# The pro-inflammatory activation of endothelial cells by complement MASP-1

Thesis

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# 1. INTRODUCTION

The antimicrobial systems of native immunity consist of soluble and cellular components. Among the soluble components there are particular cytokines (e.g. TNFα, IFNα/β, IL-6), soluble pathogen recognition receptors (PRRs) and the components of the complement system. The cellular elements of the native immunity express the pathogen recognition receptors in the cytoplasm and on the cell surface. Beside B1-cells and macrophages, the neutrophil granulocytes are the most effective effector cells of the innate antimicrobial response. The neutrophil granulocytes have crucial role in the defense against the bacterial and fungal pathogens by their phagocytic and free radical generating capacity. Neutrophil granulocytes can also present antigens to T-cells. The endothelial cells are usually not mentioned as a part of the native immune system, however, their anatomical localization, cytokine producing capacity, PRR expression and regulatory function on the blood flow, permeability and immune cell transmigration make them a very important regulator of the immune homeostasis.

Among the complement activation routes, the lectin pathway is able to recognize the carbohydrate structures on the surfaces of microbes or changed self compartments. The pattern recognition receptors of lectin pathways are called lectin pathway receptors (LPRs). The mannan-binding lectin (MBL), the ficolin-1,-2,-3 and the CL-K1 and CL-L1 proteins belong to the LPRs. Receptor associated effector enzymes and regulatory proteins are commonly designated as lectin pathway effectors (LPE). There are three MBL-associated serine proteases: MASP-1,-2,-3. Because

of its broad substrate specificity, MASP-1 can activate not only the complement system, but also the kininogen and the coagulation cascades. Our group described that MASP-1 can stimulate endothelial cells via cleavage of their protease activated receptors.

Complement activation is usually accompanied by the generation of the inflammatory response. During inflammation cytokines are produced - partially by endothelial cells - and this together with the opsonizing effect of complement may switch on the adaptive immune system as well. The lipid mediators, free radicals, cytokines, and the elements of the acut-phase reaction and complement system play important role in the generation of inflammation. The recruitment of the appropriate immune cell types in the inflammatory site is regulated by cytokines and chemokines as well as a special combination of adhesion molecules. For example IL-8 attracts mainly neutrophil granulocytes, while monocytes are recruited by monocyte chemoattractant protein-1 (MCP-1) and/or macrophage colony-stimulating factor (M-CSF). Endothelial cells can also generate these cytokines rendering them a regulatory function of homing. Beside influencing the adhesion molecule expression and the chemotaxis, cytokines also play an indispensable role in immune cell activation including phagocytic activity and reactive oxygen species production of neutrophil granulocytes.

The endothelial cells have many different functions. They can regulate the vascular tone, the transport of soluble metabolites for tissues, they form thrombo-resistant and anti-coagulant surface, they can activate/inactivate hormones and they are involved in the regulation of several immunological processes. A remarkable

manifestation of heterotype cell-cell adhesion is the selective transmigration of leukocytes through the endothelial cell layer, which is mediated by the activated endothelial cells via chemokine production (e.g. IL-8) and surface targeted adhesion molecule expression (e.g. E-selectin). The endothelial cells regulate also the inflammatory process via generating anti- and pro-inflammatory mediators. The endothelial cells respond effectively to microbial macromolecules and pro-inflammatory cytokiens (e.g IL-1β, TNFα): loosening of junctional connections and contraction of cells lead to an elevated permeability. Simultaneously, the endothelial cells generate pro-inflammatory cytokines and chemokines (e.g. MCP-1, IL-6, IL-8) and change the pattern of their adhesion molecules, which together support the maintenance of the inflammation. The production of anti-inflammatory cytokines (e.g. IL-1Ra) and mediators ensure the regulatory role of the endothelial cells. Because the endothelial cells are directly exposed to the activated component of the complement system they express high amount of complement regulatory proteins on their surface. Pro-inflammatory factors usually induce the expression of these regulatory proteins further, rendering a resistance to the endothelial cells against cytopathic effects of the activated complement system.

MASP-1 is a key player in the activation of complement lectin pathway, however, *in vitro* it can also enhance the $Ca^{2+}$ mobilization, the p38-MAPK phosphorylation and the NFκB nuclear translocation in endothelial cells via the cleavage of protease activated receptors. Nevertheless, the physiological role of the MASP-1 induced endothelial cell activation is still unclear.

# 2. OBJECTIVES

In the first part, we wanted to answer the following questions:

- Is it possible to enhance the effectiveness of the former preparation method of MASP-1 from serum, to produce sufficient amount and quality of protein for *in vitro* studies with endothelial cells?
- Are the prepared MBL-MASP complexes (i.e. MBL in complex with MASP-1, MASP-2, MASP-3, MAp44 or MAp19) able to activate the $Ca^{2+}$ mobilization of endothelial cells, and if they are, which protein structural properties are indispensable for the calcium response?

In the second part, we asked the followings:

- Which signaling pathways are activated by MASP-1 and how are these pathways involved in the cytokine and adhesion molecule production of the endothelial cells?
- Which are the main similarities and differences between the activation pattern of endothelial cells activated by MASP-1 or by other known pro-inflammatory factors?
- Is MASP-1 able to act on the chemotaxis and adhesion of neutrophil granulocytes by the activation of endothelial cells?

# 3. METHODS

**MASP-1 proteins used in our experiments**

The used recombinant MASP-1 proteins were expressed in a bacterial expression system. To enhance the purification efficiency of plasma MBL-MASP complexes, high amount of recombinant MBL had been added to the samples, before it was separated on Sepharose™ column.

**Preparation and culture of HUVECs**

Our experiments utilized human umbilical vein endothelial cells (HUVECs). After the digestion with collagenase, the endothelial cells were grown in cell culture flasks, pre-coated with 0,5% gelatin. The culture was kept in previously optimized medium until confluency and it was checked routinely with microscope. The experiments were performed between passage 2 to 4.

**Analysis of signaling pathways**

The $Ca^{2+}$ mobilization in endothelial cells was monitored by fluorescence microscopy using Fluo-4-AM loaded cells. The phosphorylation of CREB was measured also by fluorescence microscopy, based on the localization and intensity of fluorescence labeled specific antibody. The activation of CREB and JNK pathways were detected with specific antibody by Western blot analysis.

**Analysis of mRNA expression by quantitative PCR**

Specific primer pairs were designed for the cytokines and adhesion molecules, and then LightCycler® analysis was performed. The relative mRNA expression to actin and GAPDH was calculated.

**Measurement of cytokine production**

The production of cytokines in response to MASP-1 was measured by ELISA, according to the manufacturers' protocols.

**Detection of the adhesion molecules**

The expression of adhesion molecules were detected by fluorescence microscopy, using specific antibodies and by in-house cellular ELISAs.

**Neutrophil granulocyte chemotaxis assay**

Previously, the HUVECs were treated with recombinant MASP-1, then after short time the supernatant was changed for MASP-1 free buffer for 2,5 hours. This supernatant was added into the outer wells of the Transwell system, while the inner wells were filled with the neutrophil granulocytes isolated from peripheral venous blood. After incubation, the number of migrated cells were measured by alkaline phosphatase assay.

**Adhesion of differentiated PLB-985 cells to HUVECs**

The PLB-985 cells are acute myeloid leukemia cells. These cells can be differnetiated to neutrophil granulocyte like cells in the presence of DMSO. The differentiated neutrophil model cells were labeled with fluorescent dye and co-incubated with HUVECs, which had previously been treated with MASP-1 or other activators. After several washing steps, the adherent cell number was measured by fluorescence plate reader.

# 4. RESULTS

**The activation of the endothelial cells by MBL-MASP complexes**

The previous addition of recombinant MBL to the serum samples increased the efficiency of preparation of MBL-MASP complexes (*6.1*). We showed that only MASP-1 was able to induce dose dependent $Ca^{2+}$ mobilization of the endothelial cells amongst the MBL-MASP complexes (i.e. MBL in complex with MASP-1, MASP-2, MASP-3, Map19 and Map44). $Ca^{2+}$ activation was not elicited if the MBL-MASP complexes had previously been treated with C1-Inhibitor. The dose-dependence and the kinetics of endothelial cell activation by MBL-MASP complexes were similar to those of recombinant MASP-1. It verifies that the recombinant MASP-1 fragment can be a useful model of the wild type protein in *in vitro* studies.

**The impact of the molecular structure of MASP-1 on the calcium mobilization**

We had different mutants of recombinant MASP-1 proteins: 1. an inactive serine protease domain containing mutant (S/A mutant), 2. a constitutive zymogene conformation mutant (R/Q mutant). These mutant proteins could not activate the endothelial cells (*6.1*). Based on these results we can assume that the induction of $Ca^{2+}$ mobilization depends on the serine protease activity and on the activation of lectin pathway (i.e. activation of zymogene MASP-1).

**The effect of MASP-1 on the signaling pathways of endothelial cells**

We described that the recombinant MASP-1 induced not only the calcium mobilization, the NFκB nuclear translocation and the p38-MAPK activation, but it was also able to enhance the phosphorylation of CREB and JNK signaling elements (*6.2*). The p38-MAPK pathways played an important role in the MASP-1 induced cytokine production, however, the NFkB and JNK pathways also had crucial role in quick (3 hours) response(*6.2*).

**The pattern of cytokine production and adhesion molecule expression in response to MASP-1**

MASP-1 induced the generation of IL-6 and IL-8 cytokines in dose dependent manner. Beside the effect of MASP-1 on cytokines production, it enhanced the expression of E-selectin and decreased the level of ICAM-2 on endothelial cells. The MASP-1 induced activation pattern was similar to the effect of thrombin mediated pro-inflammatory activation in its most elements (*6.2*), however, there are some unique features as well. E.g MASP-1 induced MCP-1 only at mRNA level, whereas thrombin elevated MCP-1 protein production as well. MASP-1 had no effect on ICAM-1 (neither on mRNA nor protein level), while thrombin induced ICAM-1 protein expression. This unique characteristics provide a differential regulatory function for MASP-1 via endothelial cell activation.

**The effect of recombinant MASP-1 induced cytokine and adhesion molecule pattern on the activation of neutrophil granulocytes**

The pro-inflammatory activation of endothelial cells by MASP-1 suggests that the main target cells of the endothelial activation may be the neutrophil granulocytes. In consent with this hypothesis, our results indicated that MASP-1 induced the chemotaxis of neutrophil granulocytes via the cytokine production of endothelial cells (*6.2*). This effect was similar in intensity to that of IL-8, which was used as a positive control. Moreover, presumably because of the elevated expression of E-selectin, the pretreatment of HUVECs with recombinant MASP-1 enhanced the adhesion of the neutrophil granulocyte model cells to the surface of the endothelial cells. The kinetics of cell adhesion effects of MASP-1 was similar to that of MASP-1 induced E-selectin expression: MASP-1 induced only a short-term elevation of adhesion of neutrophil granulocytes to the endothelial cells, while it had no effect on long-term induction. This phenomenon was similar to the effect of thrombin but different from that of TNFα.

# 5. CONCLUSIONS

The lectin pathway of complement system is activated after the recognition of carbohydrate side chains of pathogens, which is followed by the autoactivation of zymogene MASP-1. Only the activated MASP-1 is able to activate the endothelial cells, which provides a very important regulatory role for complement activation: the anatomical localization of the endothelial cells results close contact with the components of the activated complement system.

The unique phenomenon of the MASP-1 induced cytokine production is that it has effect only on the generation of IL-6 and IL-8 but not on MCP-1 production. This indicates that the IL-8 production can specifically act on the chemotaxis of neutrophil granulocytes, while the cytokine pattern does not help recruiting monocytes. The physiological role of MASP-1 induced IL-6 production may be the triggering of the acute-phase protein expression, -including several complement components. The special pattern of the adhesion molecules and cytokines in response to MASP-1 suggests that the main target of the MASP-1 induced endothelial cells are the neutrophil granulocytes.

It is important to note that there are factors, which were affected by MASP-1 only at mRNA level but not at protein level. The physiological aspect of this phenomenon might be the synergism between MASP-1 and other activators. These factors (cytokines, microbial macromolecules and self danger signals) - which are generally released at the same time as MASP-1 is activated – could impact more efficiently even in lower concentration if they act together with MASP-1.

Summarizing the results we could assume that due to the recognition of pathogens by the complement lectin pathway MASP-1 becomes activated. Activated MASP-1 plays important role not only in the cleavage of complement components but in the activation of endothelial cells, which in turn enhances the chemotaxis and adhesion of neutrophil granulocytes. These mechanisms could connect the two most important antimicrobial components of the innate immunity, the complement system and the neutrophil granulocytes via the endothelial cells.

# 6. LIST OF PERSONAL PUBLICATIONS

## 6.1 Publications related to the dissertation

1) Megyeri M, Jani PK, Kajdacsi E, Dobo J, Schwaner E, Major B, Rigo J Jr, Zavodszky P, Thiel S, Cervenak L, Gal P,(2014) Serum MASP-1 in complex with MBL activates endothelial cells. MOLECULAR IMMUNOLOGY 59:(1) pp. 39-45.

IF: 2.645

2) Jani PK, Kajdacsi E, Megyeri M, Dobo J, Doleschall Z, Futosi K, Timar CI, Mocsai A, Mako V, Gal P, Cervenak L, (2014) MASP-1 Induces a Unique Cytokine Pattern in Endothelial Cells: A Novel Link between Complement System and Neutrophil Granulocytes. PLOS ONE 9:(1) p. e87104.

IF: 3.730

Cumulated impact factors of the publications, related to the dissertation: **6,375**

## 6.2 Other publications

1) Jani Péter Károly, Makó Veronika, Cervenak László, (2011) Természetes és szintetikus flavonoidok gyulladásgátló hatásának összehasonlítása in vitro endotélsejt modellen. ÉRBETEGSÉGEK XVIII.:(4.) pp. 89-95.

2) Kajdácsi E, Jani PK, Csuka D, Varga LA, Prohászka Z, Farkas H, Cervenak L (2014) Endothelial cell activation during edematous attacks of hereditary angioedema types I and II. J Allergy Clin Immunol. 133(6):1686
IF: 12.047 (2012)

3) Csuka D, Simon D, Hóbor R, Uray K, Prohászka Z, Bánlaki Z, Jani PK, Szilágyi A, Hudecz F, Rajczy K, Beke G, Boros Major A, Tordai A, Illés Z, Berki T, Czirják L, Füst G, (2013) Serum concentration of IgG type antibodies against the whole EBNA-1 and its aa35-58 or aa398-404 fragments in the sera of patients with systemic lupus erythematosus and multiple sclerosis. CLINICAL AND EXPERIMENTAL IMMUNOLOGY 171:(3) pp. 255-262.
IF: 3.409 (2012)

Cumulated impact factors of the publications, unrelated to the dissertation: **15,456**