Lund University

Regulation of endothelial inflammation by TAM receptors

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ABSTRACT

The TAM (Tyro3, Axl, Mer) receptors tyrosine kinases together with their ligands Gas6 and protein S play an important role in many cellular processes such as resolution of inflammation and phagocytosis. Notably, they were found to have an important function in vasculature, acting as a pro-survival factors and regulators of angiogenesis. Nevertheless, their role in endothelial inflammation is still poorly understood. Conflicting conclusions have been published, indicating both pro and anti-inflammatory properties of TAM-mediated signaling in endothelium. In this study we sought to elucidate the role of these receptors in endothelial inflammation by measuring the levels of pro-inflammatory markers, such as cytokines and adhesion molecules, in response to inflammatory stimuli in TAM and TAM ligand-deficient human umbilical vein endothelial cells (HUVEC). We found that Axl/Gas6 signaling acts as a pro-inflammatory agent by promoting an increased expression of adhesion molecules and cytokine secretion. Conversely, we suggest anti-inflammatory tendencies of the Mer/protein S axis. Moreover, we demonstrate that starvation strongly upregulates Mer expression in HUVEC cells. Finally, we propose that TAM receptors expression is differently regulated depending on cell type, as inflammatory regulators known to induce TAM-expression in immune cells fail to upregulate TAMs in HUVECs. Taken together, we present diversification and complexity of Axl and Mer receptor functions in endothelial inflammation, thus suggesting the need for further investigations.

Abbreviations and Symbols

AC	*Apoptotic cell*
aPC	*Activated Protein C*
APC	*Antigen- presenting cell*
C4BP	*C4b-binding protein*
CNS	*The central nervous system*
DC	*Dendritic cell*
Dex	*Dexamethasone*
EC	*Endothelial cells*
EGF	*Epidermal growth factor*
ELISA	*Enzyme-linked immunosorbent assay*
FACS	*Fluorescence-activated cell sorting*
Gas6	*Growth arrest specific gene 6*
GM-CSF	*Granulocyte-macrophage colony-stimulating factor*
HAEC	*Human aortic endothelial cells*
HUVEC	*Human umbilical vein endothelial cells*
ICAM-1	*Intercellular adhesion molecule-1*
IFN- γ	*Interferon- γ*
IFNAR	*Type I interferon receptor*
Ig	*Immunoglobulin*
IL-1b	*Interleukin-1 beta*
IL-6	*Interleukin-6*
IP	*Immunoprecipitation*
LG	*Laminin G domain*

LPS	*Lipopolysaccharide*
MCP-1	*Monocyte chemoattractant protein-1*
NF- κB	*Nuclear factor-kappa B*
Pro S	*Protein S*
PtdSer	*Phosphatidylserine*
qRT-PCR	*Quantitative real-time polymerase chain reaction*
RCS	*The Royal College of Surgeons rats*
RPE	*Retinal pigment epithelial cell*
SHBG	*Sex hormone binding globulin*
siRNA	*Small- interfering RNA*
SIRS	*Systemic Inflammatory Response Syndrome*
SLE	*Systemic lupus erythematosus*
SOCS	*Suppressors of cytokine signaling proteins*
TAM RTK TKO	*Tyro3, Axl, Mer receptor tyrosine kinase triple knockout*
TAM RTKs	*Tyro3, Axl, Mer receptor tyrosine kinases*
TLR	*Toll-like receptor*
TNF-α	*Tumor necrosis factor-α*
TSR	*Thrombin-sensitive region*
VCAM-1	*Vascular cell adhesion molecule-1*
VEGF-A	*Vascular endothelial growth factor A*
VEGFR2	*Vascular endothelial growth factor receptor 2*
VKD	*Vitamin K dependent*
VSMC	*Vascular smooth muscle cells*
WB	*Western blot*

Contents

1. INTRODUCTION

Inflammation is a biological process, which is initiated in response to harmful stimuli. There are many causes of inflammation in human body, such as mechanical injuries, bacterial or viral infections, or chemical intoxication. Upon inflammation, the blood flow increases and the vessels dilate, which can be observed as a typical "redness and swelling". In addition, endothelial cells produce various proteins, which promote further response and activation of specific immune cells, which can then migrate from the circulation into the tissue. Inflammation is a very complex biological process, and studying mechanism regulating this is crucial for the development of various pharmaceuticals and understanding disease pathways. In the presented study, we put the effort to better understand this process with respect to the involvement of TAM receptors expressed in the endothelium.

1.1. TAM receptors

Receptor tyrosine kinases (RTKs) have been known to science since the 1980s, thanks to the work of Hunter and colleagues (5). Nevertheless, it was not until a decade later when Lai & Lemke described three novel genes clustered together, within the RTK family, which would later on be referred to as the TAM family (6). The sequence homology of these genes -*Tyro3, Tyro7 and Tyro12*- brought the attention of many research groups. *Tyro3* was first cloned and described by Crosier et al. (7), *Tyro*7 by O'Bryan et al. (8) and was given the name Axl, originating from the Greek word *anexelekto* meaning uncontrolled. Finally, Graham et al. (9) cloned and described human *Tyro12*, naming it Mer, as it was found expressed in monocytes, epithelial and reproductive tissues. It is worth noticing that nomenclature can be confusing, as many groups put the effort describing these receptors giving them different names. However, in this work Tyro3, Axl, Mer will be used to refer to the particular receptors, often abbreviated as TAM receptors or TAM RTKs.

1.1.1. *The structure of TAMs*

All three TAM receptors share structural similarities as presented in *Figure 1A*. They consist out of an extracellular, a transmembrane and a cytosolic part. The extracellular part in the N-terminus begins with two immunoglobulin (Ig)-like domains, which promote the interaction with TAM ligands. The Ig-like domains are followed by two fibronectin type 3 domains. The extracellular and cytosolic parts are linked by a hydrophobic transmembrane

domain, leading to the C-terminal protein tyrosine kinase domain (8, 10, 11). It has been shown that binding of a ligand results in dimerization of the receptor and hence receptor activation, a common feature of RTKs. TAM-activation is associated with autophosphorylation of the tyrosine kinase domain, consequently leading to an intracellular signal transduction cascade (12, 13).

The expression of these receptors varies depending on the tissue. In humans, Tyro3 is mostly expressed in the central nervous system (CNS) as well as in kidneys, ovaries and testes (14, 15), whereas Axl is found ubiquitously (8). Mer expression is mostly limited to ovaries, prostate, lungs and kidneys as well as to a lower extent in the spleen, liver, colon and placenta (9, 16). However, these receptors have been described also in vascular cells, such as in endothelial cells and vascular smooth muscle cells (17). Moreover, the expression of TAMs in antigen-presenting cells, monocytes and natural killers cell determines their primary role in the immune system, which will be discussed further on (9, 16). Interestingly, it has been shown that Axl, Mer and Tyro3 can be present as both transmebrane proteins as well as soluble molecules in plasma (sAxl, sMer, sTyro3). Specialized metalloproteinases have been described, which cleave off extracellular domain of the receptors and release it to the system (18-20).

1.2. TAM RTKs ligands

Ligand binding is crucial for the activation of TAM RTKs. Two main ligands have been identified to date, Gas6 and protein S (21, 22). However, it is worth mentioning that recent studies suggest novel agonists for TAM receptors, such as Tubby, Galectin-3 and tubby-like protein 1 (Tulp1), activating Mer (23-25). Nevertheless, not much is known about these ligands, showing the need for further investigations.

Again, Gas6 and protein S exhibit structural similarities, as presented in *Figure 1B*. Moreover, they are homologous with around 44% sequence identity (26). Both proteins are vitamin K dependent (VKD) with a negatively charged Gla-domain in their N -terminal end. It has been shown that Gla-domain containing proteins, such as Gas6 and protein S, are constitutively γ-carboxylated in their Gla-domain by a vitamin K dependent γ-carboxylase (27). Recently, Tsou et al. showed an essential role of this modification in the full activation of TAMs by their ligands (28). The gamma-carboxylation may affect the folding of Gas6 and protein S, hence affecting their possibility to interact with TAMs. As the gamma-carboxylation is necessary for Gas6 and protein S to interact with negatively charged

phospholipids such as phosphatidylserine, the GLA-domain may also allow the ligands to cluster on a surface, thereby enhancing their activity (29, 30). In addition to the Gla-domain, TAM ligands contain a thrombin-sensitive loop, four tandem epidermal growth factor (EGF)-like domains and a sex hormone binding globulin (SHBG)-domain in the C-terminal end. The SHBG-domain includes two laminin G (LG) domains, which are involved in binding to the TAM receptors (26).

1.2.1. *Gas6*

Thanks to the work of Varnum et al. *growth arrest specific gene 6* (Gas6) was identified as a ligand for Axl (22). Nevertheless, further research proves Gas6 to serve as a ligand for all three receptors with different affinities (Axl≥Tyro3>>Mer)(31). The structure of Gas6 exhibits a multidomain organization with a molecular weight of 75 kDa.(32). Balogh et al. (33) estimated the concentration of Gas6 in human plasma to be around 13 to 23 ng/mL (0.16 to 0.28 nM). This amount is considered very low when compared to other VKD proteins involved in homeostasis. Gas6 plays an important role in many cellular processes. As reviewed by Laurance et al. (32) Gas6 is involved in cellular homeostasis, promoting leukocyte migration and sequestration as well as platelet aggregation and hematopoiesis, as a response to injury and inflammatory states. However, it is worth stressing that to our knowledge, Gas6 is always present in complex with Axl. Interestingly, the studies show that circulating Gas6 is bound to soluble Axl (sAxl), thereby inhibiting its function as TAM agonist (34).

1.2.2. *Protein S*

Protein S is a well-characterized protein mostly due to its predominant role as an anticoagulant, serving as a cofactor for activated Protein C (aPC) (35). Protein S-aPC complex is involved in the coagulation pathway, preventing thrombin formation (36). The concentration of this protein in the blood stream is around 300 nM, which makes it much more abundant than Gas6 (37). However, it has been shown that around 60% of the circulating protein S is bound to the complement protein C4b-binding protein (C4BP), thus competing with binding to TAM receptors (38, 39). Protein S is known to be a potent Tyro3 and Mer agonist (21), although its function as a ligand for Axl remains controversial (13).

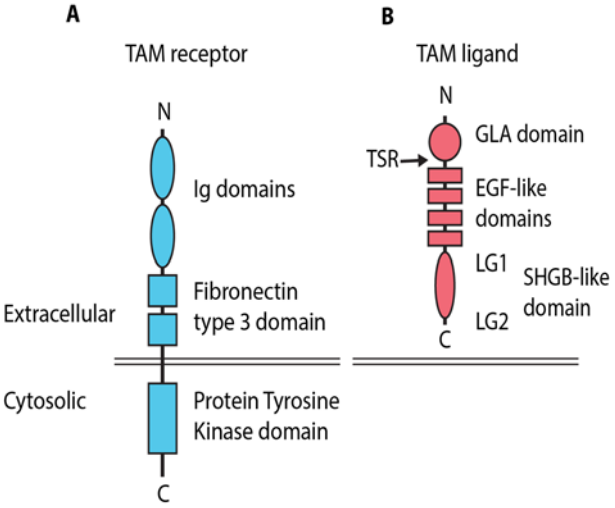

Figure 1 The structure of TAM (Tyro3, Axl, Mer) receptors and their ligands Gas6/ protein S. (A) The extracellular part of TAM receptors consists out of two immunoglobulin-like (Ig) domains, followed by two fibronectin type 3 domains. The cytosolic part is built out of a protein tyrosine kinase domain. **(B)** The TAM ligands (Gas6/protein S) consist out of a GLA domain in the N-terminus, a thrombin-sensitive region (TSR), four epidermal growth factor-like (EGF) domains and a sex hormone-binding globulin (SHBG) in the C-terminus with two laminin G (LG) domains (van der Meer et al. 2014, modified) (3).

1.3. TAM functions

TAM receptors have been studied extensively throughout past decades, due to their abundance and significance in many processes. For that reasons, many animal models have been developed and carefully investigated, thus contributing to better understanding of these multifunctional kinases.

1.3.1. *TAM/protein S/Gas6 deficient mice models*

Lu & Lemke were first to generate triple TAM knockout mice (TAM RTK TKO) (40). TAM RTK TKO mice did not develop any serious abnormalities until at the age of 4-6 weeks. At the age of 6 months, significant abnormalities, especially concerning the immune system were observed. TAM knockout's spleens and lymph nodes grew abnormally much, ranging in some cases up to ten times that of a wild type organ weight. High levels of antibodies against own tissues were detected together with elevated levels of pro-inflammatory cytokines, such as TNF-α and IL-6. It is worth noticing that the clearance of apoptotic cells (ACs) was impaired as well. Altogether, this phenotype resembles features of human autoimmune diseases such as systemic lupus erythematosus (SLE), psoriasis, and rheumatoid arthritis. Single TAM knockouts did not exhibit such dramatic phenotypes. Tyro3$^{-/-}$ mice suffered from neurological disorders, whereas Axl knockouts exhibited increased vascular permeability and dysfunctional vascular remodeling, indicating an important role of Axl in vascular inflammation (41, 42). Notably, Mer deficient mice exhibited a similar phenotype to triple knockouts, considering autoimmunity. Interestingly, only triple TAM knockout resulted in infertility, whereas double or single TAM knockout mice were both viable and fertile (41). Altogether, TAM mice knockout models confirm an important role of TAM receptors in

the AC clearance as well as in controlling immune responses (30). Protein S deficient mice cannot be bred due to lethal coagulopathy and severe vascular complications(35). Gas6 knockout mice were protected against venous and arterial thrombosis, suggesting Gas6 as a possible therapeutic target for thrombosis (43). However, a significant role of Gas6 in vasculature will be a subject of further discussion.

1.3.2. *The role of TAM RTKs in the immune response*

The first indication of an important role of TAMs in the immune response came from the work of Camenish et al. (44). They observed that Mer knockout mice are much more sensitive to lipopolysaccharide (LPS)-induced endotoxin shock compared to wild type mice. This discovery brought the attention towards the role of TAMs in controlling the immune response, as the expression of Mer in macrophages, one of the main components of the adaptive immune response, is widely known. Rothlin et al. have proposed a novel model of TAM-dependent negative regulation of the immune response (1), as presented in *Figure 2*. This model is based on the research conducted in murine-derived dendritic cells (DCs), specialized antigen- presenting cells (APCs), which work as a linker between innate and adaptive immune response. This model involves an activation of Toll-like receptors (TLRs), in response to pathogen encounter (45, 46). This leads to the activation of the transcription factor nuclear factor-kappa B (NF- κB) pathway, thereby triggering the expression of pro-inflammatory cytokines (47, 48). As a result, other stimulators of inflammatory response are activated such as type I interferon receptor (IFNAR) together with its transcription factor STAT1 (2, 49). This stimulates the expression of TAM receptors, which are activated as soon as the ligand is present. The activation of TAMs leads to the switching of the pro-inflammatory IFNAR/STAT1 signaling to inhibition of inflammation (1,49). As a result, the suppressors of cytokine signaling proteins (SOCS1 and SOCS3) are transcribed, which then block TLRs, NF- κB pathway and cytokine expression, thus resulting in inhibition of inflammation. This model reveals a significant role of TAMs in preventing autoimmunity (1, 16, 50, 51). Notably, Deng et al. (52) demonstrated that mouse-macrophage derived TLRs down-regulate Gas6 and protein S expression, hence promoting inflammatory state. Altogether, the findings show the complexity of this regulation and must be further investigated.

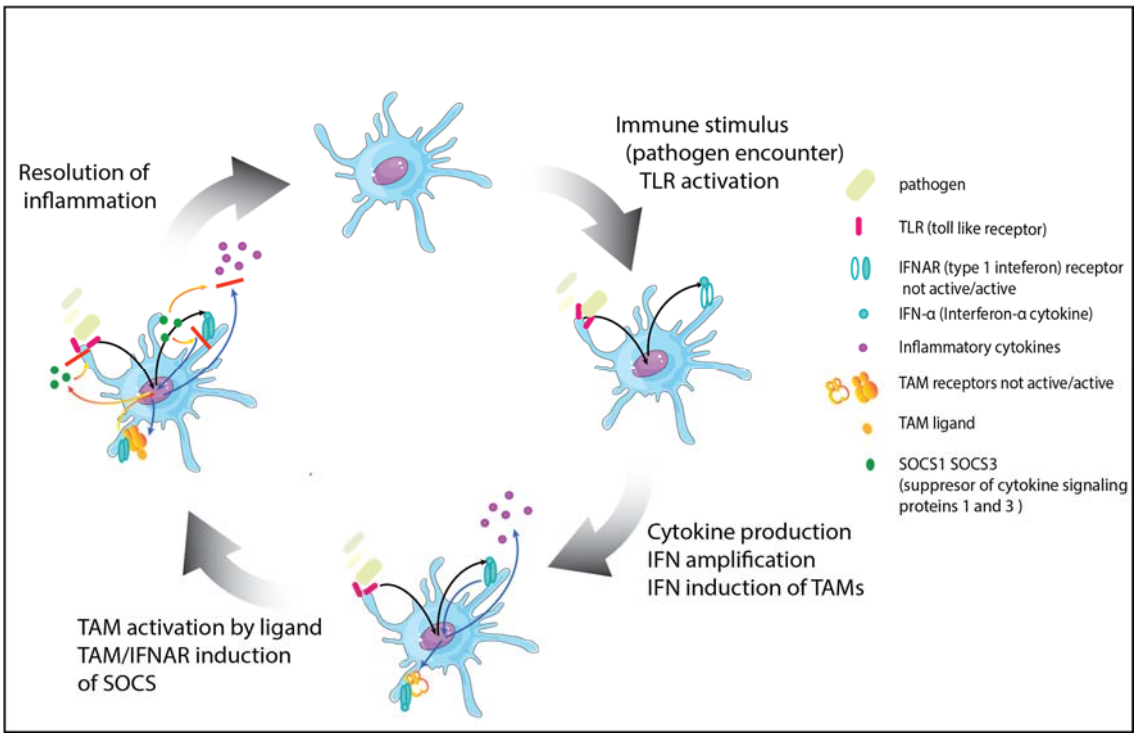

Figure 2 The key role of TAM receptors in the resolution of inflammation (murine dendritic cell model). Toll-like receptors (TLR) become activated in response to a pathogen encounter. The signal from TLR receptors is sent to the nucleus and transcription of pro-inflammatory cytokines including IFN-α is initiated (black pathway). Upon secretion, IFN-α may activate the IFNAR receptor, leading to upregulation of TAM expression (blue pathway). Upon the Gas6/protein S binding, TAMs become activated and the transcription of SOCS1 and SOCS3 cytokine and TLR-suppressor proteins is initiated (orange pathway). Upon silencing of cytokine production, the cell returns to the non-inflammatory state. (Rothlin et al. 2007, modified) (1, 2). Note that some elements in the figure were from servier.com.

Furthermore, TAM RTKs have an important role in phagocytosis of apoptotic cells (ACs) by macrophages and dendritic cells (29, 53). Briefly, this mechanism involves recognition of apoptotic cells through phosphatidylserines (PtdSer) displayed on the outer leaflet of the plasma membrane. These signals are recognized by the TAM ligands Gas6 and protein S. As mentioned before, N-terminal Gla domain binds to PtdSer, which is exposed on the apoptotic cell, thus creating "bridge" between phagocyte and apoptotic cell, leading to the TAMs activation (29, 54). The studies show that Mer is mainly involved in efferocytosis by macrophages under basal conditions (55), whereas Axl is expressed in dendritic cells, and is associated with inflammatory milieu (16, 56). Notably, the phagocytic properties of TAMs have also been observed in different cell types. Again, animal models have been extensively studied, providing numerous examples. One of them is the work of D'Cruz et al. (57), who showed that phagocytosis of outer fragments of photoreceptors by retinal pigment epithelial cell (RPE) is dependent on Mer. The studies were conducted in the Royal College of Surgeons (RCS) rats, which serve as a model of inherited blindness. As a result, the *mer* gene was mapped, by positional cloning, to the locus responsible for inherited retinal dystrophy in

this rat model. Another example is the sterility of TAM triple knockout male mice. It was concluded that TAM receptors are expressed in Sertoli cells, which are responsible for phagocytosis of apoptotic germ cells in testis. The lack of TAMs completely impaired this function, thereby proving their role in phagocytosis (41, 58). Furthermore, one of the best studied functions of TAM receptors is their role in cancer. It has been shown that both TAMs and their ligands are overexpressed in many human cancers. They are known to promote tumor angiogenesis, metastasis and immunosuppressive tumor microenvironment (59). Loges et al. (60) have observed elevated expression of Gas6 in tumor-associated macrophages compared to non-tumor associated variants, thus promoting TAM activation and proliferation of tumor cells in mice through PtdSer-based mechanism.

1.4. TAM RTKs in endothelium

Endothelial cells (ECs) line the inside of the entire network of the human vascular system. It has been shown that an adult human body contains approximately 1×10^{13} of these cells, which could be compared to an organ with the mass of 1 kg. The vasculature plays an important role in maintaining and modulating vascular tone and blood flow. Moreover, its role in the regulation of thrombosis, platelet adherence and inflammatory responses is of great importance. Chronic vascular dysfunction leads to a number of severe diseases such as atherosclerosis, thrombosis and Systemic Inflammatory Response Syndrome (SIRS)(4, 61).

1.4.1. *The role of TAM receptors in vasculature*

TAM receptors play a significant role in many vascular processes. It has been shown that TAM receptors have a significant role in angiogenesis, which is the formation of new blood vessels from pre-existing capillaries. The main known pro-angiogenic factor is vascular endothelial growth factor A (VEGF-A), which functions as a promoting agent for survival, proliferation, and migration of endothelial cells (68, 69). Studies in human endothelial cells revealed that Axl deficiency leads to the impaired cell proliferation and endothelial tube formation *in vitro*. This finding has been confirmed with the use of Axl deficient mice model, where blood vessel formation and functions were diminished, thereby proving the involvement of Gas6/Axl signaling in angiogenesis (70). Ruan et al. (71) proposed a model where Axl is ligand-independently activated by VEGF-A and VEGF-2, leading to the engagement of P13K/Akt pathway, which is crucial for VEGF-mediated angiogenesis. However, it is worth stressing that itself, Axl activation by Gas6 is not sufficient for the induction of tube formation, and to date no reports have stated pro-angiogenic functions of

Gas6 (71). Conversely, other study claims that Axl/Gas6 signaling is involved in hindering the VEGF-A-mediated angiogensis (72). Furthermore, the work of Fraineau et al. (73) proves a role of protein S in angiogenesis. According to the authors, protein S acts as an endogenous inhibitor of angiogenesis, and Mer mediates this action. They claimed the existence of protein S/Mer/tyrosine phosphatase SHP2 axis, which would inhibit VEGF receptor 2 (VEGFR2) mediated pro-angiogenic signaling pathway. This study is in agreement with Png et al.(74), who showed that tumor cells secrete sMer, which may block the normal proS/Mer-dependent inhibition of angiogenesis, thereby promoting tumor vascularization. Moreover, it has been shown that exogenous Gas6/Axl axis promotes pro-survival environment in endothelium, by acting as an antiapoptotic factor in response to TNF-α treatment (17, 75). Hasanbasic et al. concluded that this action is determined by the Gas6/Axl-mediated activation of survival pathways such as Akt phosphorylation and NF-κB (76, 77).

Notably, angiogenesis is a key process in cancer development, thus many studies report the use of small-molecule inhibitors targeting Axl signaling pathway, as a promising approach in the treatment of cancer (70).

1.4.2. *Regulation of endothelial inflammation by TAMs*

Endothelial cells become activated as a result of pro-inflammatory stimuli such as cytokines. Under inflammatory conditions, an elevated surface expression of adhesion molecules and tissue factor is initiated, leading to activation of coagulation and platelet attachment. Furthermore, activated endothelium promotes leukocyte and monocyte adhesion to the inflammatory site, triggering the innate immune response. Adhesion molecules are one of the main mediating agents of this process. One of the most quickly upregulated molecules is E-selectin, followed by intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). Together, adhesion molecules ensure the migration of leukocytes across the vasculature, which plays an important role in both chronic and acute inflammation. In practice, adhesion molecules serve as good markers for detecting endothelial inflammatory states (62, 63). Furthermore, pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) have been found to trigger strong inflammatory response in endothelial cells, resulting in elevated expression of adhesion molecules and increased endothelial permeability (4, 64), *Figure 3*.

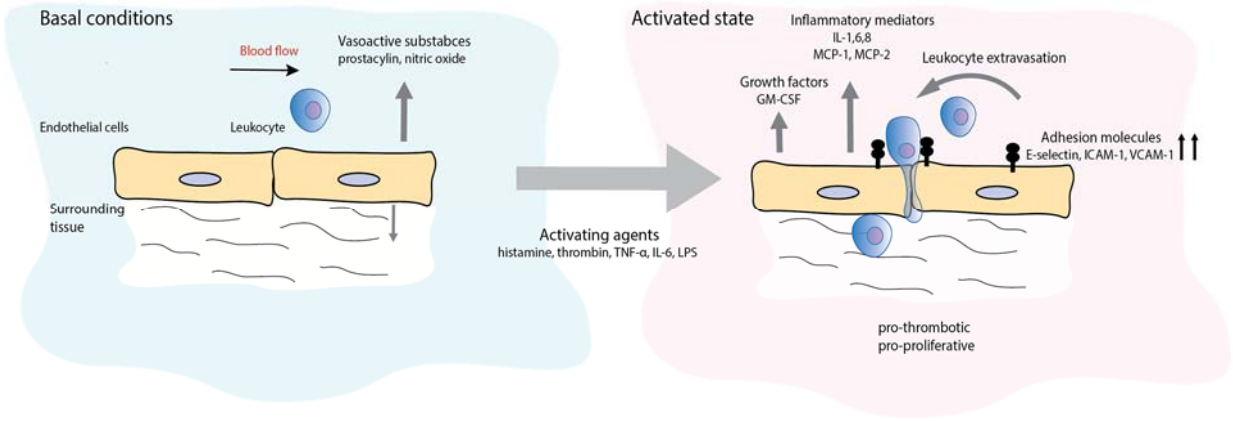

Figure 3 Endothelial inflammation. Under basal conditions endothelial cells are involved in ensuring a non-thrombogenic blood-tissue environment. They release a number of vasoactive substances such as prostacyclin and nitric oxide, which prevent platelet aggregation and regulate blood flow. Inflammatory stimuli such as histamine, thrombin, TNF-α, IL-6 or LPS activate endothelial cells, triggering immune responses. The first line of response is expression of adhesion molecules, such as E-selectin, ICAM-1 and VCAM-1, which promote leukocyte extravasation. As a result, the endothelium responds with the production of various inflammatory mediators and growth factors. Altogether, the inflammatory environment can be described as pro-thrombotic, and pro-proliferative as it promotes platelet aggregation to the site of injury and cell growth respectively (4). TNF-α, Tumor necrosis factor-α; IL-6, Interleukin-6; MCP-1 or 2, Monocyte chemoattractant protein-1 or 2; LPS, Lipopolysaccharide; GM-CSF, Granulocyte-macrophage colony-stimulating factor; ICAM-1, Intercellular adhesion molecule-1; VCAM-1, Vascular cytoadhesion molecule-1.

It has been observed that endothelial cells (ECs) express Axl and its ligand Gas6 (26). Nevertheless, whether they regulate inflammatory responses in the vasculature is still unclear, as contradictory findings have been reported. Tjwa et al. (65) concluded, based on a Gas6 knockout mice model, the involvement of Gas6 in the sequestration of leukocytes on TNF- α activated ECs. Moreover, according to this model, Gas6 amplifies endothelial activation in response to the inflammatory agents as well as is involved in graft destruction of transplanted heart in mice. Furthermore, *in vitro* studies in human umbilical vein endothelial cells (HUVEC) indicate significant reduction of the expression of TNF- α induced ICAM-1, when Gas6 and Axl have been knocked down with siRNA. Altogether, this study indicates pro-inflammatory properties of the Axl/Gas6 system. However, according to the study of Avanzi et al. (66), resting ECs (such as HUVECs) produce Gas6 as a physiologic anti-inflammatory agent. In this model, endogenously secreted Gas6 inhibits granulocyte adhesion to endothelium. The authors suggest that pro-inflammatory triggers lead to the diminishing of endothelial Gas6 and activation of proadhesive machinery. Furthermore, other animal studies indicate the role of Gas6 in promoting platelet aggregation, contributing to the venous and arterial thrombosis (43, 61). Interestingly, research in vascular smooth muscle

cells (VSMC) reveals the role of environmental alterations such as glucose concentration in modulating Axl/Gas6 signaling (67).

The presented findings show that the variation in the Gas6 concentration might influence TAM signaling in both immune cells (*Figure 2*) and in endothelium. Nevertheless, the outcome of the Gas6 /Axl signaling in vasculature remains unclear.

Despite the widely discussed role of Axl/Gas6 signaling in vasculature, one cannot underestimate the significance of Mer and Tyro3, which needs further investigations. However, as mentioned before, Tyro3 is mainly expressed in the CNS (14), thus its role in vascular endothelium might be of lesser significance.

1.5. The aim of work

The aim of this work is to elucidate the role of TAMs in inflammatory signaling in primary human endothelial cells by studying whether TAM activation by Gas6 and protein S affects the pro-inflammatory responses in endothelial cells, induced by different inflammatory stimuli, such as cytokines. Moreover, the role of TAMs will be studied by blocking the different TAM receptors and their ligands with either siRNA treatment or inhibitory antibodies, thereby providing information about their function in the inflammatory response. As readout, we will investigate the secretion of cytokines and cell surface expression of adhesion molecules.

2. MATERIALS AND METHODS

2.1. Cell culture

Human umbilical vein endothelial cells (HUVEC, Life Technologies) were cultured in M200 medium (GIBCO) supplemented with low serum growth supplement (LSGS, GIBCO), 50 U/ml penicillin and 50 µg/ml streptomycin (PS) (full/complete medium) or in serum-free medium (starvation medium). Cells were grown on 6- or 12- well plates or in T75 flasks (Thermo Scientific). Before seeding cells, culture dishes were coated with 0.1% gelatin (gelatin from porcine skin type A, Sigma-Aldrich). Cells were cultured in humidified chamber with 5% CO_2 at +37 °C. The experiments were carried out on cells in passage 2-6.

2.2. Reagents and antibodies

Recombinant Human Tumor Necrosis Factor-alpha (TNF-α) (#PHC3016) was from GIBCO. Dexamethasone (#D49002) as well as lipopolysaccharide from *Escherichia coli* (LPS) (#L3024) was purchased from Sigma-Aldrich, recombinant human Interferon gamma (IFN-γ) (#11343534) from Immunotools. For siRNA treatment control siRNA (sc-37007), human (h) Axl siRNA (sc-29763), (h) Mer siRNA (sc-37127), (h) Tyro3 siRNA (sc-36438) and (h) Gas6 siRNA (sc-35450) were from Santa Cruz Biotechnology. BSA Cohn fraction V, protease free (#B2000-100) was from Saveen Werner.

Antibodies used were as follows: anti-Mer (ab52968) from Abcam, anti-Axl (AF154), anti-Mer (AF891), anti-Tyro3 (AF859), anti-Gas6 (AB885), anti-VCAM-1 (FAB5649A), anti-ICAM-1 (BBA20), anti-E-selectin (BBA21) were from R&D Systems and anti-protein S (A0384) from Dako. Moreover, anti-Axl H-124 (sc-20741) and C-20 (sc-1096), anti-p-Tyr PY99 (sc-7020) were from Santa Cruz Biotechnology, rabbit polyclonal anti-Axl 042 (home made). Anti-β-Actin (A5441) was from Sigma-Aldrich. The secondary antibodies used for immunoblot analysis were horseradish peroxidase-conjugated anti-rabbit (P0399), anti-goat (P0449) and anti-mouse (P0447), all from Dako.

Gas6 and protein S

Recombinant human Gas6 was expressed in Hek293 cells and purified as descried in the previously published method (78) with modifications. In short, the expression media containing recombinant human Gas6 was centrifuged at 2500 rpm, 10 minutes at 4°C, and then filtered in order to remove any remaining debris. Next, Gas6-contaning media was batch purified on a DEAE Sephacel matrix (GE), followed by wash with 20 mM Tris-HCl pH 8.0, 20 mM NaCl, and elution with a 50-500 mM NaCl gradient in 20 mM Tris-HCl, pH 8.0, performed on an Äkta HPLC system (GE). The fractions with Gas6 were collected, pooled and again purified on DEAE Sephacel, with a 20-500 mM NaCl gradient in 20 mM Tris-HCl, pH 8. Further, the Gas6-containing fractions were concentrated on 10 kDa MWCO spin columns (Amicon), according to the provided manual. Next, Gas6 was purified on a Superdex 200 column, and subsequently eluted with 100 mM NaCl in 20 mM Tris-HCl, pH 8.0. Again, Gas6 fractions were collected and concentrated. Finally, 2 mg/mL BSA was added to the purified Gas6 and stored in aliquotes at -70°C.

Protein S was purified from human plasma according to (79) with the following additions: After dissolving the $BaCl_2$-precipitate in 0.2 M EDTA containing 10 mM benzamidine and 0.1 mM PMSF, ammoniumsulfate was added stepwise to 40% saturation and the mixture was incubated at +4°C o/n with stirring. Precipitated proteins were removed by centrifuging the sample at 2400 g for 20 min at +4°C. The ammoniumsulfate concentration of the supernatant was then increased to 67%, and the sample was stirred at +4°C for 1 h to allow vitamin-K dependent proteins to precipitate. The precipitate was collected by centrifuging the sample at 15 000g for 20 min at +4°C and further dissolved in 0.1M sodium phosphate pH 6.0, 10 mM benzamidine, 0.1 mM PMSF, 1% Tween-20. The sample was then dialyzed o/n against 0.1M sodium phosphate pH 6.0, 10 mM benzamidine, 0.1 mM PMSF with three buffer changes. The sample was filtered through a 0.45 μm filter and applied to a DEAE Sephacel matrix (GE HEalthcare) equilibrated with 0.1M sodium phosphate pH 6.0, 10 mM benzamidine, 0.1 mM PMSF. The column was washed with 0.1M sodium phosphate pH 6.0, 10 mM benzamidine, 0.1 mM PMSF, 1% Tween-20 followed by 0.1M sodium phosphate pH 6.0, 100 mM NaCl, 10 mM benzamidine. Next, washed with 0.1 mM PMSF before eluting bound proteins with a linear gradient of 100-700 mM NaCl in 0.1M sodium phosphate pH 6.0, 10 mM benzamidine, 0.1 mM PMSF. Fractions containing free protein S were pooled and dialyzed against 20 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM benzamidine, 0.1 mM PMSF.

The sample was passed through a Blue Sepharose column (GE Healthcare) equilibrated with 20 mM Tris-HCl pH 7.5, 1 mM EDTA, 100 mM NaCl, 10 mM benzamidine, 0.1 mM PMSF and the flow through containing protein S was collected. To ensure complete removal of C4BP-bound protein S, the flow through was passed through a 5 mL HiTrap column (GE Healthcare) coupled with an in-house monoclonal antibody against C4BP (MK104). The unbound fraction was further purified on a HiTrap-column coupled with an in-house monoclonal antibody against protein S (MK21). All the monoclonal antibody affinity columns were equilibrated with 20 mM Tris-HCl pH 7.5, 100 mM NaCl, 2 mM $CaCl_2$, 10 mM benzamidine before use, and bound proteins were eluted with 100 mM Glycine-HCl pH 2.7 into tubes containing 1M Tris-HCl pH 9.0 to neutralize the pH. The pure protein S was dialyzed against TBS containing 2 mM $CaCl_2$ and stored in aliquots at -80°C.

2.3. Cytokine stimulation

2.3.1. *Acute and chronic cytokine stimulation*

HUVECs were grown until 70% confluence. In the acute stimulation model, cells were first washed once with DPBS for cell culture (GIBCO) then transfected with siRNA against Axl/Mer/Tyro3/Gas6 with the use of Oligofectamine Transfection Reagent from Invitriogen, according to the manual provided by the producer. After incubating for 4h in the serum-free conditions, the serum was added to the transfection mixes to 1% corresponding to the amount in full medium. After 20h incubation, the medium was changed and incubation continued for additional 7 hours to allow cells to recover from the transfection. After that, cells were washed once with DPBS and stimulated for 20 hours with 10 ng/mL TNF- α in starvation medium. Supernatants were collected for ELISA analysis and stored in the -20°C. Cells were trypsinized and FACS analysis was performed as described later on.

In the chronic stimulation variant, cells were first pre-stimulated for 48h with 10 ng/ml TNF-α in complete medium, followed by wash with DPBS and transfection with siRNA against Axl/Mer/Tyro3. Again, serum was added to the transfection mix after 4 hours, and the incubation continued for 20 hours. Next, cells were washed with DPBS and stimulated for 24 hours with 10 ng/mL TNF- α in serum-free medium, followed by FACS and ELISA analysis, as in the acute stimulation variant.

2.3.2. *Cell stimulation with TAM ligands and antibodies*

HUVEC cells were grown until 90% confluence. Cells were washed once with DPBS for cell culture, then stimulated for 20 hours with 10 ng/mL TNF- α with or without either 5 µg/mL of

anti-Axl (AF154), anti-Mer (AF891) or anti Tyro3 (AF859) in serum-free medium. In another variant, HUVEC cells were grown until 90% confluence. However, prior the stimulation cells were washed once and pre-starved for 2 hours in serum-free medium. Next, cells were stimulated for 20h with 10ng/mL TNF- α supplemented with either 200 ng/mL Gas6, 5 μg/mL protein S, 2.5 μg/mL anti-Gas6 (AB885) or 50 μg/mL anti-protein S (DAKO) in serum-free medium. The stimulation was followed by FACS analysis for adhesion molecule expression as described further on.

2.3.3. *TAM upregulation upon cytokine stimulation*

HUVEC cells were grown until 90% confluence, then washed once with DPBS and stimulated with 10 ng/mL TNF- α in full medium or serum-free medium for different time points. In another variant, 90% confluent HUVEC cells were washed once as above and stimulated for 20 hours with different concentrations of LPS or IFN-γ in complete medium or stimulated with 40 ng/mL (0.1μM) dexamethasone in complete medium for different time points. After the stimulations, the cells were subjected to the protein expression analysis by western blot, as described further on.

2.4. Protein expression analysis – western blot (WB)

Cells were washed twice with ice-cold DPBS, then lysed on ice with RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, pH.8.0, 0.1% SDS, Triton X-100, 0.5% deoxycholate) supplemented with HALT protease phosphatase inhibitors (#1861281) from Thermo Scientific and 2mM sodium orthovanadate (#450243) from SIGMA. A cell scraper was used in order to detach cells from plate and lysates were transferred to pre-chilled eppendorf tubes. After 10 to 15 minutes incubation on ice, lysates were centrifuged 10 000 rpm 1 min at +4°C to remove cell residues and nuclei. The protein content in the cell lysates was measured with Pierce BCA Protein Assay Kit (#23227) from Thermo Scientific. Cell lysates (10 μg or 5 μg of total protein) was separated on 4-15% SDS-PAGE gels (Bio Rad,) under reducing conditions and blotted (Bio Rad Trans-Blot Turbo Transfer Starter System) onto PVDF membranes (Bio Rad). Membranes were blocked in blocking buffer; 3% fish gelatin from Nordland (#9000-79-8) diluted in immunowash (50 mM Tris HCl, 0.15M NaCl, 0.01% Tween-20, pH.8.0.). Next, probed for 1h at room temperature (RT) or over night at +4°C with primary antibodies in the blocking buffer. Membranes were washed with the immunowash and probed with HRP-conjugated secondary antibodies for 1h at RT. The blots were visualized with Bio Rad Molecular Imager Gel Doc XR+ system. Prior the visualization

the membranes were incubated for 1 minute with the Chemiluminescent HRP Substrate (#WBKLS0500) from Millipore. Western blot analysis and quantification was done with the use of the Bio Rad ImageLab 4.0.1 program. Note that all western blot results are presented as normalized against loading control. Membranes were stripped with 0.4 M NaOH and re-probed after a washing and blocking step as above.

2.5. Immunoprecipitation (IP)

HUVECs were grown until 90% confluence. Cells were washed once with DPBS and incubated for 2 hours in serum-free medium, followed by 15 minutes stimulation with the appropriate concentrations of either anti-Axl (AF154) or Gas6. After that, lysates and protein concentration was obtained with the same procedure as for the WB. Equal lysates amounts (at least 150 µg) were suspended in RIPA- lysis buffer supplemented with protease and phosphatase inhibitors as above. Next, rec-Protein G-Sepharose 4B conjugate beads (#101242) from Invitrogen, were added together with 40 µg of anti-Axl 042 (home made) or with 2 µg of anti-Axl (AF154). Cell lysates were incubated for at least 1.5 hours at +4°C with rotation. Immunoprecipitates were pelleted for 5 min with 1000g at +4°C, washed once with phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH. 7.4) and pelleted again. Lysates were separated on 4-15% SDS-PAGE gels under reducing conditions, followed by transfer onto PVDF membranes. The membranes were blocked in 3% fish gelatin in immunowash and first probed with anti- p-Tyr (Santa Cruz), then with anti-Axl H-124 or anti-Axl C-20 (Santa Cruz). The western blots were developed and analyzed as above.

2.6. Fluorescence-activated cell sorting (FACS)

Cells were rinsed once with DPBS and trypsinized with 200µl (6-well plate) or 100µl (12-well plate) of TrypLE Express (GIBCO), then transferred to pointy bottom mictrotiter plates (NUNC) and centrifuged at 700g for 3 minutes at +4°C. Supernatant was removed and the cells were re-suspended in 150µl full medium, centrifuged as above. Next, the cells were washed with 1% BSA in PBS, then stained with antibodies against VCAM-1, ICAM-1 or E-selectin (R&D) diluted in 1% BSA in PBS. After 1h incubation (on ice, under tin foil), the cells were pelleted as above and re-suspended in 100µl of 1% BSA in PBS. Cell suspension was transferred to FACS tubes containing 1 ml of the 1%BSA in PBS and FACS analysis was performed on the Partec Cyflow Space flow cytometer with the use of FlowMAX 2.5 program. The cells were gated, based on forward scatter (FCS) and side scatter

(SCC). ICAM-FITC and E-selecting FITC signals were monitored by measuring the geometrical mean fluorescence on channel FL1 (green), whereas VCAM-APC was measured on FL6 (red). At least 5000 cells per sample were counted. Data analysis was performed with the use of the FlowJo 8.8.7 program.

2.7. Enzyme-linked immunosorbent assay (ELISA) for cytokines

After various stimulation, cell supernatants were collected and stored at -20°C. Before use, supernatants were centrifuged 10 000 rpm for 5 minutes and transferred to fresh tubes. ELISAs were performed according to the manual provided by the R&D Systems: IL-6 (#DY206), GM-CSF (DY215) and CCL2/MCP-1 (#DY279).

2.8. Statistics

Statistical analysis was performed in the GraphPad Prism Version 6.0e. One-way ANOVA with Dunnet's multiple comparison test was used to analyze the statistical significance of differences between the groups. P-values below 0.05 were considered to be significant. Experiments were carried out on at least three different occasions unless specified differently in the figure legend.

3. RESULTS

In order to elucidate the role of TAM receptors in the endothelial inflammatory response, many different approaches have been considered. Here, some of them are presented, showing the complexity and multifunctionality of these receptors. It is worth stressing that our observations are based on Axl and Mer receptor activity, as we were unable to detect the expression of Tyro3 in human umbilical vein endothelial cells (HUVEC) (data not shown).

3.1. Axl promotes TNF-α-mediated endothelial inflammation

It has been shown that Axl and Gas6 knockdown in human primary endothelial cells causes a significant reduction of ICAM-1 expression, in response to TNF- α stimulation (65). In order to validate this finding, we performed a similar experiment investigating both the expression of adhesion molecules as well as secretion of inflammatory cytokines in response to TNF- α in TAM-deficient and TAM-sufficient cells. HUVECs treated with siRNA against the different TAM-receptors were stimulated for 20h with TNF- α as presented in *Figure 4*.

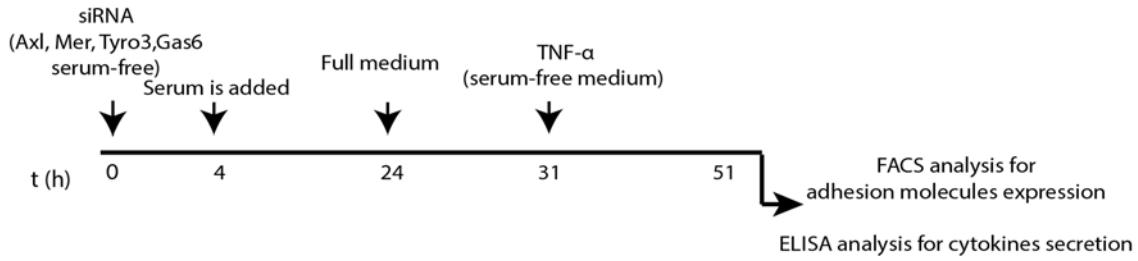

Figure 4 Experiment overview. HUVEC cells were first treated with siRNA against different TAM receptors or Gas6 in serum-free conditions. After 4 hours treatment, serum was added and the incubation continued until next day. Then, the medium was changed, and after 7 hours of recovery the cells were stimulated with 10 ng/mL TNF-α in serum-free medium for 20 hours. After that, FACS analysis for the expression of adhesion molecules on the cell surface, and ELISA analysis for secretion of the inflammatory cytokines were performed.

We confirmed the effectiveness of the TAM knockdown by western blot (*Figure 5, A*). We observed a significant reduction in the expression of ICAM-1 and VCAM-1 in response to TNF-α, in Gas6 deficient cells compared to control cells. Furthermore, Axl deficiency resulted in a significant reduction of TNF-α -induced VCAM-1 expression. We did not see any significant changes in the expression of adhesion molecules upon Mer or Tyro3 siRNA knock down. In addition, TAM knock-down did not affect the basal expression of ICAM-1,

VCAM-1 or E-selectin in the absence of TNF-α stimulation (*Figure 5, B*). Next, we evaluated the secretion of IL-6, MCP-1 or GM-CSF into the cell culture supernatants upon TNF-α stimulation (*Figure 5, C*). We observed a significantly lower secretion of IL-6 and MCP-1 by Axl deficient cells compared to control cells upon TNF- α exposure. In contrast, GM-CSF secretion in response to TNF- α was enhanced in Mer deficient cells.

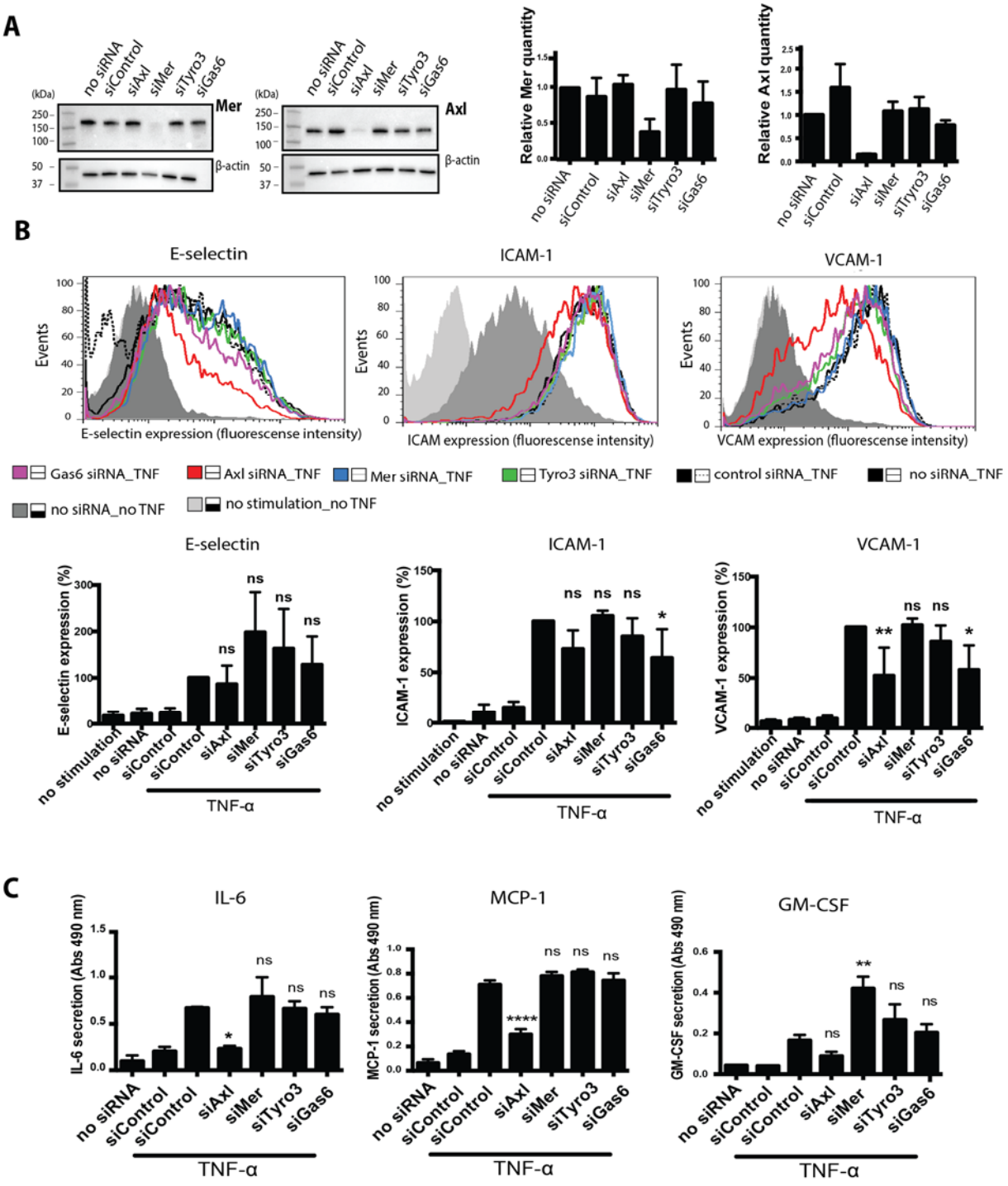

Figure 5 Axl promotes TNF-α mediated endothelial inflammation. (A) Western blot analysis for TAM knockdown efficiency. After siRNA treatment, cell lysates were prepared and blotting procedure was performed as described in the Materials and Methods. Membranes were first probed for either Axl or Mer, then re-probed for β-actin as a loading control, as presented on the left part of the panel A. Blot calculations (n=3) are presented on the right side of the panel A. **(B)** FACS analysis for the E-selectin, ICAM-1, VCAM-1 expression. FACS histograms (upper part of the panel B) as well as quantification of expression (n=3), normalized against the control siRNA, TNF-α stimulated sample, are presented in the lower part of the panel B. Please note that "no stimulation" refers to the control sample, which was incubated in full medium, "no siRNA" means that the sample was treated the same way as the stimulated variants, only with no siRNA added. **(C)** ELISA analysis for cytokine secretion (n=2). Note that raw absorbance values were plotted, due to the high signals, exceeding the standard curve. *P <0.05, **P <0.01, ***P <0.001, ****P<0.0001 (One-way ANOVA with Dunnet's multiple comparison test), ns-not significant.

3.2. Effects of TAMs during chronic inflammation

To investigate the role of TAMs on adhesion molecule expression and cytokine secretion in a more chronic inflammatory setting we set up an experiment as presented in the *Figure 6.* In this model, prior to the siRNA knock-down, we treated HUVEC cells for two days with TNF-α in complete medium. After that, we performed TAM siRNA knock-down, followed by additional stimulation with TNF- α in starvation medium for 24 hours. As readout, we monitored adhesion molecule expression by FACS and cytokine secretion by ELISA.

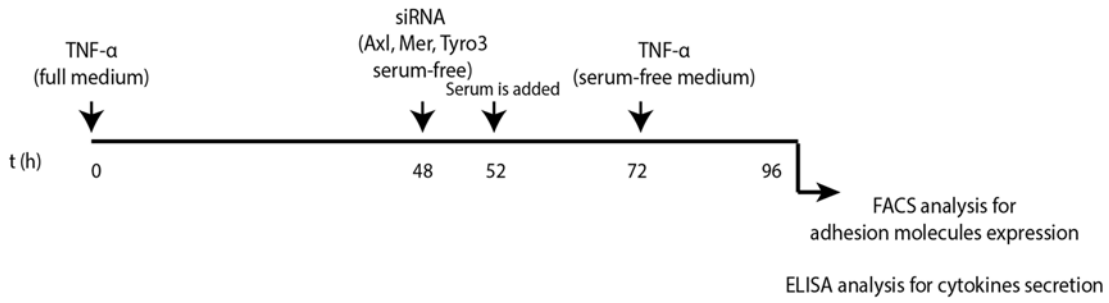

Figure 6 Experiment overview. HUVEC cells were pre-stimulated for 48 hours with 10 ng/mL TNF-α in complete medium, followed by treatment with anti-TAM siRNA in serum-free medium. After 4 hours, serum was added and the cells were allowed to recover over night. Cells were then stimulated with 10 ng/mL TNF-α in starvation medium and incubated for 24 hours. After that, the analysis for adhesion molecule expression by FACS, were carried out. Prior to the FACS analysis, supernatants were collected and subjected to the ELISA analysis, in order to check for secretion of the inflammatory cytokines.

First, we evaluated the efficiency of siRNA knock-down by western blot (*Figure 7, A*). We observed that Mer knock-down was not as efficient as that of Axl in these experiments. Next, we measured the expression of ICAM-1, VCAM-1 and E-selectin in TAM-deficient cells in response to TNF- α stimulation (*Figure 7, B*). We observed that Mer knock-down leads to a slight decrease in the expression of VCAM-1 and a significant increase in the expression of E-selectin, as compared to control cells. In contrast, Axl deficiency caused a significant decrease in the expression of E-selectin. Notably, TAM knock-down did not affect the basal

expression of ICAM-1, VCAM-1 or E-selectin in the absence of TNF- α stimulation. Further, we investigated the secretion of inflammatory cytokines by performing ELISA on supernatants collected after the treatment (*Figure 7,C*). We observed a tendency of increased GM-CSF secretion when Mer was knocked down and reduced secretion when Axl was knocked down, but this did not reach statistical significance. In order to study whether the initial 48-h TNF- α stimulation altered Mer or Axl expression in the cells, we studied Axl/Mer expression changes by western blot (*Figure 7,D*). No significant changes in either Mer or Axl expression levels were observed.

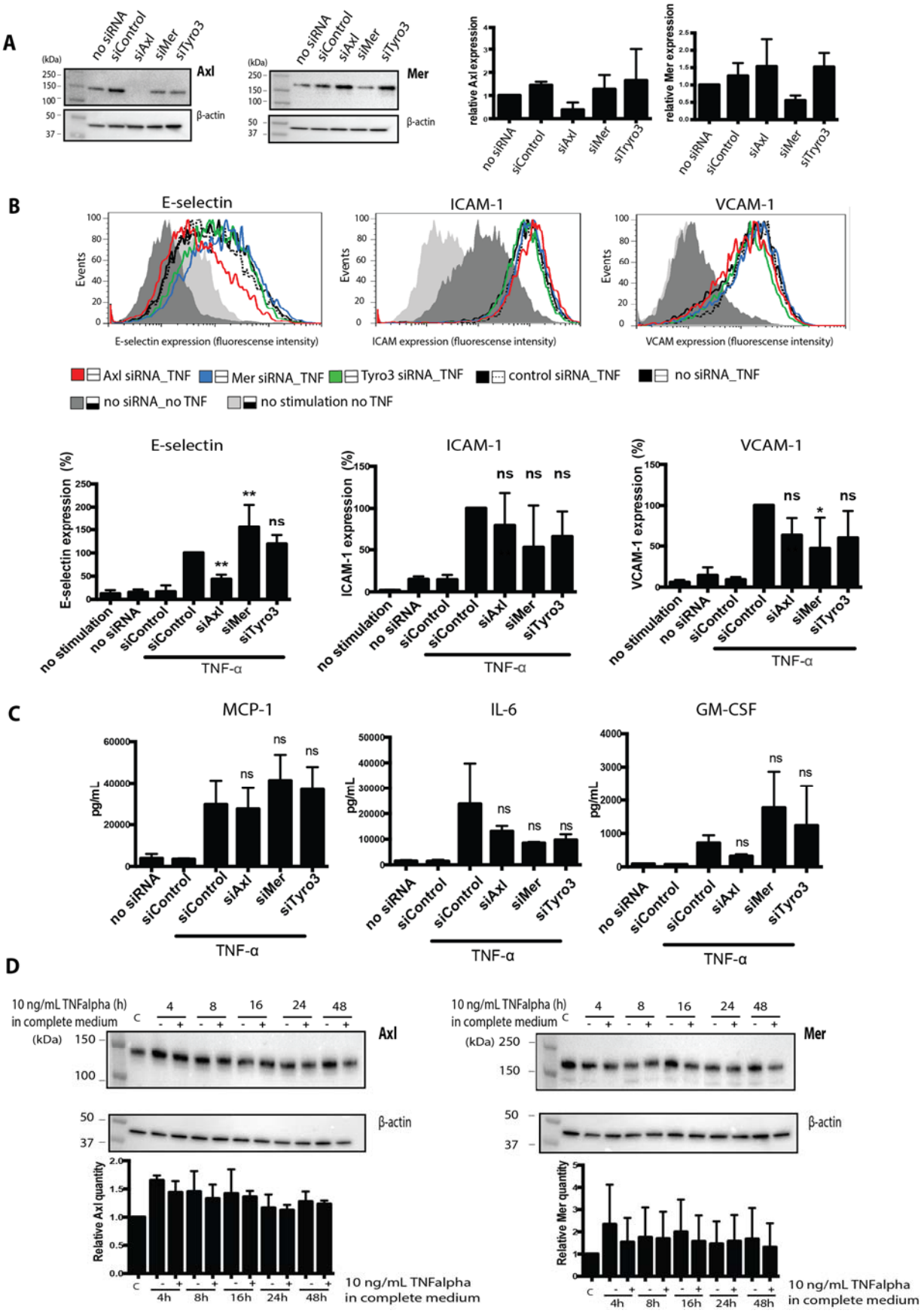
A
no siRNA
siControl
siAxl
siMer
siTyro3
(kDa)
Axl
β-actin
Mer
relative Axl expression
relative Mer expression
B
E-selectin
ICAM-1
VCAM-1
Events
E-selectin expression (fluorescense intensity)
ICAM expression (fluorescense intensity)
VCAM expression (fluorescense intensity)
Axl siRNA_TNF
Mer siRNA_TNF
Tyro3 siRNA_TNF
control siRNA_TNF
no siRNA_TNF
no siRNA_no TNF
no stimulation no TNF
E-selectin expression (%)
ICAM-1 expression (%)
VCAM-1 expression (%)
ns
no stimulation
TNF-α
C
MCP-1
IL-6
GM-CSF
pg/mL
D
10 ng/mL TNFalpha (h)
in complete medium
Relative Axl quantity
Relative Mer quantity
4h
8h
16h
24h
48h

Figure 7 Effects of TAMs during chronic inflammation. (A) The TAM knock-down efficiency was confirmed by western blot. After 96 hours, cell lysates were prepared and subjected to Axl or Mer expression analysis, with β-actin serving as a loading control; blot calculations (n=3) are presented on the right site of the panel A. **(B)** FACS analysis for adhesion molecule expression upon TNF-α stimulation. Histograms are presented in the upper part with corresponding expression quantifications (n=4), normalized against the control siRNA (siControl), TNF- α stimulated sample, placed underneath. Note that "no stimulation" refers to the sample incubated in full medium throughout the experiment. **(C)** ELISA analysis for the secretion of inflammatory cytokines. After 96 hours, cell supernatants were collected and analyzed with commercially available ELISA kits. **(D)** Axl/Mer expression analysis in HUVEC cells in response to TNF- α stimulation in full medium. After various time points, cell lysates were prepared and subjected to protein expression analysis by western blot, and probed first for Axl or Mer, then for β-actin as a loading control. Blot calculation (n=2) is presented in the lower part of the panel. *P <0.05, **P <0.01 (One-way ANOVA with Dunnet's multiple comparison test), ns-not significant.

3.3. Anti-Axl antibody activates the receptor

In order to test the effect of different external TAM-blocking agents, we treated HUVEC cells with TAM antibodies simultaneously to TNF- α stimulation, and monitored the expression of adhesion molecules. We observed no differences in expression of ICAM-1, VCAM-1 and E-selectin between cells treated with TAM-antibodies together with TNF- α and cells treated with TNF-α alone (*Figure 8, A*). A study by Zagorska et al. (56) suggests that polyclonal TAM-reactive antibodies mediated TAM-activation in mouse bone marrow-derived macrophages. To investigate whether our antibodies stimulated TAM-activation in HUVEC cells, we performed Axl immunoprecipitation from cells stimulated with either Gas6 or the same anti-Axl antibodies used in the blocking experiment (R&D Systems). The immunoprecipitated Axl was clearly tyrosine phosphorylated, indicating Axl activation, in both anti-Axl and Gas6 stimulated cells (*Figure 8, B*). This shows that the antibody treatment, at least in the case of Axl, actually activated the receptor as opposed to blocking it, which might explain the discrepancies in the regulation of adhesion molecule expression compared to Axl siRNA treated cells.

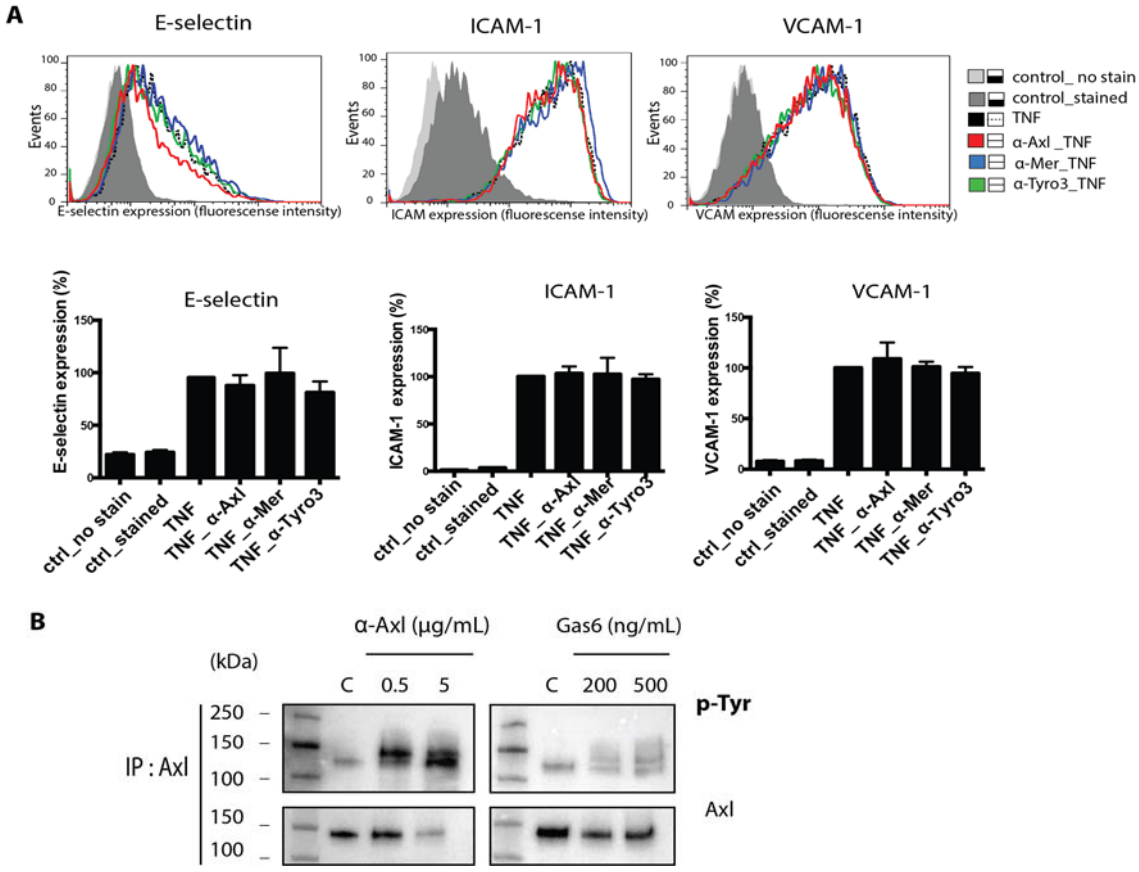

Figure 8 Anti-Axl antibody activates the receptor. (A) FACS analysis for adhesion molecule expression in HUVEC cells stimulated for 20 hours with 5μg/mL anti-Axl (AF154) (α-Axl), anti-Mer (AF891) (α-Mer) or anti-Tyro3 (AF859) (α-Tyro3) together with 10ng/mL TNF- α, or TNF- α alone, in serum-free medium. Histograms are presented in the upper panel A; note that "control, no stain" refers to a not stimulated, full medium incubated sample, where no antibody against adhesion molecules was added, unlike "stained", where this antibody was added. Lower panel A, represents adhesion molecules expression quantification (n=3), normalized against the TNF- α stimulated sample. **(B)** Axl immunoprecipitation from the anti-Axl (AF154) or Gas6 stimulated HUVEC cells. Cells were treated with either anti-Axl or Gas6 in serum free medium for 15 minutes. Lysates were prepared and blotting was performed as described in the Materials and Methods. Blots were probed first for p-Tyr, then for total Axl as a loading control.

3.4. Cell secreted protein S inhibits adhesion molecules expression

In order to study the role of TAM ligands in the endothelial inflammation, we stimulated HUVEC cells with TNF- α together with ligand or anti-ligand antibody for 20 hours in serum-free medium. This gave us an opportunity to monitor the contribution of exogenously added or endogenously produced ligand to the endothelial inflammatory response. First, we confirmed the activation of Axl receptor by Gas6 in HUVEC cells with Axl immunoprecipitation, and we observed a strong signal from tyrosine phosphorylation already after stimulating with 200 ng/ml of Gas6 (*Figure 9, A)*. Next, we performed FACS analysis for adhesion molecule expression. We did not observe any effect on adhesion molecule expression, when stimulating HUVECs with anti-Gas6 antibody (α-Gas6) together with TNF- α as opposed to TNF- α alone, however the treatment with exogenous Gas6 caused a significant reduction of TNF- α -induced E-selectin (*Figure 9,* panel *B).* We performed the same analysis for proteins S and in this case, stimulation with exogenous protein S together with TNF- α did not cause any significant changes in the expression of adhesion molecules. However anti-protein S antibody (α-protein S) treatment led to a significant increase in TNF-α mediated expression of ICAM-1, VCAM-1 and E-selecting, by around 50% (*Figure 9,* panel *C)*.

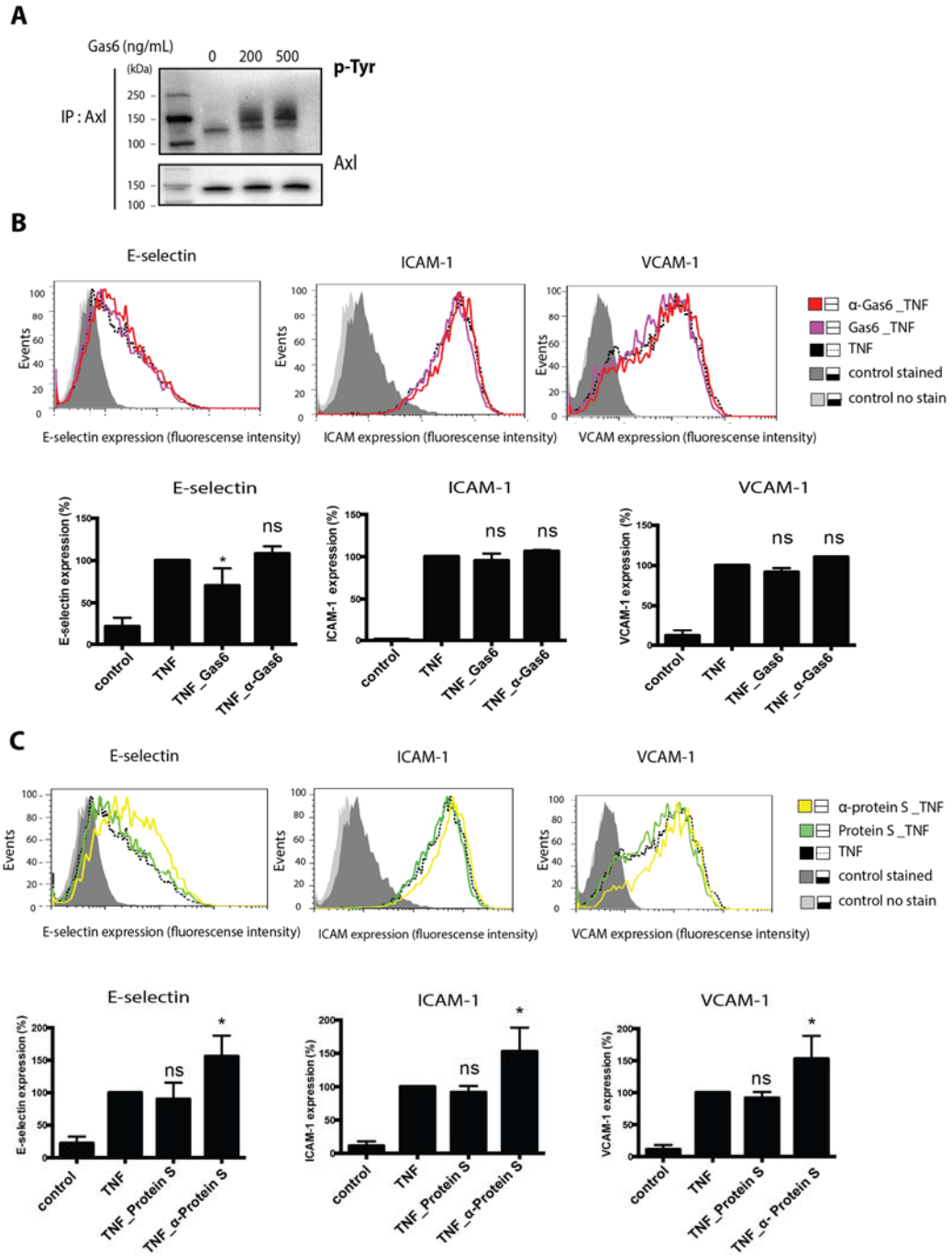

Figure 9 Cell secreted protein S inhibits adhesion molecule expression. (A) Axl activation in HUVECs pre-starved for 2 hours and then left unstimulated (control) or stimulated for 15 minutes with Gas6 in serum-free medium. Blots were first probed for p-Tyr to look for the receptor activation, then for total Axl as a loading control. **(B)** FACS analysis of adhesion molecules expression in HUVECs, pre-starved for 2 hours, then stimulated for 20 hours with 10 ng/mL TNF- α alone or with, either 200 ng/mL Gas6 or 2.5 µg/mL anti-Gas6 (α-Gas6) in starvation medium or starvation medium alone (control), n=3. **(C)** Adhesion molecule expression in HUVECs, pre-starved for 2 hours, then stimulated for 20 hours with 10 ng/mL TNF-α alone or with, either 5 µg/mL protein S or 50 µg/mL anti-protein S (α-protein S) in starvation medium or starvation medium alone (control), n=3. *P <0.05 (One-way ANOVA with Dunnet's multiple comparison test), ns-not significant.

3.5. Mer receptor expression is upregulated by starvation

It has been shown that upregulation of TAM receptor expression can be triggered by different agents, both pro and anti-inflammatory. We decided to test some of these stimuli in human endothelial primary cells, as most of the studies have been carried out in immune cells. First, we tested whether TNF- α in serum-free medium would affect Axl/Mer expression in HUVEC cells, as we could previously observe a strong inflammatory response upon this stimulation. We treated the cells with either serum-free medium alone or starvation medium supplemented with TNF- α, for 24 or 48 hours (*Figure 10, A*). We observed a strong down-regulation of Axl expression upon the TNF- α treatment; however further repetitions did not prove it as a pattern. On the other hand, Mer expression was not affected by TNF- α stimulation, nevertheless starvation potently upregulated this receptor. According to the study of Zagorska et al. (56), Axl expression is upregulated by inflammatory stimuli such as lipopolysaccharide (LPS) and interferon- γ (IFN- γ) in mouse bone marrow-derived macrophages. We performed similar analysis in HUVEC cells, stimulating with different concentrations of either LPS or IFN- γ for 20 hours in complete medium followed by western blot analysis (*Figure 10, B*). As a result, we did not see any significant changes in the expression pattern of either Axl or Mer, only slightly elevated levels of Axl upon the stimulation with high concentrations of IFN- γ. Further, we decided to investigate Axl and Mer expression in endothelial cells upon the stimulation with the immunosuppressive glucocorticoid dexamethasone (Dex), as it was reported to be a potent upregulating agent for Mer expression in human monocyte-derived macrophages (80) as well as in mouse bone marrow-derived macrophages (56) (*Figure 10 ,C*). We stimulated HUVECs with 40 ng/mL Dex in complete medium for different time points followed by western blot analysis. Dex stimulation did neither affect the expression of Mer nor Axl.

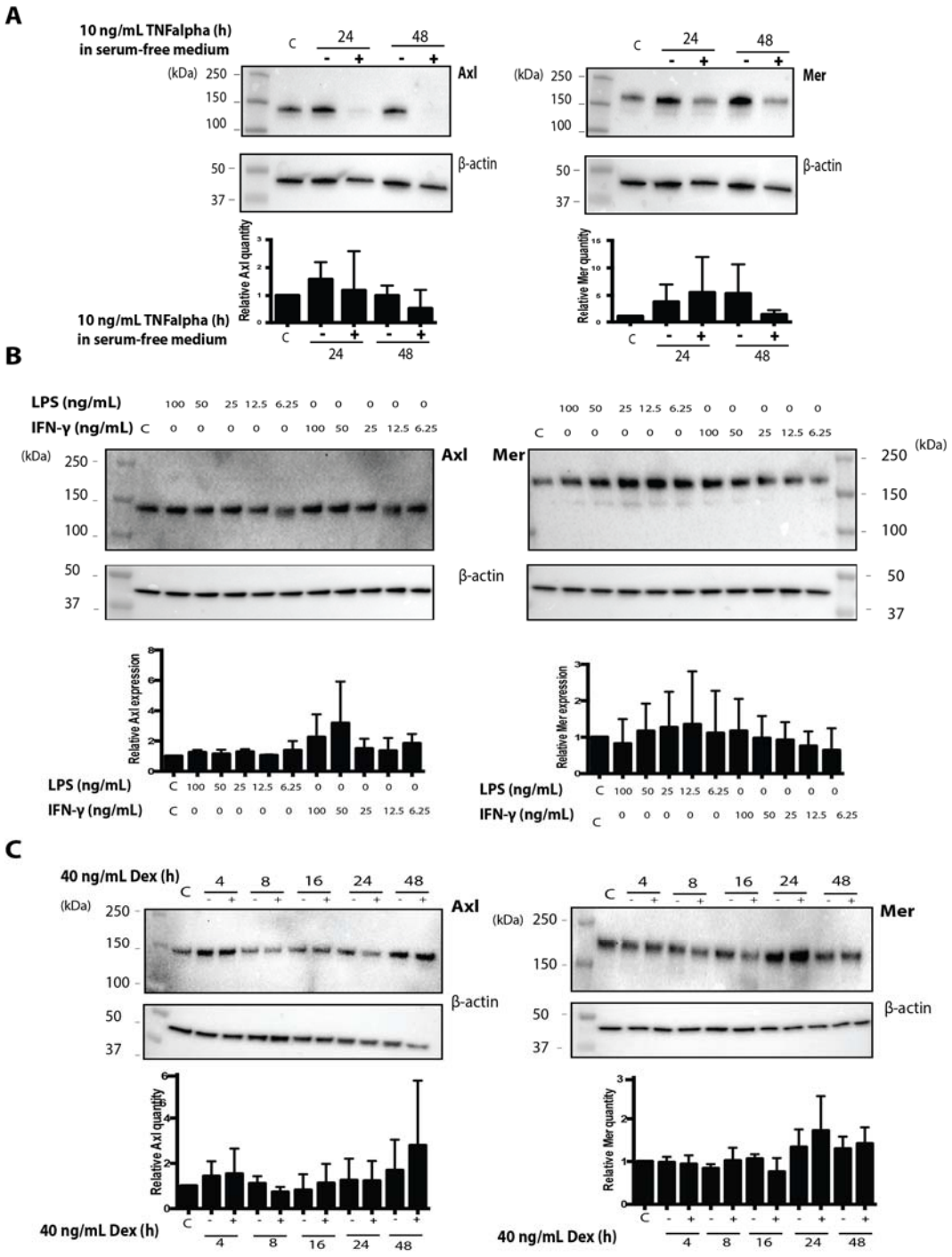

Figure 10 Starvation upregulates Mer expression. (A) Axl/Mer expression upon stimulation with 10 ng/mL TNF- α in serum-free medium or starvation medium alone in HUVEC cells. After 24 and 48 hours lysates were prepared and analyzed by western blot for Axl and Mer expression. The blot quantification (n=2) is presented in the lower part of the panel. Note that "control" corresponds to a sample incubated in complete medium throughout the experiment. **(B)** Immunoblot analysis of Axl and Mer upon 20 hours stimulation in complete medium with inflammatory agents - LPS and IFN-γ. Blot quantification (n=2) is presented underneath with "control" referring to the sample incubated in full medium. **(C)** Immunoblot analysis of Axl and Mer expression after stimulating for different time points with the immunosuppressive agent dexamethasone (Dex) in complete medium. Again, "control" refers to the complete-medium incubated sample. Note that in the presented results β-actin serves as a loading control.

4. DISCUSSION

TAM receptors are ubiquitously present in the human body, which determines their differentiated role in many processes. Their significance in restoring homeostasis has been extensively studied. Moreover, TAM RTKs play an important role in the vasculature, by affecting angiogenesis and promoting cell survival. Notably, they have been reported to modulate human endothelial inflammation, but with conflicting results provided in different studies (65, 66). In this project, we decided to further investigate the role of TAMs in inflammatory signaling in primary human endothelial cells, hence contributing to better understanding of these kinases in the inflammatory response.

4.1. Axl/Gas6 signaling plays a pro-inflammatory role in endothelial inflammation

We studied the role of Axl and Mer in the inflammatory signaling in HUVEC cells, by measuring adhesion molecule expression and inflammatory cytokine secretion. We developed two experimental models, which enabled us to monitor TAM response in the acute (*Figure 4*) and chronic (*Figure 6*) inflammatory environments. We found that Axl and Gas6 signaling exhibits pro-inflammatory properties, as their knock-down efficiently reduced the expression of adhesion molecules and secretion of inflammatory cytokines in HUVEC cells, in response to TNF-α-mediated inflammation. This conclusion is in agreement with Tjwa et al. (65). On the other hand, we observed that Mer knock-down leads to the increased expression of both adhesion molecules and inflammatory cytokines, thereby suggesting anti-inflammatory properties. It is worth noticing that conclusions regarding adhesion molecules in the acute variant are based on the observations for ICAM-1 and VCAM-1 expression, since we did not obtain any significant results for E-selectin. This might be due to the fact that E-selectin expression peak has been shown to be around 4-6 hours after the acute cytokine stimulation, whereas ICAM-1 and VCAM-1 effect persists for around 72 hours (81). This indicates that E-selectin might play a bigger role after stimulating with TNF-α for 6 hours instead of 20, which is one of our future plans. We did, however, observe significant values for the regulation of E-selectin expression by TAMs in the chronic stimulation variant (*Figure 7, B*). This might be difficult to explain, as there are many limitations regarding this model, which will be discussed later on. Further, we noticed discrepancies in the ELISA results between acute (*Figure 5, C*) and chronic (*Figure 7, C*) models. We hypothesize that low expression

level of IL-6 and MCP-1 in the chronic variant compared to the acute model, might be the result of negative feedback loop regulating cytokine expression. Cells, after being exposed to a chronic inflammatory condition, switch on various cell rescue programs, and one of them might be the down-regulation of expression of these inflammatory agents. We could see, however, a similar pattern of GM-CSF secretion in both models. This might be due to the fact that GM-CSF, which serves as a leukocyte growth factor (82), might be differentially regulated than IL-6 and MCP-1. It is worth stressing, that there are many limitations regarding the chronic inflammatory model, as to the effect of pre-stimulation step. We managed to examine the Axl/Mer expression levels after the pre-stimulation step (*Figure 7, D*), and we concluded no changes. Nevertheless, we need more controls to validate this model. One of them is to investigate the effect of pre-stimulation on adhesion molecule expression after the siRNA knock-down, which is our future plan. Altogether, these experiments reveal the heterogeneity of Axl/Mer function in endothelial inflammation. Interestingly, Zagorska et al. have reported similar conclusion (56), based on studies in mouse bone marrow-derived immune cells. In addition, Gas6 has been reported as an indicator of inflammation, as increased amounts of this protein in plasma are observed in septic patients (83, 84). Based on our and Tjwa et al. (65) observations, it is possible that increased circulating Gas6 promote endothelial inflammation in sepsis. These conclusions are suggesting Gas6 as a possible target for the treatment of sepsis and transplantation-induced organ rejection (65). Nevertheless, it is worth keeping in mind that Gas6 has been reported to have an immunosuppressive role in immune response in macrophages and dendritic cells (*Figure 2*), which could be affected by this treatment. It is also worth stressing that pro-inflammatory properties are essential for the activation of the immune response and extravasation of immune cells from the vasculature into the tissues, which is important to consider when suggesting therapeutic applications. Conversely, decreased Gas6 plasma concentrations have been detected in patients with type 2 diabetes, associating with adiposity, insulin resistance, altered glucose tolerance and inflammation (85). The role of circulating Gas6 in contrast to Gas6 produced locally by cells in the immediate vicinity on the regulation of vascular responses remains to be elucidated.

4.2. Axl receptor is ligand-independently activated by anti-Axl antibody

To date, TAM agonists, that would exhibit absolute receptor specificity in human tissues, have not been found. In the work of Zagorska et al., however, the authors described that

anti-TAM antibodies act as TAM-activating agents in mouse bone marrow-derived macrophages. Our study suggests that affinity purified polyclonal anti-Axl antibody (R&D Systems) potently activates the receptor instead of blocking it (*Figure 8, B*). This finding indicates a possibility of selective activation of endothelial expressed Axl regardless of ligand binding. This observation led us to think of possible use of Axl-activating antibodies as therapeutics, since Axl/Gas6 signaling has been shown to have a pro-survival function in endothelium, by acting as an anti-apoptotic agent (75). Notably, increased apoptosis is associated with many cardiovascular complications, such as atherosclerosis and pulmonary hypertension (86-88). Nevertheless, it is worth keeping in mind that the activation of Axl receptor might be not tissue specific, and with respect to the various functions of Axl, should be examined for side effects. Further, we did not confirm the activation of Mer receptor by anti-Mer antibody, as we currently working on the protocol for efficient immunoprecipitation of Mer receptor. However, based on our observations, we concluded that anti-Mer antibody (R&D Systems) lacks the specificity for endothelial expressed Mer (data not shown). This explains, why we did not observe any effect on adhesion molecule expression when blocking Mer with this antibody (*Figure 8, A*). We reasoned that TAM receptors are differently glycosylated, depending on a tissue as well as cell's growth phase. If the antibody is directed against the site of glycosylation, it might lose its specificity. This could be of use if one aims to produce tissue-specific TAM-activating antibodies.

4.3. Mer/protein S axis has an anti-inflammatory function in endothelial inflammation

We sought to elucidate the role of TAM receptor ligands- Gas6 and protein S in the endothelial inflammation. We found that the addition of exogenous ligand does not affect TAM response to inflammatory stimuli, regarding the adhesion molecule expression (*Figure 9*). We hypothesize that endothelial cells produce sufficient amounts of ligands to trigger TAM-activation, hence adding extra does not change their response. Notably, blocking endogenous Gas6 with anti-Gas6 antibody did not show any effect (*Figure 9*), when compared to siRNA knock-down experiment, where we concluded pro-inflammatory tendencies of Gas6 (*Figure 5*). These findings are contradictory to the work of Avanzi et al.(66), where the authors claimed anti-inflammatory properties of endogenously produced Gas6 in endothelium. However, it is worth stressing that according to their proposal, pro-inflammatory triggers would diminish Gas6 concentration in endothelium, which would consequently lead to the activation of the proadhesive machinery.

In our study, however, we did not validate this finding and therefore it might be that the TNF- α stimulation on its own blocks Gas6 secretion from the cells. Another issue to consider is the specificity of the anti-Gas6 antibody, with respect to the possible modifications of Gas6 produced by HUVEC cells. Again, if this antibody is directed to the site of modification, it might lose its Gas6-targeting function. Further, we blocked the exogenously secreted protein S with anti-protein S antibody and we observed a significant increase in the expression of adhesion molecules (*Figure 9, C*). This indicates the anti-inflammatory properties of endothelial cell secreted protein S. To date, protein S is known to be a potent ligand for Mer receptor (21), and as we previously described, Mer deficiency leads to the elevated expression of inflammatory cytokines and adhesion molecules. These observations allow us to speculate the existence of Mer/protein S-mediated anti-inflammatory signaling in endothelium. Notably, the role of Mer in suppressing inflammation has been already confirmed in mouse immune cells (44, 51).

4.4. Starvation upregulates Mer expression in endothelium

We decided to test whether stimulation with different agents would affect Axl and Mer expression in HUVEC cells. We found that Axl expression is reduced upon the TNF-α stimulation in serum-free medium, however further repetitions did not confirm this finding. In addition, Mer expression was strongly upregulated upon starvation (*Figure 10, A*). Notably, the same stimulation was performed in complete-medium conditions, showing no effect on Axl and Mer expression (*Figure 7, D*). This observations allow us to hypothesize that endothelium promotes the expression of Mer as a response to stress conditions such as starvation. This might be one of the cellular rescue mechanisms, as we previously presented the anti-inflammatory properties of Mer-mediated signaling. Another reason could be the significant role of Mer in phagocytosis of apoptotic cells (29, 53). The cells in the starvation environment might “prepare” for apoptosis by expressing more Mer, which could then promote an efficient clearance of dying cells upon extended starvation. Further, in order to explain discrepancies between the experiments, we decided to measure mRNA levels of Mer and Axl with quantitative RT-PCR (qRT-PCR). Nevertheless, we did not obtain any reliable results due to the very low signals of Axl and Mer expression (data not shown). In order to test this methods in the future, we will need to optimize the reaction conditions. In addition, we stimulated HUVECs with various agents, which were described to potently induce Axl and Mer expression in immune cells. We found that these stimuli did not cause any response with respect to Axl and Mer expression in endothelial cells (*Figure 10*). As a result of this

approach, we concluded that depending on the cell type, different agents affect TAM expression. This might be due to the distinct roles of TAM receptors in different cells, thus the pathways leading to their activation might be also diverse.

4.5. Future plans

First of all, we would like to develop an efficient method for immunoprecipitation (IP) of Mer in primary human endothelial cells. Moreover, we plan to confirm the specificity of Axl activation by anti-Axl antibody by stimulating HUVECs with anti-Axl and performing an IP analysis for Mer receptor activation. Subsequently, we will test the properties of anti-Mer antibodies to selectively activate Mer in endothelial cells. Furthermore, we would like to investigate TAM expression levels upon the stimulation with different inflammatory agents such as Interleukin-1 beta (IL-1β). In addition, we would like to test whether TAMs regulate IL-1β-mediated adhesion molecule expression and cytokine secretion, compared to TNF-α-mediated effects. Importantly, we will optimize qRT-PCR conditions and measure Axl and Mer expression in response to the treatment with various stimuli. This will complement protein expression analysis by western blot. In addition, we would like to test whether TNF-α stimulation affects the Gas6 secretion by HUVEC cells, as proposed in the Avanzi et al. (66). Finally, we plan to verify our observations in other primary human endothelial cell line such as human aortic endothelial cells (HAEC).

4.6. Final conclusions

Our study provides an important contribution to better understanding of the role of TAM receptors in endothelial inflammation, since conflicting conclusions have been reported (65, 66). We presented a pro-inflammatory role of Axl/Gas6 axis, which is in line with Tjwa.et al. (65), and anti-inflammatory properties of Mer/protein S signaling in primary human endothelial cells. We managed to prove ligand-independent activation of Axl by anti-Axl antibody (R&D Systems). Notably, we demonstrated that Mer receptor expression is potently elevated upon starvation as well as Axl and Mer upregulation by different agents is dependent on the cell type. Altogether, we presented diversification and complexity of Axl and Mer receptors functions in endothelial inflammation, hence suggesting the need for further research.

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POPULAR SCIENCE SUMMARY
Oktawia Wolanin

TAMing endothelial inflammation

TAM (Tyro3, Axl and Mer) receptors are some of the proteins attached to the cell surface. In the presented work we show an important role of these proteins in cells, which line up inner part of blood vessels (endothelial cells). We found that Axl and Mer play opposite roles. Axl receptor increases inflammation in these cells, whereas Mer decreases inflammation.

The human cell is a very complex structure, with many proteins attached to its surface, referred to as cell receptors. There are many different kinds of receptors, and one group of them is the TAM receptors-family, which stands for Tyro3, Axl and Mer. They share structural similarities, however their abundance and functions vary from cell to cell. In order to activate the TAM receptor, a so called "TAM receptor ligand" must bind to it (Figure 1).

TAM receptors are involved in various processes in the human body, such as clearance of dying cells. In this process, specialized immune cells, thanks to TAM receptors on their surface, recognize dying cells and "eat" them. This process is determined by TAM-ligand interaction, where the ligand binds to specific signals expressed on the dying cell, thus creating a "bridge" between both cells (Figure 1).

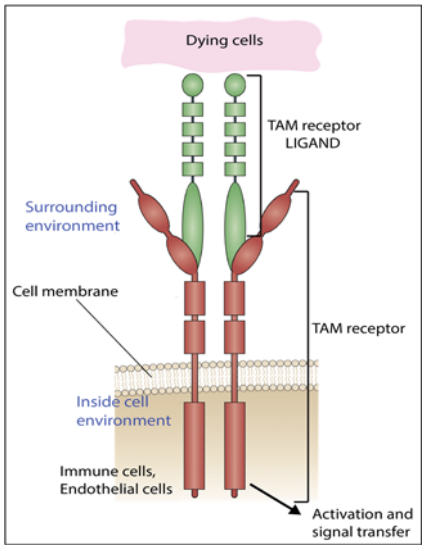

Figure 1 TAM receptors-ligand interaction (Lemke & Rothlin. Nature reviews Immunology. 2008;8(5):327-36, modified).

TAM receptors in endothelium- are they good or bad players?
TAM receptors are also expressed in endothelial cells, which line up the inner part of blood vessels, hence they are in direct contact with blood. There are many causes of inflammation in the human body, such as injuries and infections. Upon the inflammation, endothelial cells produce various proteins, which allow immune cells to become activated and help them pass through the blood vessel wall to reach the underlying tissue.

We have measured how the different TAMs regulate various proteins expressed by endothelial cells during inflammatory conditions. Previously published studies have reached opposing conclusions regarding the pro or anti-inflammatory role of TAMs in endothelium. We observed that Axl-activation seems to promote inflammation, whereas Mer-activation down-regulates it. This observation brought new light on their role in vascular inflammation, which could be both good and bad. Further, we tested different pro- and anti-inflammatory agents, which were reported to strongly induce TAM expression in immune cells. To our surprise, these agents did not affect the expression of TAM receptors in human endothelial cells. This conclusion led us to hypothesize that TAM expression is regulated differently depending on cell type.

Our study provides another step on the way to better understanding these receptors for future therapeutic use.
Our results provide the confirmation of pro-inflammatory properties of Axl in endothelium, thus suggesting this receptor as an interesting drug target for fighting inflammation.