Platelet activation at the onset of human endotoxemia is undetectable *in vivo*

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Effects of different doses of LPS on platelet activation *in vitro*

To further study the effects of LPS on platelet function *in vitro*, we aimed at stimulating platelets with similar LPS concentrations as in our endotoxemia model.

We assumed that injection of 2 ng/kg body weight (approximately 150 ng LPS per healthy, male volunteer), led to a LPS plasma concentration of approximately 50 pg/ml (150ng /3 l plasma as hydrophilic substances primarily distribute to the plasma compartment). As it takes approximately three lung passages to equally distribute a substance in blood, LPS concentrations might be altered at the beginning of infusion, while at later time points LPS levels will drop due to LPS-cell interactions. Therefore we tested the effect of 25, 50 and 100 pg/ml LPS on surface expression of P-selectin and GPIIb/IIIa activation in platelet-rich plasma (PRP). As depicted in supplemental Fig. 1 no significant differences in platelet activation were observed between 25, 50 and 100 pg/ml LPS. Of note, no significant changes in platelet activation were

observed between 0 and 60 min without addition of any agonists, indicating that untreated platelets remained in a resting state within the investigated time frame.

Supplemental figure 1: Platelet activation in response to 25 - 100 pg/ml LPS. PRP was spiked with LPS (25 pg/mL, 50 pg/mL or 100 pg/mL) for 60 min. Platelet activation was determined at basal level (0min, 60min) and after LPS spike (60 min) by measuring surface P-selectin (CD62P) expression (left panel) and GPIIb/IIIa activation (PAC-1, right panel) as described in the Materials and Methods section of the manuscript. Statistics by repeated measures ANOVA with Bonferroni's multiple comparison test. n=9 for CD62P; n=5 for PAC-1;

Effect of LPS on endothelial cell activation within 4 minutes of stimulation

To rule out that platelet adhesion to endothelial cells in response to LPS stimulation was a result of endothelial cell activation, we measured endothelial cell activation after 4 minutes of stimulation with LPS.

Due to that short time of stimulation, neither de novo protein synthesis nor integrin expression can be observed. Only rapid events like exocytosis of Weibel Palade bodies are possible. Due to flow conditions released cytokines cannot be measured. Therefore we determined endothelial cell activation after 4 minutes of LPS stimulation by P-selectin via immune histochemistry.

As shown in supplemental Figure 2 we did not observe any significant differences in VE-cadherin or P-selectin expression.

Supplemental figure 2: P-selectin expression of HUVECs after LPS (50 pg/ml) perfusion. HUVECs were perfused with 50 pg/ml LPS for 4 min, followed by a 10 min washout phase and immediate fixation with 1% PFA. Cells were stained for P-selectin (1:100, 60 min, BioLegend), VE-Cadherin and Hoechst (as described in the Materials and Methods section of the manuscript). P-selectin expression was quantified as percentage of P-selectin positive area using ImageJ. Representative pictures and mean $\pm$ SD are given. n=3