

1 **Heparanase-2 protects from endothelial injury by inhibiting TLR4 signaling**

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3 Running title: Heparanase 2 protects from microvascular injury

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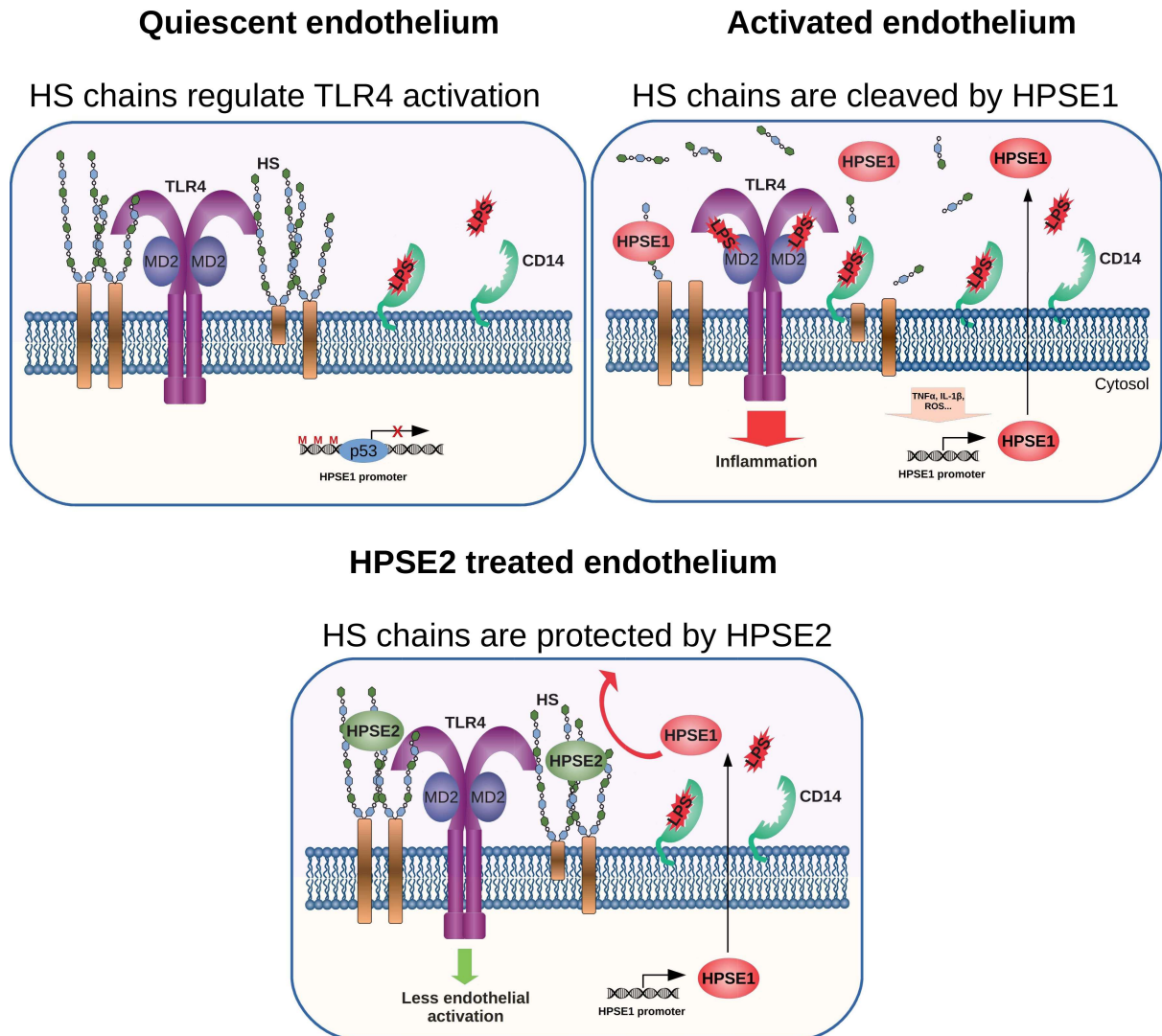
## 1 **Abstract**

2 **Objective.** The endothelial glycocalyx and the regulation of its shedding are important to  
3 vascular health. Endo- $\beta$ -D-glucuronidase heparanase-1 (HPSE1) is the only enzyme that can  
4 shed heparan sulfate. However, the mechanisms are not well understood.

5 **Approach and results.** To investigate HPSE1 and its endogenous inhibitor, heparanase-2  
6 (HPSE2), we used cell culture, lentiviral protein overexpression, a microfluidic chip model of  
7 cell culture under shear stress conditions, and lipopolysaccharide (LPS) injections in mice.  
8 We show that HPSE1 activity aggravated Toll-like receptor 4 (TLR4)-mediated response of  
9 endothelial cells to LPS. On the contrary, HPSE2 overexpression was protective. The  
10 microfluidic chip flow model confirmed that HPSE2 prevented heparan sulfate shedding by  
11 HPSE1. Furthermore, heparan sulfate did not interfere with cluster of differentiation-14  
12 (CD14)-dependent LPS binding, but instead reduced the presentation of the LPS-CD14  
13 complex to TLR4. HPSE2 reduced LPS-mediated TLR4 activation by LPS, subsequent cell  
14 signaling, and cytokine expression. Moreover, HPSE2-overexpressing endothelial cells  
15 remained protected against LPS-mediated loss of cell-cell contacts. In vivo, expression of  
16 HPSE2 in plasma and kidney medullary capillaries was decreased in mouse sepsis model. We  
17 next applied purified HPSE2 in mice and observed decreases in TNF $\alpha$  and IL-6 plasma  
18 concentrations after intravenous LPS injections.

19 **Conclusions.** Our data demonstrate the important role of heparan sulfate and the glycocalyx  
20 in endothelial cell activation and suggest a protective role of HPSE2 in microvascular  
21 inflammation. HPSE2 offers new options for protection against HPSE1-mediated endothelial  
22 damage and preventing microvascular disease.

## 1 Graphical abstract



## 3 Non standard abbreviations

HPSE1	Heparanase 1
HPSE2	Heparanase 2
LPS	Lipopolysaccharide
TLR4	Toll-like receptor 4
CD14	Cluster of differentiation 14
PDMS	Polydimethylsiloxan
CLP	Cecal ligation and puncture

## 1 Introduction

2           The endothelial glycocalyx comprises a network of core proteins and branching  
3 glycosaminoglycan polymers such as heparan sulfate, chondroitin sulfate, and hyaluronans,  
4 that cover the luminal side of endothelial cells. The glycocalyx is essential to maintain  
5 integrity and function of the endothelial layer<sup>1-3</sup>. It serves as a receptor or co-receptor for  
6 various ligands such as growth factors, cytokines and chemokines, coagulation factors,  
7 proteases, complement-system proteins, membrane receptors, and adhesion proteins<sup>4</sup>.  
8 Endothelial glycocalyx shedding occurs during many pathological conditions<sup>5</sup>, including  
9 cardiovascular, renal diseases<sup>6,7</sup> and sepsis<sup>8-10</sup>. Heparan sulfate is a highly sulfated  
10 polysaccharide element of the endothelial glycocalyx. Experiments in gene-deleted mice  
11 showed that glycocalyx disturbances are not compatible with life.<sup>11,12</sup> The heparan sulfate  
12 interactome is formed by a 300-protein network<sup>4</sup>. Thus, degradation of heparan sulfate affects  
13 numerous cellular functions. The only known endo- $\beta$ -D-glucuronidase capable of degrading  
14 heparan sulfate chains in mammals is heparanase 1 (HPSE1). HPSE1 is upregulated in many  
15 inflammatory diseases including cancer<sup>13</sup>, diabetes<sup>14</sup>, and sepsis<sup>10</sup>.

16           The Toll-like receptor-4 (TLR4) is an important inflammatory mediator<sup>15</sup>. Several  
17 reports addressed the role of heparan sulfate in the TLR4-mediated response. Soluble heparan  
18 sulfate chains serve as TLR4 ligands<sup>16,17</sup>. Cleavage of heparan sulfate by HPSE1 strongly  
19 promoted TLR4 response in macrophages<sup>18</sup>. Furthermore, TLR4 signaling was inhibited in  
20 cells cultured on heparan sulfate proteoglycans-rich matrix. This inhibition was relieved by  
21 the matrix degradation<sup>19</sup>. However in microglia, overexpression of HPSE1 led to diminished  
22 LPS-induced response<sup>20</sup>. Co-localization of TLR4 co-receptor Cluster of Differentiation 14  
23 (CD14) with heparan sulfate has been observed that let the authors to suggest that heparan

1 sulfate promotes TLR4/CD14 association. In addition, it has been demonstrated that HPSE1  
2 can act via TLRs causing pro-inflammatory response that is independent of catalytic  
3 activity<sup>16,21</sup>.

4 McKenzie and colleagues cloned and characterized the Heparanase 2 (HPSE2)  
5 protein, that exhibits a 40% homology to HPSE1<sup>22</sup>. HPSE2 has no catalytic activity, but  
6 instead can inhibit HPSE1<sup>23</sup>. An antagonistic interaction of the heparanases in cancer have  
7 been recently reviewed recently<sup>24</sup>. Although HPSE2 circulates in the human plasma<sup>13</sup>,  
8 interactions between HPSE1 and HPSE2 in endothelial cells has not been investigated.  
9 Endothelial cells express HPSE1 in vitro<sup>25,26</sup> and serve as an important local source of  
10 HPSE1 during inflammation<sup>27</sup>. We studied HPSE1 and HPSE2 in endothelial cells and animal  
11 models to elucidate the role of these enzymes in maintaining endothelial integrity

## 12 **Materials and methods.**

### 13 ***1. Cells, antibody, Real Time-PCR.***

14 Human microvascular endothelial cells line HMEC-1 was from ATCC. Cells were cultured  
15 as recommended by the supplier. Primary human dermal microvascular endothelial cells were  
16 from Promocell. Cells were cultivated as recommended by the supplier and used in the  
17 passage 4. Unless otherwise indicated, HMEC-1 cells were used through the study. The  
18 following antibodies were used: Heparan sulfate (clone 10e4) from Amsbio; HPSE1  
19 (GTX32650) from GeneTex; Heparanase 2 (NBP1-31457) from Novus Biologicals were used  
20 for western blotting; 1c7 monoclonal blocking antibody to HPSE2 was a kind gift of Prof. I.  
21 Vlodaysky; antibodies to HPSE2 purchased from Sigma (Cat. # HPA044603) were used for  
22 tissue staining; Tubulin (556321) from BD Pharmingen, P-p65 (93H1); P-p38 (D3F9), P-  
23 MEK (166M8), and cleaved Caspase 3 antibody (9665) were from Cell Signaling

1 Technologies; VE-cadherin antibody (Clone 123413) from R&D Systems; mouse CD31  
2 Dianova (Dia-310) were used for tissue staining; phalloidin (Alexa 488) was from Invitrogen.  
3 DraQ5 was from BioStatus Ltd. OGT 2115 was from Tocris. Purified heparan sulfate (10-70  
4 kDa) was from Sigma. Bovine fibronectin was from Sigma. Purified catalytically active  
5 HPSE1 was from R&D Systems.  
6 Cells proliferation was assessed by BrdU Cell proliferation ELISA from Roche. IL-6 ELISA  
7 was from Affimetrics. Western blotting was performed as established earlier<sup>28</sup>. Dot blot was  
8 performed using nitrocellulose membrane and Heparan sulfate (clone 10e4).  
9 RNA was isolated from cells using Quiagen RNAeasy kits. RT-PCR Taqman assay was  
10 performed using Roche TaqMan Master Mix and Roche LightCycler96. The sequence of the  
11 primers is given in the Supplementary Table 1. TaqMan Gene Expression Assays for  
12 RANTES, IFNB1, and actin as a housekeeper were from Thermofisher Scientific. Human  
13 Common Cytokine RT<sup>2</sup> profiler array (Quiagen) was used accordingly to manufacturer's  
14 instructions. TMB substrate kit was from Thermofisher Scientific.

### 15 ***3. Lentivirus for HPSE2 and HPSE1 overexpression.***

16 Construction of the expression vectors was performed according to the standard cloning  
17 protocols. The vectors were generated by recombination of pDEST12.2 (Invitrogen) with  
18 vectors containing full length HPSE2 and HPSE1 (pENRT223.1\_HPSE1,  
19 pENRT223.1\_HPSE2, MyBioSource, San Diego, CA, USA). Gateway LR Clonase enzyme  
20 mix (Invitrogen) catalysis the *in vitro* recombination between an entry clone  
21 pENTR223.1\_HPSE2 and destination vector pDEST12.2 to generate expression clones.  
22 Correct clones was selected by PstI (New England Biolabs) digestion and confirmed by  
23 sequencing (SeqLab). For all cloning experiments in current work subcloning efficiency DH5

1 alpha competent cells (Invitrogen) were used accordingly to the standard protocols  
2 recommended by the supplier.

### 3 ***4. Lentiviral gene transfer of HPSE2 and HPSE1.***

4 Briefly, the lentivirus packaging genome pCMV-dR8.74<sup>29</sup>, and pMG2G vectors were kindly  
5 provided by Dr. Didier Trono (Department of Genetics and Microbiology, Faculty of  
6 Medicine, University of Geneva, Switzerland). The lentivirus transfer vector used in this  
7 study was generated by Gateway cloning strategy (Invitrogen). An entry vector pENRT223.1  
8 HPSE2 (MyBioSource) was used. As Gateway Destination vector was used modified plasmid  
9 pWPTS-GFP (Tronolab). Modification plasmid pWPTS-GFP was performed by Gateway  
10 Vector Conversion System (Invitrogen). A Gateway cassette containing attR recombination  
11 sites flanking ccdB gene and a chloramphenicol-resistance gene are blunt-end cloned into  
12 cloning site pWPTS-GFP to generate a pWPTS-Dest vector. Gateway LR Clonase enzyme  
13 mix (Invitrogen) carried over the in vitro recombination between an entry clone pENTR-  
14 HPSE2 and destination vector pWPTS-Dest to generate expression clones pWPTS-HPSE2.  
15 pCMV-dR8.74, pMD2G and pWPTS-HPSE2 plasmids were purified using an EndoFree®  
16 Plasmid Maxi kit (Qiagen, Valencia, CA), and co-transfected (using ratio pWPTS-  
17 HPSE2:pCMV-dR8.74:pMD2G = 3:2:1) into 293T cells using PerFectin transfection reagent  
18 (Genlantis) as per manufacturer. After 48 h post transfection cell supernatants, containing  
19 viral particles, were filtered using 0.4 mm Steriflip vacuum filtration system (Millipore) and  
20 concentrated by ultracentrifugation at 50 000 g for 1.5h at 4°C<sup>30</sup>. Viral titer was determined  
21 by LV Lentiviral Titer kit (MoBiTec) and viruses were used at a Multiplicity of infection of  
22 1 - 5 using polybrene (H9268, Sigma) at a concentration of 2 µg/ml.

### 23 ***5. Microfluidic experiments.***



1           Microfluidic chips were fabricated of polydimethylsiloxan (PDMS) (Sylgard 184,  
2 Dow Corning) by replication of the polymeric master. The master was produced at the  
3 Institute for Quantum Optics, Leibniz University (Hannover) from diurethane dimethacrylate  
4 resin (Sigma-Aldrich) by two-photon polymerization (2PP) technology<sup>31</sup> The chips consisted  
5 of 4 parallel 10mm-long and 2mm-wide channels. The height of the channels was 1 mm.  
6 PDMS-microfluidic chips were sealed with 150  $\mu\text{m}$  thick microscopy cover glass using  
7 plasma bonding<sup>32</sup>. The diameter of the chip was matched to 35 mm diameter of the cover  
8 glass for compatibility with the Okolab incubation chamber.

9           For operating the microfluidic chips and carrying out cell culture experiments a  
10 microfluidic actuation platform was built on the basis of Okolab BOLD LINE H301BL stage  
11 incubation set, 4-channel 400VDL/VM4 OEM peristaltic pumps (Watson-Marlow GmbH).  
12 Equipment integrated into system provided incubation of the Chips, liquids circulation  
13 through the chip, loading/extraction of the cultured cells and loading specific chemicals for  
14 experiments. The operation of the microfluidic actuation platform was computer-controlled.  
15 The microfluidic interface was based on standard Teflon<sup>®</sup> tubes with outside diameter of  
16 1.8mm and standard Omni-Lok<sup>®</sup> microfluidic connectors (Diba Industries Inc.).

17           Internal surfaces of the microfluidic chip channels were coated with 2 $\mu\text{g}/\text{ml}$  bovine  
18 fibronectin to facilitate cell adhesion. Control virus-transduced cells were seeded in 2  
19 channels of the chip; whereas the other two channels were seeded with HPSE2-  
20 overexpressing HMEC-1 cells. Thus, identical experimental conditions were provided for  
21 both cell variants. Cells were allowed to adhere for 2-3 hrs, after that the medium flow was  
22 initiated in all four channels. Medium flow rate was used form 0.4 ml/h that corresponded to  
23 0.1  $\text{dyn}/\text{cm}^2$  shear stress to 159 ml/h that corresponded to 38.75  $\text{dyn}/\text{cm}^2$ . Cells were  
24 incubated under flow conditions for 3 days before the experiments. Purified active HPSE1

1 was perfused through the channels for 4 hrs. Cells were fixed by perfusion with 2% PFA and  
2 then stained.

3 Confocal microscopy was performed using Leica TCS-SP2 AOBS confocal microscope  
4 (Leica Microsystems). All the images were taken with oil-immersed x63 objective, NA 1.4.  
5 Series of z-scans were processed and quantified using ImageJ software.

## 6 **6. Biotin-LPS binding and pull-down assay.**

7 Ultrapure biotin-LPS that activates only TLR4 pathway was purchased from InVivoGen. To  
8 analyze LPS binding, cells were placed on ice to prevent receptor internalization and  
9 incubated with 50ng/ml biotin-LPS for 1 h. Then cells were washed three times with 1% BSA  
10 in PBS and incubated with streptavidin-conjugated HRP on ice for 1 h. HRP activity was  
11 quantified using TMB substrate kit. Measurements were take using Tecan plate reader.

12 For pull down assay strepavidin-magnetic beads were used. Cells were incubated on ice with  
13 50 ng/ml biotin-LPS. Then, the cells were placed at 37°C for indicated time to allow for  
14 activation of TLR4 signaling complex. Then cells were placed on ice, washed, and cell lysis  
15 was performed. Cell lysates were incubated with streptavidin-beads for 1h at 4°C on rotator,  
16 then washed three times with ice cold PBS and used for electrophoresis followed by  
17 immunoblotting.

## 18 **7. Cell surface TLR4 ELISA.**

19 Cell surface TLR4 expression was measured on live cells incubated on ice in buffer  
20 containing 0.5% BSA/PBS with 0.05% NaN<sub>3</sub> in ELISA plate<sup>33</sup>. Staining was quantified using  
21 HRP-antibody and TMB substrate kit.

## 22 **8. Immunocytochemistry.**

1 After the stipulated time, cells grown on coverslips were fixed and processed for  
2 immunostaining as we have previously described<sup>34</sup>. Briefly, cells were stained for with alexa  
3 fluor 488 phalloidin (ThermoFisher Scientific) and subsequently for VE-Cadherin or heparan  
4 sulfate. The corresponding secondary antibody conjugated with alexa fluor 594 for 1 hour at  
5 room temperature. DAPI was applied for nuclear staining. For negative controls, samples  
6 were incubated with mouse IgG. Cells were then mounted with Aqua poly mount  
7 (Polysciences) and analyzed on a Leica TCS-SP2 AOBS confocal microscope (Leica  
8 Microsystems). All the images were taken with oil-immersed x40 objective, NA 1.25 and x63  
9 objective, NA 1.4.

#### 10 ***9. HPSE2 purification.***

11 HPSE2 was purified from conditioned medium of HEK 293T cells infected with HPSE2-  
12 overexpression lentivirus. Conditioned medium was collected, centrifuged at 3500 rpm for 10  
13 min, and filtered through 0.22  $\mu$ m filter. HPSE2 was collected with flow-through from  
14 CaptoQ ion exchange column equilibrated with 20 mmol/L Tris pH 8.7. HPSE2-containing  
15 fractions were collected, buffer was changed to 20 mmol/L MES pH 6.0 containing 100  
16 mmol/L NaCl. HPSE2 was elute with 0.1 – 1 mol/L NaCl gradient. HPSE2 fractions purity  
17 was assessed by electrophoresis followed by gel staining with Colloidal Blue Staining Kit  
18 (ThermoFisher Scientific) and by western blotting. HPSE2 was collected, concentrated using  
19 centricons, and dialysed against PBS.

#### 20 ***10. Animal experiments.***

21 *Sepsis models in mice.* All procedures were carried out at Phenos GmbH (Hannover,  
22 Germany) The animal protection committee of the local authorities (Lower Saxony state  
23 department for food safety and animal welfare [LAVES]) approved all experiments

1 (approval: 33.19-42502-04-16/2255). The group size was determined using  
2 <http://powerandsamplesize.com/> to achieve a detectable difference between different groups with  
3 an 80% probability at a 5% significance level. Eight- to 10-weeks-old male C57BL6 mice (20–25  
4 g) obtained from Charles River (The Charles River Laboratories; Sulzfeld, Germany) were  
5 injected iv with purified HPSE2 (5µg/g body weight) and immediately thereafter low dose (5  
6 ng/g body weight) of LPS (E.coli O111:B4, Sigma) administration was performed by iv  
7 injection. This LPS dose was chosen in preliminary experiments ranging from 5 to 1000 ng/ g  
8 body weight. 5 ng/g body weight LPS dose was well tolerated and resulted in a transient  
9 increase of pro-inflammatory cytokine level in blood within 2-4h after injection which  
10 declines at 8h and completely normalized at 20h post injection (data not shown). The blood  
11 sample were obtained at 2h post LPS injection, the plasma levels of pro-inflammatory  
12 cytokines IL-6 and TNFα were quantified by bead-based flow cytometry assay (CBA Kit; BD  
13 Biosciences, Heidelberg, Germany) according to the manufacturer's instructions.

14 Polymicrobial sepsis in mice was induced by cecal ligation and puncture (CLP) as described  
15 previously<sup>35</sup>. Vehicle (PBS) or purified HPSE2 (100 µg in 50 µl) was administrated by  
16 intravenous injection 1h prior to CLP surgery. At 18 h after CLP or sham operation, mice  
17 were anesthetized with isoflurane for blood sampling. Subsequently animals were sacrificed  
18 and the kidneys were perfused with PBS solution via the left ventricle, removed, fixed in  
19 formalin for 24h, and processed for immunohistochemistry as described<sup>35</sup>.

20 **11. Statistics.** We used mean ± SEM throughout this study. All experiments were repeated  
21 independently at least three times. For comparing two different groups of data, Student's T  
22 test test was applied. Multiple comparisons were analyzed by using the one-way analysis of  
23 variance with the Tukey as a *post hoc* test. “\*” shows *P* value of less than 0.05; “\*\*\*” shows *P*

1 value of less than 0.01; “\*\*\*\*” shows *P* value of less than 0.001. GraphPad Prism version 5.02  
2 (GraphPad Prism Software Inc., San Diego, CA, USA) was used for data analysis.

3

#### 4 **Results**

##### 5 ***1. HPSE1 aggravates endothelial LPS responses***

6 We first treated cells with purified catalytically active HPSE1 and then stimulated the cells  
7 with LPS. Immunocytochemical staining using monoclonal 10E4 antibody confirmed  
8 removal of heparan sulfate structures by HPSE1 (Fig. 1A, B). Cells treated with HPSE1  
9 demonstrated morphological changes similar to the changes induced by LPS, namely  
10 cytoskeletal rearrangement and disruption of cell-cell contacts (Fig. 1C, D). When HPSE1-  
11 treated endothelial cells were stimulated with LPS, expression of IL-6 was significantly  
12 higher than in untreated cells (Fig. 1E). IL-6 was selected as a readout of the LPS-induced  
13 TLR4 response because IL-6 expression in endothelial cells was shown to be TLR4-  
14 dependent<sup>36</sup>. In our experiments, the LPS-dependent expression of IL-6 was abrogated by the  
15 TLR4 inhibitor Cli-095 (data not shown). In a similar fashion, conditioned medium of the  
16 HPSE1-overexpressing endothelial cells that secrete the protein to the cell culture supernatant  
17 also promoted the LPS response in comparison to conditioned medium of the cells infected  
18 with control virus (Fig. 1F). This data showed that catalytic activity of HPSE1 promotes the  
19 LPS response in endothelial cells.

20 Endothelial cells can serve as a source of HPSE1<sup>26</sup>. HPSE1 is expressed in the form of 65 kD  
21 inactive precursor that is processed to an active 50 kD isoform through cleavage by Cathepsin  
22 L (Ctsl)<sup>6</sup>. To assess whether or not endogenous HPSE1 is activated by LPS in endothelial  
23 cells, we analyzed the expression of both HPSE1 isoforms in the conditioned medium of cells

1 and cell lysates using antibody that recognizes both forms. We found 65 kD inactive HPSE1  
2 mainly in cell conditioned medium, whereas active 50 kD form was mostly associated with  
3 the cells. Furthermore, the content of the endogenous catalytically active 50 kD HPSE1 was  
4 time dependently increased after the LPS treatment (Fig. 1G-E) whereas the content of 65 kD  
5 isoform in the conditioned medium was decreasing. These data point that endogenous HPSE1  
6 can be activated after LPS treatment to provide for a positive TLR4-signaling feedback loop.

## 7 ***2. HPSE2 overexpression protects from heparan sulfate-glycocalyx shedding***

8 To visualize the role of both heparanases in the turnover of the heparan sulfate glycocalyx in  
9 the endothelial cells, we established overexpression of HPSE2 through lentiviral transduction  
10 (Suppl. Fig. S1A-C). Similar to the endogenous HPSE2, overexpressed protein was released  
11 from the cells and distributed between free soluble form in the conditioned medium and cell-  
12 surface bound form (Suppl. Fig. S1B, C). HPSE-2 overexpressing endothelial cells showed  
13 no signs of toxicity. Instead, proliferation rate was slightly increased and the cells were  
14 protected against the TGF $\beta$ -induced apoptosis (Suppl. Fig. S1D, E).

15 We next inspected HPSE2-overexpressing endothelial cells that were incubated in the  
16 Polydimethylsiloxan (PDMS) - microfluidic chips under medium flow conditions. The chips  
17 consisted of 4 parallel 10 mm-long and 2 mm-wide channels (Fig. 2A). The cells were  
18 incubated under permanent medium flow conditions for 3 days. Endothelial cells developed a  
19 more 3D heparan sulfate-enriched glycocalyx layer when were subjected to the medium flow  
20 (Fig. 2B). Orthogonal views of the 3D z-scans confirmed extracellular localization of the  
21 glycocalyx layer above the layer of cell nuclei (Fig. 2B). We then added catalytically active  
22 HPSE1 to the medium used for chips perfusion. Under flow conditions this treatment also led  
23 to the shedding of heparan sulfate layer from the cell surface (Fig. 2C, D). Remarkably, cells  
24 overexpressing HPSE2 were protected against glycocalyx shedding by the exogenous HPSE1

1 (Fig. 2C, D). Similar results were obtained when shear stress was increased up to 38.75  
2 dyn/cm<sup>2</sup> (Supp. Fig. S2A).  
3 Confirming inhibitory role of heparan sulfate on the TLR4 response, the cells cultured under  
4 flow conditions demonstrated strongly diminished response to LPS (Fig. 2E). The expression  
5 of HPSE1 in endothelial cells cultured under flow was also diminished (Suppl. Fig. S2B).  
6 To assess whether or not HPSE2 prevents also the activity of endogenous HPSE1, lentivirus-  
7 infected cells were stimulated with LPS and activation of HPSE1 was followed by the  
8 accumulation of the active 50 kD HPSE1 isoform (Fig. 2F, Suppl. Fig. S1F). The  
9 overexpression of HPSE2 not only prevented the activity of exogenous HPSE1, but also LPS-  
10 dependent increase of endogenous HPSE1 activity. This data confirmed antagonistic  
11 relationship between the heparanases in endothelial cells. Accordingly, both expression and  
12 activity of Ct1 in the HPSE2 overexpressing cells were slightly decreased (Supp. Fig. S2B,  
13 C).

### 14 **3. HPSE2 protects endothelial cells**

15 We next studied whether or not HPSE2 can also exert protective functions under pathological  
16 situations. Control and HPSE2-overexpressing endothelial cells cultured under static  
17 conditions were treated with different concentrations of LPS. Significant downregulation of  
18 the LPS-induced IL-6 expression was observed in the HPSE2-overexpressing endothelial  
19 cells at different LPS concentrations (Fig. 3A). To precisely characterize the LPS response in  
20 HPSE2 overexpressing cells, Human Common Cytokine RT<sup>2</sup> Profiler Array was performed.  
21 Expressions of several pro-inflammatory cytokines, in particular, IL-6, IFNB1, IFNA1, CSF3  
22 (G-CSF), and TNFSF10 were significantly downregulated in HPSE2-overexpressing cells  
23 (Suppl. Fig. S3A). The data of the array were verified in independent RT-PCR experiment

1 (Suppl. Fig. S3B-E). Further cytokines including CSF2, IL-1A, IL-1B, TGFB3, TNFRSF11  
2 were downregulated to a lesser degree. Together, these data demonstrated general anti-  
3 inflammatory effects of the HPSE2 overexpression in the endothelial cells. The  
4 overexpression of HPSE2 have not induced any inflammatory effect on endothelial cells.  
5 We then assessed morphological changes in the endothelial cells treated with LPS. Similar to  
6 data reported by Zheng et al.<sup>37</sup>, we observed that control cells treated with 100 ng/ml LPS lost  
7 cell-cell contacts and demonstrated decreased VE-Cadherin protein expression (Fig. 3B, C),  
8 while HPSE2-overexpressing cells were protected and preserved cell-cell contacts much  
9 better than control cells. These cells also demonstrated increased VE-Cadherin expression  
10 after the LPS treatment (Fig. 3D). LPS-induced protein phosphorylation and activation of  
11 NFκB were less activated in HPSE-2 overexpressing endothelial cells as determined by  
12 western blotting (Fig. 3E, F) and luciferase assay (Suppl. Fig. S2E). Together, these data  
13 demonstrated a strong protection of endothelial cells exposed to LPS by HPSE2.

#### 14 ***4. HPSE2 prevents HPSE1 activity***

15 To test this idea, we made use of OGT 2115, an inhibitor of HPSE1 catalytic activity. The  
16 compound decreased NFκB activation as shown in promoter activity luciferase assay (Suppl.  
17 Fig. S2E). LPS-dependent expression and secretion of IL-6 was accordingly downregulated  
18 in the presence of HPSE1 inhibitor (Fig. 4A, B). However, there was no additive effect after  
19 HPSE1 inhibition in HPSE2 overexpressing cells (Fig. 4A, B; Suppl. Fig. S2E). This finding  
20 suggested that both proteins are parts of the same molecular pathway and anti-inflammatory  
21 role of HPSE2 is most likely mediated by its antagonistic action on HPSE1. A specific  
22 protective role of HPSE2 was further confirmed using the 1c7 antibody to HPSE2<sup>23</sup> that can  
23 block HPSE2 interaction with heparan sulfate<sup>38</sup>. Application of the 1c7 antibody and thus  
24 displacement of the protein from cell surface heparan sulfate glycoalyx structures resulted in



1 abrogation of HPSE2 protective action and strong activation of LPS response (Fig. 4C). We  
2 suggest that HPSE1 regulates TLR4 response in endothelial cells. The effects are counteracted  
3 by HPSE2 bound to cell surface heparan sulfate.

#### 4 ***5. HPSE2 inhibits LPS binding to TLR4 receptor complex***

5 We next studied the mechanisms of the regulation of LPS response in endothelial cells by  
6 HPSE1. Molecular mechanisms of TLR4 signaling are extensively investigated. The LPS  
7 molecule is first bound to TLR4 co-receptor, CD14<sup>39</sup>. Thereafter, LPS is transferred to the  
8 complex of TLR4 with an accessory molecule, Lymphocyte antigen 96, (also known as  
9 MD2) to induce the activation of intracellular adapters and subsequent protein  
10 phosphorylation cascades<sup>40</sup>. Several mechanisms could account for the role of HPSE1. First,  
11 TLR4 can be further activated by heparan sulfate fragments originating from the HPSE1  
12 catalytic activity<sup>16</sup>. Second, LPS ligand binding to the CD14 receptor could be affected by  
13 heparan sulfate. Finally, the interaction of TLR4 with co-receptors on the cell membrane can  
14 be regulated by heparan sulfate. We first tested whether accumulation of heparan sulfate  
15 fragments during the cells stimulation with LPS can lead to further activation of TLR4.  
16 Exogenous heparan sulfate caused a weak increase in IL-6 expression, even at high  
17 concentrations (Fig. 5A). This response was also decreased in HPSE2-overexpressing cells  
18 (Fig. 5A). To investigate whether or not endogenously produced soluble heparan sulfate  
19 fragments can promote TLR4 activation, conditioned medium of cells treated with LPS for 3  
20 h, was used for stimulation of naïve cells. We found that supernatant of LPS-treated cells  
21 caused strong increase of IL-6 expression (Fig. 5B). Proteinase K is a serine protease used to  
22 digest proteins in biological samples. Removal of protein components from the supernatant  
23 by incubation with beads-immobilized proteinase K prevented expression of IL-6. Removal  
24 of remaining LPS using Endotoxin-Removal beads caused complete abrogation of IL-6

1 expression (Fig. 5B). Exogenous heparan sulfate at concentration of 100 ng/ml strongly  
2 potentiated endothelial cell response to LPS (Fig. 5C). This response was documented by the  
3 3-folds increase of the LPS-induced IL-6 expression in the presence of exogenous heparan  
4 sulfate. These findings suggested that concentration of endogenous heparan sulfate fragments  
5 produced during 3 hrs of LPS stimulation was not sufficient to potentiate activation of TLR4.  
6 However, in the case of significant accumulation, soluble heparan sulfate fragments can  
7 potentiate inflammatory response of endothelial cells to LPS.

8 We then investigated whether HPSE2 can affect LPS binding using biotin-LPS. Cells were  
9 incubated on ice to prevent internalization of receptors. Cell binding of biotin-LPS was not  
10 decreased by overexpression of HPSE2 (Fig. 5D), suggesting that LPS binding to CD14 was  
11 not affected. The specificity of biotin-LPS binding to CD14 was confirmed by application of  
12 CD14 blocking antibody. Over 70% of the Biotin-LPS binding was blocked by CD14  
13 antibody but not isotypic IgG.

14 TLR4 intracellular signaling is mediated by the recruitment of two adapter proteins, MyD88  
15 and TIR-domain-containing adapter-inducing interferon- $\beta$  (TRIF)<sup>41</sup>. We observed diminished  
16 LPS-induced expression of IL-6 suggesting impaired MyD88-pathway activation in HPSE2-  
17 overexpressing cells. We further investigated the expression of IFNB1 and chemokine ligand-  
18 5 (CCL5), also known as RANTES that is mediated by TRIF adapter pathway activation (Fig.  
19 6A). Our data show that both, MyD88 and TRIF-mediated signaling pathways of TLR4 are  
20 inhibited by HPSE2. Since LPS binding was not impaired by the overexpression of HPSE2,  
21 we reasoned that HPSE2 most likely interfered with the LPS transfer from CD14 to  
22 TLR4/MD2 complex. In order to verify this hypothesis, a pull-down assay using biotin-LPS  
23 and streptavidin magnetic beads was performed. Cells were incubated for 1 h on ice in the  
24 presence of 50 ng/ml biotin-LPS to allow its binding to the cell surface. Then, the cells were

1 transferred to 37°C for the indicated time to enable further signaling events to take place.  
2 The results supported our hypothesis (Fig. 6B, C) as less TLR4 was detected in the biotin-  
3 LPS complexes from HPSE2-overexpressing endothelial cells. Regulation of CD14/TLR4  
4 association by HPSE1 and protective role of HPSE2 was confirmed by the Duolink proximity  
5 ligation assay – a method to detect protein-protein interaction (Fig. 6D, E). To investigate the  
6 endocytosis of TLR4, cell surface staining on living cells was performed. To stop endocytosis  
7 and prevent staining of intracellular TLR4, cells were placed on ice after LPS stimulation and  
8 stained with anti-TLR4 antibody (Fig. 6F, G). Decreased staining suggested active  
9 endocytosis of the receptor complex in control cells. However, in HPSE2-overexpressing  
10 cells TLR4 remained on the cell surface. These findings imply that membrane-bound heparan  
11 sulfate prevents LPS transfer from CD14 to TLR4/MD2 complex and its removal by HPSE1  
12 promotes the LPS response of endothelial cells.

### 13 **6. HPSE2 is protective in vivo**

14 To investigate how expression of endogenous HPSE2 is regulated during sepsis, we used the  
15 mouse CLP polymicrobial sepsis model. We observed decreased HPSE2 expression in serum  
16 of CLP mice (Fig. 7A, B). In the kidney we observed HPSE2 expression in medullary  
17 capillaries. This expression of HPSE2 was strongly downregulated in sepsis (Fig. 7C, D).  
18 Negative control staining is shown in supplementary Fig. S3F.

19 We next purified HPSE2 from conditioned medium of HEK293T cells transduced with LV-  
20 HPSE2 overexpression virus. Application of the exogenous HPSE2 diminished LPS response  
21 of the endothelial cells in vitro in a similar fashion to HPSE2 overexpression (Supplementary  
22 Fig. S3G). When co-injected intravenously along with LPS in mice, HPSE2 had similar  
23 protective effects and caused statistically significant decrease in the plasma content of TNF $\alpha$

1 and IL-6 2 h after injection (Fig. 7E, F). This data suggest that HPSE2 has also an endothelial  
2 protective function in microvasculature in vivo and can have some therapeutic potential.

### 3 **Discussion**

4 We demonstrated an important protective role for HPSE2 both in vivo and in vitro. Using  
5 different approaches we showed that HPSE1 activity aggravates endothelial response to LPS,  
6 whereas HPSE2 prevents activation of HPSE1, heparan sulfate shedding, and inflammatory  
7 responses mediated by TLR4. Our findings support an essential role for an intact glycocalyx  
8 in the endothelium. Glycocalyceal shedding occurs in many diseases and pathological insults.  
9 However, the mechanisms how glycocalyceal turnover is regulated and what the  
10 consequences are when the process is perturbed is still imperfectly understood. A high  
11 expression of HPSE1, the sole enzyme capable of cleaving heparan sulfate, is damaging for  
12 the endothelium<sup>42</sup>. Although the role of HPSE2 as an antagonist of HPSE1 is well appreciated  
13 in cancer, how the interaction of these proteins can affect endothelial cells is less clear.  
14 The role of heparan sulfate in the TLR4-mediated LPS response is not well understood.  
15 Soluble heparan sulfate fragments generated by HPSE1 can serve as ligand for TLR4<sup>16</sup>.  
16 Treatment of macrophages with HPSE1 facilitated an inflammatory response. On the  
17 contrary, in microglia heparan sulfate promoted TLR4/CD14 complex formation and HPSE1  
18 transgenic cells showed less inflammatory response to LPS<sup>20</sup>. Our data suggest that heparan  
19 sulfate prevents the activation of TLR4 signaling and inflammatory response of endothelium.  
20 HPSE1 is an important component of the TLR4 signalosome as heparan sulfate cleavage  
21 facilitates cellular response and promotes inflammatory reaction of the endothelial cells.  
22 Both heparanases are present in human plasma. HPSE1 upregulation is associated with many  
23 disease states. Our data showed decreased plasma HPSE2 expression in mouse sepsis models

1 indicating that cross-talk of the heparanases is important in vivo. Our data suggest that  
2 endothelial cell express and release HPSE1 and HPSE2 for local regulation of heparan sulfate  
3 turnover. Our mechanistic studies showed that HPSE2 bound to heparan sulfate on the  
4 endothelial cell surface prevents activation of HPSE1 and heparan sulfate shedding. We  
5 found that HPSE2 displacement from the cell surface by the 1c7 antibody<sup>38</sup> led to the  
6 abrogation of HPSE2 protective function. Taking into account poor development of the  
7 heparan sulfate glycocalyx under normal static cell culture conditions we applied  
8 microfluidic chip model to demonstrate the protective role of HPSE2<sup>43,44</sup>. In the microfluidic  
9 experiments, HPSE2 overexpressing cells were strongly protected from heparan sulfate loss  
10 by HPSE1. This observation further confirmed that HPSE2 expressed by endothelial cells  
11 partially remained bound to the heparan sulfate glycocalyx and counteracted its cleavage by  
12 HPSE1. Inhibiting role of the glycocalyx in TLR4 receptor signaling was confirmed by  
13 diminished LPS response of the cells incubated under flow conditions.

14 In macrophages HPSE1 itself was shown to induce TLR dependent signaling<sup>21</sup>. In endothelial  
15 cells HPSE1 induced rather weak inflammatory response by itself and only by the prolonged  
16 incubation (data not shown). Thus, in the context of TLR4 activation on the endothelium,  
17 remodeling of the glycocalyx on the cell surface seems more important. Interestingly,  
18 exogenous heparan sulfate strongly potentiated cell response to LPS. The finding can  
19 probably be explained by competitive binding of HPSE2 to soluble heparan sulfate resulting  
20 in its' removal from the cell membrane-associated glycocalyx.

21 We used biotin-LPS to demonstrate that HPSE2 does not interfere with LPS binding to the  
22 CD14 but rather prevents LPS transfer to TLR4 and activation of the cellular signaling. As a  
23 result, the inflammatory response was reduced. Early reports suggested that endothelial cells  
24 express only the soluble form of CD14<sup>45,46</sup>. On the contrary, more recent reports showed the

1 expression and the role for membrane CD14 in endothelial response to LPS<sup>47,48</sup>. Lloyd-Jones  
2 et al. showed that membrane-bound CD14 is indispensable for the activation of TRIF-  
3 mediated pathway in endothelial cells<sup>48</sup>. We showed that both, MyD88- and TRIF-dependent  
4 pathways are affected by HPSE2. Therefore, we assume that membrane bound form of CD14  
5 is regulated by heparan sulfate. However, involvement of soluble CD14 can not be excluded.  
6 In vivo co-injection of HPSE2 along with LPS in mice led to decreased cytokines expression  
7 in plasma. In polymicrobial CLP mouse sepsis model the expression of HPSE2 in renal  
8 medullary capillaries was decreased. Thus, loss of HPSE2 can promote microcirculatory  
9 disorder in sepsis leading to organ failure.

10 Our results suggest that HPSE2, expressed locally by the endothelial cells or delivered with  
11 blood, fulfills protective role in microvasculature via protection from heparan sulfate  
12 shedding and anti-inflammatory regulation of TLR4 signaling. HPSE2 is a novel molecule  
13 which exerts a direct protective effect on the endothelial glycocalyx thereby maintaining  
14 microvascular function and stability as well as protecting the endothelium from damage. Our  
15 results in vivo suggest that HPSE2 supplementation may be beneficial for the protection of  
16 the microvasculature. This novel mechanism supports therapeutic strategies to stabilize the  
17 endothelial glycocalyx.

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2 c) Disclosures: none.

3

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5

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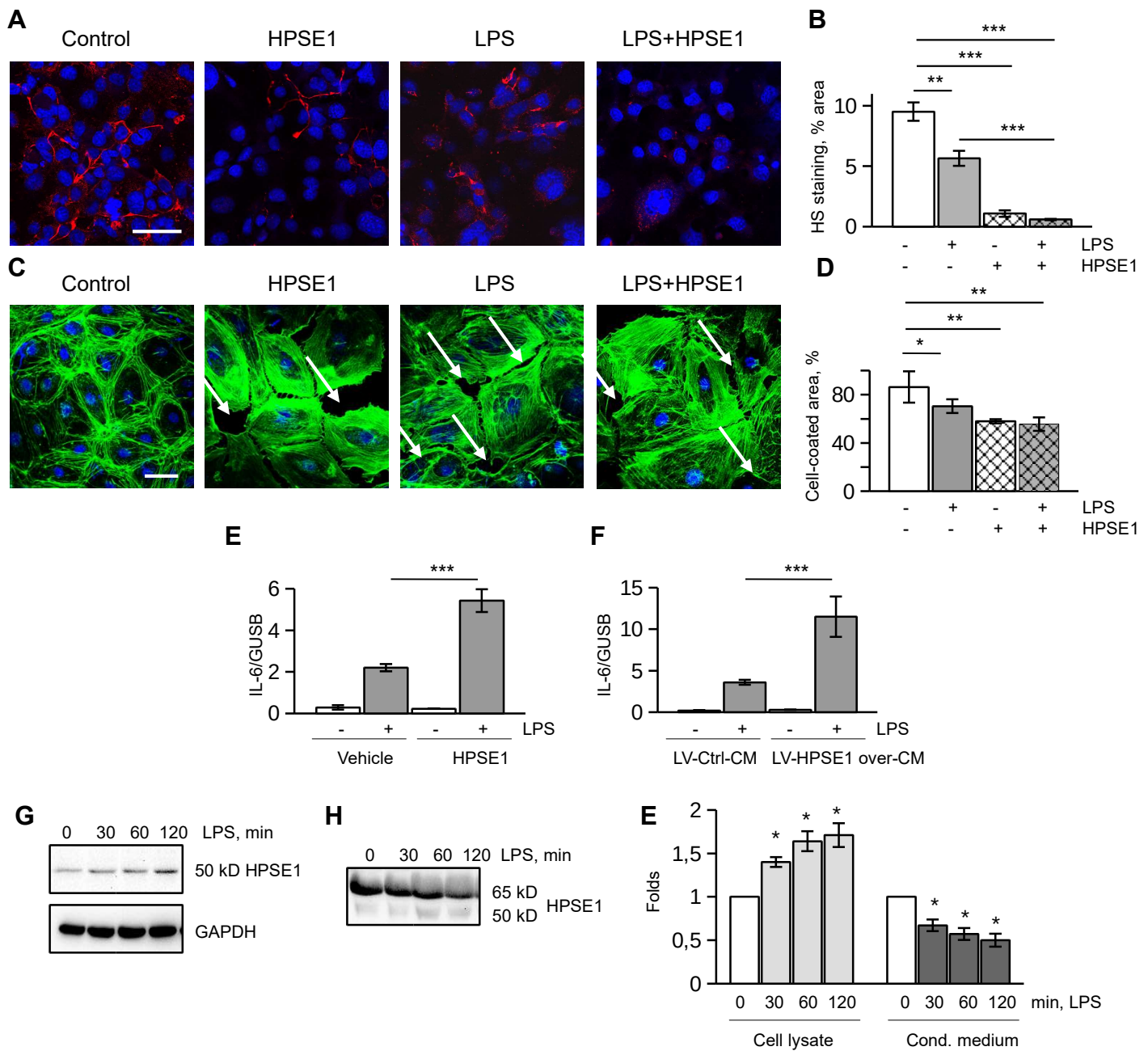
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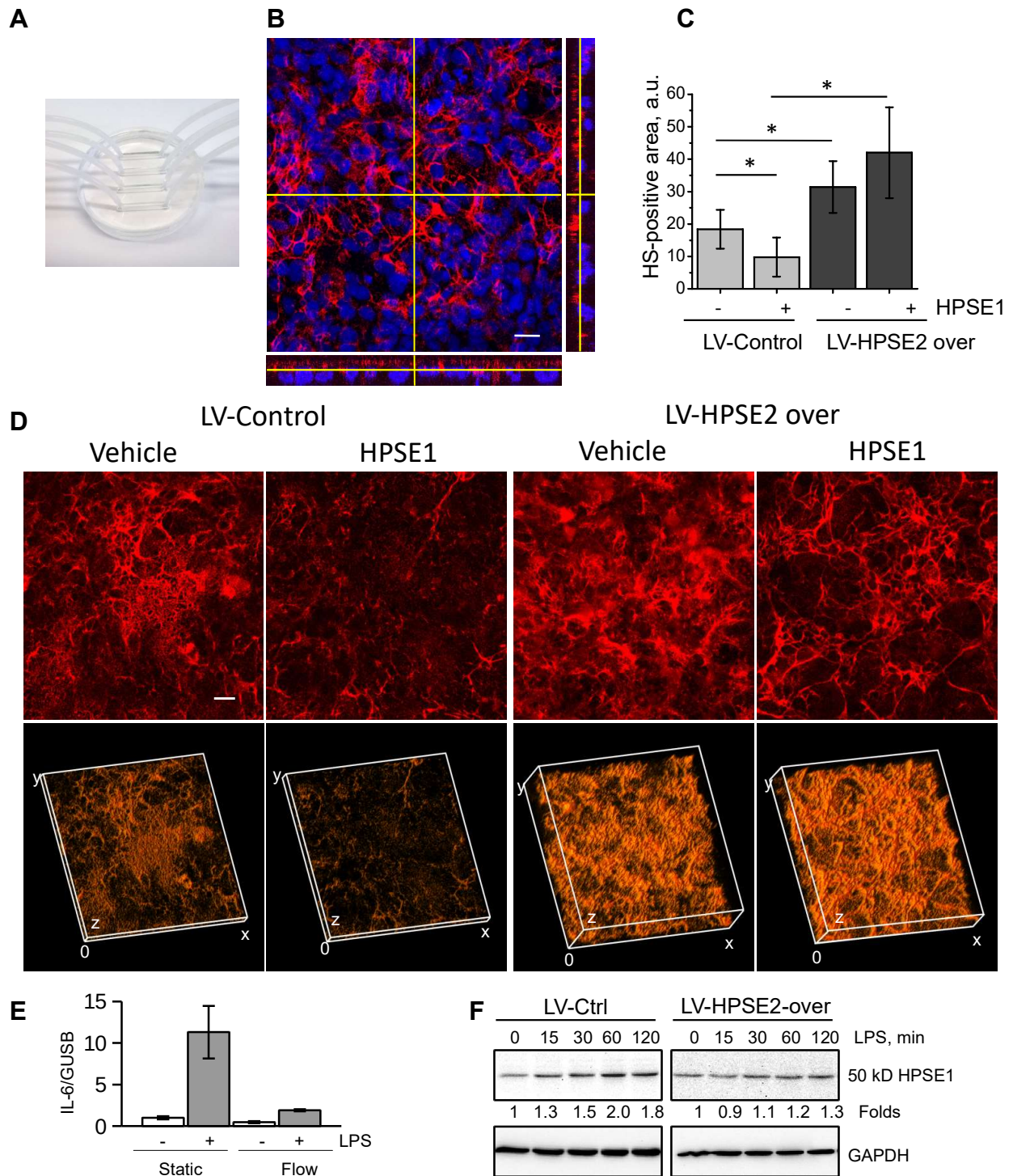
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- 10

1 **Highlights.**

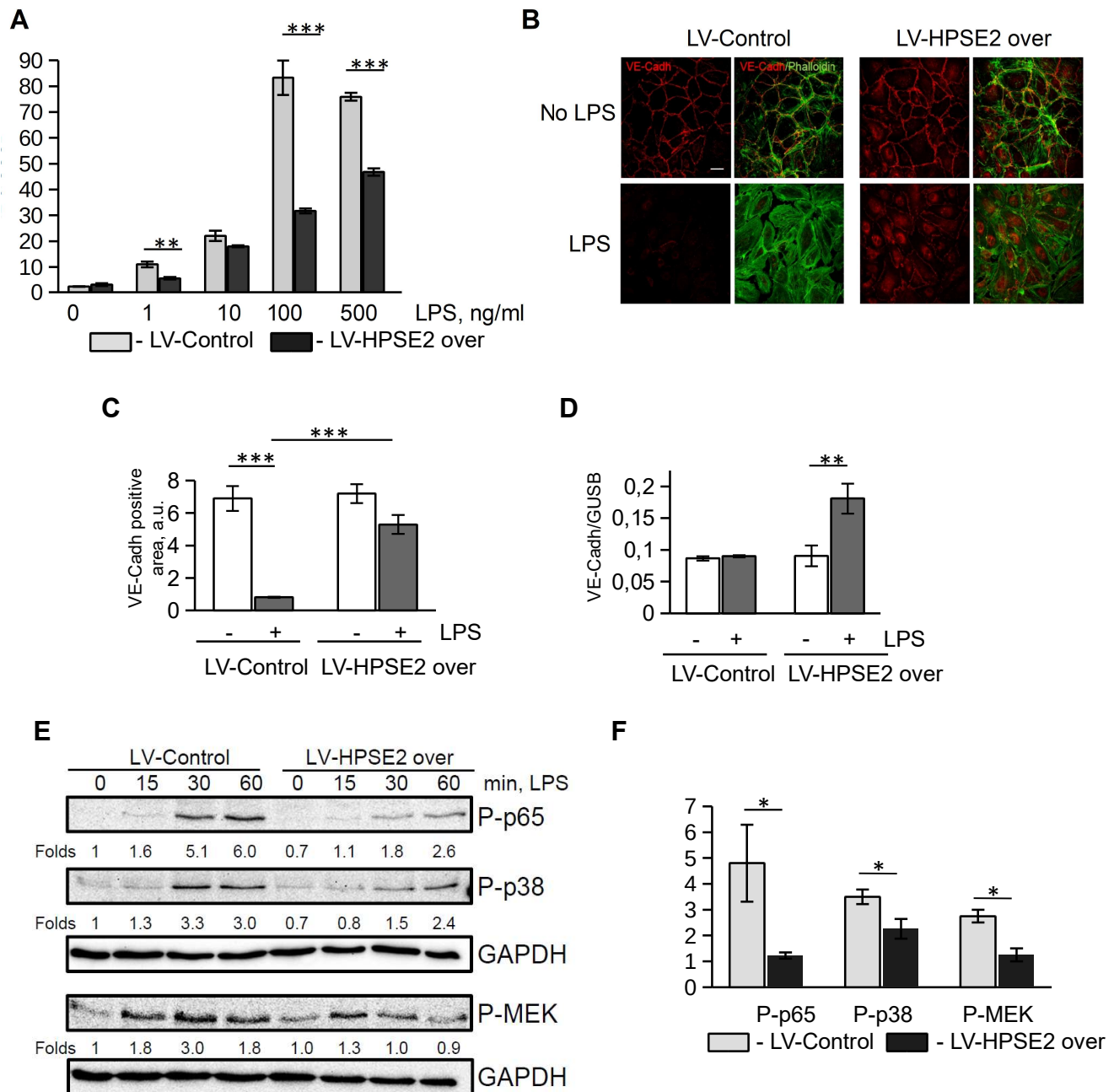
- 2       ➤ Catalytic activity of Heparanase 1 aggravates TLR4 signaling and endothelial  
3       response to LPS
- 4       ➤ Heparanase 2 diminishes endothelial response to LPS
- 5       ➤ Heparanase 2 does not affect LPS binding to CD14 but inhibits LPS presentation to  
6       TLR4
- 7       ➤ Expression of HPSE2 in vivo is decreased in sepsis.



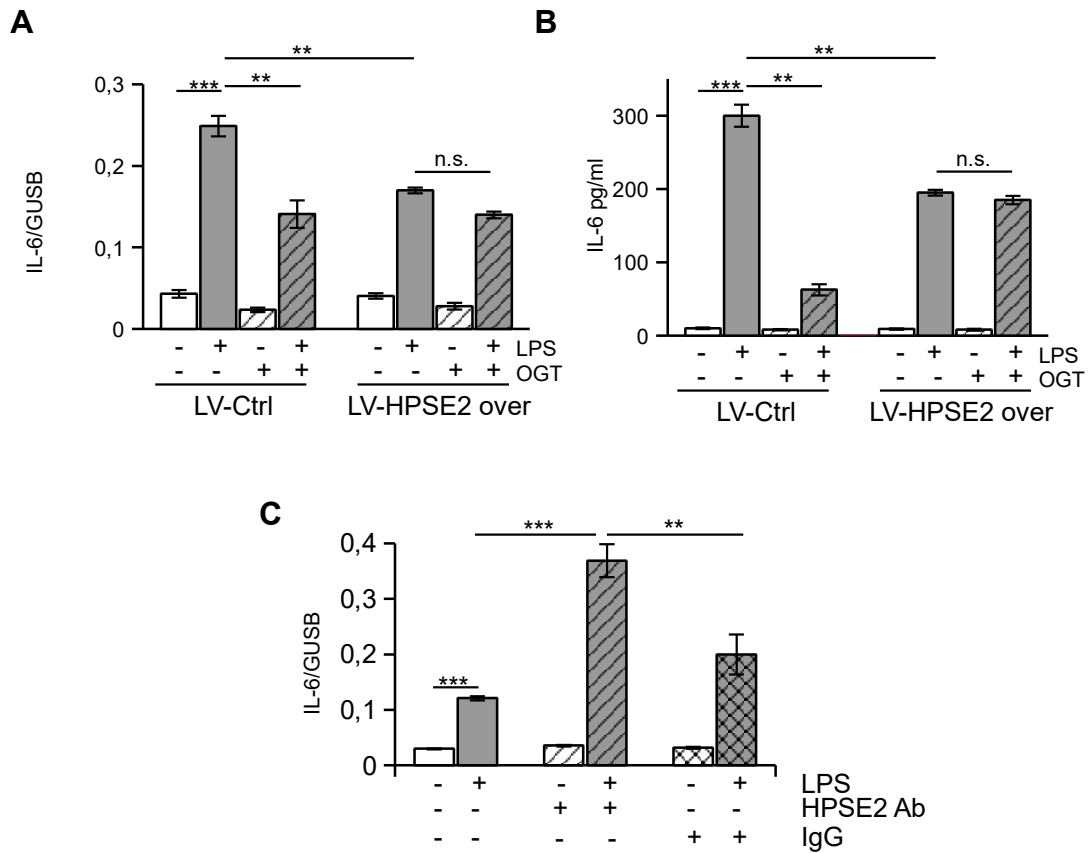
**Figure 1. HPSE1 aggravates endothelial cell response to LPS.** **A.** HMEC-1 cells were treated with active HPSE1 for 1 h prior to LPS stimulation. Then, cells were treated with 100 ng/ml LPS for 3 hrs. Cells were fixed and stained with 10A4 antibody for heparan sulfate (Alexa 594). Nuclei were stained with DAPI. Scale Bar 50 $\mu$ m **B.** Quantification of heparan sulfate stainings. Percentage of stained areas was calculated using ImageJ. **C.** Primary endothelial cells treated as in A were stained with Alexa 488-Phalloidin (green) and DAPI. White arrows show loss of cellular contacts. Scale Bar 50 $\mu$ m. **D.** Quantification of phalloidin images. Percentage of cell-coated areas was calculated using ImageJ. **E.** HMEC-1 cells were treated with catalytically active HPSE1 and then stimulated with LPS. IL-6 expression was assessed by RT-PCR. **F.** Cell were treated with conditioned medium from control (LV-Ctrl-CM) and HPSE1 overexpression (LV-HPSE1 over-CM) lentivirus-transduced HMEC-1 cells for 1h prior to LPS stimulation. IL-6 expression was assessed by RT-PCR. **G.** Expression of 50 kD active HPSE1 after LPS stimulation of endothelial cells assessed in cell lysates by western blotting. **H.** Expression of 65 kD and 50 kD isoforms of HPSE1 was assessed in conditioned medium of LPS-stimulated endothelial cells. **E.** Quantification of three independent western blotting experiments as shown in G (Cell lysate) and H (Cond. Medium). Data were quantified using QuantityOne software (BioRad).



**Figure 2. HPSE2 overexpression protects endothelial heparan sulfate glycoalyx in a microfluidic chip model.** **A.** 4-channels PDMS-microfluidic chip. **B.** Development of 3D heparan sulfate glycoalyx layer after endothelial cells cultivation under flow conditions (3 days, 0.1 dyn/cm<sup>2</sup>) was assessed by 3D confocal microscopy using 10E4 antibody to heparan sulfate followed by image reconstruction using ImageJ software. DraQ5 was used as a nuclear stain. Yellow lines show position of orthogonal slices. Bottom and right panels show corresponding orthogonal slices of the sum images. **C.** Control and HPSE2 overexpression-lentivirus-infected endothelial cells were cultivated in the microfluidic chip for 3 days, and then perfused with recombinant HPSE1 for 4 hrs. Cells were fixed by perfusion with PFA as described in the methods section. Heparan sulfate content was quantified using ImageJ software after 3D confocal microscopy. Experiment was independently repeated in 4 chips. **D.** 3D reconstruction of heparan sulfate glycoalyx layer images of lentivirus-infected endothelial cells after incubation in the microfluidic chip and recombinant HPSE1 treatment. The 3D reconstruction was performed using ImageJ software. Scale bar 20µm. **E.** Endothelial cells incubated in microfluidic chips under static and flow conditions were stimulated with 100ng/ml LPS for 3 hrs. IL-6 expression was assessed by TaqMan RT-PCR. **F.** 50 kD HPSE1 expression in lysates of LPS-stimulated endothelial cells.

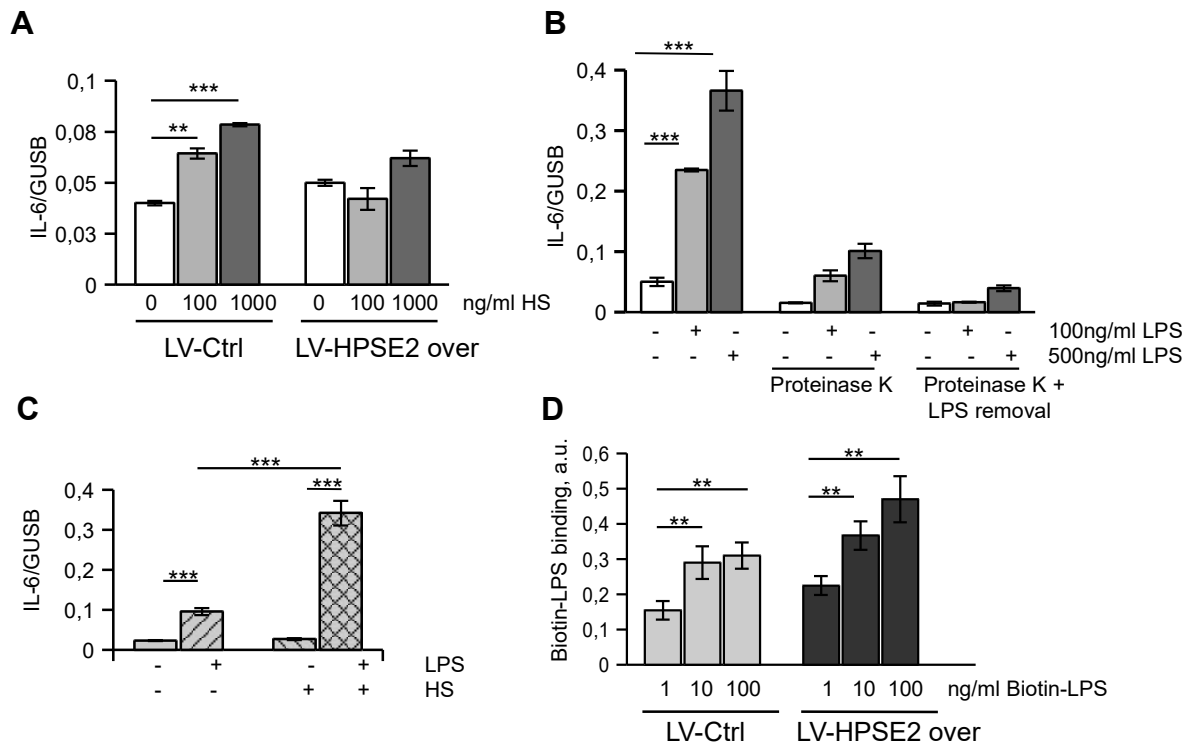


**Figure 3. HPSE2 protects endothelial cells from LPS-induced damage.** **A.** Endothelial cells infected with Control and HPSE2- overexpression lentivirus were treated with different concentrations of LPS for 3 hrs as indicated. IL-6 expression was assessed by TaqMan RT-PCR. **B.** Primary endothelial cells treated with LPS for 24 hrs cells were fixed and immunostained for VE-Cadherin (Alexa 594) and Phalloidin (Alexa 488). **C.** The percentage of VE-Cadherin positive areas was calculated using ImageJ. **D.** Expression of VE-Cadherin mRNA in LPS treated cells was quantified by TaqMan RT-PCR. **E.** Endothelial cells were stimulated with 1  $\mu$ g/ml LPS for indicated times. Protein phosphorylation was assessed by western blotting. **F.** Quantification of phosphorylation of p65, p38, p-MEK at 30 min of LPS stimulation quantified from three independent western blotting experiments.

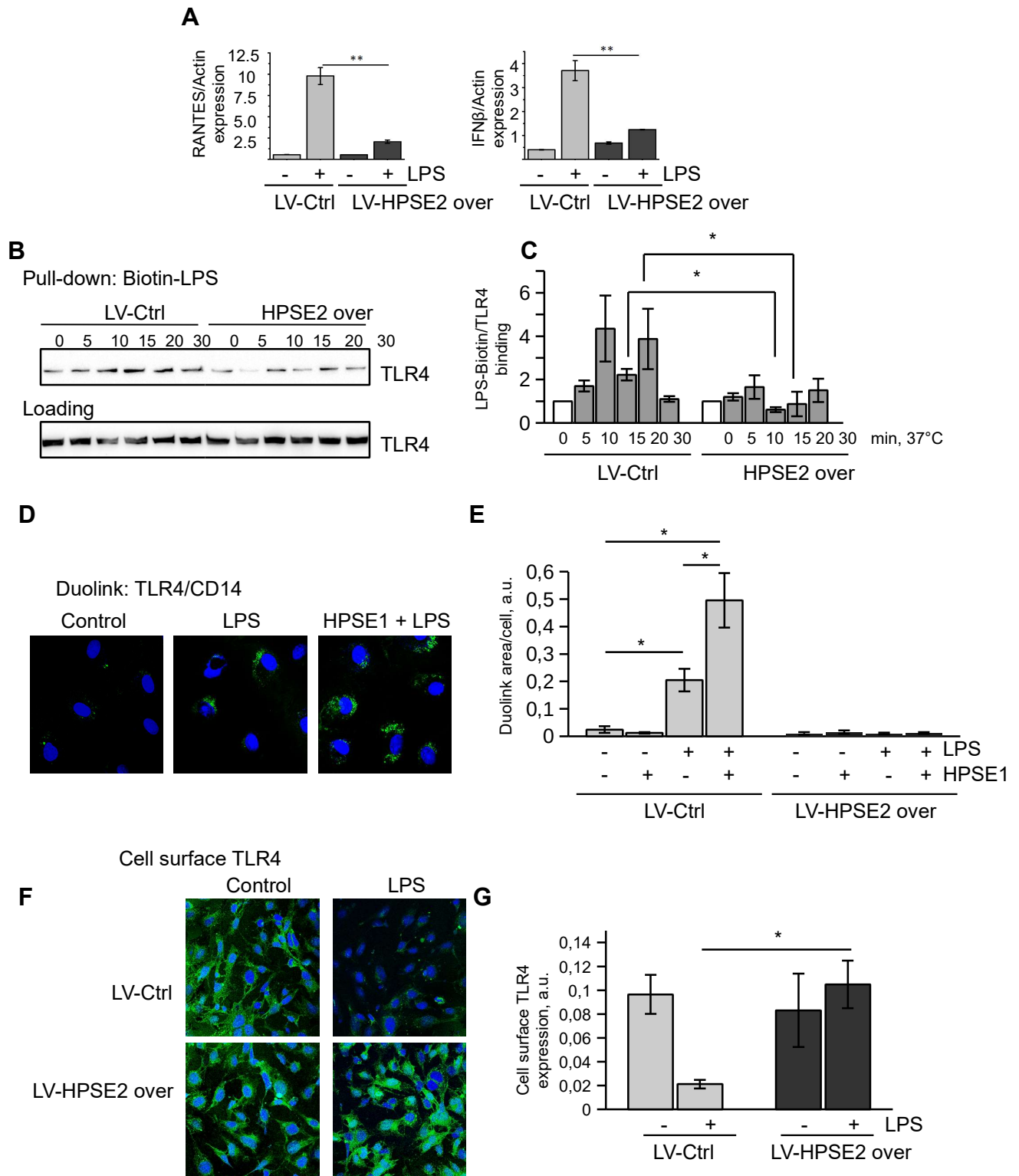


**Figure 4. HPSE2 prevents HPSE1 activity.** A. Endothelial cells were pre-treated with inhibitor of HPSE1 catalytic activity, OGT 2115, and then stimulated with 100 ng/ml LPS for 3 hrs (A) and overnight (B). IL-6 expression was analyzed by RT-PCR (A) and ELISA (B). C. Endothelial cells were pre-incubated with mouse IgG or anti-HPSE2 antibody 1c7 for 30 min prior to LPS stimulation and then stimulated with 100 ng/ml LPS for 3 hrs. IL-6 expression was assessed by RT-PCR.

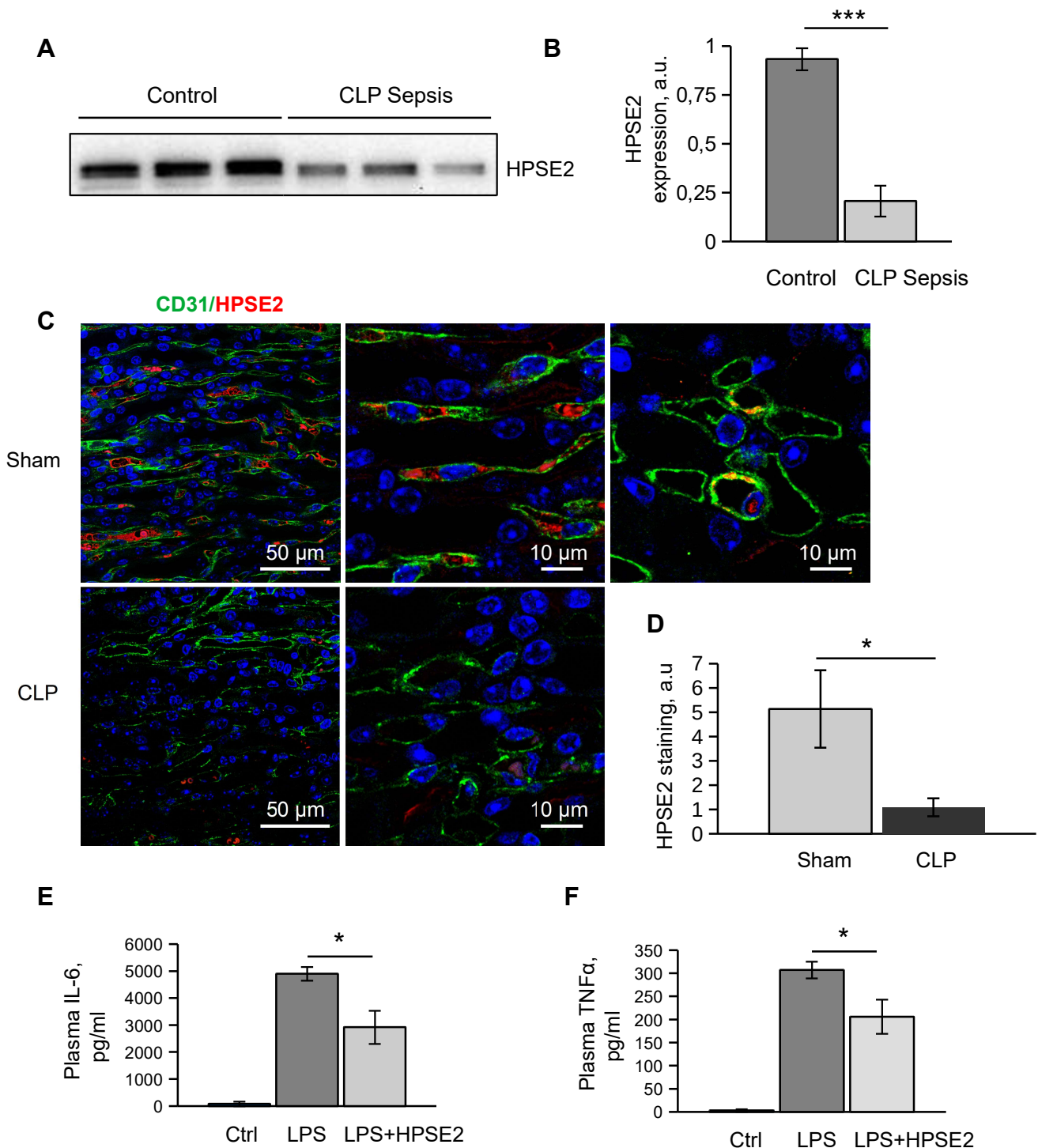




**Figure 5. Heparan sulfate fragments cause weak activation of endothelial cells.** **A.** Endothelial cells were treated with the indicated concentration of heparan sulfate (HS) for 3 hrs. IL-6 expression was assessed by RT-PCR. **B.** Conditioned medium of endothelial cells treated for 3 hrs with 100 ng/ml and 500 ng/ml LPS was used for stimulating naïve cells. Conditioned medium was treated with Proteinase K immobilized on agarose beads and remaining LPS was removed by incubation with LPS removal beads, as indicated. IL-6 expression was assessed by RT-PCR. **C.** Endothelial cells were stimulated with 100 ng/ml LPS in the absence and in the presence of 100 ng/ml heparan sulfate (HS). **D.** Cell-based ELISA for Biotin-LPS binding. Lentivirus-transduced endothelial cells were incubated on ice for 1h in the presence of biotin-LPS as indicated. After washing cells were incubated with SV-HRP. Biotin-LPS binding was quantified using TMB substrate kit.



**Figure 6. HPSE2 overexpression prevents TLR4 activation by LPS.** **A.** Endothelial cells infected with Control and HPSE2 overexpression lentivirus were treated with 100 ng/ml LPS for 3 hrs. Expression was analyzed by TaqMan RT-PCR. **B.** TLR4 pull-down using biotin-LPS was performed as described in the Materials and Methods section. TLR4 was detected by immunoblotting. **C.** Quantification of independent TLR4 pull-down assay experiments. **D.** Duolink proximity ligation assay revealing direct interaction of TLR4 and CD14 was performed on endothelial cells stimulated with 100 ng/ml LPS for 20 min. Treatment with catalytically active HPSE1 was performed for 1 h prior to LPS stimulation. **E.** Duolink signal stained as in F was quantified using ImageJ as described in Materials and Methods. **F.** To detect cell surface TLR4 lentivirus-infected endothelial cells were stimulated with 500 ng/ml LPS for 1h, then placed on ice and incubated with TLR4 antibody. Then, the cells were washed, fixed, and stained with secondary antibody (green) and DAPI. **G.** Cell surface TLR4 expression of lentivirus-infected and LPS-stimulated endothelial cells was quantified using cell-based ELISA as described in Materials and Methods.



**Figure 7. HPSE2 protects against inflammation in vivo.** **A.** Serum level of HPSE2 expression in mouse CLP sepsis model was assessed by western blotting. **B.** Quantification of western blotting data. **C.** Expression of HPSE2 in mouse kidney medullary capillaries in CLP sepsis model. Tissue sections were stained with CD31 (Alexa 488) and HPSE2 (Alexa 594). Nuclei were stained with DAPI. **D.** Quantification of HPSE2 immunohistochemistry stainings in mouse kidney. **E, F.** Mice were iv injected with 5 $\mu$ g/g body weight LPS alone or LPS alone with 5 $\mu$ g/g body weight purified HPSE2. After 2hrs plasma cytokine level was measured using cytometric bead array. (E) IL-6 expression; (F) TNF $\alpha$  expression. Mean  $\pm$ s.e.m. is shown.