Science B, 23*(5), 407–422.

1.2.2 Macrophages in Airway Inflammation

Macrophages are key players in the inflammatory mechanisms of asthma [218-220], a chronic lung condition characterised by inflammation, airway remodelling and increased reactivity to stimuli [221]. These immune cells are present in higher numbers in the lung tissue of asthmatics, where they play a role in both initiation and maintenance of inflammation. Macrophages produce pro-inflammatory cytokines such as TNF, IL-1β and IL-6, which are essential for the initiation of the inflammatory response. TNF increases blood vessel permeability, allowing immune cells to enter [222, 223]. IL-1β causes fever and induces adhesion molecules on the cells lining the blood vessels to attract immune cells [224]. IL-6 triggers the immediate response and adds to the widespread inflammation [225]. These cytokines recruit additional immune cells, such as neutrophils and eosinophils, to further amplify the inflammatory response in the airways.

Asthmatics have both M1 and M2 macrophages in the lungs. M1 macrophages are associated with Th1-type inflammation and are known to stimulate inflammation and attract neutrophils [226, 227]. M2 macrophages are associated with Th2-type inflammation and contribute to tissue repair and eosinophil recruitment. However, the importance of macrophages in asthma lies more in their function rather than their numbers, as changes in macrophage activity, such as polarization, play a significant role in the development of asthma [226, 227]. Macrophage polarization can influence asthma, where M1 macrophages tend to support neutrophil-driven inflammation, and M2 macrophages support eosinophil-driven inflammation. This means these macrophage types can contribute to different subtypes of asthma.

Macrophages in asthma have impaired phagocytic function [228, 229], which is a key feature of their dysfunction. Macrophages typically eliminate infections, apoptotic cells and debris by engulfing them through processes called phagocytosis and efferocytosis [230]. This is important for resolution of inflammation and tissue homeostasis. However, in asthmatics, these functions are often impaired. Defective efferocytosis has been associated with secondary necrosis and the release of danger signals, which can exacerbate inflammation [231, 232]. Macrophages are key players in efferocytosis, but this function is impaired in asthma, especially in macrophages from individuals with severe asthma [229][233]. A study found that macrophages isolated from the sputum of severe asthma patients demonstrated a reduced ability to engulf apoptotic bronchial epithelial cells compared to those from patients with mild to moderate asthma [234]. Elevated oxidative stress in macrophages is linked to their classical activation and a diminished capacity to clear apoptotic cells. Additionally, the prevalence of classically activated macrophages is higher in people with severe asthma than in those with mild to moderate asthma, potentially contributing to the observed decrease in efferocytosis [235].

Additionally, the activation of inflammasomes in macrophages, particularly the NLRP3 inflammasome, has been linked to asthma [236, 237]. Inflammasomes are complex structures of many proteins that sense stress and microbial signals and produce pro-inflammatory cytokines like IL-1β. NLRP3 activation in macrophages is associated with severity of asthma and corticosteroid resistance [238]. Various triggers such as house dust mites [239], uric acid [239] and Charcot-Leyden crystals [240] can activate NLRP3 in the lungs of asthmatics. The inflammasome pathway is another way macrophages contribute to persistence of inflammation in asthma.

Macrophages are involved not only in inflammation but also in airway remodelling, which is a feature of persistent asthma [241, 242]. Airway remodelling includes changes in the airway structure such as increased collagen deposition, goblet cell proliferation and airway wall thickening. M2 macrophages, which are involved in tissue repair and remodelling, control the synthesis of ECM and interact with growth factors and MMPs [243]. Although there is not much direct evidence of macrophages in airway remodelling in asthmatics, studies in mouse models show that inhibiting M2 polarization decreases collagen deposition and increases airway hyperresponsiveness [244]. This means macrophages are likely involved in the structural changes seen in asthma.

In summary, macrophages are involved in asthma by producing pro-inflammatory cytokines, impaired phagocytic function, activating inflammasomes, and restructuring the airway. Their importance is not only due to their presence in the lung, but also by their functional changes, specifically in their polarization and ability to remove apoptotic cells and pathogens. Macrophage driven processes play a key role in the inflammatory environment in asthma and could be targeted therapeutically to control chronic airway inflammation and remodeling in asthmatics.

Alveolar macrophages

Within the lung there are two main types of macrophages with separate but complementary functions: alveolar macrophages (AMs) and interstitial macrophages (IMs). These cells play a key role in maintaining lung homeostasis and coordinating the immune response to environmental pathogens and particles.

AMs are situated on the epithelial surface of the alveoli and are in close proximity to inhaled substances such as infections, pollutants and macrophages in the interstitial spaces [245]. They are mostly derived from fetal monocytes that settle in the lung shortly after birth and can self-renew through local growth [245]. However, blood born monocytes can replenish them, especially after injury or depletion. The dual origin of AMs ensures a constant and adaptable population throughout an individual's lifetime.

AMs play a key role in maintaining the alveoli homeostasis by removing waste and pathogens through phagocytosis [230]. They have surface markers like CD206 and MHC Class II, which are important for identifying pathogens and presenting antigens to T cells and coordinating the immune response.

AMs have altered function in asthma. As previously described, studies have shown that AMs from asthmatics have impaired phagocytic function and are unable to remove pathogens and waste [228, 229][246, 247]. This makes them more susceptible to respiratory infections and perpetuates inflammation. Asthmatics also have impaired efferocytosis in their AMs, which impairs the ability to remove apoptotic cells and results in prolonged inflammation and tissue damage. In asthma there is often the failure of the normal shift of AM function from pro-inflammatory to anti-inflammatory state during the resolution phase of inflammation. This dysregulation leads to chronic inflammation and airway remodeling [248].

Interstitial Macrophages

IMs are located in the lung interstitium, which is the tissue surrounding the airways and blood vessels [249]. Because of their location, they are in close proximity to many cell types,

such as epithelial cells, fibroblasts and endothelial cells, which allows for diverse immunological activities. While AMs mainly do phagocytosis and clear debris, IMs regulate the function of other immune cells. They produce anti-inflammatory cytokines such as IL-10, which is important in reducing excessive immune response and protecting tissues from damage during inflammatory responses [248-251].

IMs come from early developmental precursors and monocytes produced from bone marrow after birth [252]. They have variable functions and can adapt. In steady-state conditions, IMs can self-renew within their local environment [253]. However, during inflammation or injury, monocytes in the circulation can also replenish IMs. The dual origin ensures a strong and adaptable immune cell population that can respond to a wide range of physiological and pathological stresses.

IMs can adapt their function to the lung tissue needs. For example, some immune cells can increase their ability to engulf and kill pathogens and strengthen their anti-infectious and anti-allergic response. Others can reduce inflammation and facilitate tissue repair.

IL-10 reduces excessive inflammatory response in airway inflammation such as asthma [254]. This minimizes tissue damage and facilitates resolution of inflammation and tissue repair. Asthmatic patients have higher levels of IL-10 in their immune cells, including Tregs, macrophages, and DCs [255], which suppress Th2 and Th17 responses and reduce inflammation and tissue damage [256]. However, in severe asthma, the function of IMs can be dysregulated and lead to chronic inflammation and airway structural changes. Understanding the biology of the immune system in the lung and its function can provide opportunities for new treatments for lung diseases such as asthma, COPD and pulmonary infections.

1.2.3 Macrophages in Sepsis

Macrophages have a key role in the immune response during sepsis, a life-threatening condition where the immune system is hyperreactive in response to infection and can lead to organ dysfunction and failure. Macrophages are involved in both innate immunity and inflammation. They are the first cells to respond to invading pathogens, coordinating the early defence mechanisms by recognizing PAMPs through PRRs such as TLRs [257]. During sepsis, macrophages are hyperactivated and produce excessive pro-inflammatory cytokines such as TNF, IL-1β and IL-6. Excessive cytokine production can lead to a cytokine storm, an intense inflammatory response that causes tissue damage, vessel leakage and failure of many organs.

In the early stage of sepsis, macrophages switch to M1 phenotype which is important for pathogen elimination. M1 macrophages produce high levels of pro-inflammatory cytokines, generate ROS, and activate NF-κB pathway to amplify the immune response. Although this initial inflammatory response is important for managing the infection, it must be tightly regulated. Prolonged or excessive stimulation of M1 macrophages is a contributor to the hyperinflammatory state in septic shock [257]. In this environment, macrophages can be sources of harmful cytokines and intensify the inflammatory cycle, causing tissue damage and organ failure.

As sepsis progresses, macrophages undergo a significant change in their function, switching from M1 to M2 phenotype [258-260]. This is important for reducing inflammation and tissue repair. M2 macrophages produce immunosuppressive cytokines such as IL-10 and TGF-β, that dampen the immune response and facilitate tissue repair. But in severe sepsis, this compensatory anti-inflammatory response can get out of control and lead to immunosuppression

[258, 260]. This makes the patient more susceptible to secondary infections. This phase is called the 'immunoparalysis' phase of sepsis, where the immune system is unable to fight infections and patients are very susceptible to opportunistic infections.

The dual role of macrophages in sepsis is evident in their ability to initiate the inflammatory response and then resolve it. Researchers are trying to modulate macrophage activity and their switch between M1 and M2 stages as a therapeutic approach to regulate the immune response in sepsis [261]. Therapies that aim to regulate macrophage polarization and cytokine production target specific signaling pathways (e.g., NF-κB, STAT3) and surface receptors (e.g., TLR4) involved in macrophage activation [50]. Additionally, approaches that block or enhance cytokine activity, such as IL-10 or TNF inhibitors, are being studied to shift macrophages toward an anti-inflammatory M2 phenotype or reduce excessive M1-driven inflammation [262]. This would reduce the harm of excessive inflammation, while still allowing control of infection. Developing drugs that target macrophages in sepsis is still a challenge despite the abundance of research. This is because the timing and management of macrophage function is crucial in determining patient outcomes.

1.3 IL-10

IL-10 is an anti-inflammatory cytokine that plays a key role in regulating many aspects of the immune system [52][263]. Controlling hyperactive immune responses and maintaining immunological homeostasis is important to prevent inflammatory and autoimmune diseases. IL-10 works in multiple ways, mainly by modulating macrophages and other immune cells.

1.3.1 IL-10 Production by Innate Immune Cells

IL-10 is produced by a wide range of immune cells from both the adaptive and innate immune system. This includes various types of immune cells such as T helper cells (Th1, Th2, Th17), Tregs, CD8+ T cells, B cells, DCs, macrophages, mast cells, NK cells, eosinophils and neutrophils [52][263-266]. The wide distribution of IL-10 shows its importance as a feedback controller in many immune responses. Each of these cells produce IL-10 when exposed to different stimuli, highlighting the cytokine's adaptability and importance in maintaining immunological balance. In adaptive immunity, T and B cells produce IL-10, which is important in maintaining a balance between immune activation and suppression. IL-10 produced by macrophages, DCs and other cells regulates the early immune response to pathogens in innate immunity. Its role is to ensure the response is efficient and not too damaging. IL-10 can regulate both immediate and long-term immune responses in different tissues and infections.

Pathogen recognition by PRRs, such as TLRs on DCs and macrophages, induces IL-10 expression. TLR2 is important in triggering IL-10 production by APCs, such as macrophages and DCs, when they are triggered by pathogens like *Mycobacterium TB and Yersinia pestis* [267]. These PRRs recognize PAMPs and trigger signalling pathways that result in IL-10 release. This ensures the immune system can respond to different infections, while controlling the inflammatory response, to prevent harm to the host tissues. Other PRRs such as TLR4 and TLR9 also have a role in IL-10 activation [268], highlighting the overlap and complexity of the immune system. This complexity allows the immune system to fine tune its response based on the pathogen and the site of infection.

To achieve maximum IL-10 production in macrophages when stimulated by LPS through TLR4, both MyD88-dependent and TRIF-dependent pathways need to be activated [268, 269], which, in turn, activate MAPKs and NF-κB [270] (Figure 10). Activation of these pathways is important in regulating IL-10 and pro-inflammatory cytokine synthesis. The MyD88 pathway activates NF-κB and MAPKs and leads to early cytokine production. The TRIF pathway maintains these signals and induces IFN synthesis [269-274]. Stimulation of these two pathways ensures a strong but controlled response. Moreover, the involvement of certain kinases like ERK, JNK and p38 MAPK in this process shows the complexity of intracellular signalling in regulating cytokine synthesis. By integrating signals from multiple pathways, macrophages can dynamically adjust their response and balance the need to eliminate the pathogen while preventing excessive inflammation.

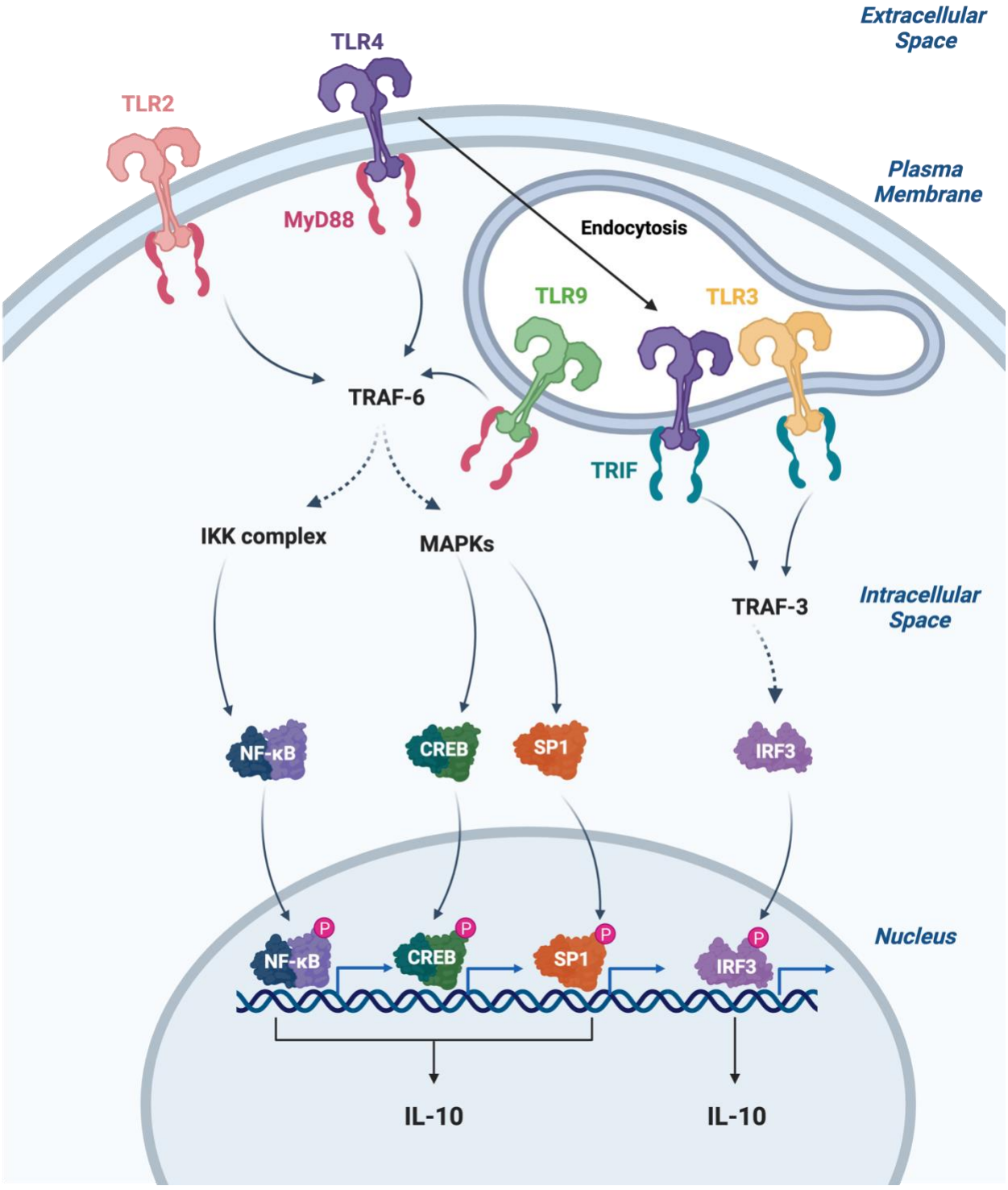

Figure 10. Signals that induce IL-10 in macrophages following TLR activation

Different TLRs contribute to IL-10 production in macrophages through distinct signalling pathways. TLR4, located on the cell surface and within endosomes, utilizes both MyD88 and

TRIF adaptor proteins. Surface TLR4, TLR2, and TLR9 recruit MyD88, leading to the activation of TRAF6 and subsequent phosphorylation of NF-κB and MAPKs (ERK, JNK, p38). These pathways drive IL-10 transcription through the activation of transcription factors NF-κB, CREB, and SP1. Endosomal TLR4 and TLR3 recruit TRIF, activating TRAF3, which phosphorylates IRF3 to promote IL-10 transcription. Together, these pathways integrate signals from various TLRs to fine-tune IL-10 production, ensuring an effective yet controlled immune response.

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Adapted from: Saraiva, M., & O'Garra, A. (2010). The regulation of IL-10 production by immune cells. *Nature Reviews Immunology, 10*(3), 170–181.

1.3.2 IL-10 Regulation Mechanisms

Chromatin Remodeling

Chromatin remodelling at the *IL10* locus is involved in IL-10 production [275-278]. Multiple DNase I hypersensitivity sites in the mouse *IL10* locus are shared by IL-10 producing cells like macrophages and DCs, indicating a chromatin structure that favors IL-10 expression. Chromatin remodeling is an essential process that allows transcription factors to access DNA and start transcription. The regulation of this process is controlled by histone modifications like acetylation and methylation, which modify chromatin structure. Macrophages have distinct histone modification sites that are linked to active IL-10 transcription. These sites may be different in different cell types, suggesting that there are different mechanisms to activate the *IL10* gene in each cell type. Knowing these epigenetic changes will give us a better understanding of how *IL10* is precisely regulated, and the role of its dysregulation in inflammatory and autoimmune diseases.

Transcription Factors

Multiple transcription factors bind to the *IL10* promoter to regulate transcription. The proteins mentioned in the literature are SP1 [279], SP3 [280], CCAAT/enhancer binding protein-β (C/EBPβ) [281, 282], IRF1 and STAT3 [283]. The p50 subunit of NF-κB has been shown to initiate IL-10 transcription in primary macrophages [284]. The interaction of these transcription factors is often controlled by upstream signalling pathways that are activated by different stimuli. For example, NF-κB activation is a common pathway that occurs after TLR ligation and induces IL-10 and other cytokines. The interaction of these transcription factors and the chromatin structure at the *IL10* gene is crucial for precise IL-10 regulation in response to various immunological stimuli. These transcription factors not only induce IL-10 transcription but also integrate inputs from multiple pathways to fine tune in different immunological contexts.

Post-Transcriptional Regulation

IL10 mRNA stability also regulates its expression after transcription [285]. The mRNA has adenosine-uridine-rich regions (AREs) which direct it to be degraded quickly. AREs bind to proteins like tristetraprolin (TTP) which facilitates mRNA degradation [286]. Activation of pathways like p38 can stabilize IL-10 mRNA by inhibiting TTP activity [287]. This layer of regulation allows for rapid modulation of IL-10 synthesis in response to changing environmental signals. Presence of inflammatory stimuli stabilizes IL-10 mRNA, allowing for continuous cytokine production. Absence of these stimuli leads to rapid degradation of IL-10 mRNA to prevent excessive anti-inflammatory responses. This highlights the importance of post-transcriptional regulation in fine tuning cytokine levels during immune responses.

MicroRNAs (miRNAs) regulate IL-10 expression [288]. For example, miR-106a which is present in both lymphoid and myeloid cells can bind to the 3' UTR of IL-10 mRNA and degrade it to regulate the amount of IL-10 produced [289]. miRNAs are short RNA molecules that control gene expression after transcription by binding to complementary sequences in target mRNA and causing them to be degraded or suppressed in translation. The involvement of miRNAs in IL-10 regulation adds another layer of complexity to allow for rapid and reversible IL-10 modulation in response to different stimuli. This is crucial to maintain the balance between pro-inflammatory and anti-inflammatory signals during immune responses.

Feedback Regulation

IL-10 production is regulated by both positive and negative feedback loops to maintain a balanced immune system and prevent excessive inflammation. One of the processes is IL-10 inducing the production of dual-specificity protein phosphatase 1 (DUSP1). DUSP1 removes phosphate groups from and deactivates p38 MAPK, a key signalling protein that produces pro-inflammatory cytokines. DUSP1 limits p38 phosphorylation and thus reduces IL-10 production and creates a negative feedback loop to prevent excessive IL-10 production and maintain immune response balance [290-292].

Conversely, IL-10 can create a positive feedback loop by increasing tumour progression locus 2 (TPL2). TPL2 is an activator at an earlier stage of the MAPK pathway, activating ERK and p38 MAPKs. These proteins are involved in IL-10 synthesis [293]. IL-10 boosts its own production by activating these MAPKs through TPL2 upregulation. This feedback loop ensures production of enough IL-10 during immune responses to combat inflammation and resolve

immune responses. The feedback mechanisms show the fine tuning of IL-10 regulation. IL-10 uses a balance of positive and negative feedback loops to regulate the immune response. This ensures the response is strong enough to manage inflammation but not too strong to suppress or hinder pathogen elimination.

IFN-γ inhibits cellular activity [294]. It's a cytokine mostly produced by Th1 cells, NK cells and cytotoxic T lymphocytes. It's important in immune response against intracellular pathogens and in macrophage activation. However, IFN-γ also has a suppressive effect on IL-10 synthesis, a regulatory mechanism to control the anti-inflammatory property of IL-10. IFN-γ blocks IL-10 production by preventing MAPK activation, specifically ERK and p38, which are involved in IL-10 synthesis. This suppression is done by activating glycogen synthase kinase 3 (GSK3). GSK3 inhibits activator protein 1 (AP1) binding to IL-10 promoter and thus reduces IL-10 production. AP1 is a transcription factor complex activated by MAPKs and involved in the transcription of many cytokines including IL-10.

IFN-γ blocks IL-10 production to maintain a strong pro-inflammatory response to fight infections. This is important in the early stage of the disease where a strong inflammatory response is needed to manage and eliminate infections. But IL-10 control by IFN-γ must be carefully regulated to avoid excessive inflammation and tissue damage, demonstrating the fine tuning of immune regulation.

1.4 Sex as a Modifier of Immune Response

Sex is a biological factor that has a big impact on immune responses to many stimuli, including fungus, viruses, bacteria, parasites and allergies [295]. Biological sex is determined by variations in chromosomes, reproductive organs, and the levels of sex hormones such as

estrogen, progesterone and testosterone. These variations affect many physiological and anatomical aspects of immune function including, pathogen recognition, infection clearance and overall inflammatory control. We need to distinguish sex from gender since gender includes behaviors and cultural factors that can impact immune responses, such as exposure to infections, access to healthcare, and health seeking behaviors. However, this thesis will focus on the biological aspects of sex and its effects on immunological function.

There is a lot of evidence that immunological responses can differ significantly between males and females. These differences include vulnerability to infections, autoimmune diseases and inflammatory diseases. For example, in many contexts, females have stronger and more effective adaptive immune responses than males [295]. This can help them fight off some infections but also make them more prone to autoimmune diseases. Males, on the other hand, have a stronger and more robust inflammatory response, which can protect them from some diseases, but also make them more susceptible to inflammatory diseases. The sex differences are determined by an intricate interplay of genetic, hormonal and environmental factors, largely the effect of sex hormones on immune cells and cytokine production.

Although sex is increasingly recognized as an important factor in immune responses, immunology has traditionally not considered sex in trial design and data analysis. Underreporting of sex-based differences in immune function studies is a large problem. This means we might be missing out important findings that can be used to inform personalized treatments and interventions. Less than 10% of immunology studies report sex-based data, which is the lowest among all biological disciplines [296]. The lack of information in the existing literature emphasizes the need to prioritize sex in immune function.

This section will discuss the biological factors that contribute to the differences in inflammation and immune function between males and females. It will focus on how sex hormones, genetics and immune cell regulation differ between the two sexes.

1.4.1 Sex Differences in Inflammation and Immune Function

The sex differences in immunological responses have multiple interconnected pathways. Although females have a stronger immune response overall, males have more intense inflammatory response in specific situations, especially during bacterial infections. For example, males produce more pro-inflammatory cytokines such as TNF by activating TLR4 signalling [297]. This results to increased inflammation but not necessarily better pathogen elimination. Females on the other hand, have higher expression levels of specific PRRs, such as TLR7, which amplifies IFN production during viral infections and boosts their antiviral response [298]. This allows females to detect and eliminate infections early on. However, their increased immune reactivity, especially in the adaptive immune system, makes them more prone to autoimmune diseases as their immune system is more likely to attack self-antigens.

Hormones also play a role in regulating immune responses. Higher estrogen levels in females stimulate type I interferon and pro-inflammatory cytokine production, which makes the body better at fighting viruses [295][299]. But this also increases the risk of chronic inflammation and autoimmune diseases. In males, testosterone inhibits immune activity by producing pro-inflammatory cytokines [295][300]. This protects against autoimmune diseases, but also makes males more prone to infections. Males also have higher levels of inflammatory cytokines at baseline.

1.4.2 Sex Differences in Innate Immunity

Sexual dimorphism is quite pronounced in the innate immune system. An example is the different expression of PRRs, such as TLRs, which recognize microbial components. Women have higher TLR7 expression compared to men, which results in increased IFN-α production during viral infections [298]. The sex-specific immune response is affected by the activation of some immune-related genes on the X chromosome, such as TLR7, which are not inactivated in females. Hence, immune cells from females produce more cytokines and have stronger inflammatory response compared to immune cells from males in this scenario.

Apart from TLR7, other PRRs also show sex differences. For example, in response to TLR9 activation, male immune cells, such as monocytes and plasmacytoid dendritic cells, produce more IL-10, associated with higher androgen levels [301]. Following TLR4 stimulation with LPS, male PBMCs and neutrophils produce more TNF than female cells [302, 303]. Conversely, female immune cells produce more anti-inflammatory molecules, such as prostanoids, in response to the same stimulus. Male macrophages also show higher TLR4 surface expression than female macrophages, potentially explaining the stronger pro-inflammatory response observed in males [297][304] (Figure 11). The heightened pro-inflammatory response in males, mainly through TLR4 signalling, appears testosterone-dependent.

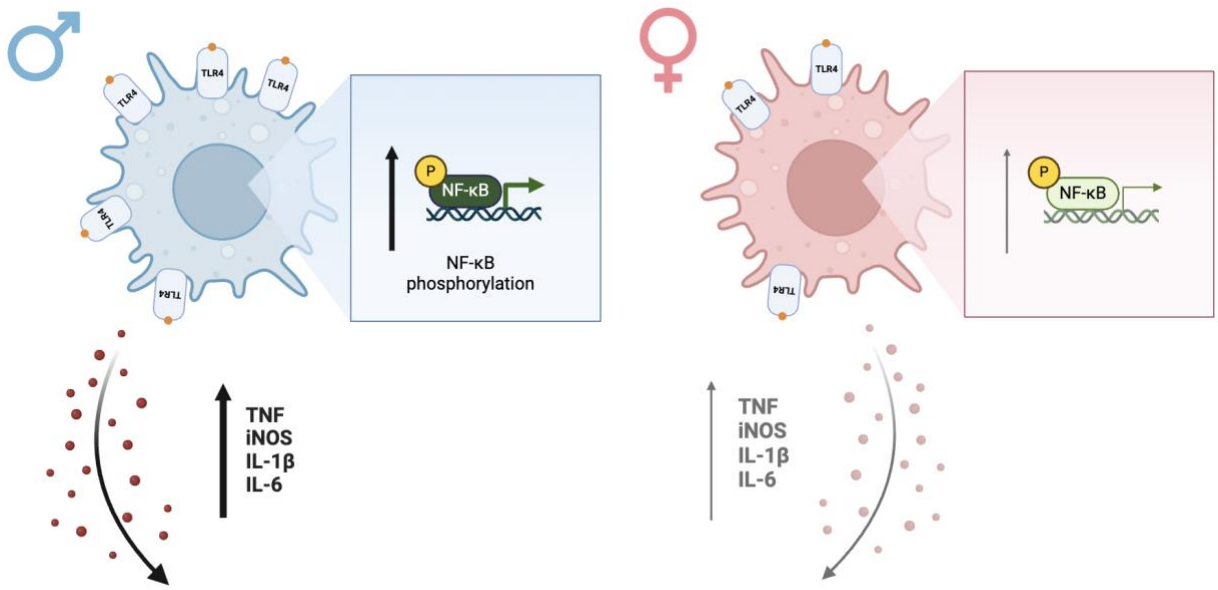

Figure 11. Sex differences in inflammatory responses in macrophages after TLR4 activation

Compared to females, male macrophages have stronger inflammatory response, characterized by higher baseline TLR4 surface expression, higher NF-κB phosphorylation and more production of pro-inflammatory molecules such as TNF, IL-1β, ROS and MCP-1. This means male macrophages are more prone to M1 phenotype which is associated with excessive pro-inflammatory activity. The M1 polarization in males leads to exaggerated inflammatory response, which can contribute to increased susceptibility to inflammatory diseases and infection related complications. The excessive production of pro-inflammatory molecules in males may be beneficial for pathogen clearance but also increases the risk of tissue damage and chronic inflammation.

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Information from: Barcena, M. L., Niehues, M. H., Christiansen, C., Estepa, M., Haritonow, N., Sadighi, A. H., Müller-Werdan, U., Ladilov, Y., & Regitz-Zagrosek, V. (2021). Male macrophages and fibroblasts from C57/BL6J mice are more susceptible to inflammatory stimuli. *Frontiers in Immunology*, *12*, 758767.

Apart from PRRs, other components of innate immunity also show sex differences, though the extent of these differences can vary across individuals and populations. For example, males generally have more NK cells than females [305]. Females tend to exhibit higher

phagocytic activity in neutrophils and macrophages, which may enhance their ability to clear infections [306]. Female antigen-presenting cells (APCs) are often more efficient in delivering antigens to T lymphocytes than male APCs [307]. Additionally, sex-specific variations are observed in innate lymphoid cells (ILCs)—lymphocyte-like cells that regulate immunological responses in tissues. Females typically have lower numbers of type 2 ILCs, which could be associated with increased susceptibility to demyelination in autoimmune diseases [308]. These findings are generally observed across studies but may differ due to genetic, ethnic, or environmental factors.

The presence of sex-specific differences in quantity and function of innate immune cells and PRRs emphasizes the importance of considering sex as a biological variable in studies of immune function and inflammation.

1.4.3 Factors Affecting Sex Differences in Immune Responses

The reported sex differences in immune responses are influenced by genetic, hormonal and environmental factors. The X chromosome plays a major role in genetic mediation, as it has multiple immune related genes that are expressed differently in males and females [309]. Genes like TLR7 and forkhead box p3 (FOXP3), which control immune responses, are located on the X chromosome, and have a significant impact on shaping the immune system. Females with two X chromosomes may have an advantage in immune control, since some genes on the X chromosome can escape inactivation and result in higher immune responses.

Hormonal mediators such as estrogen, progesterone, and testosterone significantly impact immune function, with all three present in both males and females at varying levels. Estrogen, for example, enhances the activity of innate immune cells and promotes pro-inflammatory

cytokines, but its effects are modulated by other hormones [310-320]. Progesterone has anti-inflammatory properties and promotes the resolution of inflammation, while testosterone, the primary male sex hormone, generally suppresses immune responses. This balancing effect of these hormones may help explain the differing immune responses and the lower incidence of autoimmune diseases in males than females [321-333]. Understanding the complex interplay of these genetic and hormonal factors is critical to understanding the mechanisms behind sex differences in immune function.

Environmental factors play a major role in shaping the sex differences in immune responses. Genetic and hormonal factors can be influenced by exposure to pathogens, nutrition, stress, lifestyle and microbiome composition [334-346], which, in turn, affect immune responses in both males and females. For example, microbiome composition of males and females may differ due to lifestyle factors which can affect the immune system's ability to respond to infections or inflammation [295]. Environmental stressors, such as chronic stress, can affect immune function in many ways by altering hormone levels like cortisol, which interacts with sex hormones and can either enhance or suppress inflammatory responses. Moreover, the impact of work and social roles on pathogen exposure may differ between males and females, resulting to differences in immune responses and susceptibility to diseases [295].

Age is a major factor in sex differences in immune function. 'Immunosenescence' refers to the many changes that happen in the immune system as we age. Age related changes are more pronounced in males than in females, especially in older males who experience more decline in immune function. Females generally have stronger and longer lasting immune responses [347]. However, after menopause, they may have higher risk of autoimmune diseases, due to a decrease

in estrogen levels [348]. The cumulative effect of hormonal changes throughout one's life amplifies the effect of age on immune responses. Puberty starts the production of sex hormones, which has a big impact on immune function.

1.5 Sema3E/PlexinD1 Axis

1.5.1 Semaphorins: General Classification and Structure

Semaphorins were first found to guide axons during neuron development [349, 350]. Now, we know semaphorins have many functions in developing and maintaining organs and tissues in normal and abnormal conditions. Semaphorins are found in circulatory, endocrine, gastrointestinal, musculoskeletal, immunological and respiratory systems [350, 351]. There are over 20 semaphorins classified into eight families based on sequence similarity and structural properties [352, 353], including secreted, cell surface attached and transmembrane-bound forms. Invertebrates have only Sema1 (A, B) and Sema2 (A, B), while vertebrates have Sema3 (A-G), Sema4 (A-G), Sema5 (A, B), Sema6 (A-D), Sema7 (A) and viruses have Sema8 (A, B) [351] (Figure 12). Semaphorins generally activate signalling pathways that control cell adhesion and cytoskeleton formation. These activities are important for body structure development, new blood vessel formation, and cell specialization, growth, proliferation, movement, and survival [350, 351].

Semaphorins are classified based on their amino acid sequence and structural components. These proteins have 400-1000 amino acids and are named by a letter code such as semaphorin3E (Sema3E). Semaphorins have a 500 amino acid extracellular domain called the "Sema" domain with a 7-blade-β-propeller fold [354, 355]. They also have a receptor-binding domain called plexin semaphorin and integrin (PSI), which are rich in cysteine. The protein

Sema3E, which is this study's main subject, has a single immunoglobin (Ig)-like domain [350]. This domain is proteolytically cleaved, resulting in the production of its active form [356-358]. Studies show that the Sema domain interacts with receptors and promotes homodimerization, which combines two semaphorin proteins. Semaphorins can generate signals that either repel or attract cells, guiding their movement and behaviour based on the specific receptors and co-receptors expressed on the cell surface. This means that semaphorins can direct cells toward or away from certain areas, influencing processes like cell migration, tissue development, and immune responses, depending on the cell type and the receptors involved.

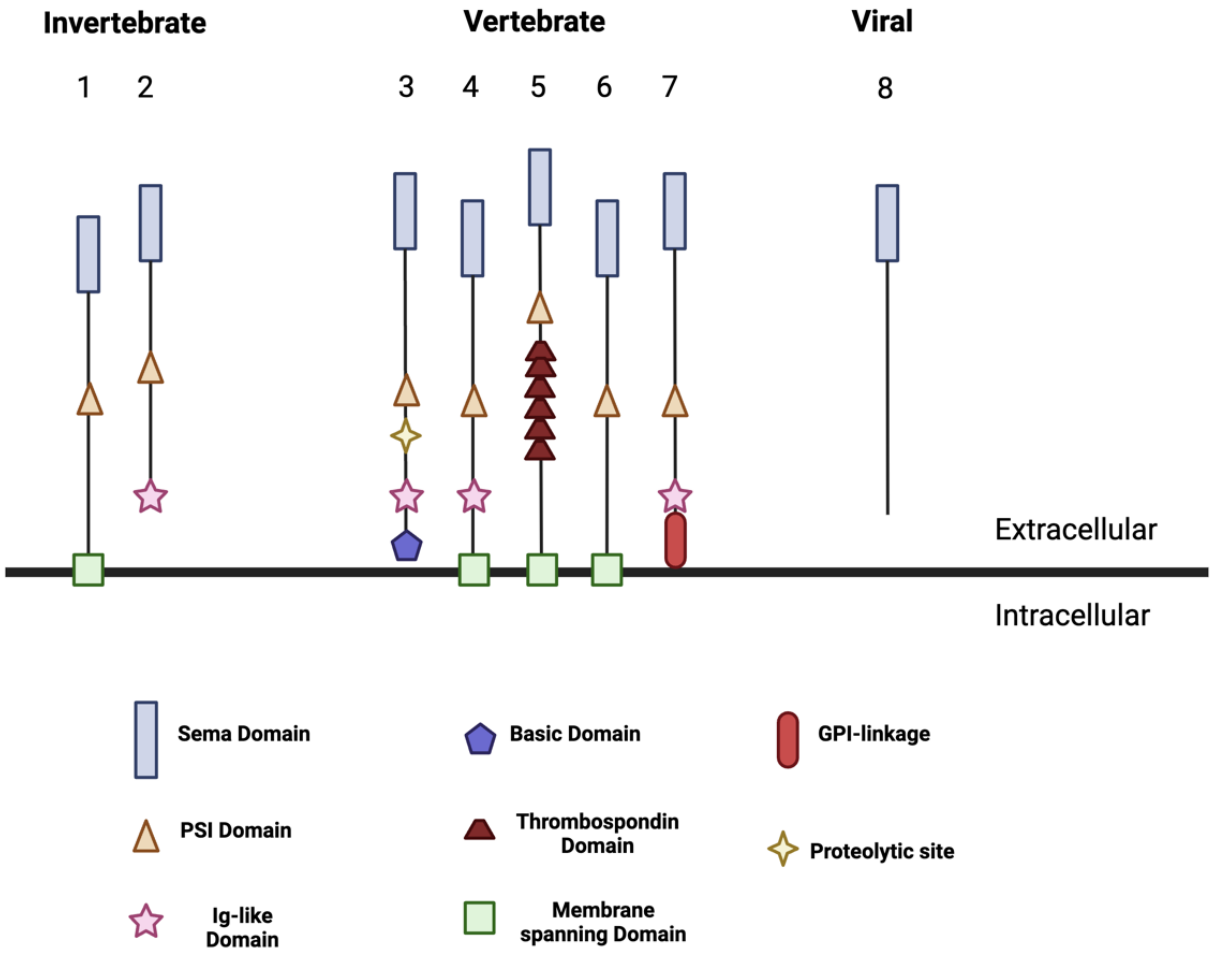

Figure 12. The semaphorin family

Semaphorins are divided into 8 classes. Classes 1 and 2 are found in invertebrates, classes 3-7 are found in vertebrates and class V (sema-8) is found in viruses. All semaphorins have a conserved region called the Sema domain. Semaphorins 2, 3 and 8 are secreted proteins, while classes 1, 4, 5, 6 and 7 are membrane-bound proteins. Some semaphorins (classes 2, 3, 4 and 7) have immunoglobin (Ig)-like domains, while class 5 has a thrombospondin domain. Class 7 semaphorin is anchored to the membrane via a GPI linkage at the carboxyl terminus.

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Adapted from:

Takahashi, T., Fournier, A., Nakamura, F., Wang, L. H., Murakami, Y., Kalb, R. G., Fujisawa, H., & Strittmatter, S. M. (1999). Plexin-neuropilin-1 complexes form functional semaphorin-3A receptors. *Cell, 99*(1), 59–69.

Hu, S., & Zhu, L. (2018). Semaphorins and their receptors: From axonal guidance to atherosclerosis. *Frontiers in Physiology, 9*, 1236.

1.5.2 Semaphorins and their Receptors

Semaphorins interact with two primary receptors, plexins and neuropilins, to initiate various intracellular signalling [359, 360]. Membrane-bound semas can directly bind and signal through plexins, while secreted class 3 semas cannot [361, 362]. Semas bind to neuropilins, the subunits that bind to the ligand. Plexins also have a role in this receptor complex, specifically in signal transduction. Sema3E is an exception, where it can directly bind to its receptor plexinD1 without needing the co-receptor neuropilins [361].

Plexins are classified into four classes, A-D based on their homology. There is one A-type, three B-types, one C-type and one D-type [362]. Plexins have structural similarities to semaphorins and have a sema domain in their extracellular region, but plexins do not dimerize [363]. There is a cysteine-rich plexin-semaphorin-integrin (PSI) domain and three immunoglobin-plexin-transcription (IPT) domains [364]. Ligand binding occurs at the Sema domain, and protein interactions at the PSI and IPT domains ensure perfect ligand binding [365]. When no ligand exists, plexins are not activated, and the Sema domain acts as an autoinhibitory domain [366]. Signal transduction occurs in the cytoplasm upon ligand binding. The cytoplasmic domain has no kinase activity, but plexins can transmit their signal to the cytoplasm by binding to tyrosine kinase through phosphorylation [367]. The cytoplasmic region of plexins has GTPase-binding and GTPase-activating protein (GAP) domains, which trigger the response when activated [368].

1.5.3 Sema3E/PlexinD1 Axis

Sema3E

Sema3E was initially identified as a regulator of neuronal axon growth [349, 350]. It is a secreted protein that undergoes proteolytic cleavage by furin convertase, resulting in two main fragments: an inactive form, P87, and an active isoform, P61. The P61 fragment, which retains the ability to interact with receptors, is considered the biologically active form of Sema3E [361-365]. This cleavage removes a roughly 20 kDa fragment from the C-terminus, creating P61, which engages in functional interactions with its receptor, PlexinD1. Sema3E, like other members of the Sema3 family, has a Sema domain, a PSI domain (plexin-semaphorin-integrin), an immunoglobulin domain, and a C-terminus tail.

PlexinD1 is the primary receptor that Sema3E interacts with. Under normal physiological conditions, Sema3E functions as a repellent to PlexinD1, primarily influencing neuronal and vascular systems. However, when neuropilin-1 (Nrp1) and PlexinD1 are co-expressed, as seen in some contexts of neuronal guidance, Sema3E acts as an attractive factor [361]. This dual role of Sema3E is attributed to structural changes upon cleavage and the presence of specific receptors or co-receptors. In terms of expression, Sema3E is primarily produced by endothelial cells, neurons, and certain immune cells, although its expression profile may vary based on the tissue context and physiological state [369].

PlexinD1

PlexinD1 is one of the class 4 plexins. It has structural diversity and is expressed in many cell types, including neurons, endothelial cells, airway smooth muscle cells, adipose cells, thymocytes, B cells, neutrophils, DCs and macrophages [359]. This protein has 1879 amino

acids and, like semaphorins, has an extracellular N-terminal sema domain [364]. The protein has three PSI repeats, four IPT domains and a transmembrane domain. The cytoplasmic region has the Sex and Plexins (SP) domain, which includes the GTPase activation protein (GAP) domain (Figure 13A). The GAP domain has a Rho-GTPase-binding domain (RBD) between the C1 and C2 regions, which are highly conserved. The C1 and C2 regions have the Ras GAP motif 1 (RasGAP1) and Ras GAP motif 2 (RasGAP2), respectively. These have arginine residues, which are essential for Ras protein inhibition. PlexinD1 has a GAP domain and a terminal region called T-segment. The T-segment is linked to a short PDZ-binding motif called D1-PBM43 [368].

PlexinD1 is the only receptor that Sema3E interacts with, as mentioned above [361]. The PlexinD1 receptor has an active and inactive state. Without the Sema3E ligand, the receptor is inactive with the Sema domain in a specific conformation, and the other parts of the extracellular region are unfolded (Figure 13B). The cytoplasmic C1 and C2 regions are also folded. Upon Sema3E binding, the GAP domain is activated, there is a conformational change, and Ras-mediated downstream signalling is suppressed [368]. This axis is crucial for the body's homeostasis as it regulates essential processes such as vascular development , neuronal development and hormone control [360][369].

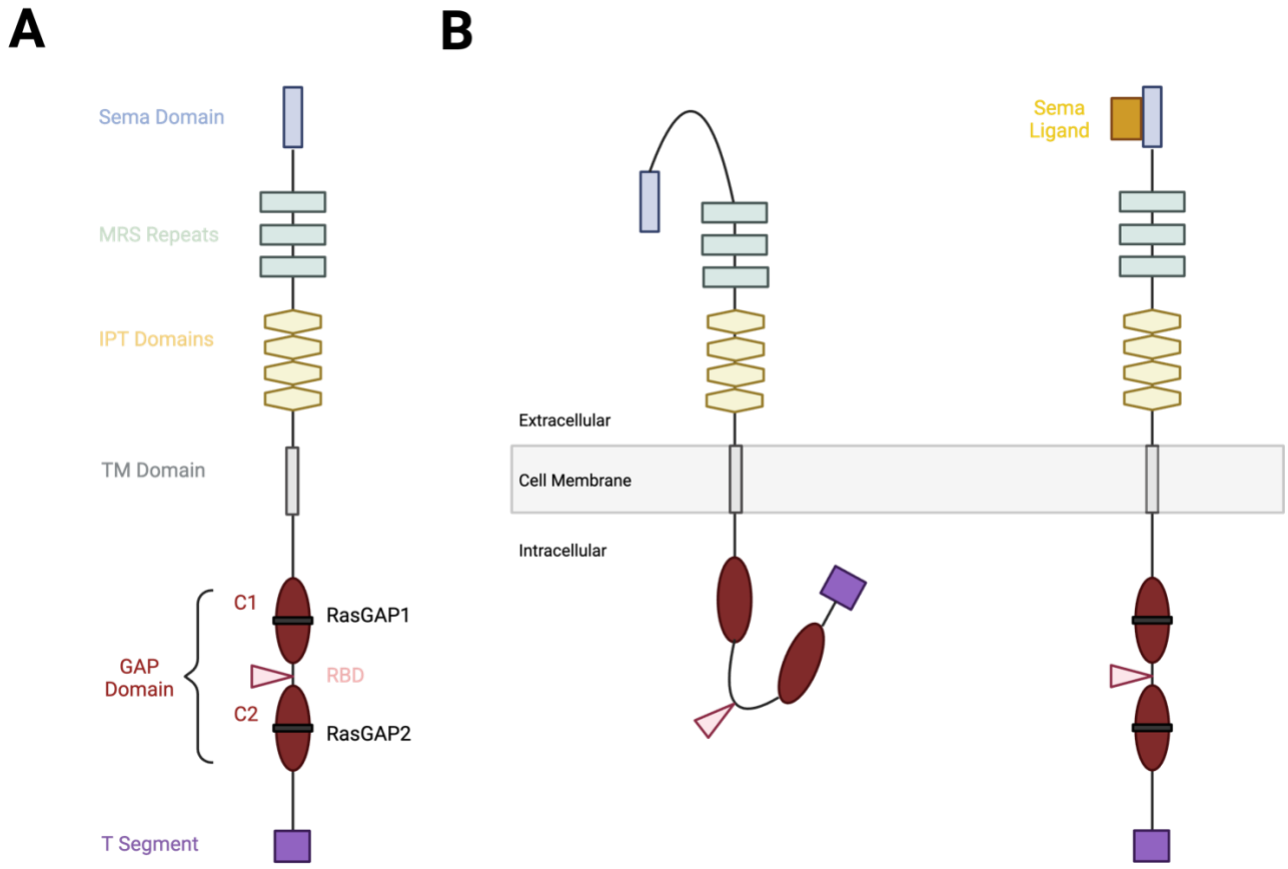

Figure 13. PlexinD1 structure and activation

(A) PlexinD1 is a type I transmembrane protein. The extracellular N-terminal region has a Sema ligand binding domain followed by three MRS repeats, four IPT domains and a transmembrane domain. The cytosolic tail is called the Sex and Plexins domain and has a split GAP domain with conserved regions C1 and C2, each having Ras GAP motif, which is essential for Ras protein inhibition. Between C1 and C2 is a RBD. The GAP domain has a conserved terminal T segment. **(B)** Inactive PlexinD1 has the Sema domain binding to the rest of the extracellular region, and the GAP domain is non-functional. Upon Sema ligand binding, PlexinD1 undergoes conformational changes that activate the GAP domain and allow additional protein interactions.

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Adapted from: Gay, C. M., Zygmunt, T., & Torres-Vázquez, J. (2011). Diverse functions for the semaphorin receptor PlexinD1 in development and disease. *Developmental Biology*, *349*(1), 1–19.

Sema3E/PlexinD1 signalling mechanisms

The Sema3E/PlexinD1 axis has multiple roles in controlling various biological processes, such as immunological responses, cell migration, angiogenesis and neural guidance. However, the exact mechanisms by which Sema3E and PlexinD1 regulate their functions are still unknown. Multiple signalling pathways and molecular interactions are involved in Sema3E/PlexinD1 signalling. These pathways and interactions vary depending on the cell type and biological context.

PlexinD1 was found to have an early signalling pathway involving its R-Ras GAP activity, which inhibits cell migration [370]. In COS-7 cells, it was shown that PlexinD1 inhibits migration by using its GAP activity, which is Rnd2 dependent. Sema3E/PlexinD1 signalling in cortical neurons is Rnd2 dependent, suppressing axon extension by reducing R-Ras activity [371]. Other plexins, such as Plexin-C1, also have Ras GAP activity [368]. But Rnd proteins are not required for their function. Therefore, different plexins control Ras activity differently depending on the cell type.

The Sema3E/PlexinD1 interaction in smooth muscle cells removes phosphate groups from RAC and RAS GTP molecules through the GAP domain. This deactivates the AKT and ERK1/2 signalling pathways. This inhibits cell movement and may control cell cycle signalling pathways, preventing cell division and smooth muscle cell growth [372] (Figure 14). In endothelial cells, the Sema3E/PlexinD1 interaction exposes the GAP domain to adaptors such as RAS-GTP, which converts GTP to GDP [373]. This removes alpha-beta integrins from the extracellular matrix. RAS-GDP then activates phosphatidylinositol-4-phosphate 5-kinases

(PIP5K), which activates ADP-ribosylation factor 6 (Arf6) and internalizes integrin [371, 372].

The endothelial cells collapse and inhibit angiogenesis.

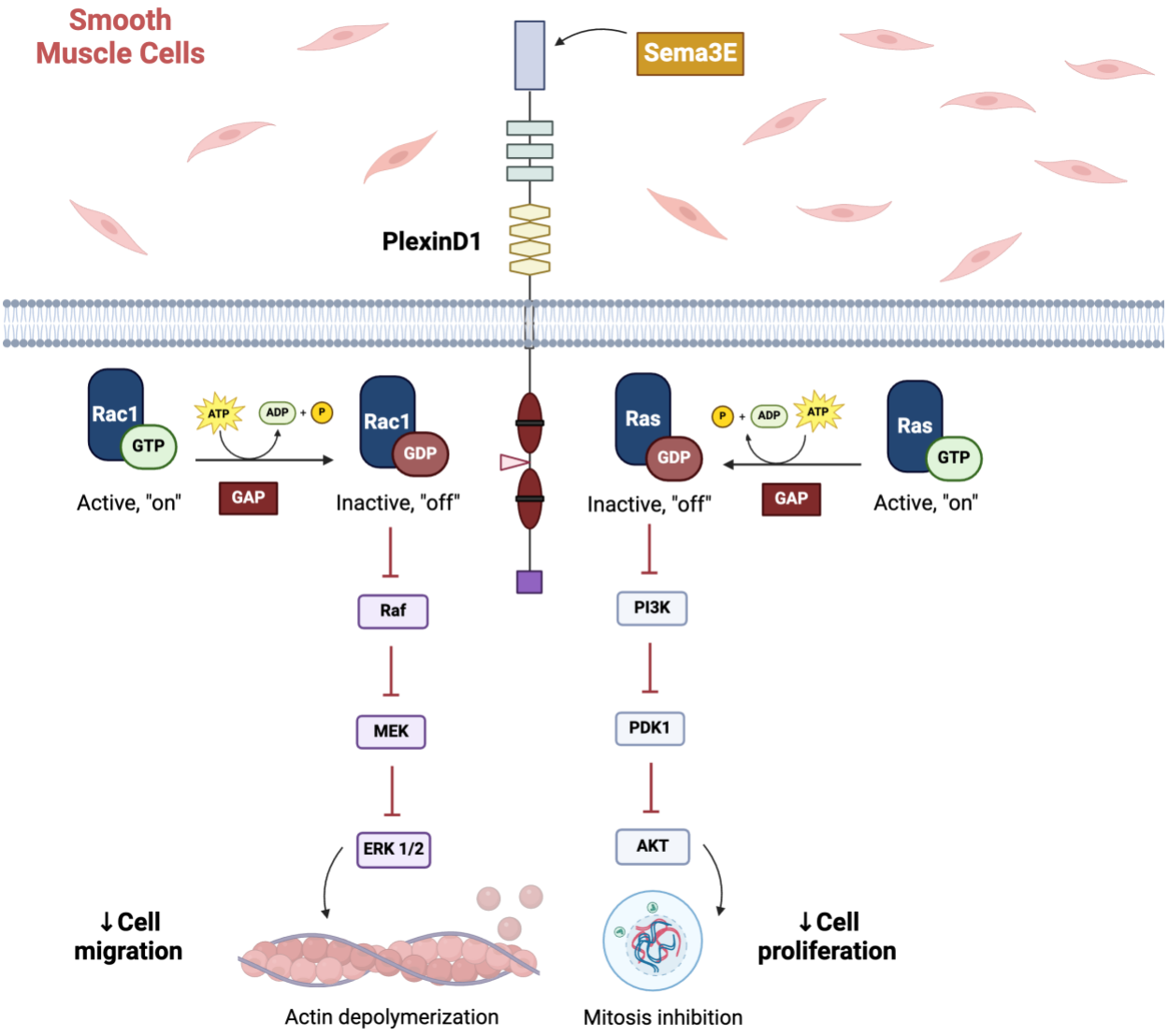

Figure 14. Sema3E/PlexinD1 in smooth muscle cells

Sema3E binding to PlexinD1 activates the GAP domain in smooth muscle cells, which promotes the hydrolysis of RAC and RAS-bound GTP to GDP, inactivating these small GTPases. This signalling cascade suppresses AKT and ERK1/2 pathways, resulting in reduced smooth muscle cell migration, inhibition of mitosis, and decreased cell proliferation. Additionally, this mechanism may regulate cell cycle progression, contributing to the anti-proliferative effects of Sema3E/PlexinD1 signalling.

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Adapted from: Matloubi, M., Koussih, L., Shan, L., Lukawy, C., & Gounni, A. S. (2023). Targeting the Semaphorin3E-plexinD1 complex in allergic asthma. *Pharmacology & Therapeutics, 242,* 108351.

The Sema3E/PlexinD1 axis controls multiple cellular processes and has therapeutic potential. Sema3E inhibits VEGF-induced angiogenesis in retinal vascular models by activating Dll4-Notch signalling [374]. Studies with human umbilical vein endothelial cells (HUVECs) showed that Sema3E inhibits VEGF-induced angiogenesis by blocking ERK and Akt signalling pathways [375]. Furthermore, the GTPase Ras homolog family member J (RhoJ) controls the communication between Sema3E/PlexinD1 and VEGF signalling and RhoJ is a potential therapeutic target in ischemic retinas [376]. The suppressive function of Sema3E in new vessel formation is essential under ischemic conditions when Sema3E is upregulated in a p53 dependent manner in response to tissue ischemia. Inhibiting Sema3E in ischemia models enhances the response to VEGF and is a potential therapeutic approach to promote angiogenesis in ischemic conditions.

The Sema3E/PlexinD1 axis works not only in endothelial and neuronal cells but also in immune control. The Sema3E/PlexinD1 signalling pathway regulates immune cell function by modulating macrophages and other immune cells. In the LPS-induced acute inflammatory sepsis model, deletion of Sema3E in macrophages resulted in better temperature regulation, reduced iNOS production and reduced levels of pro-inflammatory cytokines TNF and IL-6 [377]. Macrophages lacking Sema3E exhibited a skewing toward the M2 phenotype, which is associated with anti-inflammatory functions. Sema3E deficiency was associated with decreased

phosphorylation of key signalling molecules like ERK1/2, AKT, STAT3 and NF-κB, indicating its role in suppressing inflammatory signalling [377]. Deleting PlexinD1 in macrophages rescued the survival of mice after the LPS challenge, which further supports the role of Sema3E/PlexinD1 in macrophage-mediated inflammation [377].

Sema3E/PlexinD1 signalling is involved in multiple biological processes and cell types. In endothelial cells it controls angiogenesis, in macrophages it controls inflammation and polarization. The wide range of functions suggests that the signalling pathways triggered by Sema3E/PlexinD1 are context-dependent and cell type and process-specific. More studies are needed to understand the complex molecular mechanisms of Sema3E/PlexinD1 signalling. This can be therapeutically targeted in multiple diseases such as ischemic disorders, sepsis and chronic inflammatory conditions.

1.5.4 Sema3E/PlexinD1 and Immune Responses

Sema3E and PlexinD1 control cell movement within the immune system. Immune cells, such as T cells and dendritic cells (DCs), rely on precise navigation to effectively perform their surveillance and pathogen elimination functions. The Sema3E/PlexinD1 signaling pathway plays a key role in this process. Sema3E is typically secreted by endothelial cells, neurons, and certain immune cells, while PlexinD1, the receptor for Sema3E, is expressed on T cells, DCs, and some macrophages. This interaction enables immune cells to respond to signals that either attract or repel them, guiding them through complex tissue environments. In cancer, the Sema3E/PlexinD1 axis has been shown to direct immune cells to the tumor site. Interestingly, PlexinD1 can exhibit both pro- and anti-tumor effects depending on factors such as cancer type, location, and its interaction with different isoforms of Sema3E. For example, the full-length form of Sema3E,

P87-Sema3E, does not promote metastasis [378]. However, P61-Sema3E is associated with metastatic activity. PlexinD1 expression is also positively correlated with tumor progression and metastasis [378]. In human colon cancer, PlexinD1 and Sema3E levels are elevated in metastatic cells compared to primary tumor cells, with Sema3E signaling through PlexinD1 linked to the advancement of metastasis [379]. Furthermore, deleting PlexinD1 in tumor cells has been shown to block the metastatic effects of P61-Sema3E.

Additionally, Sema3E contributes to the growth of intestinal, gastric, and pancreatic cancers, with higher Sema3E levels correlating with gastric cancer metastasis and poorer survival rates in pancreatic cancer patients [380, 381]. In ovarian cancer, Sema3E triggers epithelial-to-mesenchymal transition (EMT), enhancing cell migration and malignant progression via PlexinD1 [382]. In breast cancer, Sema3E/PlexinD1 signalling has been found to inhibit apoptosis and promote metastasis, while blocking PlexinD1 signalling induces cancer cell apoptosis [383]. Conversely, a mutated, furin-resistant isoform of full-length Sema3E has been shown to inhibit tumour angiogenesis and metastatic spread [384].

Sema3E acts as a chemotactic factor in the immune system, controlling the movement and positioning of NK cells. Research has shown that Sema3E can provide repulsive signals to guide NK cell migration, directing them toward infection or tumor sites as needed [385] (Figure 15). In this context, immature DCs are a primary source of Sema3E, secreting it to limit NK cell migration and prevent premature interactions. As DCs mature, they reduce Sema3E secretion, allowing NK cells to approach and interact with them more readily. This interaction between Sema3E and PlexinD1 on NK cells is essential for NK cell migration to specific tissues where their cytotoxic activity is needed, while also ensuring that NK cell-DC interactions occur at the

appropriate time. In cancer, Sema3E has been shown to regulate NK cell infiltration into the tumour microenvironment, which can slow down tumour progression by limiting NK cell's ability to kill cancer cells. Sema3E also regulates NK cell activation. Sema3E signalling via PlexinD1 inhibits NK cell activation, reduces their cytotoxicity and is an inhibitory factor for NK cell responses [386]. This regulatory function limits excessive inflammatory response and tissue damage during immune responses. But in cancer, the suppressive effect of Sema3E on NK cells can help cancer cells to evade the immune system, highlighting the complex role of Sema3E in maintaining the balance between immune activation and suppression.

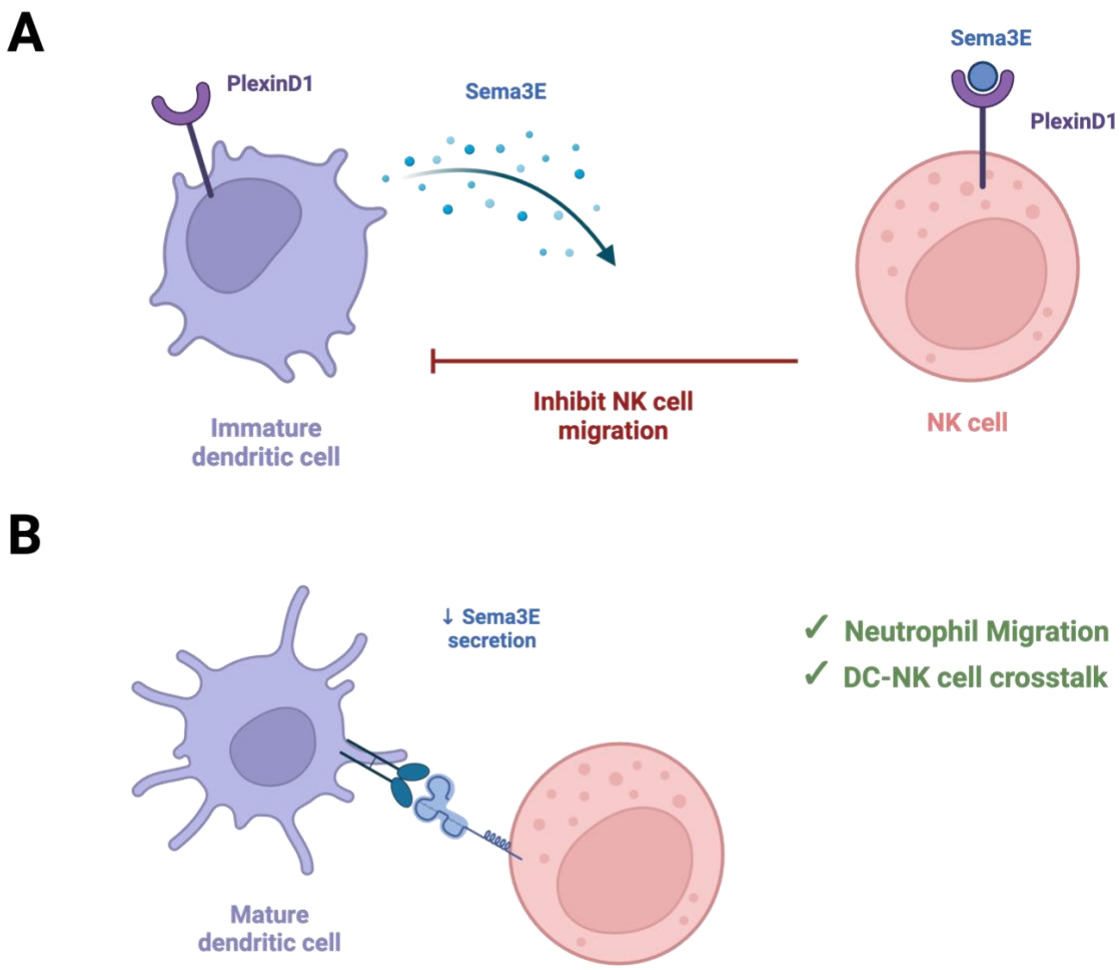

Figure 15. Sema3E as a chemotactic regulator of NK cell migration and DC-NK interactions

(A) Immature DCs secrete Sema3E, providing repulsive signals that limit NK cell migration and prevent premature interactions. This mechanism ensures proper NK cell positioning and minimizes unnecessary crosstalk with immature DCs. **(B)** As DCs mature, Sema3E secretion decreases, enabling NK cells to approach and interact with mature DCs. This Sema3E/PlexinD1 signalling axis is critical for directing NK cell migration to specific tissues and coordinating their cytotoxic activity with the immune response.

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Information from: Alamri, A., Rahman, R., Zhang, M., Alamri, A., Gounni, A. S., & Kung, S. K. P. (2018). Semaphorin-3E produced by immature dendritic cells regulates activated natural killer cells migration. *Frontiers in Immunology, 9*, 1005.

Sema3E and PlexinD1 regulate airway inflammation and immune cell recruitment in allergic conditions such as asthma [387-392]. Sema3E controls the influx of immune cells into the lungs, including eosinophils, neutrophils, and T cells, thereby influencing the severity of allergic inflammation. Research has shown that asthma symptoms worsen when Sema3E/PlexinD1 signalling is not correctly regulated. These symptoms include airway hyperresponsiveness, excessive mucus production, and higher levels of pro-inflammatory cytokines [387-392]. By controlling the movement and activation of immune cells such as eosinophils and T cells in the airways, Sema3E and PlexinD1 help maintain immune balance in allergic conditions.

Furthermore, Sema3E/PlexinD1 axis is involved in cardiovascular health as it controls endothelial and smooth muscle cells. PlexinD1 regulates angiogenesis and vascular remodelling which is essential for vessel integrity [361][393]. Abnormalities in Sema3E/PlexinD1 signalling can lead to vessel growth and is involved in cardiovascular disorders, including atherosclerosis [394]. PlexinD1-mediated signalling regulates immune cell entry into vascular tissues and thus reduces chronic inflammation and prevents vessel damage.

In summary, Sema3E and PlexinD1 are involved in immunological responses in different disease conditions. Their ability to control immune cell movement, inflammation and tissue modification makes them important in maintaining immune balance and potential targets for therapeutic intervention in immune-dysregulated disorders.

Sema3E/PlexinD1 and Macrophages

Sema3E has been shown to control macrophage function in several conditions, such as systemic inflammation, atherosclerosis and allergic asthma. For example, our group demonstrated that Sema3E controls macrophage response to LPS-induced systemic inflammation [377]. In the absence of Sema3E in a global knockout model, macrophages produce higher levels of pro-inflammatory cytokines such as IL-6 and TNF, exacerbating systemic inflammation. This shows that Sema3E is a suppressor of excessive inflammatory signals, potentially through its interaction with PlexinD1.

Additionally, research by our group demonstrates that the absence of PlexinD1 in lung IMs worsens airway inflammation and hyperresponsiveness, highlighting the importance of PlexinD1 in allergic asthma [395]. *Plexind1* knockout mice have higher levels of Th2 and Th17 cytokines and increased leukocyte infiltration, which contributes to mucus formation and airway blockage. This shows the importance of PlexinD1 in maintaining a healthy immune system and macrophage-induced inflammation in respiratory conditions.

PlexinD1 is involved in macrophage polarization, particularly in promoting the pro-inflammatory M1 phenotype. In models of atherosclerosis, PlexinD1 is upregulated in macrophages exposed to 'disturbed flow'—a type of irregular blood flow pattern commonly found at arterial branch points and curvatures, where blood flow becomes turbulent or oscillatory rather than steady. This disturbed flow environment is associated with increased shear stress, which activates macrophages toward an M1 pro-inflammatory state, contributing to the development of atherosclerotic plaques [396]. The study’s findings indicate that PlexinD1

signalling promotes this inflammatory response under disturbed flow conditions, enhancing inflammation and accelerating plaque formation in chronic cardiovascular disease [396].

In obesity, Sema3E/PlexinD1 signalling facilitates the recruitment of macrophages into adipose tissue, contributing to a heightened inflammatory state [397, 398]. This pathway plays a critical role in worsening inflammation within adipose tissue, which is associated with insulin resistance and other metabolic dysfunctions commonly seen in obesity. Blocking Sema3E/PlexinD1 signalling can reduce the infiltration of pro-inflammatory macrophages, thereby alleviating inflammation and leading to an improved metabolic profile.

2. RATIONALE

Sema3E and its receptor PlexinD1 have been implicated in various immune responses, including allergic inflammation. Decreased expression of Sema3E in severe asthmatic patients and animal models of asthma suggests its importance in maintaining airway homeostasis. The recent finding that deletion of PlexinD1 in lung IMs exacerbates allergic responses indicates that PlexinD1 signalling protects against allergen-induced inflammation. IL-10 is a potent anti-inflammatory cytokine produced by various immune cells, including macrophages. It plays a critical role in dampening inflammatory responses and promoting the resolution of inflammation. In allergic asthma, IL-10 produced by lung IMs has been shown to inhibit allergen-loaded DC maturation and migration, subsequently reducing Th2 responses and allergic inflammation [399]. IMs are a subset of lung macrophages replenished from bone marrow-derived monocytes. Under conditions of inflammation or injury, circulating monocytes from the bone marrow can be recruited to the tissue, where they differentiate into macrophages (BMDMs) to replenish the interstitial macrophage population and support the immune response. Understanding the function and regulation of BMDMs is crucial because they serve as precursors to IMs, which are directly involved in the immune responses within the lung environment. Given the protective role of IL-10 in allergic asthma and the regulatory role of Sema3E/PlexinD1 in immune responses, we hypothesized that the deletion of PlexinD1 may disrupt IL-10 production by IMs (and, by extension, BMDMs). Investigating IL-10 levels in Sema3E/PlexinD1 KO models can provide insights into the mechanisms through which Sema3E/PlexinD1 signalling modulates inflammation.

Moreover, it is important to consider sex differences in the immune response, especially in macrophages, where males and females differ significantly in their innate immune responses.

Previous evidence has shown that immune cells, such as macrophages, display sex-specific differences in cytokine production and surface receptor expression. These differences can influence how males and females respond to inflammatory stimuli, with male macrophages generally exhibiting a more pro-inflammatory phenotype and females tending toward an anti-inflammatory response. Given the involvement of Sema3E/PlexinD1 in immune regulation, understanding the role of sex differences is essential for uncovering the mechanisms by which Sema3E/PlexinD1 signalling influences IL-10 production and macrophage polarization. This research aims to examine how Sema3E/PlexinD1 deletion affects IL-10 production and inflammatory responses in a sex-specific manner, contributing to a deeper understanding of the interplay between immune regulation and sex in macrophage-dependent inflammation.

3. HYPOTHESIS

BMDMs from Sema3E knockout mice exhibit sex-dependent alterations in TNF and IL-10 cytokine production in response to LPS stimulation, suggesting that Sema3E/PlexinD1 signalling differentially modulates cytokine responses in male and female macrophages. These variations may contribute to distinct inflammatory outcomes between the sexes, potentially influencing the balance between pro- and anti-inflammatory cytokine production.

4. AIMS

- Investigate whether Sema3E/PlexinD1 deletion on IMs affects IL-10 expression *in vivo*, using an HDM model of allergic asthma.

- Investigate whether Sema3E/PlexinD1 deletion on BMDMs affects IL-10 expression following TLR4 activation *in vitro*, with a focus on identifying potential sex differences in cytokine responses.

- Investigate the mechanism by which Sema3E/PlexinD1 signalling affects IL-10 expression in BMDMs following TLR4 activation *in vitro*, exploring how sex differences may influence this regulatory pathway and whether Sema3E plays a role in the modulation of these differences.

5. MATERIALS & METHODS

5.1 Animal Housing and Breeding

The B6.129P2 Sema3E global KO mice were obtained from an in-house breeding colony maintained by Central Animal Care Services at the University of Manitoba. Dr. F. Mann initially provided the parent breeders for these mice from Université de la Méditerranée, Marseille, France. WT littermate controls used in this study were B6.129P2 mice.

For additional genetic models, $CX3CR1^{ERT2}$ mice (B6.129P2(C)-$CX3CR1^{tm2.1(cre/ERT2)Jung/}$J) were sourced from Jackson Laboratory, while Floxed PlexinD1 mice (B6.129-$Plxnd1^{tm1.1Tmj/}$J), initially developed by Dr. T.M. Jessell (Columbia University/Howard Hughes Medical Institute, New York, NY), were kindly provided by Dr. Cheghua (Harvard Medical School, Cambridge, MA). $CX3CR1^{ERT2}$ mice were hemizygous, while PlexinD1 floxed mice ($PlexinD1^{fl/fl}$) were homozygous. To generate the desired genetic background, $CX3CR1^{ERT2}$ mice and $PlexinD1^{fl/fl}$ mice were crossed at the Genetic Models Center at the University of Manitoba, resulting in offspring that were homozygous for $PlexinD1^{fl/fl}$ and carried the $CX3CR1^{creERT2}$ allele.

All mice were housed in individualized ventilated cages with wooden chip bedding within an enriched environment at the University of Manitoba's animal care facility. Each cage housed a maximum of four animals. The animals were maintained on a 12-hour light/dark cycle and had access to standard chow and water ad libitum.

The mice were kept in a pathogen-free facility managed by Central Animal Care at the University of Manitoba. They were used following ethical guidelines set forth by the Canadian Council for Animal Care (Protocol Number 23-042).

5.2 HDM-induced airway inflammation model

5.2.1 PlexinD1 deletion from IMs (CX3CR1^{+} cells)

Tamoxifen (Tam) (Cat T5648; Sigma) was prepared by dissolving it in corn oil to a concentration of 8 mg/100 µL. *CX3CR1^{creERT2}-Plxnd1$^{fl/fl}$* mice received 100 µL of Tam via oral gavage. This treatment was administered every other day for one week to achieve selective deletion of PlexinD1 in CX3CR1-expressing cells, generating *CX3CR1^{creERT2}-Plxnd1* KO mice. Untreated *CX3CR1^{creERT2}-Plxnd1$^{fl/fl}$* mice served as WT controls. This model utilizes the Cre-LoxP system, where the *CX3CR1creERT2* allele contains a Tam-inducible Cre recombinase, specifically expressed under the CX3CR1 promoter [400]. In the presence of Tam, Cre recombinase is activated, allowing for the excision of loxP-flanked (floxed) sequences, explicitly targeting the *Plxnd1* gene in CX3CR1-expressing cells such as macrophages. This system enables the controlled, tissue-specific deletion of the *plxnd1* gene in response to Tam. The efficacy of PlexinD1 deletion from CX3CR1 cells was previously confirmed through flow cytometry and quantitative real-time PCR analysis of lung CX3CR1+ cells.

5.2.2 HDM-induced allergic asthma

PlexinD1 KO Mice

Following the deletion of PlexinD1 on CX3CR1-expressing cells, as described previously, mice were subjected to an HDM-induced allergic asthma protocol. Three days after tamoxifen treatment, mice were exposed to 60 µg of HDM extract administered intranasally. This exposure was followed by a 72-hour period during which the mice were allowed to recover. At the end of this period, the mice were euthanized, and lung tissues were collected for further analysis.

Sema3E KO Mice

For the Sema3E knockout experiments, Sema3E KO and WT mice were intranasally exposed to 25 µg of HDM extract in 35 µL of saline. This exposure regimen was administered over two weeks, with mice receiving HDM exposure five consecutive days weekly. Control groups were treated with saline only. After exposure, the mice were euthanized, and lung tissues were collected for further analysis.

5.2.3 Collection of Bronchoalveolar Lavage Fluid (BALF)

BALF was collected from *CX3CR1*^{creERT2}*-Plxnd1*$^{fl/fl}$ and *CX3CR1*^{creERT2}*-Plxnd1* KO mice to assess airway cytokine responses. Following euthanasia, 1 mL of sterile phosphate-buffered saline (PBS) containing 0.05M Ethylenediaminetetraacetic acid (EDTA) was instilled into the trachea, and the BALF was collected into Eppendorf tubes. This process was repeated for a second instillation, with the collected fluid separated into two Eppendorf tubes corresponding to each instillation. After centrifugation at 400 x g for 5 minutes, the supernatant from the first 1 mL instillation was stored at -80°C for later cytokine analysis. IL-10 protein levels in the BALF were subsequently measured using a Mesoscale Discovery assay, following the manufacturer's protocol.

5.2.4 Processing of cells

Lungs were collected from saline- and HDM-challenged *CX3CR1*^{creERT2}*-Plxnd1* KO, Sema3E KO, and WT mice. Each lung was minced and enzymatically digested in Roswell Park Memorial Institute (RPMI) 1640 medium containing 1 mg/mL collagenase IV (Worthington Biochemical, Lakewood, NJ) at 37°C for 1 hour. Following digestion, red blood cells (RBCs) were lysed using 5 mL of ammonium-chloride-potassium lysing buffer (ACK) buffer for 4

minutes. The cell suspension was then washed with flow buffer and counted using a hemocytometer with trypan blue staining for viability assessment, which under optimal conditions showed approximately 85% viability.

5.2.5 Surface and intracellular staining of cells with fluorochrome-conjugated antibodies

To prepare the cells for flow cytometry analysis, they were first blocked with Fc Blocker for 10 minutes to reduce nonspecific binding. After blocking, the cells were washed with flow buffer and stained with anti-mouse antibodies in 20 µL of flow buffer per tube. The antibody mixture included: Fixable Viability Dye-eFluor 780 (eBioscience), CD45-PB (clone 30-F11, Biolegend), CD11c-FITC (clone N418, Biolegend), CD11b-PE-Cy7 (clone M1/70, Biolegend), CD64-Percp-Cy5.5 (clone X54-5/7.1), MerTK-APC (clone 2B10C42, Biolegend), IL-10-PE (clone JES5-16E3, Biolegend). After a 30-minute incubation with the antibody mixture, cells were washed, fixed, and resuspended in 200 µL of flow buffer. The samples were then acquired using a BD FACSCanto-II flow cytometer.

Fixed and surface-stained lung cells were permeabilized for intracellular cytokine staining with 0.1% saponin in flow buffer (1 mL/tube) for 15 minutes. Flow buffer was prepared by combining 20 mL fetal bovine serum (FBS), 100 mL 10x PBS, and 880 mL distilled water to a total volume of 1 litre. After permeabilization, cells were washed with 0.1% saponin-flow buffer and stained with 0.5 µL of anti-mouse IL-10 antibody (eBioscience) per 20 µL of 0.1% saponin-flow buffer per tube. After a 30-minute incubation with the antibody mixture, cells were washed with saponin buffer first, followed by flow buffer. After removing the supernatant, cells were resuspended in 200 µL of flow buffer. The samples were then acquired using a BD FACSCanto-II flow cytometer.

Table 1. Flow Cytometry Antibodies for Lung Macrophage Staining to Assess Intracellular IL-10 Production

Target Antigen	Fluorophore	Clone Number	Company
Fixable Viability Dye	eflour 780		eBioscience
CD45	PB	30-F11	Biolegend
CD11c	FITC	N418	Biolegend
CD11b	PE-Cy7	M1/70	Biolegend
CD64	Percp-Cy5.5	X54-5/7.1	Biolegend
MerTK	APC	2B10C42	Biolegend
IL-10	PE	JES5-16E3	Biolegend

5.2.6 Flow cytometry analysis

Following standard instrumental protocols, cell analysis was conducted using a BD FACSCanto II flow cytometer (BD Biosciences, USA). Stained cells were acquired, with at least 50,000 events collected for each sample. To ensure accurate gating, Fluorescence Minus One (FMO) control were used for each fluorochrome-conjugated antibody specific to the biomarkers. Flow data were analyzed using FlowJo software.

5.3 Generation of Bone Marrow-Derived Macrophages

5.3.1 Collection and preparation of bone marrow cells

BMDMs were generated from PlexinD1 KO, Sema3E KO, and WT mice. The mice were euthanized, and bone marrow cells were collected from the tibias and femurs. RPMI 1640 medium was flushed through the marrow cavities to create a cell suspension. The resulting suspension was treated with ACK buffer for 5 minutes at room temperature to lyse RBCs. Following lysis, the suspension was centrifuged at 300 × g for 5 minutes, and the supernatant was discarded. The cell pellet was then resuspended in PBS. Subsequently, the cells were

suspended in 1 mL of BMDM medium, which consisted of RPMI 1640 medium supplemented with 10% FBS, 1% penicillin-streptomycin, 25 ng/mL macrophage colony-stimulating factor (M-CSF), and 50 mM β-mercaptoethanol (β-ME). This cell suspension was plated in 12-well plates at a density of 3.5×10^5 cells per well. The plates were incubated at 37°C in a 5% CO_2. On Day 3, the medium was refreshed with 1 mL of fresh BMDM medium per well to support continued cell growth. The BMDMs were allowed to grow for 6 days, reaching approximately 80% confluency by the end of the culture period [401].

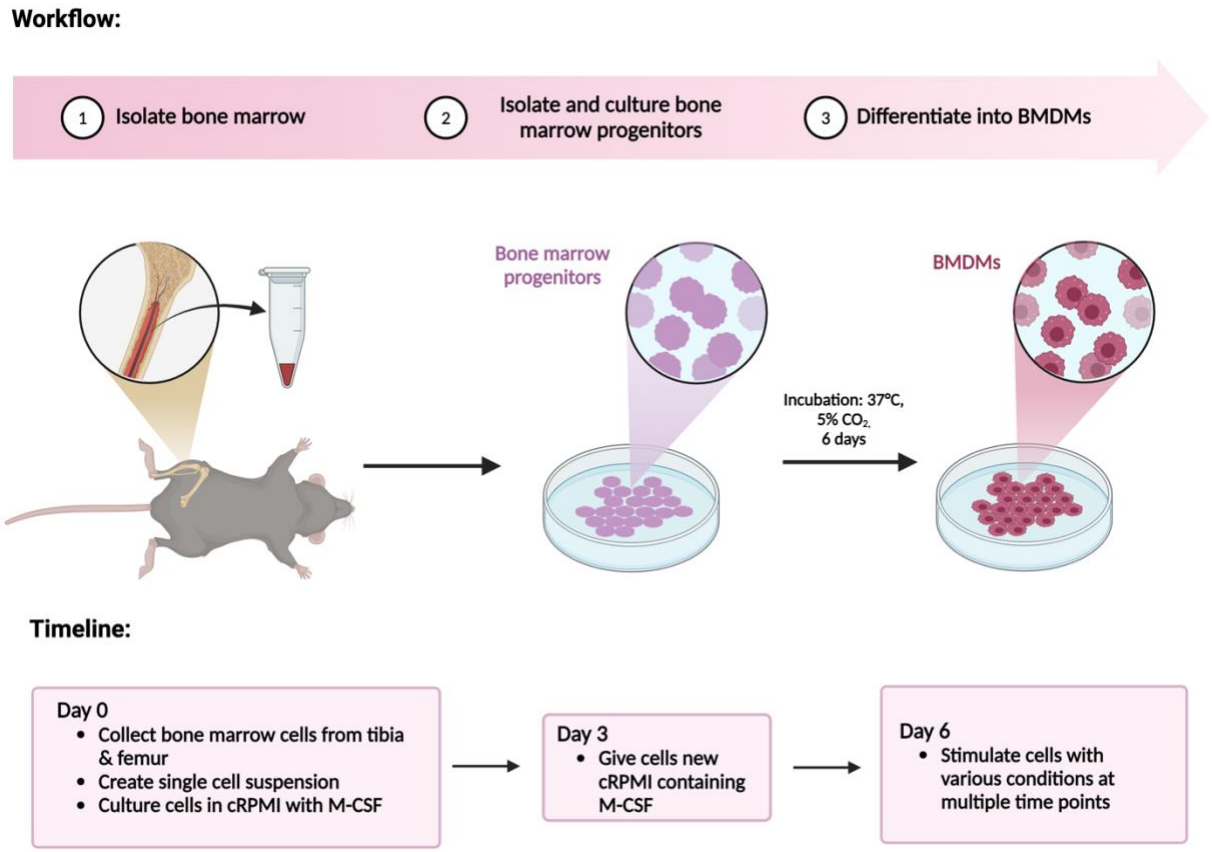


Figure 16. BMDM cell culture workflow

Figure created with *BioRender.com*

5.3.2 PlexinD1 deletion from BMDMs (CX3CR1^{+} cells)

On Day 6, to induce the knockout of PlexinD1 in the PlexinD1 KO BMDMs, cells were treated with Tam at a concentration of 5 µg/mL. The treatment was administered for 24 hours to remove PlexinD1 from the floxed cells effectively, using a similar Cre-LoxP system as previously described for the *in vivo* IM experiments. Following the Tam treatment, the BMDMs were serum-starved by incubating them in media containing 1% FBS for two hours. The cells were washed twice with sterile 1X PBS to remove residual serum and Tam. Fresh media was added to the wells, and the cells were subjected to stimulation under the previously described conditions at various times. After stimulation, cell culture supernatants were collected, centrifuged at 1200 rpm for 5 minutes at 4°C to remove cell debris, and aliquoted and frozen for future analysis.

5.4 Experimental Procedures and Analysis of BMDMs

5.4.1 LPS-induced inflammation of BMDMs

On Day 6, BMDMs adhered to the culture plates. To standardize cell conditions before stimulation, they were serum-deprived by incubating in media containing 1% FBS for two hours. This serum deprivation helps synchronize the cells, allowing them to be in similar metabolic and proliferative stages before stimulation. Following this step, the cells were washed twice with sterile 1X PBS to ensure residual serum was removed. Fresh cRPMI media was added to the wells to support cell health during the 24-hour stimulation period. This approach avoids additional stress from prolonged serum deprivation, allowing any effects observed to be attributed to the LPS treatments. Cells were exposed to 100 ng/mL LPS from *Escherichia coli* 0111: B4 (Sigma-Aldrich, Mississauga, ON, Canada), 100 ng/mL of Sema3E-Fc, or a combination of 100 ng/mL LPS and 100 ng/mL Sema3E-Fc. Sema3E-Fc was administered as a

pretreatment for the combined treatment for two hours before LPS was added. After the appropriate stimulation times, cell culture supernatants were collected, centrifuged at 1200 rpm for 5 minutes at 4°C to remove cell debris, and then aliquoted and frozen for future analysis.

5.4.2 Sandwich enzyme-linked immunosorbent assays (ELISA)

ELISA tests were conducted on the cell culture supernatant of BMDM cells to measure the protein concentration of IL-10, TNF, and IL-1β. The procedure followed the manufacturer's instructions and is briefly described below. ELISA plates (Immunol VWR, Mississauga, ON) were coated with a primary antibody (50 µL/well) and incubated at 4°C overnight. The following day, plates were blocked by adding 100 µL/well of diluent buffer to prevent non-specific binding. After 2 hours of blocking, plates were washed four times with washing buffer (0.05% Tween-20 in 1X PBS). Standards and samples were diluted in assay diluent according to their respective concentration range. Then, they were added to each well in a volume of 50 µL. The plates were incubated at room temperature with shaking (200 rpm) for 2 hours and then washed four times with washing buffer. After adding the detection antibody (50 µL/well), plates were incubated at room temperature with shaking for 1 hour. Plates were washed four times with washing buffer, and 50 µL of diluted Avidin-HRP solution was added to each well. After incubation at room temperature for 30 minutes with shaking, plates were washed four times with washing buffer. A mixture (A+B) of TMB Substrate Solution (50 µL/well) was added and kept in the dark for 15-20 minutes. Then, the reaction was stopped by adding a stop solution (50 µL of 1M H_2SO_4 /well). Finally, plates were read at 450 nm by Spectra Max 190 ELISA reader (Molecular Devices, CA, USA) after appropriate colour development. Data were analyzed using SoftMax Pro software (Molecular Devices). All cytokine ELISA kits were obtained from

BioLegend (San Diego, CA). The sample concentration was calculated by subtracting blank OD from sample OD.

5.4.3 Flow cytometry analysis

To prepare BMDMs for flow cytometry analysis, cells were first blocked with Fc Blocker for 10 minutes to reduce nonspecific binding. After blocking, they were washed with flow buffer and stained with the following antibodies: F4/80-FITC (clone BM8, Biolegend), CD11b-PE (clone CBRM1/5, eBioscience), TLR4-PE-Cy7 (clone HTA125, Biolegend), and CD14-APC (clone Sa14-2, Biolegend) in 20 µL of flow buffer per tube. After a 30-minute incubation, cells were washed, fixed, and resuspended in 200 µL of flow buffer. Samples were then acquired using a BD FACSCanto-II flow cytometer (BD Biosciences, USA), and flow data was analyzed using FlowJo software.

Table 2. Flow Cytometry Antibodies for BMDM Staining to Analyze TLR4 and CD14 Receptor Expression

Target Antigen	Fluorophore	Clone Number	Company
F4/80	FITC	BM8	Biolegend
CD11b	PE	CBRM1/5	eBioscience
CD14	APC	Sa14-2	Biolegend
TLR4	PE-Cy7	HTA125	Biolegend

5.5 Statistical Analysis

This study's results are expressed as mean ± SEM. The statistical analysis was designed to determine potential significant differences between experimental groups in cytokine protein levels, receptor expression, and other measured outcomes.

A two-way analysis of variance (ANOVA) was used to compare cytokine protein levels from BMDMs across different groups. After the ANOVA, Tukey's post hoc test was applied for multiple group comparisons. Each ELISA assay used in this analysis had a specific detection limit, and any values falling below this limit were considered undetectable. For cases where baseline protein production values consistently fell below the detection limit across all samples in a group, these groups were excluded from statistical analysis.

Baseline receptor expression of TLR4 and CD14 in BMDMs was analyzed using unpaired t-tests. This approach was chosen to compare two independent groups (e.g., WT vs. KO) to determine whether significant differences in receptor expression levels exist between different genotypes. Receptor internalization was further analyzed using a two-way ANOVA, followed by Tukey's post hoc test for multiple comparisons.

For the IL-1β recovery experiments, the Kruskal-Wallis Test was used. This nonparametric test is the equivalent of a one-way ANOVA. However, it does not assume normal distribution or homogeneity of variance, making it suitable for my data, which did not meet these assumptions due to a lack of variance in some groups. The Mann-Whitney Test was used to compare sex differences in IL-1β secretion at a single time point (24 hours) between two groups (e.g., WT male vs. WT female and 3E KO male vs. 3E KO female). This nonparametric test was chosen because WT BMDMs showed zero variance, necessitating a comparison method that does not rely on the assumptions of parametric tests.

Additionally, correlation matrices were used to explore the relationships between variables, such as cytokine levels and receptor expression. The correlation matrix provided a quantitative measure of the strength and direction of these relationships, helping to identify potential interactions between the variables being studied. The Pearson correlation coefficient (r)

was calculated for each pair of variables within the matrix to quantify the strength and direction of the linear relationships. The Pearson coefficient ranges from -1 to 1, where values close to 1 indicate a strong positive correlation, values close to -1 indicate a strong negative correlation, and values around 0 indicate no linear relationship.

All data were analyzed using GraphPad Prism 7.0 (GraphPad Software, Inc., La Jolla, CA). The alpha level for determining statistical significance was set at 0.05.

6. RESULTS

6.1 PlexinD1 deletion from CX3CR1+ expressing lung cells

In this study, we investigated the effects of PlexinD1 deletion on IMs using a well-established protocol previously validated in our lab [394]. Although direct validation of PlexinD1 knockout in IMs was not performed in this current study, prior work by a former lab member demonstrated the efficacy of Tam treatment in achieving significant PlexinD1 deletion in IMs. Specifically, *CX3CR1*^{creERT2}*-Plxnd1*$^{fl/fl}$ mice were treated with Tam every other day for one week, followed by the sorting and analysis of lung IMs two days post-treatment (Figure. 17A). This earlier study confirmed approximately 60% deletion of PlexinD1 in lung IMs of Tam-treated mice (Figure. 17B), as verified through real-time reverse transcription-polymerase chain reaction (RT-PCR). We confidently applied the same approach in our current work, presuming similar levels of PlexinD1 deletion in our IMs.

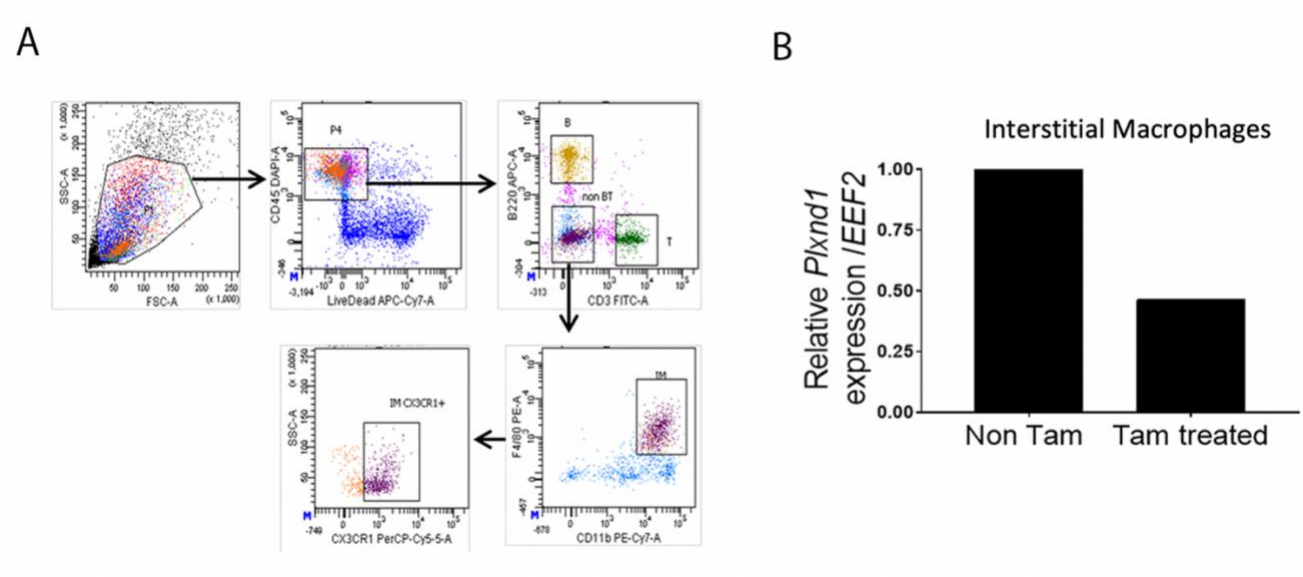


Figure 17. Deletion of PlexinD1 from CX3CR1+ Cells

CX3CR1^{creERT2}*-Plxnd1*$^{fl/fl}$ mice were either treated with Tam or left untreated to induce the deletion of PlexinD1 from CX3CR1+ cells. Data is representative of 3 mice per group.
(A) FACS analysis was conducted to sort IMs from the lungs of both Tam-treated and control mice.
(B) The deletion of PlexinD1 in these sorted IMs was confirmed using RT-PCR, demonstrating successful knockout in the Tam-treated samples.

Figure courtesy: Amena Aktar and Lianyu Shan

6.2 PlexinD1 deficient IMs have increased IL-10^{+} cells

Previous studies in our lab have demonstrated that the absence of Sema3E exacerbates asthma features in both acute and chronic asthma models [387-392]. Furthermore, we have shown that the lack of PlexinD1 in IMs leads to increased airway hyperresponsiveness, elevated airway leukocyte numbers, enhanced allergen-specific IgE production, goblet cell hyperplasia, and a heightened Th2/Th17 cytokine response in an HDM-induced allergic asthma model [395]. Given these findings, we hypothesized that PlexinD1 may also regulate IL-10 secretion, with decreased IL-10 levels potentially contributing to the exacerbated allergic asthma features observed in HDM murine models. To test this hypothesis, we examined IL-10 levels in PlexinD1-deficient IMs following a protocol previously described [402]. This protocol uses a single, large dose of HDM to trigger a robust, acute allergic airway response, focusing on early immune activation (Figure. 18A).

Contrary to our hypothesis, PlexinD1-deficient IMs exhibited increased intracellular IL-10 levels following HDM sensitization (Figure 18C). Interestingly, when comparing IMs to AMs, we found that IMs consistently produced more intracellular IL-10 than AMs after HDM exposure, regardless of whether they were WT or PlexinD1 KO. In contrast, AMs showed minimal IL-10 production (Figure 18D). Additionally, to further assess a longer-term effect of PlexinD1 deficiency, we transitioned to an acute two-week HDM model, in which PlexinD1 KO

mice exhibited higher IL-10 levels in BALF following HDM challenge compared to WT mice (Figure 18E).

These findings suggest a complex regulatory role for PlexinD1 in modulating IL-10 production, particularly in interstitial macrophages. The increased IL-10 production in IMs, both intracellularly and systemically, could indicate a compensatory anti-inflammatory response that counteracts the exacerbated inflammatory features observed in PlexinD1 KO models. This raises important questions about the balance between pro-inflammatory and anti-inflammatory signalling in these cells and whether IL-10 elevation in PlexinD1-deficient IMs serves to mitigate excessive tissue damage or contributes to immune dysregulation.

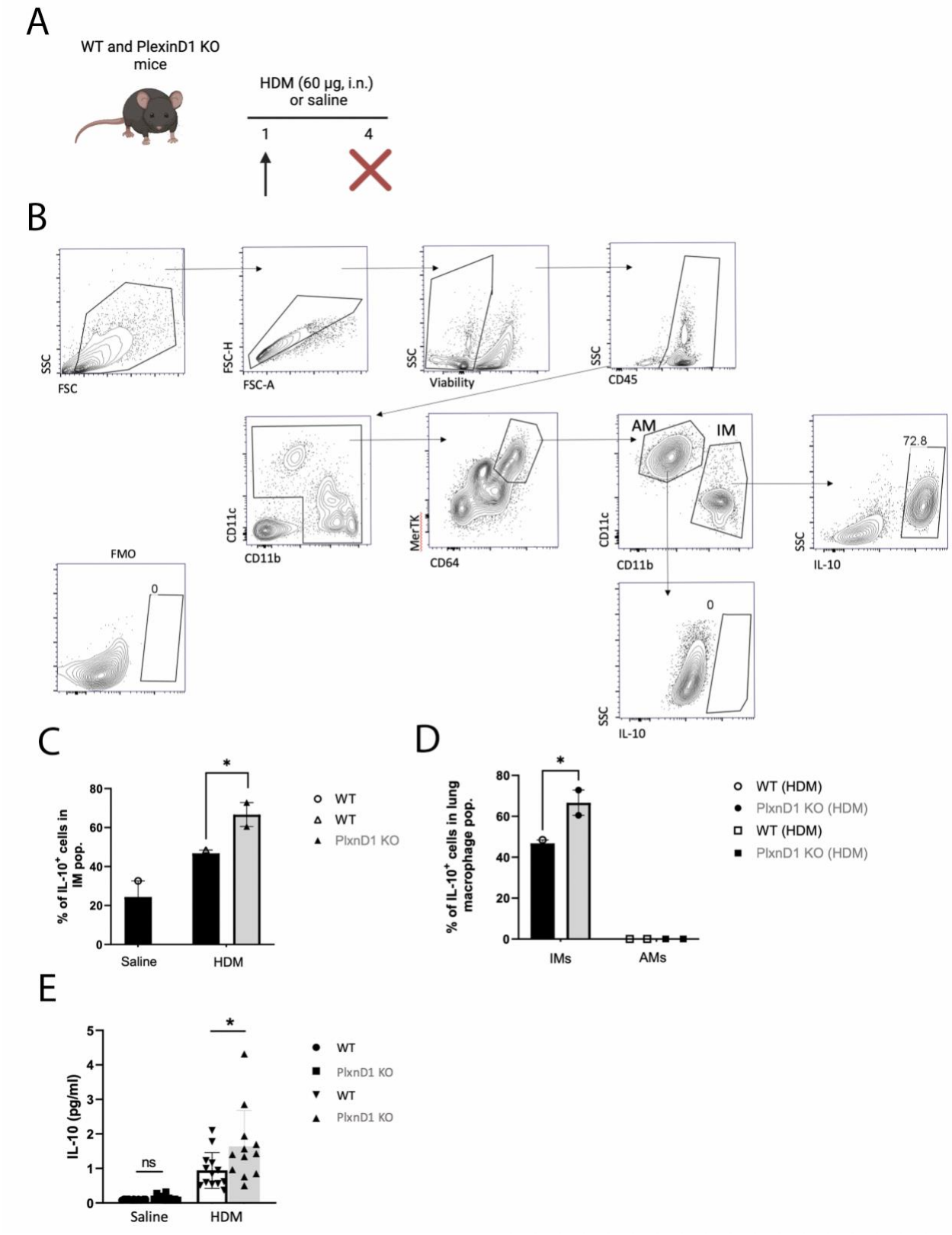


Figure 18. PlexinD1 KO mice IMs display increased IL-10^{+} cells

(A) HDM sensitization model: PlxnD1 KO and WT mice received 8 mg of Tam orally for three alternating days. After three days, mice were given 60 µg of HDM, and 72 hours later, FACS was performed to assess IL-10 levels in AMs and IMs from lung digests. Data is representative of 2-3 mice per group.
(B) FACS gating strategy used to identify pulmonary AMs and IMs in PlxnD1 KO and WT mice. Initially, debris was excluded by gating on the forward scatter (FSC) area versus the side scatter (SSC) area, followed by doublet exclusion using a plot of FSC height versus FSC area. Live cells were identified by gating on a plot of SSC versus viability dye. CD45+ cells were selected by plotting SSC against CD45, ensuring the inclusion of immune cells. Myeloid cells were further defined by gating on CD11c versus CD11b. Macrophages were identified within the total myeloid population by plotting MerTK versus CD64, isolating the MerTK+CD64+ macrophage population. Subsequently, AMs and IMs were distinguished within this macrophage gate by plotting CD11c versus CD11b. Finally, IL-10 expression levels were assessed in alveolar and interstitial macrophages by gating on a plot of IL-10 versus SSC in pre-gated CD11c+ CD11b- and CD11c- CD11b+ cells [403].
(C) Flow cytometry analysis of IL-10+ IMs in PlxnD1 KO and WT mice after HDM sensitization.Percentages of IL-10+ cells within the gated IM population were calculated. Data were analyzed using a one-way ANOVA with Tukey's post-hoc test for multiple comparisons (*$p<0.05$).
(D) Flow cytometry analysis of IL-10+ cells in AMs and IMs from PlxnD1 KO and WT mice after HDM sensitization.Percentages of IL-10+ cells were calculated for both the gated IM and AM populations. Data were analyzed using a one-way ANOVA with Tukey's post-hoc test for multiple comparisons (*$p<0.05$).
(E) IL-10 levels in BALF from PlxnD1 KO and WT mice following saline or HDM challenge. IL-10 levels in the BALF were measured using a Mesoscale assay. Data represent pooled results from multiple experiments with 12 mice per group. Statistical analysis was performed using a one-way ANOVA with Tukey's post-hoc test (*$p<0.05$).

Figure prepared in collaboration with Lianyu Shan

6.3 PlexinD1 deletion from CX3CR1+ expressing BMDMs

As we observed a significant increase in IL-10 production in lung IMs following PlexinD1 deletion, we aimed to investigate whether similar results could be observed in BMDMs. BMDMs are commonly used to study macrophage mechanisms *in vitro*, providing insights into *in vivo* phenomena and offering a valuable model for studying signalling pathways

[404]. Additionally, IMs are replenished by bone marrow-derived monocytes [252], further supporting the relevance of our BMDM experiments.

During my initial experiments, I observed distinct morphological changes in BMDMs treated with 20 µg/ml of Tam. While untreated BMDMs appeared numerous and elongated and adhered well to the culture plate, Tam-treated cells were fewer, lost their elongated form, and were often found floating, indicative of reduced viability [405] (Figure. 19A). These unexpected morphological changes raised concerns about the potential impact of Tam on the cells.

To further investigate, I treated WT BMDMs with 20 µg/ml of Tam 24 hours before LPS stimulation and assessed IL-10 production. ELISA analysis revealed that WT BMDMs treated with Tam produced significantly less IL-10 than untreated WT BMDMs, suggesting a negative impact of this Tam dose on IL-10 production (Figure. 19B). Given these findings, I explored lower Tam doses. ELISA results demonstrated no significant differences in IL-10 production between WT BMDMs treated with 5 µg/ml Tam and untreated controls, indicating that this dose does not adversely affect IL-10 production (Figure. 19C).

Next, I confirmed the effective knockout of PlexinD1 using RT-PCR, which showed that the five µg/ml dose successfully knocked out most PlexinD1 receptors in BMDMs (Figure. 19D).

To assess the impact of Tam on cell viability, I performed flow cytometry using Annexin V and DAPI staining. The gating strategy revealed that most of the cell culture was healthy when treated with five µg/ml of Tam: 81.2% of the cells were alive (Annexin V-, DAPI-), 1.88% were dead (Annexin V-, DAPI+), 8.70% were in early apoptosis (Annexin V+, DAPI-), and 8.18% were in late apoptosis or necrosis (Annexin V+, DAPI+). These results indicate high viability and low cell death, with a moderate level of apoptosis (Figure. 19E).

Based on these findings, I used the five µg/ml dose of Tam for future experiments. This dose successfully showed no PlexinD1 protein while maintaining cell viability and the typical appearance of WT BMDMs. Importantly, no morphological changes were observed, and the cells remained adhered to the plate, providing a secure experimental condition.

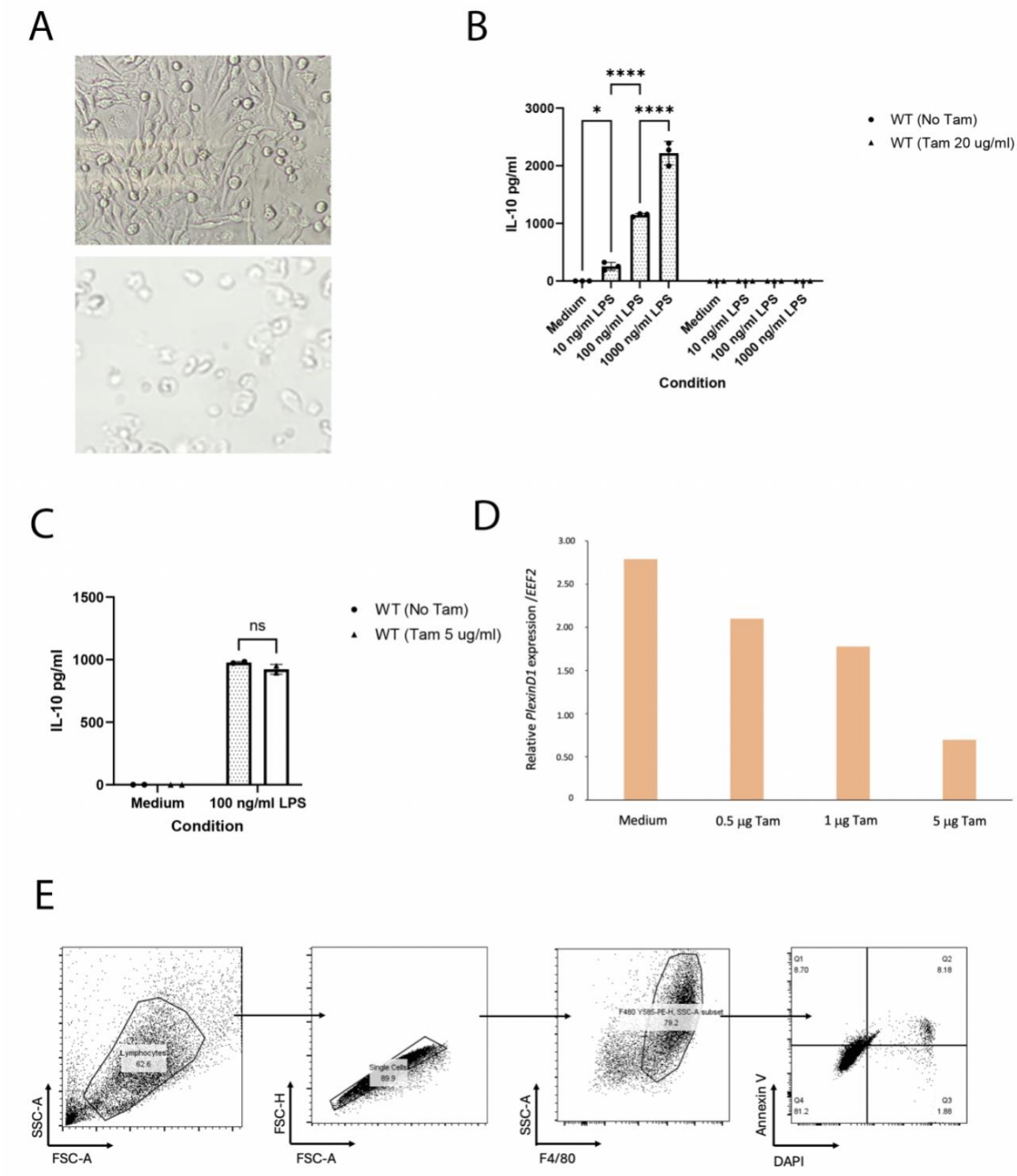


Figure 19. Validation of PlexinD1 deletion in CX3CR1+ BMDMs using *in vitro* models

(A) Morphological changes observed in WT BMDMs naïve (top) or treated with 20 µg/ml of Tam (bottom). Tam-treated cells appeared fewer, lost their elongated form, and were often found floating, indicative of reduced viability.
(B) Treatment with 20 µg/ml of Tam decreased IL-10 production in WT BMDMs. ELISA analysis revealed significantly lower IL-10 levels in treated cells than untreated controls. Statistical significance was determined using two-way ANOVA followed by Tukey's post hoc test (*$p<0.05$, ****$p<0.0001$).
(C) Treatment with five µg/ml Tam did not affect BMDMs, as IL-10 levels remained consistent between treated and untreated WT groups, and cell morphology was unchanged. Statistical analysis was conducted using two-way ANOVA with uncorrected Fisher's LSD.
(D) Tam-induced deletion of PlexinD1 was validated in *CX3CR1^{creERT2}-Plxnd1* KO BMDMs using RT-PCR.
(E) Gating strategy in *CX3CR1^{creERT2}-Plxnd1* KO BMDMs following five µg/ml Tam treatment. Flow cytometry analysis using Annexin V binding and PI uptake, with the gating strategy outlined to differentiate live, apoptotic, and necrotic cell populations. The quadrants are defined as follows: Q1 (upper left) represents early apoptotic cells (Annexin V-positive, DAPI-negative), Q2 (upper right) indicates late apoptotic or necrotic cells (Annexin V-positive, DAPI-positive), Q3 (bottom right) corresponds to necrotic cells (Annexin V-negative, DAPI-positive), and Q4 (bottom left) represents viable cells (Annexin V-negative, DAPI-negative).

6.4 PlexinD1 deficiency increases IL-10 and TNF levels in BMDMs following LPS stimulations

Next, I utilized BMDMs to investigate the regulatory role of PlexinD1 on IL-10 expression in macrophages. The TLR4 agonist LPS is a well-established inducer of IL-10 in macrophages [406]. By exposing BMDMs to 100 ng/ml LPS at various time points, I aimed to elucidate the involvement of PlexinD1 in this process. PlexinD1 KO BMDMs exhibited significantly increased IL-10 levels following LPS stimulation, suggesting a regulatory role for PlexinD1 in IL-10 expression (Figure. 20A).

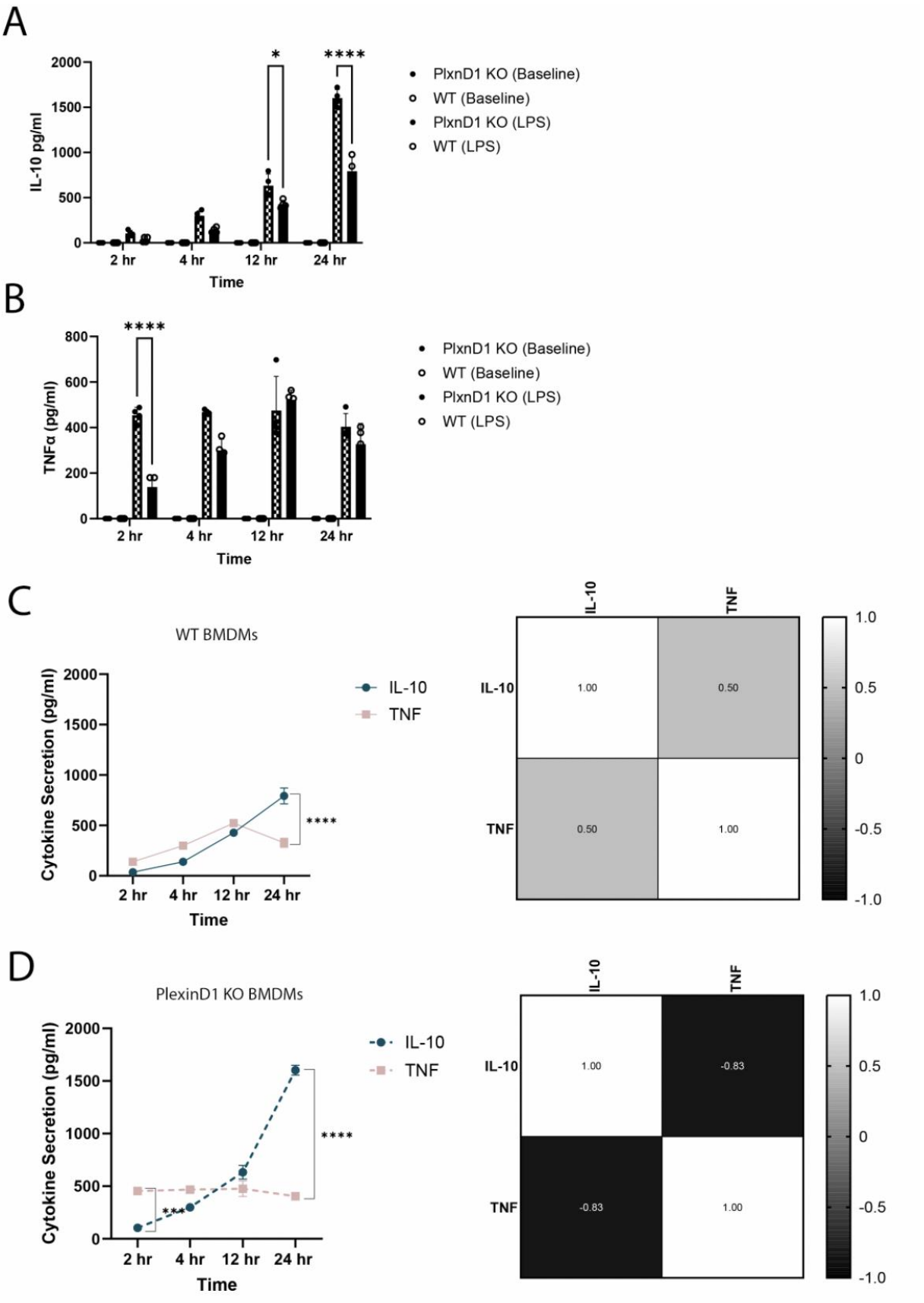
Interestingly, PlexinD1 KO BMDMs showed significantly increased IL-10 levels following LPS stimulation compared to WT BMDMs, while no such increase was observed in the medium alone. This suggests that the absence of PlexinD1 in BMDMs enhances IL-10

production, specifically in response to inflammatory stimuli like LPS. In contrast, IMs exhibited elevated IL-10 levels regardless of HDM exposure. *These findings suggest that PlexinD1 deficiency may differentially impact IL-10 regulation across various macrophage populations.*

Macrophages are primary responders to LPS *in vivo* and significant producers of TNF following LPS exposure. TNF has been shown to promote IL-10 production in BMDMs following high LPS stimulation. Subsequently, IL-10 acts as a negative feedback mechanism, reducing TNF levels [407]. We hypothesized that the differences in IL-10 levels between PlexinD1 KO and WT mice following LPS exposure were due to variations in macrophage TNF production. Investigating TNF is critical because it plays a crucial role in cytokine crosstalk, influencing the production of IL-10 [408][409]. After an initial inflammatory response, TNF can stimulate pathways leading to IL-10 production, which helps regulate the immune response and prevent excessive damage. Elevated TNF levels may signal the need for counter-regulatory mechanisms, resulting in increased IL-10 production to balance the immune response. By studying TNF, we can gain insights into the mechanisms that regulate the shift from pro-inflammatory to anti-inflammatory states during immune responses.

At 2 hours post-stimulation, BMDMs from PlexinD1 KO mice displayed significantly higher TNF levels than WT mice (Figure. 20B). I then examined the relationship between TNF and IL-10 in the TLR4 response. In WT BMDMs, a time-dependent relationship between IL-10 and TNF was observed. Both cytokines showed a concurrent increase at early time points (2 hr., 4 hr., 12 hr.). However, by 24 hours, while IL-10 continued to rise, TNF levels sharply declined, resulting in significantly higher IL-10 levels than TNF (Figure. 20C). The associated correlation matrix for these observations was 0.50, indicating a moderate positive correlation between IL-10 and TNF during the earlier time points but a divergence at later stages.

In contrast, PlexinD1 KO BMDMs displayed a distinct pattern. At 2 hours, TNF levels were significantly higher than IL-10, coinciding with a significant decrease in TNF as IL-10 rose (Figure. 20D). By 24 hours, a similar pattern to WT BMDMs was observed, where IL-10 levels increased while TNF levels decreased. However, this pattern was more pronounced in PlexinD1 KO BMDMs, with the correlation matrix showing a strong negative correlation of -0.83. *These findings suggest that IL-10 may play a role in downregulating TNF at later time points, a mechanism that appears to be exacerbated in the absence of PlexinD1*.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IL-10 pg/ml
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Time
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-0.83
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0
-0.5
-1.0

Figure 20. Loss of PlexinD1 enhances IL-10 and TNF production in BMDMs stimulated with LPS

BMDMs were generated from the bone marrow of *CX3CR1^{creERT2}-Plxnd1$^{fl/fl}$* mice. BMDMs from *CX3CR1^{creERT2}-Plxnd1$^{fl/fl}$* and *CX3CR1^{creERT2}-Plxnd1* KO were given tamoxifen (5 µg/ml) for 24 hours to knock out PlexinD1 and were subsequently stimulated with LPS (100 ng/ml) at various time points. Protein expression of IL-10 (A) and TNF (B) was measured using ELISA. Data are representative of four mice per group. *BMDMs were derived from both male and female mice; however, data are presented without separation of sexes.*
(A) Two-way ANOVA with Tukey's post hoc test was performed to determine the significance between the groups at various time points (*p<0.05, ****p<0.0001). PlexinD1 (Baseline) and WT (Baseline) served as baseline controls with 0 values across each sample; therefore, these groups were excluded from the statistical analysis.
(B) Two-way ANOVA with Tukey's post hoc test was also performed, with significance levels ****p<0.0001. PlxnD1 KO (Baseline) and WT (Baseline) served as baseline controls with 0 values across each sample; therefore, these groups were excluded from the statistical analysis.
(C) Time course of TNF and IL-10 secretion following LPS stimulation of WT BMDMs. Protein secretion was measured by ELISA and is presented as a mean ± SEM of 4 biological replicates. Two-way ANOVA with Tukey's post hoc test revealed significance levels ****p<0.0001. The correlation matrix associated with this time course is r=0.50, *p<0.05, indicating a moderate positive correlation between IL-10 and TNF at earlier time points with divergence at later stages.
(D) Time course of TNF and IL-10 secretion following LPS stimulation of PlexinD1 KO BMDMs. Protein secretion was measured by ELISA and is presented as a mean ± SEM of 4 biological replicates. Two-way ANOVA with Tukey's post hoc test revealed significance levels ***p<0.001, ****p<0.0001. The correlation matrix associated with this time course is r=-0.83, *p<0.05, suggesting a robust negative correlation between IL-10 and TNF, particularly at later times when IL-10 appears to downregulate TNF more significantly in the absence of PlexinD1.

6.5 Sema3E deficiency increases percentage of IL-10^{+} cells in IM population

Following the observation of increased IL-10 levels in PlexinD1-deficient IMs, I hypothesized that Sema3E, a known ligand for PlexinD1, might play a role in this regulatory mechanism. To explore the potential contribution of the Sema3E/PlexinD1 axis in IL-10 regulation, we examined IL-10-producing cells in Sema3E KO IMs following exposure to a two-week HDM model (Figure. 21A). This HDM model was selected as it mimics an acute allergic sensitization and airway inflammation response, allowing us to capture the early stages of immune activation. Consistent with the findings in PlexinD1-deficient IMs, Sema3E KO IMs also exhibited elevated percentage of IL-10^{+} cells. However, interestingly, this was also seen in the saline group (Figure. 21C). This increase in IL-10 suggests that Sema3E, like PlexinD1, may be involved in modulating immune responses, potentially through a coordinated regulation of IL-10 production. However, these results also indicate that the Sema3E may play a broader role in maintaining immune homeostasis and controlling inflammation, particularly in allergic asthma, as Sema3E KO IMs have elevated IL-10 regardless of HDM exposure.

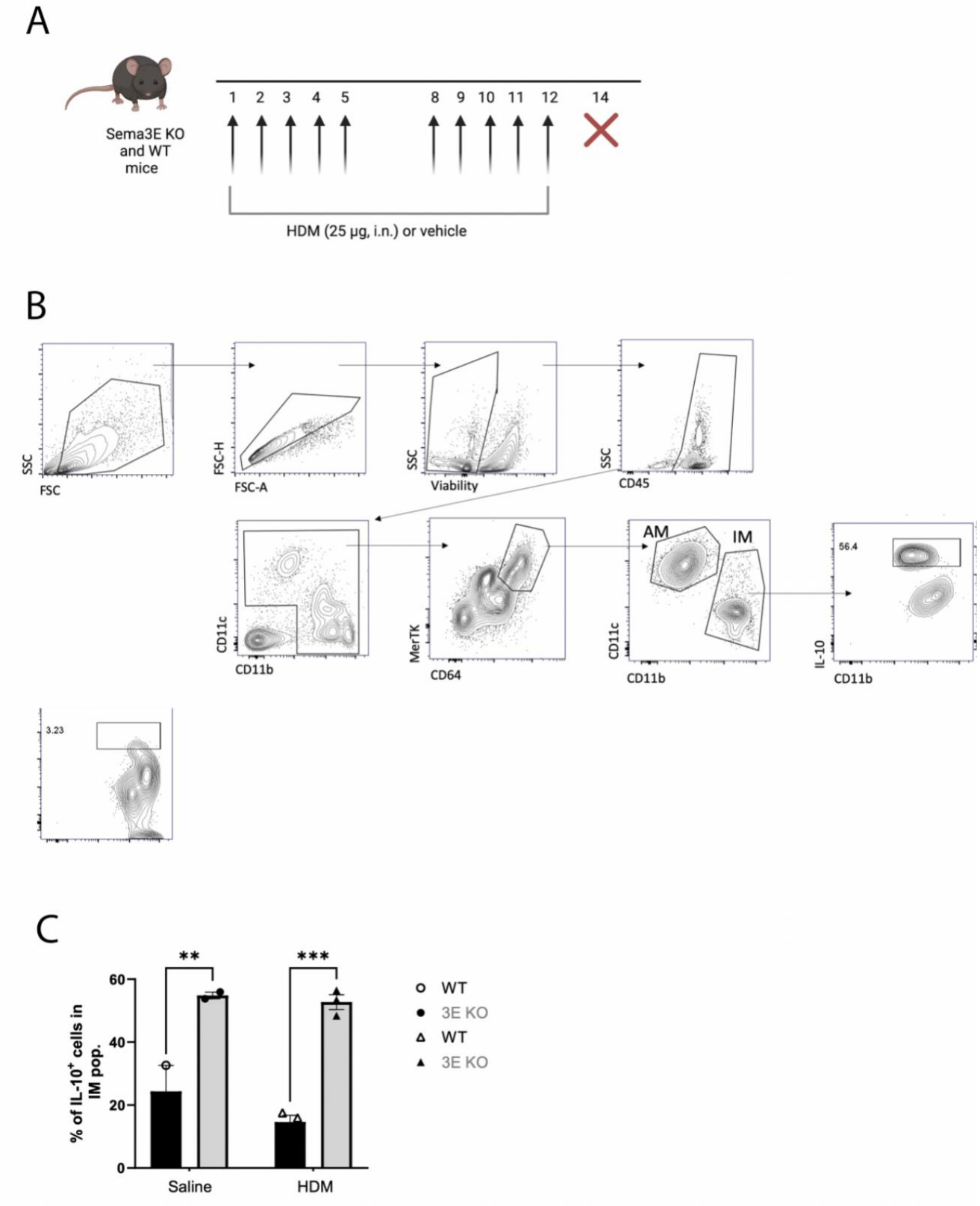


Figure 21. IMs from Sema3E KO mice exhibit elevated IL-10^{+} cells

(A) Acute HDM model: Sema3E and WT mice were intranasally exposed to 25 µg of HDM extract in 35 µl saline over two weeks, with five consecutive days of exposure per week. Control groups received saline only. Mice were sacrificed two days after the final challenge, and IL-10 levels in CD11b+ IMs were determined via FACS analysis of non-auto fluorescent CD11c- CD11b+ cells isolated from digested lung tissue. Data represents two to three mice per group. *IMs were isolated from male and female mice; sex differences were not analyzed in this study.* **(B)** FACS gating strategy used to identify pulmonary IMs in Sema3E KO and WT mice. **(C)** Flow cytometry analysis of IL-10+ IMs in Sema3E KO and WT mice after 2-week HDM model. Percentages of IL-10+ cells within the gated IM population were calculated. Data was analyzed using a one-way ANOVA with Tukey's post-hoc test for multiple comparisons (**p<0.01, ***p<0.001).

Figure prepared in collaboration with Lianyu Shan

6.6 Sema3E deficiency modulates IL-10 and TNF secretion in BMDMs, with enhanced differences between males and females

To further investigate the role of Sema3E in IL-10 and TNF regulation, I repeated the experiments conducted with PlexinD1 KO BMDMs, this time using Sema3E KO BMDMs. I stimulated my BMDMs with 100 ng/ml of LPS at various time points—2 hours, 4 hours, 12 hours, and 24 hours. Following each simulation, the cell supernatant was collected for cytokine analysis, and the cells were collected to assess other relevant markers in later experiments. After analyzing IL-10 (Figure. 22A) and TNF (Figure. 22B) protein levels in Sema3E KO and WT BMDMs using ELISA, I found no significant differences between the two groups. This finding suggests that Sema3E might not regulate IL-10 and TNF, implying that another semaphorin could interact with PlexinD1 in this context. However, during the analysis, I noticed a distinct clustering of responses within the WT and Sema3E KO groups. I wondered if these clusters corresponded to the sex of the mice, with half of the BMDMs derived from males and the other half from females. Given the existing literature on sex differences in immune cells, including in WT BMDMs, following the LPS challenge [297][299] [304], I was interested by the possibility

that these differences might be masking essential data when the results from both sexes are combined. This observation prompted me to consider analyzing male and female BMDMs separately to uncover potential sex-specific regulatory mechanisms.

I observed distinct sex-specific differences when comparing IL-10 and TNF levels between WT and 3E KO BMDMs. At 12 and 24 hours, 3E KO males exhibited significantly higher IL-10 levels than WT males (Figure. 22C), whereas 3E KO females showed lower IL-10 levels than WT females. However, this was insignificant (Figure. 22D). Additionally, TNF levels were significantly lower in 3E KO males than WT males at 12 and 24 hours (Figure. 22E), while 3E KO females had higher TNF levels than their WT counterparts (Figure. 22F). These findings suggest that Sema3E deficiency may have contrasting effects on inflammation depending on sex, potentially being less inflammatory in males but more inflammatory in females.

When analyzing the relationship between IL-10 and TNF according to sex in WT BMDMs, I found that WT males and WT females had significantly higher TNF levels than IL-10 at 2 hours post-stimulation. However, the patterns diverged at later time points. In WT males, TNF levels continued to increase, remaining significantly higher than IL-10 at 24 hours (Figure. 22G). In contrast, WT females did not show a steady increase in TNF; by 24 hours, IL-10 levels appeared to be higher than TNF, although this difference was not statistically significant (Figure. 22H). These findings suggest potential sex-specific differences in regulating pro-inflammatory and anti-inflammatory cytokines in WT BMDMs, with TNF playing a more dominant role in males. At the same time, females might exhibit a more balanced anti-inflammatory response over time.

Next, I analyzed the relationship between IL-10 and TNF according to sex in 3E KO BMDMs. In 3E KO males, IL-10 levels started higher. They continued to increase steadily over

time, while TNF levels decreased, resulting in IL-10 being significantly higher than TNF at later time points (Figure. 22I). Conversely, in 3E KO females, IL-10 levels increased only moderately and remained lower overall, while TNF levels increased instead of decreasing, with TNF consistently remaining significantly higher than IL-10 across all time points (Figure. 22J). These observations suggest a possible sex-specific regulatory mechanism where Sema3E deficiency may lead to a more anti-inflammatory profile in males but a more pro-inflammatory response in females, as indicated by the inverse relationship between IL-10 and TNF in males compared to females.

To further explore the relationship between IL-10 and TNF in Sema3E-deficient macrophages, I performed a correlation matrix analysis on the data, separated by sex. The study revealed striking differences in the correlation between these cytokines across the groups. In 3E KO females, there was a perfect positive correlation between IL-10 and TNF ($r=1.00$, $p<0.001$), indicating that as IL-10 levels increased, TNF levels rose consistently across all time points (Figure. 22J). This strong correlation suggests that in the absence of Sema3E, female macrophages exhibit a tightly linked, potentially pathological pro-inflammatory response. The synchronized increase in both IL-10 and TNF may indicate a dysregulated immune response that could contribute to the heightened inflammatory profile observed in these cells.

Conversely, in 3E KO males, the correlation between IL-10 and TNF was weak ($r=0.12$) and not statistically significant, suggesting that these cytokines are not closely related in this group (Figure. 22I). This finding aligns with the observed pattern where IL-10 levels steadily increased. In contrast, TNF levels decreased over time, indicating that 3E KO males may mount a more anti-inflammatory response, with IL-10 acting independently of TNF.

In WT males and females, the correlations between IL-10 and TNF were moderate (r=0.65, r=0.74, respectively), but these relationships were not statistically significant (Figure. 22G-H). While there is some correlation between these cytokines in WT BMDMs, it is much less pronounced than in 3E KO females. This suggests that in the absence of Sema3E deficiency, IL-10 and TNF levels are somewhat coordinated but do not exhibit the extreme correlation seen in 3E KO females.

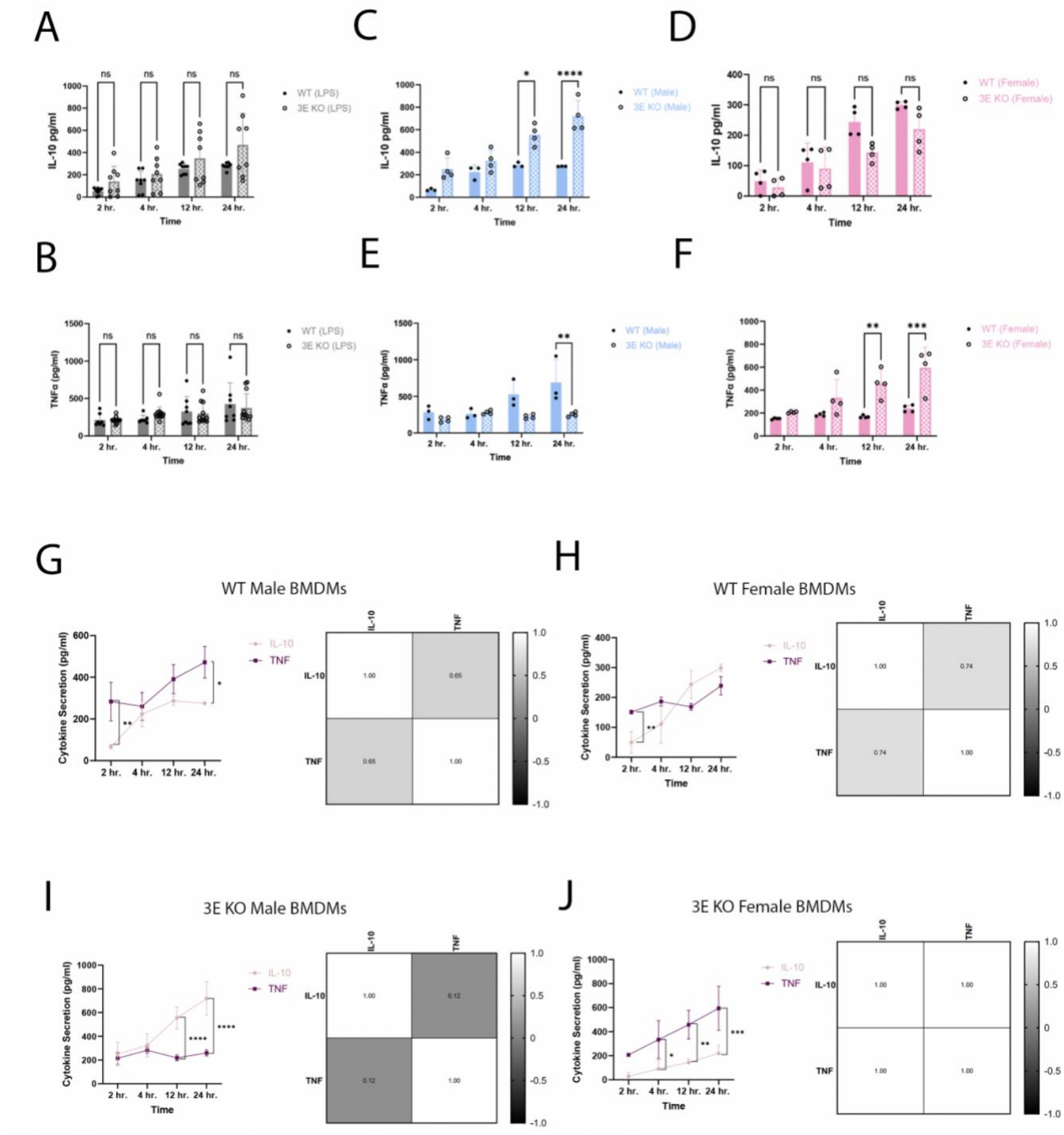


Figure 22. Sex-specific modulation of cytokine secretion in Sema3E deficient BMDMs stimulated by LPS

BMDMs were generated from the bone marrow of Sema3E KO and WT mice and stimulated with LPS (100 ng/ml) at various times.
(A-B) IL-10 **(A)** and TNF **(B)** protein expression were measured using ELISA. Data are representative of four mice per group. Two-way ANOVA with Tukey's post hoc test was performed to determine the significance between the groups at various time points ($p>0.05$ for both A and B).
(C-F) Time course of TNF and IL-10 secretion following LPS stimulation in 3E KO and WT BMDMs, separated by sex. **(C)** IL-10 levels in 3E KO males vs. WT males (*$p<0.05$, ****$p<0.0001$); **(D)** IL-10 levels in 3E KO females vs. WT females ($p>0.05$); **(E)** TNF levels in 3E KO males vs. WT males (**$p<0.01$); **(F)** TNF levels in 3E KO females vs. WT females (**$p<0.01$, ***$p<0.001$).
(G-J) Analysis of the relationship between TNF and IL-10 secretion in WT and 3E KO BMDMs, separated by sex. **(G)** TNF and IL-10 levels in WT males (*$p<0.05$, **$p<0.01$, correlation matrix $r=0.65$, $p>0.05$); **(H)** TNF and IL-10 levels in WT females (**$p<0.01$, correlation matrix $r=0.74$, $p>0.05$); **(I)** TNF and IL-10 levels in 3E KO males (****$p<0.0001$, correlation matrix $r=0.12$, $p>0.05$); **(J)** TNF and IL-10 levels in 3E KO females (*$p<0.05$, **$p<0.01$, ***$p<0.001$, correlation matrix $r=1.00$, *****$p<0.0001$).

6.7 Increases in IL-10 and TNF in Sema3E deficiency are not strain-specific

Genetic background is crucial when investigating immune responses, as different mouse strains can exhibit varying immune profiles. C57BL/6 and Balb/c mice are commonly used in immunological research due to their distinct immune response characteristics. C57BL/6 mice typically exhibit a Th1-biased response, whereas Balb/c mice have a Th2-biased response [410, 411]. To ensure that the observed phenomena were not strain-specific, I tested Sema3E KO BMDMs from both strains.

I focused on comparing male Balb/c mice with male C57BL/6 mice. The experiments performed on BMDMs from male Balb/c mice yielded results like those observed in earlier studies with male C57BL/6 mice, with Sema3E deficiency significantly increasing IL-10 (Figure. 23A) and decreasing TNF (Figure. 23B) cytokine levels compared to WT counterparts. However, this phenomenon had a greater fold increase in IL-10 levels in Balb/c mice than C57BL/6 mice, likely due to their inherent Th2 bias, which supports enhanced production of

anti-inflammatory cytokines like IL-10. The increase in TNF levels observed in Sema3E KO Balb/c BMDMs is interesting, as TNF is typically associated with Th1 responses. One possibility is that Sema3E deficiency disrupts the balance of cytokine regulation in macrophages, leading to dysregulated TNF production independent of the broader Th1/Th2 bias.

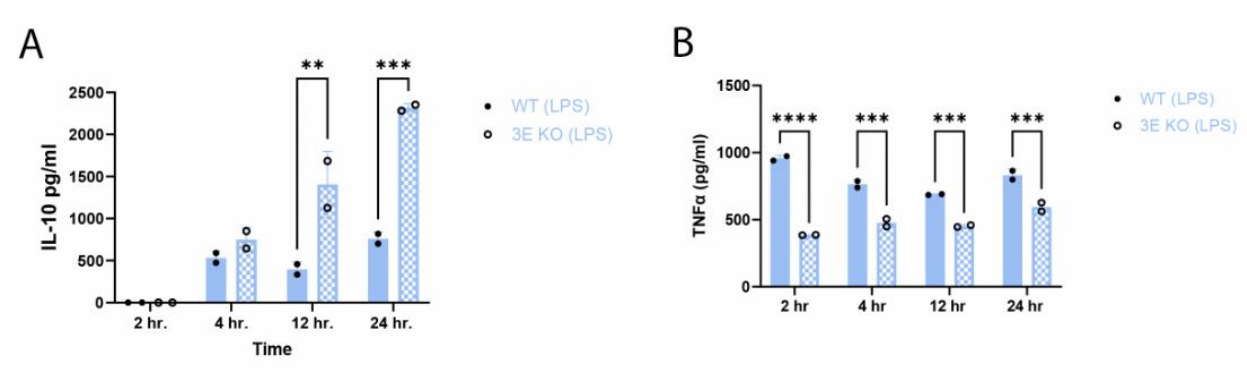


Figure 23. Increases in IL-10 and TNF in Sema3E deficiency are not strain-specific in males

BMDMs from WT and Sema3E KO male Balb/c mice were treated with LPS (100ng/ml) at various times. Protein expression of IL-10 **(A)** and TNF **(B)** was measured using ELISA. Data is representative of two mice per group. Two-way ANOVA with Tukey's post hoc test was performed to determine the significance between the groups at various time points ***p<0.01, ***p<0.001, ****p<0.0001.*

6.8 Sema3E deficiency modulates TLR4 and CD14 expression, with enhanced differences between males and females

To better understand the molecular mechanisms underlying the heightened inflammatory response observed in female Sema3E KO BMDMs and the decreased inflammatory response in male Sema3E KO BMDMs, I investigated TLR4 surface expression. TLR4 is a critical receptor in the LPS-induced signalling pathway, and its regulation is essential for modulating downstream cytokine production [412]. Increased surface expression of TLR4 enhances the ability of

macrophages to detect and respond to LPS, potentially amplifying signalling cascades such as NF-κB, which regulates the production of critical cytokines like TNF and IL-10. By examining TLR4 expression and internalization, I aimed to determine whether these processes contribute to the distinct inflammatory phenotypes observed in Sema3E KO BMDMs and to elucidate their role in shaping cytokine production dynamics.

Similar to the analysis of cytokine levels, no significant differences were observed in baseline TLR4 expression between 3E KO and WT BMDMs (Figure. 24B). However, distinct clustering emerged when separating the data by sex. WT males exhibited higher TLR4 expression than 3E KO males (Figure. 24C). In comparison, WT females showed lower TLR4 expression than 3E KO females (Figure. 24D). These findings suggest that the regulation of TLR4 may differ based on sex in the context of Sema3E deficiency, potentially contributing to the observed sex-specific inflammatory responses. The differences in TLR4 expression indicate that Sema3E plays a role in fine-tuning the TLR4-mediated signalling pathways, influencing how male and female macrophages respond to inflammatory stimuli.

To further investigate the mechanisms behind the sex-specific inflammatory responses observed in Sema3E KO BMDMs, I also analyzed the expression of CD14. This co-receptor works with TLR4 to recognize LPS and initiate downstream signalling [104, 105]. CD14 is crucial for the efficient activation of the TLR4 pathway, and its expression levels can influence the sensitivity of macrophages to LPS, thereby modulating the magnitude of the immune response.

I found that WT males exhibited decreased CD14 expression compared to 3E KO males (Figure. 24F), while WT females had increased CD14 expression compared to 3E KO females (Figure. 24G). Notably, there were no significant differences in CD14 expression when all

Sema3E KO and WT BMDMs were grouped (Figure. 24E); the differences became apparent only when the sexes were analyzed separately. These findings suggest that CD14, like TLR4, may be differentially regulated in a sex-specific manner in the context of Sema3E deficiency. The differences in TLR4 and CD14 expression between males and females in both WT and Sema3E KO BMDMs indicate that Sema3E plays a role in fine-tuning the TLR4-CD14 axis. This fine-tuning may be critical for maintaining balanced immune responses, with Sema3E deficiency leading to divergent effects in male and female macrophages, ultimately influencing their inflammatory profiles.

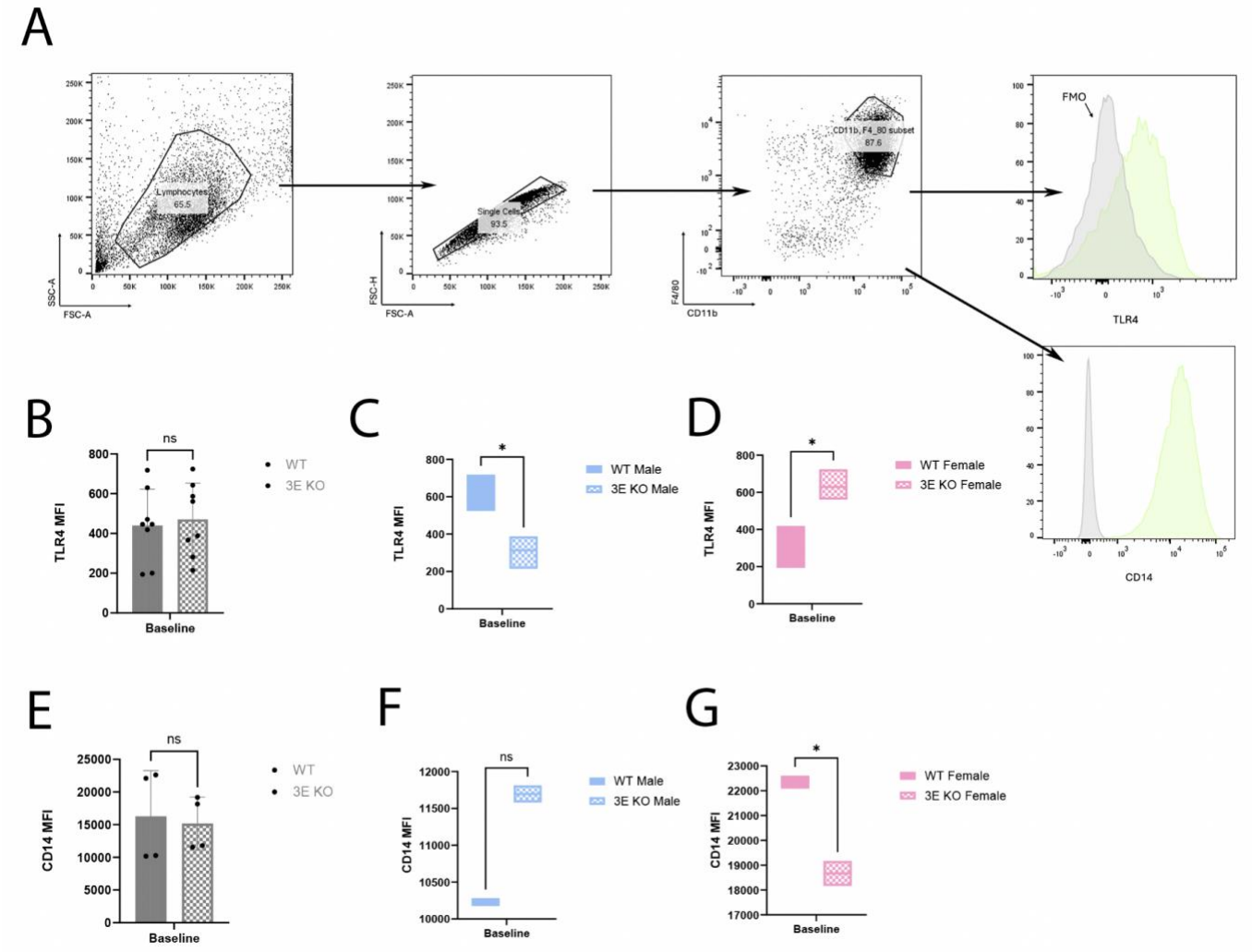


Figure 24. Sex-specific modulation of TLR4 and CD14 expression in Sema3E deficient BMDMs

(A) Flow cytometry gating strategy for analyzing TLR4 and CD14 expression in BMDMs. Initial gating was performed to exclude debris and doublets using SSC-A vs. FSC-A and FSC-H vs. FSC-A plots. Macrophages were then identified based on F4/80 and CD11b expression. TLR4 and CD14 expression levels were subsequently analyzed within the gated macrophage population.
(B-D) Analysis of TLR4 surface expression in Sema3E KO and WT BMDMs. **(B)** Baseline TLR4 expression shows no significant differences when all BMDMs are grouped ($p>0.05$). **(C)** TLR4 expression is higher in WT males than 3E KO males ($*p<0.05$). **(D)** TLR4 expression is lower in WT females than 3E KO females ($*p<0.05$). Data is representative of four mice per group. Net MFI was calculated by subtracting the MFI of the FMO sample's MFI from the experimental sample to correct for any background fluorescence or non-specific binding.

(E-G) Analysis of CD14 expression in Sema3E KO and WT BMDMs. **(E)** Baseline CD14 expression shows no significant differences when all BMDMs are grouped ($p>0.05$). **(F)** CD14 expression is decreased in WT males compared to 3E KO males ($p>0.05$). **(G)** CD14 expression is increased in WT females compared to 3E KO females ($*p<0.05$). Data is representative of two mice per group. Unpaired t-tests were performed to determine the significance between the groups.

6.9 WT BMDMs exhibit sex-specific variations in cytokine secretion, TLR4, and CD14 dynamics

The literature has consistently demonstrated sex differences in cytokine secretion and immune responses across various immune cell types. These differences are often attributed to hormonal influences and genetic factors that modulate immune cell function. Studies have shown that male innate immune cells, including macrophages, exhibit more robust pro-inflammatory responses than female immune cells. For example, studies have demonstrated that LPS elicits a more robust pro-inflammatory response in male BMDMs than in female BMDMs, characterized by higher levels of cytokine secretion [297][299][304]. Given these findings, I investigated whether similar sex differences existed in my data. To do this, I separated my groups by sex, aiming to uncover any sex-specific patterns in cytokine production and TLR4 dynamics.

At 4 hours post-LPS stimulation, WT male BMDMs displayed higher IL-10 levels than WT female BMDMs (Figure. 25A). However, this difference was only observed at this single time point, indicating that the sex-specific differences in IL-10 production may be transient or context-dependent. Interestingly, by 24 hours, WT male BMDMs exhibited increased TNF levels compared to WT female BMDMs (Figure. 25B), suggesting that the male BMDMs may sustain a more prolonged pro-inflammatory response.

Further analysis of CD14 revealed that WT males had significantly lower baseline CD14 expression than WT females (Figure. 25C). In contrast, baseline TLR4 expression was higher in

WT males compared to WT females (Figure. 25D). This difference in receptor expression may contribute to the observed variations in cytokine production. When examining CD14 internalization, WT female BMDMs showed significant internalization from 15 minutes to 1 hour post-LPS stimulation (Figure. 25E). At 2 hours, CD14 surface expression in females increased slightly. However, this change was not statistically significant. In contrast, CD14 expression in WT male BMDMs remained constant, with no observable decrease in surface expression over time. However, this could be due to continuous turnover of CD14 at the surface, maintaining a steady MFI rather than indicating a lack of internalization.

Regarding TLR4 internalization, WT males and WT females exhibited significant internalization following LPS exposure (Figure. 25F). However, in males, TLR4 was 100% internalized within two hours. In contrast, TLR4 was 100% internalized in females within 15 minutes. These findings suggest that WT males may have a higher inflammatory response than WT females, as they not only have higher baseline TLR4 expression but are also slower to internalize TLR4. This delay could allow more TLR4 to remain on the surface, increasing the likelihood of detecting LPS and mounting a more significant inflammatory response.

To further explore the relationship between CD14 and TLR4 expression at baseline, I conducted a correlation matrix analysis using MFI values from WT male and female BMDMs. At baseline, females exhibited significantly higher CD14 expression (22088 and 22607 MFI) compared to males (11573 and 12185 MFI), while males showed significantly higher TLR4 expression (718 and 630 MFI) compared to females (194.6 and 200.6 MFI). To analyze this relationship, I averaged the MFI values of side-by-side replicates for each group and calculated the Pearson correlation coefficient. The analysis revealed a perfect negative correlation of -1.00 between CD14 and TLR4 (Figure 25G).

This strong inverse relationship suggests that as CD14 expression increases, TLR4 expression decreases (female BMDMs), and as CD14 decreases, TLR4 increases (male BMDMs). Running the correlation matrix was helped quantify the interaction between these two receptors, as their coordinated expression may play a role in modulating the inflammatory response in a sex-specific manner. The inverse correlation highlights a potential mechanism where the balance between CD14 and TLR4 expression could fine-tune the sensitivity of macrophages to LPS, contributing to the distinct inflammatory profiles observed between male and female BMDMs.

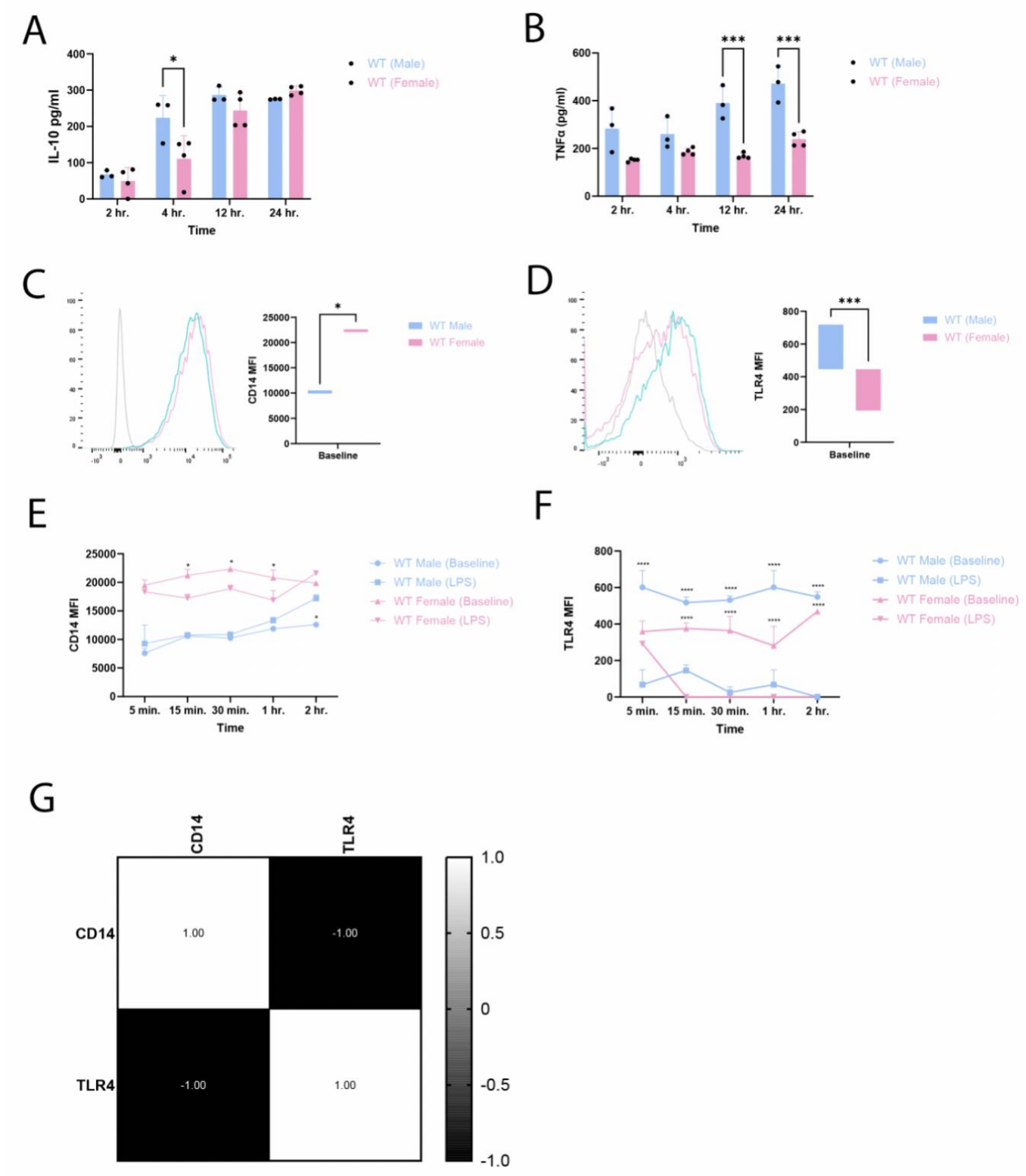


Figure 25. Analysis of sex-specific differences in cytokine production, CD14 expression, and TLR4 dynamics in WT BMDMs following LPS stimulation

(A-B) Analysis of cytokine levels in WT BMDMs following LPS stimulation. **(A)** IL-10 levels at 4 hours post-LPS stimulation show higher levels in WT males than WT females (two-way ANOVA with Tukey's post hoc, *$p<0.05$). **(B)** TNF levels at 24 hours post-LPS stimulation, with WT males exhibiting increased levels compared to WT females (two-way ANOVA with Tukey's post hoc, ***$p<0.001$). Data is representative of 3-4 mice per group.
(C-D) Analysis of baseline receptor expression in WT BMDMs. **(C)** Baseline CD14 expression indicates lower levels in WT males than WT females (paired t-test, *$p<0.05$). **(D)** Baseline TLR4 expression shows higher levels in WT males than WT females (paired t-test, ***$p<0.001$). Data represents two mice per group for CD14 and 4 for TLR4.
(E-F) Internalization dynamics of CD14 and TLR4 in WT BMDMs following LPS stimulation. **(E)** CD14 internalization over time in WT females, with significant internalization observed between 15 minutes and 1-hour post-LPS stimulation, followed by a slight increase in surface expression at 2 hours (two-way ANOVA with Tukey's post hoc, ***$p<0.001$). **(F)** TLR4 internalization in WT BMDMs, with males showing complete internalization by 2 hours and females by 15 minutes (two-way ANOVA with Tukey's post hoc, ****$p<0.0001$). Data is representative of 4 mice per group.
(G) Correlation matrix analysis between baseline CD14 and TLR4 expression revealed a perfect negative correlation of -1.00, suggesting an inverse relationship between these two markers ($r=-1.00$, **$p<0.01$).

6.10 The absence of Sema3E disrupts CD14 and TLR4 dynamics in BMDMs, leading to sex-specific changes in inflammatory responses

To further elucidate the sex-specific differences in immune response observed in Sema3E-deficient macrophages, I compared 3E KO males to 3E KO females. This comparison aimed to uncover any differential regulation of cytokine production, receptor expression, and internalization dynamics between the sexes without Sema3E. Since Sema3E plays a crucial role in modulating immune responses, understanding how its deficiency impacts male and female macrophages differently could provide insights into sex-specific mechanisms underlying inflammatory responses.

This comparison revealed significant differences between 3E KO males and 3E KO females. Across all time points (2 hours, 4 hours, 12 hours, 24 hours), 3E KO males exhibited significantly higher IL-10 levels in culture supernatants than 3E KO females (Figure. 26A).

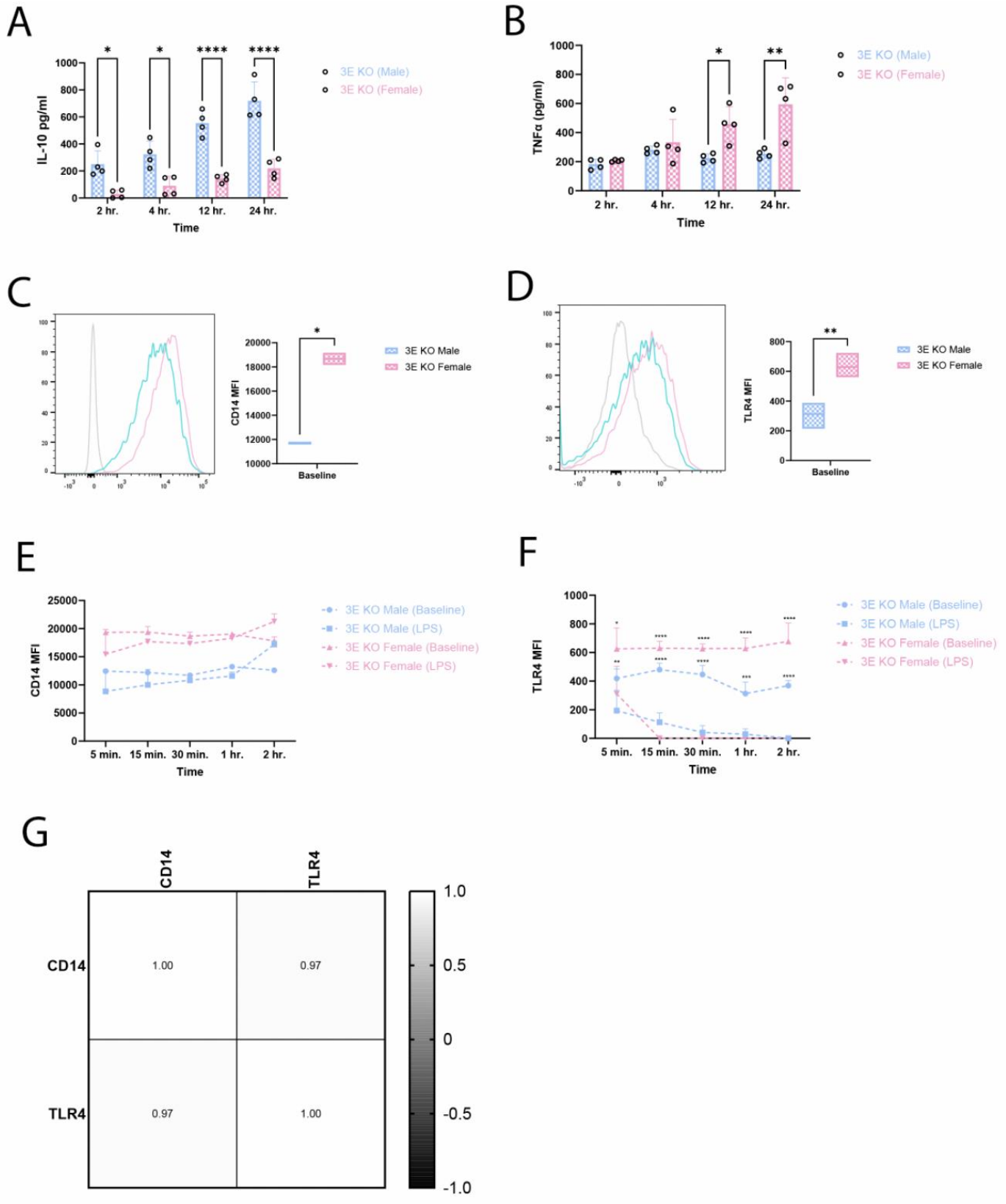
Conversely, 3E KO females showed increased TNF in culture supernatants at 12 and 24 hours post-LPS stimulation (Figure. 26B), suggesting a more pronounced pro-inflammatory response in females.

Baseline receptor expression also varied between the sexes. 3E KO males had significantly lower CD14 expression than 3E KO females (Figure. 26C). In contrast, 3E KO females exhibited significantly higher TLR4 expression at baseline (Figure. 26D). Interestingly, neither 3E KO males nor 3E KO females showed significant CD14 internalization following LPS stimulation (Figure. 26E). However, both groups demonstrated substantial TLR4 internalization, with a notable difference in the timing: 3E KO females achieved 100% TLR4 internalization within 15 minutes, whereas 3E KO males took up to 2 hours to reach complete internalization (Figure. 26F).

As with the WT analysis, I first averaged side-by-side replicates for each sample and used the resulting values for a Pearson correlation calculation in Prism. The analysis revealed a strong positive correlation 0.97 between CD14 and TLR4 expression at baseline (Figure 26G). This positive correlation sharply contrasts the inverse correlation (-1.00) observed in WT BMDMs, suggesting that the absence of Sema3E shifts the relationship between CD14 and TLR4 expression. Specifically, in female 3E KO BMDMs, as CD14 expression increases, TLR4 expression also increases, which may reflect a disruption of the regulatory balance seen in WT macrophages.

This shift in receptor dynamics could have significant implications for the inflammatory response, particularly in the context of sex differences. The positive correlation in 3E KO macrophages aligns with the hypothesis that Sema3E deficiency disrupts the coordinated

regulation of CD14 and TLR4, contributing to altered sensitivity to LPS and distinct cytokine profiles between male and female BMDMs.


A
IL-10 pg/ml
1000
800
600
400
200
0
*
*
****
****
2 hr.
4 hr.
12 hr.
24 hr.
Time
3E KO (Male)
3E KO (Female)
B
TNFα (pg/ml)
1000
800
600
400
200
0
*
**
2 hr.
4 hr.
12 hr.
24 hr.
Time
3E KO (Male)
3E KO (Female)
C
CD14 MFI
20000
18000
16000
14000
12000
10000
*
Baseline
3E KO Male
3E KO Female
D
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800
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Baseline
3E KO Male
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CD14 MFI
25000
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0
5 min.
15 min.
30 min.
1 hr.
2 hr.
Time
3E KO Male (Baseline)
3E KO Male (LPS)
3E KO Female (Baseline)
3E KO Female (LPS)
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TLR4 MFI
1000
800
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200
0
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30 min.
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2 hr.
Time
3E KO Male (Baseline)
3E KO Male (LPS)
3E KO Female (Baseline)
3E KO Female (LPS)
G
CD14
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CD14
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1.00
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1.00
1.0
0.5
0
-0.5
-1.0

Figure 26. Disruption of CD14 and TLR4 dynamics and sex-specific changes in inflammatory responses in Sema3E-deficient BMDMs

(A-B) Cytokine levels in 3E KO BMDMs following LPS stimulation. **(A)** IL-10 levels at multiple time points (2 hours, 4 hours, 12 hours, 24 hours) showed significantly higher levels in 3E KO males compared to 3E KO females (two-way ANOVA with Tukey's post hoc, *$p<0.05$, ****$p<0.0001$). **(B)** TNF levels at 12 and 24 hours post-LPS stimulation, with 3E KO females exhibiting increased levels compared to 3E KO males (two-way ANOVA with Tukey's post hoc, *$p<0.05$, **$p<0.01$).
(C-D) Baseline receptor expression in 3E KO BMDMs. **(C)** Baseline CD14 expression is significantly lower in 3E KO males compared to 3E KO females (paired t-test, *$p<0.05$). **(D)** Baseline TLR4 expression is significantly higher in 3E KO females compared to 3E KO males (unpaired t-test, **$p<0.01$).
(E-F) Receptor internalization dynamics in 3E KO BMDMs following LPS stimulation. **(E)** CD14 internalization shows no significant changes in 3E KO males or females (two-way ANOVA with Tukey's post hoc, $p>0.05$). **(F)** TLR4 internalization dynamics reveal that 3E KO females achieve 100% internalization within 15 minutes, whereas 3E KO males take up to 2 hours to internalize fully (two-way ANOVA with Tukey's post hoc, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$).
(G) Correlation matrix analysis between CD14 and TLR4 internalization in 3E KO BMDMs demonstrated a strong positive correlation ($r=0.97$, **$p<0.01$), in contrast to the inverse correlation (-1.00) observed in WT BMDMs, suggesting altered dynamics in the absence of Sema3E.

6.11 Restoration of Sema3E in 3E KO BMDMs Reverses Cytokine Alterations

To further investigate the role of Sema3E in modulating cytokine production, I conducted ligand recovery experiments by adding Sema3E-Fc back into 3E KO BMDMs before LPS stimulation. The goal was to determine whether the observed cytokine alterations in the absence of Sema3E could be reversed with the reintroduction of the ligand. This approach helps to confirm whether Sema3E directly influences cytokine dynamics in macrophages and whether its absence is responsible for the sex-specific differences in cytokine production observed in 3E KO BMDMs.

When analyzing the combined data from both sexes, no significant differences were observed in IL-10 levels in cell culture supernatants following ligand recovery with Sema3E (Figure. 27A). To ensure the Fc region of Sema3E-Fc was not responsible for altering cytokine production, I included an IgG-Fc control. Stimulating cells with IgG-Fc alone did not significantly impact IL-10 levels in WT or 3E KO male BMDMs compared to LPS alone, confirming that the effects observed were due to the Sema3E fragment (Figure. 27B).

When the data was split by sex, distinct patterns emerged. As previously observed, 3E KO males exhibited significantly higher IL-10 supernatant levels than WT males. These elevated IL-10 levels significantly decreased following Sema3E pre-treatment (Figure. 27C). In 3E KO female supernatants, IL-10 levels were lower than WT females. However, this difference was not statistically significant (Figure. 27D). Sema3E pre-treatment increased IL-10 levels in 3E KO females, with a significant rise observed at 12 hours.

Regarding TNF, no significant differences were noted in the combined analysis of both sexes after ligand recovery (Figure. 27E). Again, an IgG-Fc control was used to confirm that the Fc region of the Sema3E-Fc construct was not responsible for altering TNF levels. Stimulating cells with IgG-Fc alone had no significant effect on TNF levels in WT or 3E KO male BMDMs compared to LPS alone (Figure. 27F).

When separating the sexes, 3E KO males displayed significantly lower TNF levels in culture supernatants than WT males at 24 hours, with a similar trend at 12 hours (Figure. 27G). Sema3E pre-treatment resulted in a significant increase in TNF levels in 3E KO male supernatants. Conversely, 3E KO female supernatants exhibited significantly higher TNF levels compared to WT females at 12 and 24 hours, with Sema3E pre-treatment leading to a significant decrease in TNF at 24 hours (Figure. 27H).

These results suggest that Sema3E plays a critical role in regulating cytokine production in a sex-specific manner. The reversal of cytokine levels following Sema3E reintroduction indicates that the absence of Sema3E is likely responsible for the altered cytokine profiles observed in 3E KO BMDMs. This highlights the potential of Sema3E as a modulator of immune responses, particularly in balancing pro-inflammatory and anti-inflammatory cytokines in male and female macrophages.

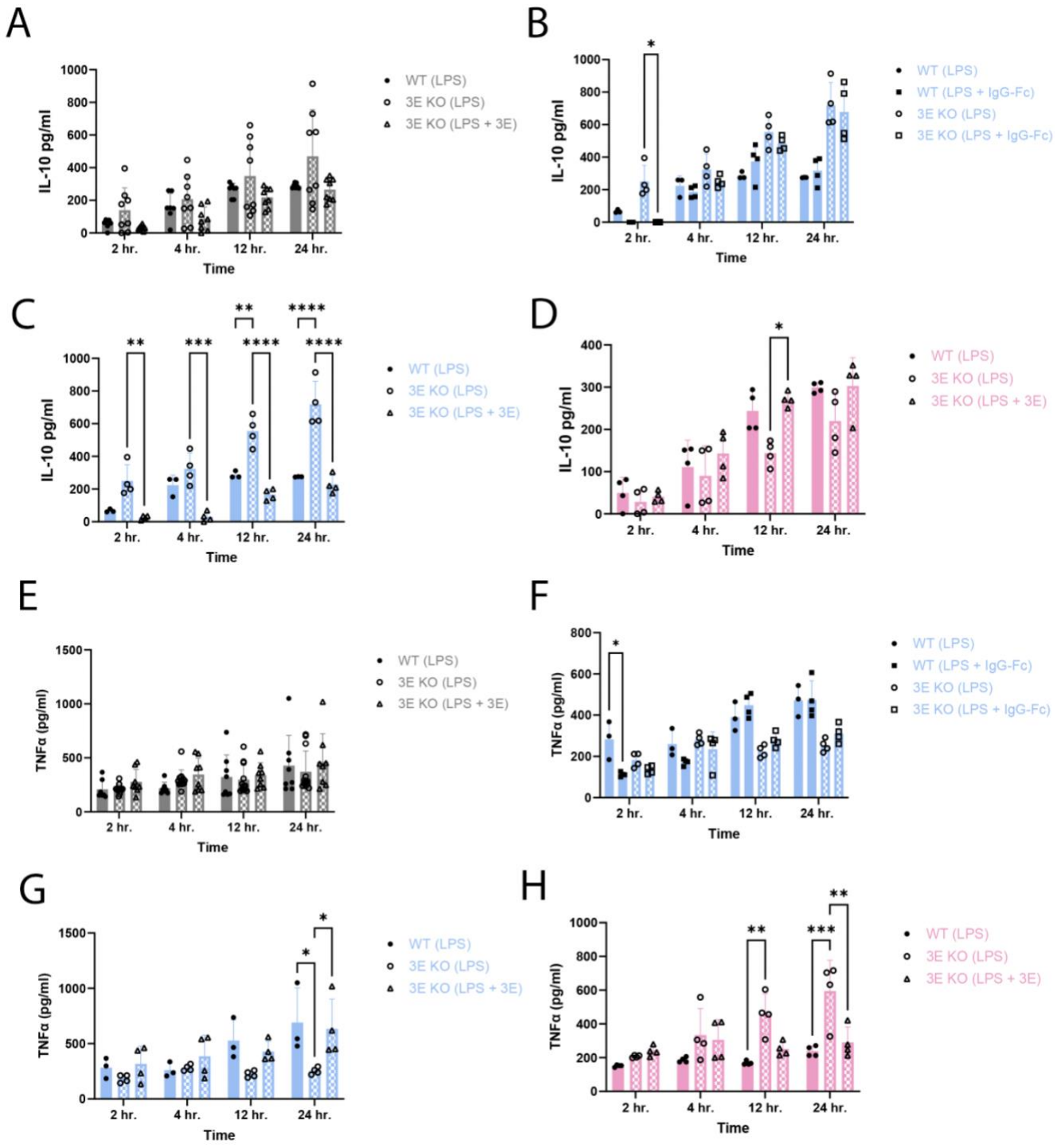

Figure 27. Restoration of Sema3E in 3E KO BMDMs modulates IL-10 and TNF production in a sex-specific manner following LPS stimulation

(A-D) Analysis of IL-10 levels in 3E KO BMDMs after Sema3E-Fc reintroduction before LPS stimulation. BMDMs were pre-treated with 100 ng/ml Sema3E-Fc for 2 hours before 100 ng/ml

LPS. **(A)** Combined data from both sexes shows no significant differences in IL-10 levels following ligand recovery (two-way ANOVA with Tukey's post hoc, $p>0.05$). **(B)** IL-10 levels in the supernatant following LPS and IgG-Fc stimulation in WT and 3E KO male BMDMs. This figure acts as a control for Sema3E-Fc stimulations to ensure the Fc portion is not causing the differences in cytokine production. There were no significant differences between WT LPS and WT LPS + IgG, and no significant differences between 3E KO LPS and 3E KO LPS + IgG at 4, 12, and 24 hours (two-way ANOVA with Tukey's post hoc, $p>0.05$). **(C)** In male 3E KO BMDM supernatant, IL-10 levels are significantly higher than in WT male cell supernatant, with a significant decrease following Sema3E pre-treatment (two-way ANOVA with Tukey's post hoc, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$). **(D)** In female 3E KO BMDM supernatant, IL-10 levels are lower than in WT female cell supernatant, with an increase following Sema3E pre-treatment, significant at 12 hours (two-way ANOVA with Tukey's post hoc, $^{*}p<0.05$).
(E-H) Analysis of TNF levels in 3E KO BMDMs after Sema3E-Fc reintroduction. **(E)** Combined data from both sexes shows no significant differences in TNF levels following ligand recovery (two-way ANOVA with Tukey's post hoc, $p>0.05$).
(F) TNF levels in the supernatant following LPS and IgG-Fc stimulation in WT and 3E KO male BMDMs. There were no significant differences between WT LPS and WT LPS + IgG, and no significant differences between 3E KO LPS and 3E KO LPS + IgG at 4, 12, and 24 hours (two-way ANOVA with Tukey's post hoc, $p>0.05$).
(G) TNF levels are significantly lower in male 3E KO BMDM supernatant than in WT male supernatant at 24 hours, significantly increasing following Sema3E pre-treatment (two-way ANOVA with Tukey's post hoc, $^{*}p<0.05$).
(H) In female 3E KO BMDMs, TNF levels are significantly higher than in WT females at 12 and 24 hours, with a significant decrease following Sema3E pre-treatment at 24 hours (two-way ANOVA with Tukey's post hoc, $^{**}p<0.01$, $^{***}p<0.001$).

6.12 Sema3E/PlexinD1 deficiency increases IL-1β levels in BMDMs following LPS stimulations

IL-1β production and secretion in macrophages are tightly regulated and require two distinct signals. The first signal is the activation of TLRs, such as TLR4, by ligands like LPS. This priming signal triggers the NF-κB pathway, leading to the transcription and accumulation of pro-IL-1β, the inactive precursor of IL-1β, within the cytoplasm. However, this signal alone is insufficient for IL-1β secretion [47, 413].

The second signal, necessary for converting pro-IL-1β into its mature, active form, typically involves the activation of the NLRP3 inflammasome [47, 414]. This step is often triggered by cellular stress or damage signals, such as extracellular ATP, which binds to the

P2X7 receptor on the surface of macrophages [415]. Activation of the NLRP3 inflammasome subsequently leads to caspase-1 activation, which cleaves pro-IL-1β into its active form, allowing it to be secreted and initiate an inflammatory response [47].

In vivo, macrophages can secrete IL-1β in response to LPS stimulation as the complex environment within a living organism often provides all the necessary conditions, including the extracellular ATP required for NLRP3 inflammasome activation. However, under *in vitro* conditions, macrophages typically do not secrete IL-1β in response to LPS alone. While direct evidence linking this observation to the absence of extracellular ATP is limited, many studies show that WT BMDMs require both LPS and extracellular ATP to trigger NLRP3 inflammasome activation and IL-1β secretion [416, 417].

Given this, I was interested in investigating whether Sema3E plays a role in inflammasome activation in BMDMs, notably since my previous experiments suggested an enhanced inflammatory response in Sema3E KO female BMDMs. In a highly inflammatory environment, the increased ROS production, higher extracellular ATP levels released from dying or stressed cells, or other stress signals could provide the necessary conditions to activate the NLRP3 inflammasome. If Sema3E typically acts to regulate or dampen inflammatory responses in females, its absence might result in an unchecked or excessive inflammatory state. This heightened inflammatory response could, in turn, generate sufficient stress signals to trigger NLRP3 inflammasome activation, leading to the processing and secretion of IL-1β. Therefore, I hypothesized that the excessive inflammation observed in Sema3E KO female BMDMs might provide the conditions for IL-1β secretion via NLRP3 inflammasome activation in these cells.

As expected, WT BMDMs did not secrete IL-1β at any of the time points analyzed (2 hours, 4 hours, 12 hours, 24 hours) following LPS stimulation (Figure. 28A). This finding

validates my research and suggests that the conditions necessary for inflammasome activation and subsequent IL-1β secretion were not met in WT BMDMs. In contrast, PlexinD1 KO BMDMs began secreting IL-1β at 12 hours. They continued 24 hours post-LPS stimulation (Figure. 28A), indicating that the absence of PlexinD1 may create an environment conducive to inflammasome activation.

Similarly, Sema3E KO BMDMs exhibited IL-1β secretion at 24 hours post-LPS stimulation. In contrast, their WT counterparts did not (Figure. 28B). This suggests that Sema3E may play a role in preventing excessive inflammation and subsequent IL-1β production. Interestingly, when analyzing potential sex differences, I found no significant differences in IL-1β secretion between WT males and females or between 3E KO males and females (Figure. 28C). Both WT males and females did not secrete IL-1β. 3E KO males and females BMDMs secreted similar levels of IL-1β at 24 hours, indicating that sex may not significantly influence IL-1β production under these conditions.

However, a ligand recovery experiment revealed intriguing sex-specific effects. When 3E KO BMDMs were pretreated with Sema3E before LPS stimulation, female 3E KO BMDMs showed decreased IL-1β secretion (Figure. 28E), suggesting that the reintroduction of Sema3E could suppress inflammasome activation in females. Conversely, male 3E KO BMDMs exhibited a further increase in IL-1β secretion, indicating that Sema3E might enhance IL-1β production in males under these conditions (Figure. 28D). Notably, male WT BMDMs showed increased IL-1β production when stimulated with both LPS and Sema3E, while female WT BMDMs did not produce IL-1β under the same conditions. These findings suggest a potential sex-specific regulatory role for Sema3E in inflammasome activation and IL-1β production,

highlighting the complex interplay between Sema3E, inflammasome activation, and the regulation of inflammatory responses.

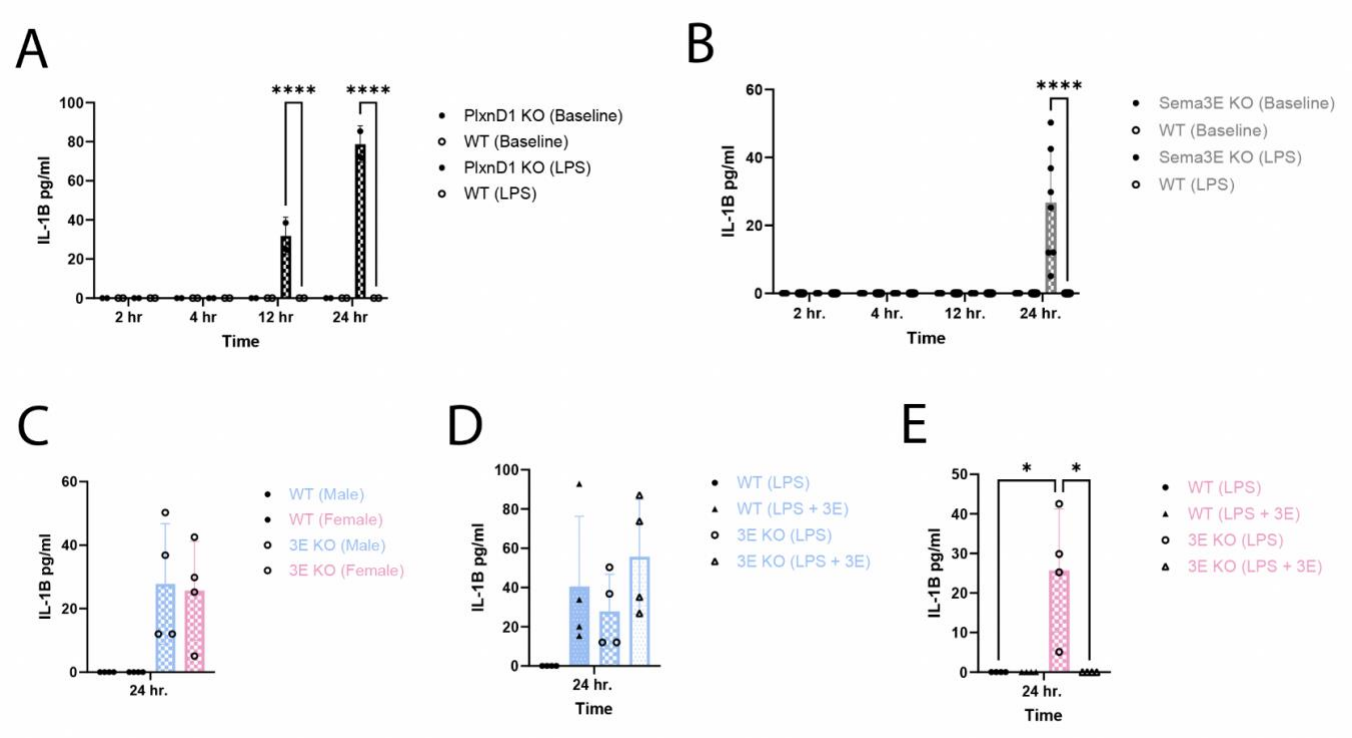

Figure 28. Sema3E and PlexinD1 deficiency influence IL-1β secretion in BMDMs following LPS stimulation

(A-B) Analysis of IL-1β secretion in WT and PlexinD1 KO BMDMs following 100 ng/ml LPS stimulation. **(A)** WT BMDMs did not secrete IL-1β at any of the analyzed time points (2, 4, 12, and 24 hours), whereas PlexinD1 KO BMDMs began secreting IL-1β at 12 hours and continued at 24 hours post-LPS stimulation (two-way ANOVA, ****$p<0.0001$). PlexinD1 KO (Baseline) and WT (Baseline) served as controls, with consistent zero values across all time points, and were excluded from statistical analysis. **(B)** Sema3E KO BMDMs showed IL-1β secretion 24 hours following LPS stimulation, while their WT counterparts did not (two-way ANOVA, ****$p<0.0001$). Sema3E KO (Baseline) and WT (Baseline) served as controls, with consistent zero values across all time points, and were excluded from statistical analysis.
(C-E) Sex-specific analysis of IL-1β secretion in 3E KO and WT BMDMs. **(C)** Comparison between WT males and females and 3E KO males and females showed no significant sex differences in IL-1β secretion at 24 hours (Mann-Whitney Test, $p>0.05$). **(D)** In male 3E KO BMDMs, Sema3E pre-treatment before LPS stimulation further increased IL-1β secretion

(Kruskal-Wallis Test, $p>0.05$). **(E)** In female 3E KO BMDMs, Sema3E pre-treatment decreased IL-1β secretion following LPS stimulation (*$p<0.05$, Kruskal-Wallis Test).

7. SUMMARY OF RESULTS

- Knocking out PlexinD1 and Sema3E in IMs leads to increased IL-10^{+} cells, suggesting that the Sema3E/PlexinD1 axis plays a role in restraining IL-10 production and maintaining immune homeostasis.

- PlexinD1 deficiency in BMDMs significantly increased TNF production at early times and IL-10 production at later times, suggesting that enhanced TNF response in PlexinD1 KO BMDMs may drive increased IL-10 as a counter-regulatory mechanism.

- Sema3E KO BMDMs exhibit distinct cytokine production patterns depending on sex, with males showing higher IL-10 levels and females exhibiting higher TNF levels, indicating that Sema3E deficiency leads to sex-specific cytokine regulation.

- The increase in IL-10 and decrease in TNF observed in male Sema3E KO BMDMs is consistent across different mouse strains, indicating that these cytokine changes are not strain-specific but rather a general effect of Sema3E deficiency.

- WT male and female BMDMs show distinct temporal patterns of IL-10 and TNF production following LPS stimulation, with males sustaining higher TNF levels and females shifting towards higher IL-10 levels at later time points.

- Significant sex-specific differences in TLR4 and CD14 expression and internalization dynamics were identified in 3E KO BMDMs, indicating that Sema3E deficiency leads to distinct receptor dynamics based on sex.

- The inverse correlation between TLR4 and CD14 in WT BMDMs becomes a positive correlation in 3E KO BMDMs, highlighting a novel disruption in receptor dynamics due to the absence of Sema3E.

- Reintroducing Sema3E-Fc into 3E KO BMDMs reversed the altered cytokine profiles in a sex-specific manner, decreasing IL-10 levels in males and increasing IL-10 levels in females, highlighting the role of Sema3E in cytokine regulation.

- Unlike WT BMDMs, Sema3E KO and PlexinD1 KO BMDMs secreted IL-1β following LPS stimulation, suggesting that the absence of Sema3E or PlexinD1 may facilitate NLRP3 inflammasome activation and IL-1β production.

- Reintroduction of Sema3E-Fc in 3E KO BMDMs resulted in decreased IL-1β secretion in females and increased IL-1β secretion in males, suggesting a sex-specific regulatory role of Sema3E in inflammasome activation.

8. DISCUSSION

Role of the Sema3E/PlexinD1 Axis in Immune Regulation

Previous studies in our lab have demonstrated that the absence of Sema3E exacerbates asthma features in acute and chronic asthma models [387-392]. Sema3E, a member of the semaphorin family, is known to play a role in various physiological processes, including immune regulation. Semaphorins typically signal through their receptors, plexins, which guide immune cell migration and modulate immune responses [359, 360]. In line with this, our lab has shown that the lack of PlexinD1 in IMs leads to increased airway hyperresponsiveness, elevated airway leukocyte numbers, enhanced allergen-specific IgE production, goblet cell hyperplasia, and a heightened Th2/Th17 cytokine response in an HDM-induced allergic asthma model [394]. These findings suggest that the Sema3E/PlexinD1 axis is crucial for maintaining airway immune homeostasis.

The primary aim of this study was to explore the role of the Sema3E/PlexinD1 axis in modulating immune responses, mainly focusing on the regulation of cytokine production in macrophages. My study aimed to investigate how the deletion of PlexinD1 and the absence of Sema3E influence the expression of key cytokines, such as IL-10 and TNF, and the dynamics of critical receptors like TLR4 and CD14, in a sex-dependent manner. The investigation involved multiple models, including IMs and BMDMs, and examined sex-specific differences in these immune processes to understand how males and females may respond differently to inflammatory stimuli and PlexinD1 signalling.

Impact of PlexinD1 deficiency on IL-10 Regulation in Macrophages

The main results show a complex interplay between Sema3E/PlexinD1 and immune regulation. I found that PlexinD1 deficiency in IMs and BMDMs leads to a significant increase in IL-10 (Figure. 18, Figure. 20A), demonstrating that PlexinD1 has a new role in modulating anti-inflammatory responses. I also found that the dynamics between TLR4 and CD14 are different in Sema3E-deficient conditions, from an inverse relationship in WT BMDMs (Figure. 25G) to a strong positive correlation in 3E KO BMDMs (Figure. 26G). This is even more pronounced when looking at sex differences, therefore, there are likely sex-specific mechanisms in immune regulation.

I found that deletion of PlexinD1 affects IL-10 production in both lung IMs and BMDMs. Furthermore, PlexinD1-deficient IMs have high IL-10 regardless of HDM exposure (Figure 18C). I also found that BMDMs lacking PlexinD1 have high IL-10 after LPS stimulation. Therefore, PlexinD1 is critical for modulating the anti-inflammatory cytokine IL-10 in different macrophage populations, a new aspect of immune regulation.

Previous studies on PlexinD1 focused on its role in axonal growth and vascular development [360][369] and very few on immune cells. Some studies showed that PlexinD1 might influence immune responses, especially T-cell activation and dendritic cell function [402][418]. However, our finding of elevated IL-10 production in the absence of PlexinD1 adds a new dimension to understanding this receptor in macrophages. Unlike other studies that linked PlexinD1 to pro-inflammatory signaling, primarily in T cells and dendritic cells [402][418], my results indicate that PlexinD1 acts as a negative regulator of IL-10 in macrophages, a key cytokine involved in controlling inflammation and promoting immune tolerance. This

observation contrasts with previous findings and opens new avenues for studying PlexinD1's role across different cellular contexts.

The high IL-10 following PlexinD1 deletion could be due to several mechanisms. One possibility is that PlexinD1 suppresses IL-10 by modulating the TLR4-NF-κB signalling axis, which is well-established in regulating inflammatory cytokine production [47][419]. Upon PlexinD1 deletion, this suppression may be lost, leading to enhanced NF-κB activity and increased IL-10 production. Additionally, PlexinD1 could interact with other pathways that converge on the NF-κB signalling node, such as the PI3K-Akt or MAPK pathways, which are known to influence both pro-inflammatory and anti-inflammatory cytokine production. Loss of PlexinD1 might disrupt the balance of these pathways, specifically tipping the scale toward increased IL-10 as a counter-regulatory mechanism to control the heightened immune activation in macrophages.

While IL-10 levels are elevated, other pro-inflammatory cytokines, such as TNF and IL-6, may also increase due to enhanced NF-κB activation, as they are similarly regulated through this pathway. The exact node where PlexinD1 modulates these pathways remains unclear; however, PlexinD1 deletion may enhance upstream signaling, potentially through MyD88, leading to more robust transcription of IL-10 and possibly other NF-κB-dependent cytokines. Further studies are needed to determine the precise molecular interactions between PlexinD1 and these signaling networks and to clarify how the absence of PlexinD1 shifts the cytokine balance in macrophages. PlexinD1 may also have a broader role in immune homeostasis, where it suppresses IL-10 and other cytokines to maintain a balanced immune response, and its absence disrupts this balance, leading to uncontrolled cytokine production.

Altered Dynamics Between TLR4 and CD14 in Sema3E Deficiency

My results show new and interesting interactions between TLR4 and CD14 in BMDMs, especially in 3E KO BMDMs. In WT BMDMs, I saw an inverse relationship between TLR4 and CD14 expression, meaning that these receptors are likely balanced to modulate the macrophage response to stimuli like LPS (Figure 25G). However, in 3E KO BMDMs this relationship is lost (Figure 26G). Therefore, the absence of Sema3E changes the regulation of these receptors and how possibly how macrophages perceive and respond to inflammatory signals.

The interaction between TLR4 and CD14 is well described in macrophage activation, especially in response to LPS, where CD14 acts as a co-receptor to facilitate TLR4 signalling. However, most studies have focused on the cooperative function of these receptors during LPS binding and the initiation of downstream signalling cascades, rather than their baseline expression dynamics [420]. The inverse relationship in WT BMDMs adds a new layer of understanding on how these receptors are regulated under normal conditions, possibly a homeostatic mechanism to prevent macrophage overactivation. The positive correlation in 3E KO BMDMs is new, and Sema3E plays a critical role in this balance. This disruption in 3E KO BMDMs could be due to the loss of Sema3E's modulatory role in signalling pathways that influence receptor expression. While the exact mechanism remains unclear, Sema3E has been shown to modulate immune signalling through pathways like NF-κB and MAPK [377][421], which could indirectly affect the expression of receptors such as TLR4 and CD14. Without Sema3E, these pathways may be differentially activated, leading to the observed positive correlation between TLR4 and CD14 in 3E KO BMDMs. Further investigation is required to elucidate the specific role of Sema3E in regulating these receptors.

The Sema 3E KO BMDM TLR4 and CD14 dynamics could have broader implications for the immune response. The inverse relationship in WT BMDMs might be a regulatory mechanism to prevent excessive inflammation, so macrophages do not overreact to minor stimuli. However, the positive correlation in 3E KO BMDMs means these cells might be more sensitive to LPS and, therefore, more prone to exaggerated inflammatory responses. This sensitivity could contribute to the dysregulated cytokine production in 3E KO BMDMs, especially the high levels of IL-10 and TNF and might influence the progression of inflammatory diseases.

Recent work from our lab supports these findings. PlexinD1 deficiency in lung IMs led to increased airway inflammation and hyperresponsiveness in a house dust mite-induced asthma model [394]. PlexinD1-deficient Ims showed elevated expression of Th2/Th17 cytokines and upregulation of mucus-associated genes such as *Muc5ac*, contributing to airway remodeling and exaggerated inflammatory responses. These changes reflect the critical role of PlexinD1 in regulating immune responses and maintaining airway homeostasis by controlling the expression of pro-inflammatory mediators and tissue remodelling factors. Additionally, our lab has shown that Sema3E deficiency exacerbates airway inflammation, remodeling, and hyperresponsiveness in a mouse model of allergic asthma [391][392]. Sema3E KO bone marrow progenitors transferred to WT recipients worsened airway inflammation and hyperresponsiveness, further highlighting the regulatory role of Sema3E in controlling inflammatory responses and tissue remodelling.

Furthermore, our lab previously demonstrated that Sema3E deficiency in BMDMs leads to impaired signalling through the MAPK, STAT3, AKT, and NF-κB pathways following LPS stimulation, with significantly reduced phosphorylation of these signalling molecules in

Sema3E-deficient BMDMs, correlating with decreased production of pro-inflammatory cytokines such as TNF and IL-6 [377] (Figure 29.). However, it is worth noting that this earlier work used L929-conditioned media for BMDM differentiation, whereas my study utilized recombinant M-CSF. Differentiation conditions can impact macrophage polarization and responsiveness, potentially affecting the activation of NF-κB and other pathways [422]. Additionally, the previous study focused exclusively on male BMDMs, which may not fully capture the sex-dependent differences observed in my study. These differences in methodology provide context for the observed variations in NF-κB activation and cytokine production between studies, highlighting how Sema3E deficiency may influence signalling differently under different conditions. Additionally, Sema3E-deficient BMDMs shifted towards an anti-inflammatory M2 phenotype, with reduced iNOS expression, suggesting that Sema3E modulates cytokine production and macrophage polarization [377].

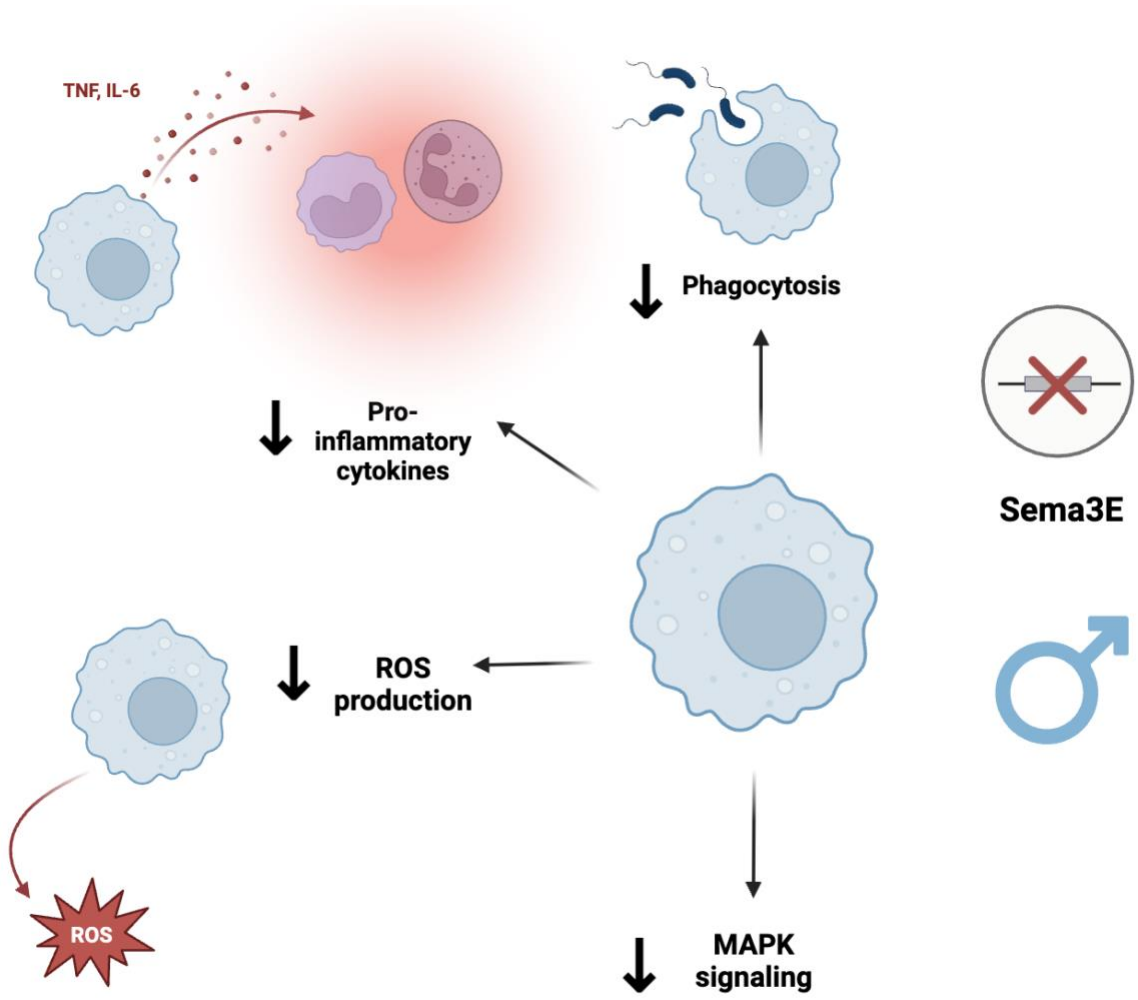

Figure 29. Sema3E deficiency in male BMDMs leads to an anti-inflammatory phenotype

Male macrophages from Sema3E global KO mice exhibit a shift toward an anti-inflammatory phenotype. These macrophages demonstrate decreased phagocytosis, reduced production of ROS, and decreased pro-inflammatory cytokine secretion (e.g., TNF and IL-6) following LPS stimulation. Additionally, impaired MAPK signalling, characterized by reduced phosphorylation of key signalling molecules, further supports the diminished pro-inflammatory response. This phenotype suggests that Sema3E deficiency influences macrophage polarization, favouring an anti-inflammatory M2-like profile.

Created with *BioRender.com*

Information from: Mohammed, A., Okwor, I., Shan, L., Onyilagha, C., Uzonna, J. E., & Gounni, A. S. (2020). Semaphorin 3E regulates the response of macrophages to lipopolysaccharide-induced systemic inflammation. *The Journal of Immunology*, *204*(1), 128–136.

These findings suggest that Sema3E, through its regulation of TLR4 and CD14, plays a critical role in maintaining immune balance and preventing excessive macrophage activation. The dysregulated receptor dynamics and cytokine production in Sema3E KO BMDMs may reflect the broader role Sema3E plays in modulating immune responses across different tissues and inflammatory contexts.

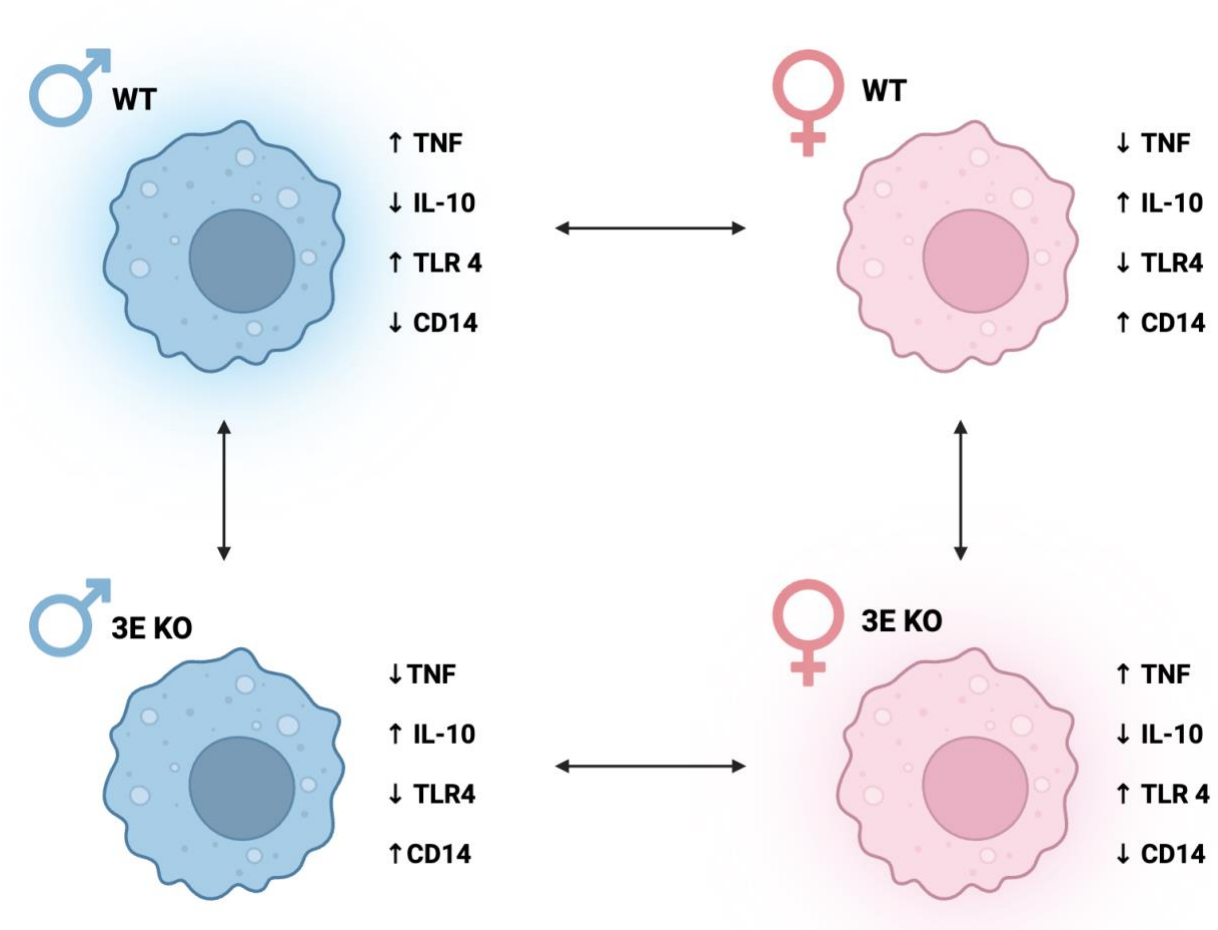

Figure 30. Disrupted TLR4 and CD14 expression dynamics in 3E KO BMDMs leads to altered cytokine production

There are contrasting dynamics of TLR4 and CD14 expression in WT and 3E KO BMDMs under baseline conditions. In WT BMDMs, an inverse relationship between TLR4 and CD14 expression is observed, reflecting a homeostatic mechanism that regulates macrophage activation and prevents excessive inflammatory responses to LPS. In contrast, 3E KO BMDMs display a

positive correlation between TLR4 and CD14, suggesting that the absence of Sema3E disrupts this balance, leading to heightened sensitivity to LPS and dysregulated cytokine production, particularly IL-10 and TNF.
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Sex-specific Differences in Macrophage Responses

My results show sex-specific differences in cytokine production and receptor dynamics in BMDMs. Sema3E KO male BMDMs had more IL-10 in culture supernatants than female 3E KO BMDMs (Figure. 26A), whereas TNF was higher in 3E KO females (Figure. 26B). These sex-specific patterns extended to TLR4 and CD14 expression and internalization. In WT BMDMs, males had higher baseline TLR4 and lower CD14 than females (Figure. 25C, 25D). TLR4 internalization was slower in male BMDMs, taking 2 hours to be fully internalized, whereas in females, it was fully internalized within 15 minutes (Figure. 25F). Therefore, male and female BMDMs respond differently to inflammatory stimuli.

Previous work in our lab demonstrated that Sema3E-Fc alleviates allergic asthma by reducing neutrophil recruitment and airway inflammation [389] (Figure 31). This effect was linked to decreased Rac1 activity, which is essential for cytoskeletal rearrangements that enhance neutrophil motility, and increased RhoA activity, which stabilizes the cytoskeleton and inhibits neutrophil migration. Notably, these studies were conducted exclusively in female mice. This observation aligns with my findings, where Sema3E deficiency in female BMDMs results in a stronger pro-inflammatory response, including elevated secretion of pro-inflammatory cytokines like TNF in the cell culture supernatants and increased TLR4 surface expression, which may amplify the inflammatory response. When Sema3E was reintroduced into these deficient female BMDMs, pro-inflammatory cytokine levels were significantly reduced. Interestingly, my data suggest that Sema3E deficiency has the opposite effect in males, where it appears to have anti-

inflammatory properties, with reduced secretion of pro-inflammatory cytokines. These findings suggest that Sema3E's role in inflammation is highly context-dependent and influenced by sex, with Sema3E deficiency exacerbating inflammation in females while dampening it in males. This data complements earlier studies focused on females and highlights the importance of investigating sex differences in Sema3E-mediated immune regulation.

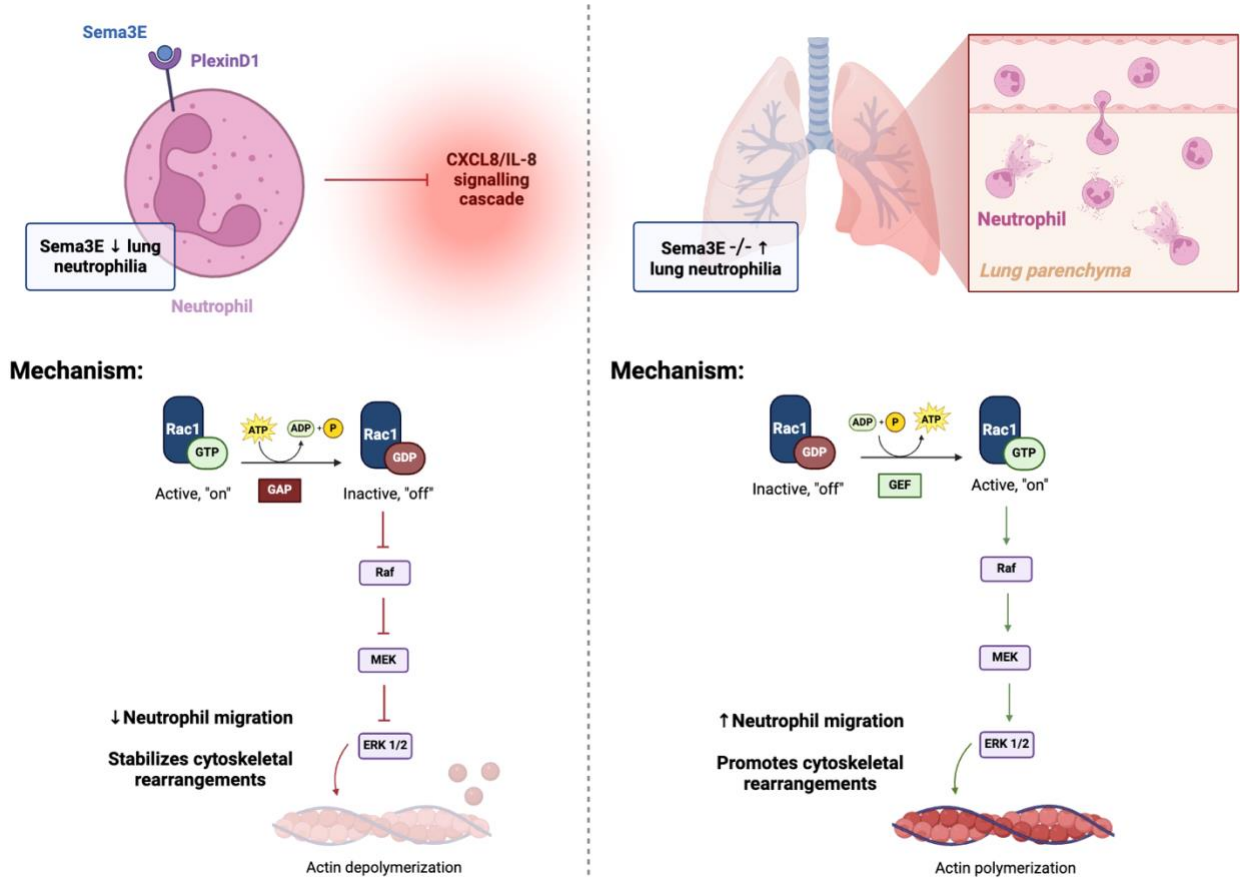

Figure 31. Role of Sema3E/PlexinD1 in regulating Rac1 activity and neutrophil migration in allergic asthma

Sema3E binding to PlexinD1 activates the GAP domain, dephosphorylating Rac1-GTP to Rac1-GDP. This inhibits the CXCL8/IL-8 signaling cascade, leading to suppression of downstream ERK1/2 activation. As a result, neutrophil migration is reduced, cytoskeletal rearrangements are stabilized, and actin depolymerization occurs, preventing excessive motility. In the absence of Sema3E, the GEF domain phosphorylates Rac1-GDP to Rac1-GTP, activating the ERK1/2

pathway. This promotes cytoskeletal rearrangements, increased actin polymerization, and enhanced neutrophil migration in response to the CXCL8/IL-8 signalling cascade.

Created with *BioRender.com*

Information from: Movassagh, H., Shan, L., Sharma, P., & Halayko, A. J. (2017). Chemorepellent Semaphorin 3E negatively regulates neutrophil migration in vitro and in vivo. *Journal of Immunology, 198*(3), 1023–1033.

The sex specific regulation by Sema3E makes it a potential therapeutic target for modulating immune responses in inflammatory diseases, which have sex differences in prevalence and severity. Sema3E likely has a dual role in immune responses; it suppresses female macrophages and promotes inflammatory responses in male macrophages. Hormones like estrogen and testosterone could influence this differential regulation and modulate immune cell function. Sema3E might interact with different signalling pathways in males and females to produce the sex-specific effects. Understanding these mechanisms is key to develop sex-specific therapies.

Sex hormones, including estrogen and testosterone, can modulate immune responses and immune cell function. Estrogen has anti-inflammatory properties; it enhances anti-inflammatory cytokines and reduces pro-inflammatory cytokines. For example, estrogen can promote a shift in M2-like anti-inflammatory phenotypes in macrophages [423-425]. Testosterone, on the other hand, is pro-inflammatory. Testosterone can upregulate TLR4 and signalling and increase TNF and IL-6 [304]. This hormonal influence might explain the sex-specific differences I saw in my study. *Sema3E might interact with testosterone-driven pathways in male macrophages to promote inflammation, and Sema3E might modulate estrogen-driven pathways to suppress inflammation in female macrophages.*

Sema3E's differential regulation also might involve interaction with different signalling pathways in male and female macrophages. One possible mechanism is the modulation of the NF-κB pathway, which is crucial for producing pro-inflammatory cytokines. NF-κB activation leads to the transcription of pro-inflammatory genes like TNF and IL-6 [426]. The delayed NF-κB phosphorylation in male Sema3E KO BMDMs [377] suggests that Sema3E might facilitate early NF-κB activation in males and rapid inflammation. In females, Sema3E might interact with other regulatory proteins or signalling molecules through its receptor, PlexinD1, which is known to activate downstream signalling cascades that can influence inflammatory responses. One possible mechanism is that PlexinD1 activation by Sema3E could recruit or modulate interactions with signalling adapters or regulatory proteins that impact NF-κB activity. For instance, Sema3E/PlexinD1 signalling might interface with estrogen receptor signalling pathways, as estrogen receptors are known to inhibit NF-κB activity [427-429]. This interaction could contribute to a dampening effect on NF-κB activation in females. In the absence of Sema3E, as seen in female Sema3E KO BMDMs, this modulatory effect would be lost, potentially leading to increased NF-κB activity and higher pro-inflammatory cytokine production.

Sex differences in immune responses are well established, with many studies showing that male innate immune cells are more pro-inflammatory than female cells [297][299][304]. This is often attributed to hormonal effects and genetic factors such as immune-related genes on the X chromosome [309]. My results align with this literature but also provide new insights into the role of Sema3E in modulating these sex-specific differences. Notably, Sema3E has been linked to male-specific diseases, such as Kallmann syndrome, where its deficiency leads to hypogonadotropic hypogonadism due to disrupted gonadotropin-hormone releasing hormone

(GnRH) neuron function, underscoring its regulatory role in male physiology [430, 431]. The increased IL-10 observed in 3E KO males suggests that Sema3E may act as a brake on anti-inflammatory responses in males, a function that is less pronounced in females. Meanwhile, higher TNF levels in 3E KO females indicate that Sema3E deficiency may lead to a loss of pro-inflammatory regulation in females. These findings contribute to the growing evidence that sex differences in immune regulation are complex and influenced by multiple factors, including specific signalling pathways like the Sema3E/PlexinD1 axis.

Furthermore, my study showed that female BMDMs have higher baseline CD14 surface expression than males (Figure. 25C, 26C). This finding could relate to previous results from our lab showing M2 skewing in Sema3E KO macrophages, as CD14 expression levels may influence macrophage polarization. Higher CD14 expression in female BMDMs could contribute to a distinct polarization profile, potentially favouring specific immune pathways or responses that differ between sexes. While a study has shown that stimulating murine macrophages with estrogen increases CD14 expression [432], further work is needed to clarify the mechanisms behind these sex-specific differences in CD14 expression and how they may intersect with macrophage polarization pathways. Understanding how estrogen and other factors regulate CD14 in immune cells could have large implications for sex-specific immune responses and disease outcomes.

Additionally, genetic factors could play a role, especially immune-related genes that are differentially expressed or regulated in males and females. Another possibility is that the TLR4 and CD14 signalling pathways are differentially modulated in male and female BMDMs, with Sema3E deficiency exacerbating these differences. For example, the slower TLR4 internalization in males could lead to prolonged signalling and increased cytokine production, while the faster

internalization in females might limit the duration of the inflammatory response. These mechanistic insights suggest that the Sema3E/PlexinD1 axis could serve as a critical modulator of sex-specific immune reactions, and further research is needed to elucidate these complex interactions fully.

Sema3E/PlexinD1 and the Inflammasome

This thesis shows that Sema3E and its receptor PlexinD1 are involved in IL-1β secretion, a key cytokine in inflammation and immune responses, particularly through NLRP3 inflammasome activation or altering IL-1β release mechanisms. The data suggests Sema3E has a regulatory role in the inflammasome and IL-1β production in a context and sex-dependent manner.

In both PlexinD1 KO (Figure. 28A) and Sema3E KO BMDMs (Figure. 28B) IL-1β was secreted following LPS stimulation. Therefore, these molecules seem to be important regulators of inflammasome activation. WT BMDMs did not secrete IL-1β under the conditions tested, so the second signal (e.g. extracellular ATP) was likely absent. However, deletion of PlexinD1 or Sema3E may create a more permissive environment for inflammasome activation and IL-1β production, potentially by altering cellular stress responses, increasing mitochondrial ROS, or promoting other intracellular changes that mimic the effects of a secondary signal. This aligns with the literature that inflammasome activation requires a secondary danger signal, in addition to TLR priming [47][413, 414]. *In vivo*, such conditions can arise from increased ROS production or extracellular ATP levels during cellular stress or tissue damage.

I was surprised by the lack of sex differences in IL-1β secretion between WT and KO male and female BMDMs (Figure. 28C). I had expected the inflammatory response in Sema3E KO females, based on previous experiments showing enhanced inflammatory responses, to result

in more IL-1β secretion. However, the lack of sex differences in IL-1β production following LPS stimulation suggests that the regulatory mechanisms of IL-1β secretion do not differ much between sexes or that other factors besides Sema3E are involved in generating these differences. This contrasts the sex differences in cytokine production for other inflammatory mediators like TNF and IL-10. Therefore, IL-1β might be uniquely regulated through the inflammasome.

The ligand recovery experiment gave further insight into the sex-specific role of Sema3E in IL-1β production. Sema3E pretreatment reduced IL-1β secretion in female Sema3E KO BMDMs (Figure. 28E) but increased IL-1β production in male BMDMs (Figure 28D), suggesting Sema3E might have opposite effects on inflammasome activation in males and females. This is interesting as it suggests Sema3E has a dual function: anti-inflammatory in females and pro-inflammatory in males. These sex differences might be due to different signalling pathways downstream of PlexinD1 or different interactions between Sema3E and other immune regulators in male and female macrophages.

Furthermore, male WT BMDMs secreted IL-1β when co-stimulated with LPS and Sema3E (Figure. 28D), demonstrating that Sema3E can enhance pro-inflammatory responses in males under certain conditions. This supports the idea that Sema3E can modulate inflammasome activation in a context-dependent manner, possibly depending on other danger signals like extracellular ATP or ROS. Female WT BMDMs didn't secrete IL-1β under the same conditions. Therefore, Sema3E's role in inflammasome regulation might be sex-specific. Furthermore, male WT BMDMs secreted IL-1β when co-stimulated with LPS and Sema3E, demonstrating that Sema3E can enhance pro-inflammatory responses in males under certain conditions (Figure 28D). This supports the idea that Sema3E can modulate inflammasome activation in a context-dependent manner, possibly depending on other danger signals like extracellular ATP or ROS.

Female WT BMDMs did not secrete IL-1β under the same conditions. This adds to the emerging body of literature suggesting that Sema3E plays a complex role in immune regulation. Although its specific role in inflammasome activation is not fully understood, recent studies hint at its potential involvement in modulating macrophage polarization and inflammatory signalling pathways such as NF-κB and MAPK [377], which are key inflammasome regulators. Sema3E is pro- and anti-inflammatory, depending on the context and cell type. Therefore, further investigation is warranted to determine whether Sema3E's modulation of these pathways directly impacts inflammasome activation in a context- and sex-dependent manner.

The results here suggest Sema3E's role in inflammasome activation and IL-1β production is complex and depends on sex, cellular stress and receptor signalling. Future studies could look at the molecular mechanisms of Sema3E modulation of inflammasome activation, especially the downstream pathways that differ between males and females. Additionally, the reintroduction of Sema3E in female 3E KO BMDMs reduces IL-1β production, so this could be a therapeutic approach to regulate excessive inflammation in diseases where IL-1β is a key player, like autoinflammatory disorders or chronic inflammatory diseases. Knowing how Sema3E modulates these pathways in a sex-specific manner could lead to sex specific therapies.

In addition to its activation, the release of IL-1β from cells is a highly regulated process that is important for its function. The release often depends on the formation of gasdermin-D pores in the plasma membrane, driven by inflammasome activation, which allows IL-1β to exit the cell in response to signals such as extracellular ATP or mitochondrial ROS. The deletion of Sema3E or PlexinD1 may not only affect IL-1β production but could also influence its release by altering cellular stress responses or membrane dynamics. This interplay between IL-1β activation and release highlights the complexity of Sema3E's role in inflammasome regulation and raises

important questions about whether the observed changes in IL-1β secretion are linked to differences in gasdermin-D activation or other release mechanisms. Further studies are needed to explore these pathways in greater detail.

Therapeutic Potential of Targeting the Sema3E/PlexinD1 Axis

Given that asthma and ulcerative colitis (UC) have been linked to Sema3E deficiency [421] targeting this pathway could offer broad therapeutic potential. While UC is often more prevalent in males [433-435] hormonal fluctuations, such as those occurring during menstruation, can worsen symptoms in females [436], highlighting the complex interplay between sex hormones and inflammatory diseases. The gastrointestinal tract is constantly exposed to various food antigens and microbes, requiring strict immune regulation to prevent chronic inflammation. When this regulation fails, due to genetic predisposition or environmental triggers, inflammatory bowel disease (IBD) emerges, with a distinct sex bias. My findings that female 3E KO BMDMs are hyper-inflammatory, characterized by increased TNF production and altered receptor dynamics, provide a potential mechanism explaining why Sema3E deficiency might exacerbate disease severity in females, particularly during hormonal changes. Recent studies have shown that estrogen receptor pathways play a significant role in these sex-specific differences, with estrogen receptor (ER) α silencing reducing inflammation in female mice, while ERβ knockout worsens it [437]. Therefore, therapies aimed at restoring Sema3E function or modulating the PlexinD1 pathway could be particularly beneficial for female patients, especially those experiencing hormonally driven disease flares, helping to counteract the excessive inflammatory response that fuels disease progression.

These findings have important implications for understanding and treating diseases with an inflammatory component. Sema3E deficiency has been linked to worse asthma symptoms and some studies suggest females are more severely affected in certain inflammatory conditions. While the relationship between Sema3E deficiency, worse asthma outcomes and female sex has not been established, my findings suggest this is an area to explore further. My findings suggest the hyper-inflammatory response in female 3E KO BMDMs may mirror the inflammatory state in female asthma patients with low Sema3E. This highlights the need to consider sex differences when developing therapies targeting the Sema3E/PlexinD1 pathway, and this could apply to other diseases where chronic inflammation is a key player. In these conditions, restoring Sema3E function or modulating its downstream signalling may help alleviate the hyper-inflammatory state, particularly in female patients who seem more severely affected.

Importantly, my study highlights the need to consider sex in data analysis and to separate data by sex, as combining data could mask important information for understanding disease mechanisms and developing therapies. Sex differences are an understudied but critical area of research as the immune systems of males and females are different. By acknowledging and exploring these differences, we can gain more insight into disease processes and improve the precision and efficacy of treatments. The hyper-inflammatory phenotype in female 3E KO BMDMs shows we need to further investigate how Sema3E deficiency contributes to disease and how this pathway can be targeted therapeutically.

This study has shown the Sema3E/PlexinD1 pathway regulates immune cells, however, many questions remain. One of the key ones is how Sema3E deficiency leads to the changes in TLR4 and CD14 dynamics, especially the switch from inverse to positive correlation in receptor

expression. Additionally, how do these receptor dynamics contribute to the differential regulation of IL-10 and TNF in Sema3E KO macrophages.

We have previously shown that 3E KO macrophages, especially from male mice, have reduced TNF and IL-6, iNOS and decreased phosphorylation of STAT3, NF-κB and ERK 1/2 [377]. This is consistent with the reduced inflammation I see in male 3E KO BMDMs. It would be interesting to see if the signalling changes are also present in female 3E KO macrophages. Given the hyper-inflammatory phenotype in female 3E KO BMDMs with increased TNF, these signalling molecules' phosphorylation status might differ between sexes. Future studies could investigate this by looking at the phosphorylation and total levels of STAT3, NF-κB, and ERK1/2 in female 3E KO macrophages compared to males. Examining the overall expression levels of these transcription factors and their phosphorylation status would provide a more comprehensive understanding of how Sema3E deficiency affects these signalling pathways. This could reveal whether sex differences in cytokine production and receptor dynamics are also reflected in the baseline levels and activation states of key signalling molecules, offering deeper insights into how Sema3E deficiency influences immune regulation in a sex-dependent manner.

Furthermore, it is unclear how these receptor dynamics contribute to the differential regulation of IL-10 and TNF in Sema3E KO macrophages. Future studies could investigate this by dissecting the signalling pathways downstream of TLR4 and CD14 in WT and 3E KO conditions using specific inhibitors or genetic modifications targeting key signalling molecules such as NF-κB or MAPKs.

Another area to be explored is the role of PlexinD1 in sex-specific immune regulation. Although this study was on the effects of Sema3E deficiency, it would be great to see if similar sex differences exist in PlexinD1 expression and function. Future studies could look at PlexinD1

expression levels and receptor dynamics in male and female macrophages under normal and Sema3E KO conditions using flow cytometry and immunohistochemistry to compare expression patterns and functional outcomes. This would give a better understanding of how PlexinD1 and Sema3E regulate immune responses in males and females.

Given these findings, there is therapeutic potential in targeting the Sema3E/PlexinD1 axis to modulate immune responses, especially in chronic inflammation. Future studies could focus on ways to enhance Sema3E signalling or replace its function where it is deficient. This would be particularly beneficial for female patients who are more susceptible to the hyper-inflammatory effects of Sema3E deficiency. And exploring PlexinD1 as a therapeutic target could give new avenues for intervention if future studies show that PlexinD1 also has sex specific regulation like Sema3E.

It would also be interesting to see how the Sema3E/PlexinD1 axis could be combined with existing asthma and UC treatments. For example, Sema3E enhancing treatments with anti-inflammatory treatments could give a synergistic effect and reduce inflammation and address the underlying immune dysregulation. And how these treatments could be sex based would give more personalized and effective treatment approaches. In summary future studies should aim to understand the mechanisms behind the sex specific effects in this study and modulate the Sema3E/PlexinD1 axis to improve outcomes in inflammatory diseases.

This study has shown the importance of the Sema3E/PlexinD1 axis in immune regulation and how it modulates cytokine production and receptor dynamics in macrophages. The sex specific effects, especially the hyper-inflammatory phenotype in female 3E KO BMDMs adds a new layer of complexity to how this axis regulates immune homeostasis. And the altered TLR4 and CD14 dynamics in Sema3E KO macrophages, from inverse to positive correlation, shows

the complex regulation of Sema3E and PlexinD1 in maintaining balanced immune responses. This adds to the field of immunology by showing new aspects of immune regulation that could be important for understanding inflammatory diseases.

This has implications for future research and potentially clinical applications. By showing how the Sema3E/PlexinD1 axis regulates immune responses mainly through sex specific mechanisms this study highlights the need to consider sex differences more in immune regulation. These differences could give us answers why certain inflammatory diseases present and progress differently in males and females.

9. LIMITATIONS

One limitation of this study is the specificity of the results to *in vitro* conditions. While the *in vitro* findings are significant, *in vivo* validation is needed. The results obtained from BMDMs may only partially replicate the complex *in vivo* environment. This complex environment can significantly influence cytokine production and cellular behaviour in ways that cannot be fully recapitulated *in vitro*. For example, other immune cells can modulate the response of macrophages to stimuli like LPS. *In vivo* studies are crucial for understanding the physiological relevance of the observed *in vitro* phenomena. The immune system's response in a living organism involves a coordinated interplay between various cell types, tissues, and organs, which cannot be fully replicated in vitro. *In vivo* validation in a model organism would strengthen the conclusions by confirming whether the observed increases in cytokine levels, such as IL-10, and the proposed compensatory mechanisms are relevant under physiological conditions. Further studies are needed to confirm these findings in the context of a whole organism, considering factors such as tissue-specific responses and systemic immune regulation.

Another limitation of this study involves using PlexinD1 KO on CX3CR1+ cells. *In vivo*, CX3CR1 is not exclusively expressed by macrophages; other cell types, including monocytes, DCs, NK cells, microglia, and specific subsets of lymphocytes, also express it [438]. This broad expression pattern means that knocking out PlexinD1 in CX3CR1+ cells *in vivo* might affect various cell types, not just the macrophages of interest. In my BMDM cell cultures, 87% were differentiated bone marrow-derived macrophages, leaving 13% as other cell types. These different cells could include monocytes from the blood, which also express CX3CR1. Thus, the observed effects in the PlexinD1-deficient BMDMs might not be solely due to the macrophages but could involve these other CX3CR1+ cells. However, the primary observations still hold,

given that most cells were BMDMs. Nonetheless, it is essential to consider the potential contributions of these other cells when interpreting the results, underscoring the need for careful interpretation of the data.

Additionally, a high dose of LPS (100 ng/ml) might have impacted the results by potentially overstimulating the cells and skewing the natural cytokine production balance. High concentrations of LPS can induce a robust and artificial inflammatory response, which may not accurately reflect physiological conditions typically encountered in the body. *In vitro*, lower and more variable concentrations of LPS might elicit a more subtle immune response. The high-dose LPS stimulation in this study likely led to a hyper-inflammatory state, characterized by an exaggerated production of pro-inflammatory cytokines such as TNF and anti-inflammatory cytokines like IL-10. This effect is particularly relevant in sepsis, where high levels of endotoxins like LPS enter the bloodstream, leading to systemic inflammation. Sepsis is characterized by an overwhelming and dysregulated immune response to infection, often driven by high concentrations of bacterial components such as LPS [439]. In such cases, the body's immune cells, including monocyte and macrophages, are exposed to large amounts of LPS, resulting in a cytokine storm, an excessive and uncontrolled release of pro-inflammatory cytokines [440, 441]. Therefore, the findings from this study might be more pertinent to understanding the mechanisms underlying inflammation and cytokine regulation in severe inflammatory conditions like sepsis. In sepsis, high levels of LPS can lead to sustained activation of TLR4 and NF-κB signalling pathways, causing prolonged MAPK activation and excessive cytokine production [440, 44 This hyper-inflammatory state can cause widespread tissue damage and organ failure and, if not managed effectively, can be fatal. By using high-dose LPS stimulation, this study may have inadvertently modelled aspects of the immune response seen in

sepsis rather than the more controlled and balanced inflammatory responses typical of milder infections or chronic inflammatory conditions. This distinction is crucial as it underscores the need to interpret the results within the appropriate physiological context. Future studies could benefit from using a range of LPS concentrations to mimic physiological conditions better and delineate the dose-dependent effects of LPS on cytokine production and signalling pathways. Understanding how different doses of LPS affect the immune response can provide insights into the threshold levels that distinguish between beneficial inflammatory responses and detrimental hyper-inflammatory states. It can also help identify potential therapeutic targets for modulating the immune response in sepsis and other severe inflammatory conditions, aiming to restore balance and prevent excessive inflammation.

Another limitation is the possibility of compensatory mechanisms. Without Sema3E/PlexinD1, other signalling pathways or molecules might be upregulated to compensate for their loss, thereby influencing IL-10 production. For instance, other semaphorin family members, such as Sema3A or Sema3C, might interact with plexins or receptors to modulate immune responses [442]. Furthermore, feedback mechanisms within the immune system might be triggered to counterbalance the heightened inflammatory state induced by the absence of Sema3E/PlexinD1, promoting IL-10 production as a regulatory response.

Moreover, a limitation of my research is the reliance on correlation matrices to infer relationships between cytokine production, such as TNF and IL-10, and receptor dynamics, like TLR4 and CD14 expression. While these matrices are valuable for identifying potential associations and can reveal complex interactions between different variables, they do not establish causation. The correlations observed in this study, including the inverse and positive relationships between TLR4, CD14, and cytokines like TNF and IL-10, suggest intriguing

connections. However, these correlations alone cannot definitively prove that one variable directly influences another. To validate these correlations experimentally, future studies should consider using specific inhibitors that target vital signalling pathways associated with TLR4, CD14, TNF, and IL-10. For instance, inhibitors of NF-κB, MAPKs, or other signalling molecules downstream of TLR4 could disrupt these pathways and observe the effects on cytokine production. We can more accurately determine whether these relationships are causative by inhibiting specific pathways and analyzing how these changes impact the correlations observed in the matrices. By combining these experimental approaches with the insights gained from correlation matrices, future research can more definitively establish the roles of specific signalling pathways in modulating immune responses. This would validate the correlations observed in this study and offer a clearer understanding of the underlying mechanisms driving the immune functions associated with the Sema3E/PlexinD1 axis.

Another limitation of my study on sex differences is that I did not control for the estrous cycle in the female mice. The estrous cycle, which consists of proestrus, estrus, metestrus, and diestrus phases [443] can significantly influence immune responses due to fluctuating levels of sex hormones such as estrogen and progesterone. These hormonal variations could affect cytokine production, TLR4 expression, and overall immune cell function. By not accounting for the estrous cycle, results may not fully capture the variability in immune responses that occur naturally within female mice. This could lead to an underestimation or overestimation of the inflammatory responses in female BMDMs, potentially masking significant sex-specific differences. Future studies should consider synchronizing the estrous cycle during experiments to better compare male and female immune responses. This approach would help clarify how hormonal fluctuations specifically impact the role of Sema3E in immune regulation.

10. FUTURE DIRECTIONS

Characterizing Macrophage Polarization Using Flow Cytometry

A critical future step is to characterize macrophage polarization in a sex-specific manner, mainly focusing on the effects of Sema3E KO. Flow cytometry could assess M1 (pro-inflammatory) and M2 (anti-inflammatory) markers. Based on current predictions, WT male macrophages will likely exhibit more M1 markers following LPS stimulation than WT females, who may have these markers to a lesser extent. This differential expression would be evident in markers such as iNOS for M1 macrophages. In contrast, Sema3E KO males are expected to shift towards an M2 phenotype, exhibiting markers such as Arginase-1 and demonstrating decreased levels of pro-inflammatory cytokines like IL-6 and TNF, along with lower CD14 and enhanced IL-10 protein levels. On the other hand, Sema3E KO females might retain more M1 characteristics, with increased TNF production and reduced IL-10 levels. This polarization shift would provide strong evidence of Sema3E's involvement in sex-specific immune responses, which can be further validated through additional experiments focusing on specific polarization markers.

Investigating Extracellular ATP Levels and IL-1B Secretion

Another interesting avenue involves studying the role of extracellular ATP in driving IL-1β secretion in macrophages following LPS stimulation. In WT BMDMs, IL-1β secretion typically does not occur within 24 hours due to low extracellular ATP levels. However, I predict that in Sema3E KO BMDMs, ATP levels will be significantly elevated, contributing to earlier IL-1β secretion. One possible reason for higher ATP levels in the absence of Sema3E could be related to changes in metabolic pathways, as Sema3E signalling might normally inhibit ATP release or influence mitochondrial function and energy metabolism. Without Sema3E, these

regulatory effects may be diminished, resulting in increased ATP production or release. This could be tested by measuring ATP levels in cell culture supernatants post-LPS stimulation and correlating them with IL-1β levels. By proving that ATP changes occur in Sema3E KO macrophages, this study would provide crucial insights into the metabolic shifts induced by the absence of Sema3E, potentially linking Sema3E to macrophage metabolism and immune responses.

Metabolomic Profiling to Explore Metabolic Pathways

Given the potential involvement of Sema3E in altering cellular metabolism, metabolomic studies are a promising direction for future research. Sending out samples for metabolomic profiling could help identify the metabolic pathways altered in Sema3E KO macrophages. One key metabolic shift in macrophages following LPS exposure is the transition from oxidative phosphorylation (OXPHOS) to glycolysis, known as the "Warburg effect" or "aerobic glycolysis." This metabolic reprogramming supports the energetic and biosynthetic demands of pro-inflammatory M1 macrophages, promoting rapid ATP production and the synthesis of macromolecules necessary for inflammation [444].

Investigating whether Sema3E influences this metabolic switch is crucial. By altering key metabolic pathways, Sema3E KO macrophages might exhibit differences in how they handle ATP production and energy metabolism, potentially modulating the balance between glycolysis and OXPHOS. This could have significant implications for cytokine production and immune response, as metabolic reprogramming is closely linked to macrophage function. Profiling these pathways could reveal specific metabolic markers associated with Sema3E deficiency, providing insights into how changes in metabolism contribute to altered cytokine secretion and macrophage polarization.

NLRP3 Inflammasome Activation and IL-18 Production

To further explore the role of Sema3E in inflammasome activation, it would be valuable to assess the activation of the NLRP3 inflammasome in Sema3E KO BMDMs. Measuring IL-18 levels, which are regulated similarly to IL-1β, could provide additional evidence of inflammasome activation in Sema3E-deficient macrophages. Moreover, western blot analyses could investigate caspase-1 activity, which cleaves pro-IL-1β into its active form. An increase in active caspase-1 and IL-1β in Sema3E KO BMDMs would further support the hypothesis that Sema3E regulates inflammasome activation. This would provide a more comprehensive understanding of how the absence of Sema3E contributes to heightened inflammatory responses.

Hormonal Influences and the Estrus Cycle

An exciting area for future exploration is how sex hormones, specifically estrogen and progesterone, modulate macrophage responses in relation to Sema3E. Collecting blood samples from female mice to monitor hormone levels across different phases of the estrus cycle could help control for these variables and reveal how Sema3E affects macrophage polarization and cytokine production during each stage. Understanding whether females are more inflammatory during the estrus (high estrogen) or diestrus (low progesterone) stages would provide valuable insight into sex-specific immune regulation. These experiments could also explore how Sema3E deficiency impacts these hormone-mediated responses and whether Sema3E plays a role in balancing pro- and anti-inflammatory signals depending on the hormonal milieu.

Blocking TNF or IL-10 to Assess Cytokine Crosstalk

There is an observed correlation between TNF and IL-10 levels, particularly in female macrophages, where an increase in one cytokine often corresponds with an increase in the other.

Future experiments could focus on blocking either TNF or IL-10 to assess how this affects the production of the other cytokine. For instance, blocking TNF might reduce IL-10 levels and vice versa, providing insights into the regulatory feedback mechanisms between these cytokines. This would also clarify the role of Sema3E in modulating this cytokine crosstalk and whether Sema3E acts as a mediator of this relationship in a sex-dependent manner.

RNA Sequencing to Identify Broader Changes

Lastly, RNA sequencing could be a powerful tool for exploring the broader transcriptional changes induced by Sema3E knockout in a sex-dependent manner. This approach would allow identifying all cytokines and immune-related genes that are altered in response to Sema3E deficiency. Such data could provide a comprehensive view of how the absence of Sema3E affects macrophage function at the transcriptomic level, revealing potential new pathways and interactions that are influenced by Sema3E and contribute to the observed sex differences in immune responses.

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