Title

Histidine rich protein II, from malaria diagnostic marker to strong proinflammatory and risk factor for severe malaria outcomes

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Abstract

Malaria is a parasitic disease that prevails in the world's poorest regions severely hampering socio-economic development. In humans, it is caused by five protozoan species belonging to the genus *Plasmodium* of which *P. falciparum* accounts for 99% of all malaria-associated deaths. Strong correlations have been established between high concentrations of Histidine-rich protein II (HRPII), produced by *P. falciparum*, and higher mortality and severity of malaria outcomes, including cerebral malaria.

The neuropathogenesis of cerebral malaria remains unclear and it presents as a multifactorial pathogenesis depending from the patient status and multiple parasitic virulence mechanisms. Recent data identify HRPII as one of the critical virulence factors for the onset of cerebral malaria as its ability to disrupt the Blood-Brain Barrier by itself. The suggested pathogenic mechanism includes activating the NF-kB pathways and the inflammasome in brain endothelial cells (even in the absence of other parasite factors, or immune cells, or astrocytes) with the release of hIL1β. These events then trigger the loss of integrity of the BBB, both in *in vitro* model systems and *in vivo*. It remains largely unknown how these cellular responses are mediated by HRPII binding to the cell and which HRPII-binding molecules are required to trigger this response. In fact, HRPII has shown high affinity towards some glycosaminoglycans, divalent cations, heme, and others, all of which could play binding, structural or cofactorial role. This study aims to investigate the early brain endothelial response of HRPII through the analysis of gene activation/repression using RNAseq data and provide a better understanding of the metabolite patterns related to HRPII-mediated effects.

The disruption of the BBB due to HRPII at concentrations comparable to its serum levels during infection has been previously shown using directly HRPII-producing parasites or purified HRPII either from parasites and parasite-conditioned media or from *E. coli* recombinant expression. Using RNA-seq and *in-vitro* BBB model system, we have investigated the HRPII-triggered transcriptional changes in endothelial cells. We used HRPII protein either produced and purified from standard *E. coli* BL21(DE3) or a BL21(DE3)-derived mutant lacking lipopolysaccharide endotoxin, called Clear-coli® (Lucigen). This comparison aimed to rule out any contribution to the cellular responses of traces of endotoxin

eventually carried over during purification from *E. coli* and bound to HRPII, possibly via the repetitive glycan polymer, commonly referred to as O antigen. An extensive LPS-removal step using Triton-X114 was performed in both purifications, and the purified protein was negative at the LAL-test. We have then analysed the cellular transcriptional responses over three time points, 0, 3h, and 12h. By including gene responses to LPS only, we have also analysed the differences between endotoxin and HRPII activation of the endothelial cells.

The RNA was extracted from eight conditions in quadruplicate, and the libraries were prepared, assessed for high quality, and then sequenced using an Illumina HiSeq. On average, a sequence depth of 26.9 ± 0.8 million reads per sample was obtained and about 99% mapped to the human genome. The average length-mapped of ~255bp was higher than expected (~200bp), and the sequencing depth per library achieved in this experiment was sufficient for downstream differential expression analyses. Our analysis of the RNAseq data revealed that HRPII purified from Clear coli® did not trigger the activation of the innate immune response, supporting the hypothesis that endothelial response to HRPII requires costimulatory molecules. Surprisingly, it seems to promote cytoprotective pathways. However, when potential traces of LPS, less than 0.01 EU (detection limit of the LAL-test), could not be ruled out, strong transcriptional changes and inflammation response, ultimately causing BBB disruption was detected. The activated pathways included TNF signaling, IL-17 signaling, NF-kappa B signaling, toll-like receptor signaling, cytokine-cytokine receptor interaction, NOD-like receptor signaling, and chemokine signaling with marked upregulation of genes, like PTGS2, TNFAIP3, CXCL-10, and MMP3, associated with HRPII treatment. The activation of the inflammatory responses was more efficient in the cells treated with HRPII than LPS-treated cells. The analysis of the activated pathways suggests that HRPII stimulation of the cells might engage with activation of MAPK, JAK/STAT1 signaling, and PI3K-Akt signaling in our experimental setting.

We have further investigated the structural features of HRPII required for cell binding and activation of the inflammasome identifying that HRPII can act as a cytoprotective or pro-inflammatory stimulus in dependence on its binding molecules and, therefore, its conformation. Our data strongly indicate that further exploring in which physiologic conditions HRPII act as virulence factors detrimental to the integrity of the blood barriers, especially the BBB, and that immunosuppressant therapeutic interventions could have a key role in the containment of the inflammatory responses and the cytokine storm associated with cerebral malaria.