Cytoadhesion of *Plasmodium falciparum*-infected red blood cells changes the expression of cytokine-, histone- and antiviral protein-encoding genes in brain endothelial cells

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Keywords: *Plasmodium falciparum*, malaria, endothelial cells, cytoadhesion, transcriptome.

Abstract

Introduction: Malaria remains a significant global health problem, particularly due to the human malaria parasite *Plasmodium falciparum*, which is responsible for most fatal infections. Infected red blood cells (iRBCs) evade spleen clearance by adhering to endothelial cells (ECs), triggering capillary blockage, inflammatory cytokine release, endothelial dysfunction, and altered vascular permeability, prompting an endothelial transcriptional response.
Methods: The iRBCIT4var04/HBEC-5i model, where iRBCs present IT4var04 (VAR2CSA) on their surface was employed to analyse the effects of iRBC binding on ECs. We used this model to investigate how cytoadhesion of iRBCs to ECs influences their expression profile depending on the temperature (37°C vs 40°C).
Results: Binding of non-infected RBCs (niRBCs) and fever alone significantly changes expression of hundreds of genes in ECs. Comparing the expression profile of HBEC-5i cultured either in the presence of iRBCs or in the presence of niRBCs, genes encoding proteins assigned to the GO terms immune response, nucleosome assembly, NF-kappa B signaling, angiogenesis, and antiviral immune response/interferon-alpha/beta signaling pathway were significantly up-regulated. If the cultivation temperature is increased from 37°C to 40°C, which simulates fever, a further significant increase in expression can be observed for most regulated genes, especially for genes coding for cytokines and proteins involved in angiogenesis.
Conclusion: The presence of iRBCs leads to the stimulation of ECs, activating several immunological signaling pathways and affecting antiviral (-parasitic) mechanisms and angiogenesis. Furthermore, to our knowledge, the induction of the interferon-alpha/beta signaling pathway in ECs in response to iRBCs has been described for the first time.

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1 Introduction

Despite recent success stories in vaccine development and roll-outs, malaria remains one of the world's most dangerous infectious diseases. In 2022, there were about 249 million malaria cases, including 608,000 deaths (WHO, 2023). Most fatal malaria infections are caused by *Plasmodium falciparum*. Parasite and host responses contribute to the development of complications characteristic of severe malaria infections. A vital aspect of the pathogenesis of *P. falciparum* infection is the cytoadhesion of infected red blood cells (iRBCs) to the vascular bed of vital organs such as the brain, heart, lungs, stomach, skin, and kidneys (Taylor et al., 2004; Milner et al., 2013; Milner et al., 2015). In addition to capillary blockage, iRBC cytoadhesion induces inflammatory cytokine production, endothelial dysfunction, and vascular permeability in the affected tissue (Lyke et al., 2004). As a result of the immune responses triggered by parasite growth and endothelial cytoadhesion, patients may develop fever, headache, myalgia, and muscle stiffness (Hasday et al., 2001; Oakley et al., 2007; Oakley et al., 2011; Cunnington et al., 2013; Phillips et al., 2017). Depending on the age and immune status of the patient, severe to life-threatening complications may develop, including cerebral malaria, organ failure, acidosis, and severe anemia (Cunnington et al., 2013; Gazzinelli et al., 2014; Phillips et al., 2017).

Cytoadhesion is primarily mediated by members of the *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) family, which is encoded by approximately 45-90 *var* genes per parasite genome (Otto et al., 2019). The expression of *var* genes is mutually exclusive in ring-stage parasites, so only one *Pf*EMP1 variant is present on the surface of trophozoite- and schizont-stage iRBCs at any one time. The *var* genes encoding *Pf*EMP1 are highly variable from parasite to parasite, resulting in a vast repertoire of *var* genes in nature (Kraemer and Smith, 2006; Voss et al., 2006; Kyes et al., 2007; Pasternak and Dzikowski, 2009; Deitsch and Dzikowski, 2017). *Pf*EMP1s consist of an intracellular and a transmembrane domain, several extracellular Duffy binding-like (DBL) domains (α, β, γ, δ, ζ, and ε), and cysteine-rich interdomain regions (CIDR; α, β, and γ) (Smith et al., 2000a; Smith et al., 2000b; Gardner et al., 2002; Rask et al., 2010) and can be classified into groups A, B, C, and E (Kraemer and Smith, 2003; Lavstsen et al., 2003; Kraemer et al., 2007; Rask et al., 2010). *Pf*EMP1s are concentrated in electron-dense protrusions of the erythrocyte plasma membrane called knobs. Knobs are composed of several submembrane structural proteins, including the major protein of this structure, the knob-associated histidine-rich protein (KAHRP). Moreover, other proteins are required for knob formation, including *Pf*EMP3, ring-infected red cell antigen (RESA), mature parasite-infected red cell surface antigen (MESA)/*Pf*EMP2, and *Pf*332 (Gruenberg et al., 1983; Maier et al., 2009; Tilly et al., 2015; Alampalli et al., 2018). Knobs play an essential role in binding iRBCs to the endothelium (Crabb et al., 1997; Horrocks et al., 2005; Cutts et al., 2017; Dorpinghaus et al., 2020; Lubiana et al., 2020). If iRBCs do not form knobs, e.g. due to loss of genes encoding knob structural proteins, adhesion under flow conditions is significantly impaired. Especially at febrile temperatures, they play a crucial role by stabilizing the binding interaction. Under these conditions, knob-negative iRBCs are no longer able to bind to various endothelial cell receptors (ECRs) (Lubiana et al., 2020).

Cytoadhesion of iRBCs also leads to a change in gene expression in endothelial cells (ECs) (Tripathi et al., 2006; Tripathi et al., 2009). Stimulation of primary human brain microvascular endothelial cells (HBMECs) through cytoadhesion of iRBCs resulted in increased ICAM-1 expression on the surface of HBMECs, which in turn resulted in increased cytoadhesion. This observation was specific to HBMECs, in contrast to human umbilical vein endothelial cells (HUVECs), where no significant changes in gene expression were observed (Tripathi et al., 2006). Cytoadhesion induced nuclear translocation of NF-κB in HBMECs, which was associated with ICAM-1 expression (Tripathi et al., 2006). In another study, the influence of cytoadhesion on HBMEC gene expression was investigated by microarray analysis (Tripathi et al., 2009). The effect of binding of iRBCs with enhanced binding

93 on HBMECs induced by panning was compared with the effects of binding of unpanned iRBCs. For
94 both populations, a 4-fold increase in the amount of ICAM-1 presented on the HBMEC surface was
95 detected after 6 h of co-incubation. Interestingly, there were no significant differences in gene
96 expression between the two parasite populations. In total, the authors identified 231 up-regulated and
97 155 down-regulated genes. Using GO term analysis, the proteins encoded by the regulated genes were
98 shown to be involved in inflammatory response, apoptosis, anti-apoptosis, cell-cell signaling and
99 transduction, and NF-κB activation cascade. The expression of some genes encoding pro-inflammatory
100 cytokines such as IL-6, CXCL8, CXCL1, CXCL2, and CCL20 was increased more than 100-fold, and
101 for some of them, the increase was also detected at the protein level (Tripathi et al., 2009). Recently,
102 the transcriptome profile of HBMECs co-cultured for different time points with iRBCs presenting
103 IT4_var14 (strong binding to ICAM-1) or IT4_var37 (strong binding to CD36) on their surface was
104 analysed. Genes coding for proteins involved in many of the pathways described above, such as
105 inflammation, cell-cell interaction, and apoptosis, were identified as differentially expressed. However,
106 significant differences were also found between $iRBC^{IT4var14}$ and $iRBC^{IT4var37}$. Among others, atonal
107 bHLH transcription factor 8 (*ATOH8*), *CXCL8*, *CXCL10*, *VCAM-1*, radical s-adenosyl methionine
108 domain containing 2 (*RSAD2*), and IL6 were found to be up-regulated in the presence of $iRBC^{IT4var14}$
109 after 6 h of co-cultivation, whereas the expression of these genes was down-regulated in $iRBC^{IT4var37}$
110 (Othman et al., 2023).

111 Chakravorty and colleagues also investigated the influence of cytoadhesion on HUVEC gene
112 expression (Chakravorty et al., 2007). They confirmed the observation of Tripathi and colleagues that
113 cytoadhesion of iRBCs alone is not sufficient to stimulate HUVEC cells (Tripathi et al., 2006).
114 However, when co-incubated in the presence of 5 pg/mL of TNF, there is an increase in the presentation
115 of ICAM-1 on the cell membrane. Using microarray analysis, they showed that genes encoding proteins
116 involved in cell communication, cell adhesion, signal transduction, and immune response were
117 regulated. Thus, HUVEC can mobilize immune and adhesion responses, but only when co-incubated
118 with low concentrations of TNF in addition to iRBCs cytoadhesion (Chakravorty et al., 2007).
119 However, there is another study in this context that has shown stimulation of HUVECs even without
120 the addition of TNF (Viebig et al., 2005). They were able to show that stimulation of HUVECs with
121 iRBCs of the *P. falciparum* isolate IT4 results in more ICAM-1 being presented on the surface of the
122 ECs and increased amounts of IL-6 being secreted, whereas this is not the case for VCAM-1.
123 Furthermore, significantly increased expression of various cytokines such as CXCL8, CCL20, CXCL1,
124 and CCL2 was detected (Viebig et al., 2005).

125 In previous studies, an immortalized human cerebral microvascular endothelial cell line (HBEC-5i)
126 was used to characterize the cytoadhesion of iRBCs (Dorpinghaus et al., 2020; Lubiana et al., 2020).
127 HBEC-5i cells are derived from the microvasculature of the human cerebral cortex and therefore have
128 essential characteristics of cerebral ECs. HBEC-5i cells present Willebrand factor (vWF), VE-
129 cadherin, occludin, VCAM-1, ICAM-1, CD54, CD40, and chondroitin-sulfate A (CSA) on the cell
130 surface. However, the binding of iRBCs of the *P. falciparum* isolate IT4 to HBEC-5i cells was shown
131 to occur mainly via CSA. Parasites enriched for binding to HBEC-5i cells present IT4var04 (var2CSA)
132 on the surface of iRBCs ($iRBCs^{IT4var04}$), of which CSA is known to be the ligand. Thus, CSA seems to
133 be dominant on HBEC-5i cells and that binding of iRBCs to other receptors such as ICAM-1 is shielded
134 (Dorpinghaus et al., 2020). Using this model ($iRBC^{IT4var04}$/HBEC-5i), it was shown that binding to
135 CSA occurs only at a low shear stress of 0.9 dyne/cm^2 with a static binding characteristic. iRBC
136 adhesion to CD36 on the other hand displayed a rolling behavior. Additionally, it could be shown that
137 binding at febrile temperatures can only occurs when the iRBCs have formed knobs (Lubiana et al.,
138 2020).

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139 In the present study, the iRBC^{IT4var04}/HBEC-5i model was used to investigate whether stimulation of
140 ECs by binding of iRBCs to CSA is possible and, if so, whether and how this stimulation differs from
141 that of HBMEC and HUVEC and what happens in the presence of fever. The aim was to determine
142 whether cytoadhesion in general elicits similar responses in ECs, independent of the receptor.
143 Moreover, we also studied the effects of febrile temperatures due to the co-incubation with iRBCs.

144 Our results show that cytoadhesion of iRBCs increases the expression of genes in HBEC-5i cells
145 encoding proteins involved in immune response, angiogenesis, antiviral mechanisms and nucleosome
146 assembly. In particular, under febrile conditions, the expression of some cytokines is strongly
147 increased, suggesting the formation of a cytokine storm.
148

149 **2 Materials and methods**

150

151 **2.1 Cell culture**

152

153 *P. falciparum* isolate IT4 (FCR3S1.2) was cultured in human 0+ erythrocytes at a hematocrit of 5% in
154 the presence of 10% human A+ serum according to standard procedures (Trager and Jensen, 2005).
155 Parasites were synchronized once a week with 5% sorbitol (Lambros and Vanderberg, 1979). Every
156 two weeks, iRBCs were enriched for the presence of knobs. IRBCs were resuspended in two volumes
157 of pre-warmed gelatin (1%, 37°C) and incubated at 37°C for 45 min (Waterkeyn et al., 2001). The
158 supernatant containing knob-positive iRBCs was washed with GibcoTM RPMI 1640 medium (Gibco,
159 Thermo Fisher Scientific, Bremen, Germany) and cultured as usual.
160 The HBEC-5i cell line (ATCC CRL-3245) was cultured in DMEM/F12 (Gibco, Thermo Fisher
161 Scientific, Bremen, Germany) medium supplemented with L-glutamine and sodium bicarbonate (2.438
162 g/l) at 37°C in 5% CO_2 and passaged every 2-4 days at 70-90% confluence.
163

164 **2.2 Enrichment of iRBCs that bind to HBEC-5i**

165

166 To enrich iRBCs that bind to HBEC-5i cells, synchronized iRBCs at the trophozoite stage (5-10%
167 parasitemia, 1% hematocrit) were resuspended in binding medium (RPMI 1640 medium, 2% glucose,
168 pH 7.2; Gibco, Thermo Fisher Scientific, Bremen, Germany) and co-incubated with HBEC-5i cells
169 (70-90% confluence) for 90 min at 37°C and 5% CO_2. After 60, 75, and 90 min, the cultures were
170 gently shaken orbitally. This was followed by five washes with a binding medium to remove any
171 unbound iRBCs. The remaining parasites were further cultured with 10 ml of RPMI 1640 medium and
172 erythrocytes (5% hematocrit) to the ring stage and harvested. Any detached HBEC-5i cells were
173 removed with Biocoll separation solution (Merck Biochrom, Berlin, Germany). The parasites were
174 then grown under standard conditions to a parasitemia of 5-10%, and the whole procedure was repeated
175 seven times. Parasites enriched for binding to HBEC-5i cells were shown to express Var2CSA
176 (IT4_var04), which binds to CSA presented on HBEC-5i cells (Dorpinghaus et al., 2020).
177

178 **2.3 Infected RBC-HBEC-5i co-incubation, RNA isolation, and RNAseq**

179

180 To analyse the influence of cytoadhesion on HBEC-5i cell metabolism, HBEC-5i cells (70-90%
181 confluence) were co-incubated with HBEC-5i-enriched trophozoite-stage iRBCs at a parasitemia of
182 5% (hematocrit 1%) in binding medium (Dorpinghaus et al., 2020). Co-incubation was performed in
183 T-25 flasks at either 37°C or 40°C (iRBC$^{37°C}$/iRBC$^{40°C}$). HBEC-5i cells cultured at 37°C or 40°C +/-
184 niRBCs (hematocrit 1%) served as controls. After seven h of co-incubation, cells were collected in 4
185 ml of pre-warmed TRIzol (Thermo Fisher Scientific, Bremen, Germany), thoroughly resuspended,
186 transferred to a 15 ml reaction tube, and incubated in a water bath at 37°C for 5 min. Samples could

then be stored at -80°C or processed immediately. For RNA isolation, samples of 1 ml each were divided into 4 RNase-free reaction tubes, supplemented with 200 µl of chloroform cooled to 4°C, shaken well, and incubated for 3 min at room temperature (RT). Samples were then centrifuged at 4°C for 30 min at 12,000 *x g*, and the supernatant was transferred to a new reaction tube. RNA was precipitated by the addition of an equivalent volume of 70% ethanol. RNA was then isolated using the PureLink RNA Mini Kit (Thermo Fisher Scientific, Bremen, Germany) according to the manufacturer's instructions. Possible contamination with genomic DNA was removed using the TURBO DNA-free kit (Fisher Scientific, Thermo Fisher, Hampton, NH, USA). RNA was then purified using the Agencourt RNAClean XP Kit (Beckham Coulter, Brea, CA, USA). Library preparation and subsequent RNA sequencing was performed on an Illumina HiSeq 4000 PE100 platform from BGI (BGI Group, Shenzhen, China).

2.4 Bioinformatic analyses/Statistics

The RNAseq data were mapped to the human reference genome (GRCh38.109) using CLC Genomics Workbench 21.0 (QIAGEN, Aarhus, Denmark). The Voroni diagram was created using Python 3.11 and matplotkib 3.7.2. The interaction network analysis was performed with the database STRING, version 12.0 (Szklarczyk et al., 2023). The following setting was used: Active interaction sources: text mining, experiments, databases, co-expression; Meaning of network edges: high confidence 0.700. Network display mode: Interactive; Network display options: Hide disconnected nodes in the network; Clustering Options: MCL clustering, inflation parameter 3; Edges between clusters: Solid lines.

3 Results

3.1 Effects of niRBCs, iRBCs, and fever on the expression of HBEC-5i cells

By performing a full transcriptome analysis of the ECs, we examined the impact of niRBCs, iRBCs, and fever on the expression profile of HEBEC-5i cells. For these studies, as mentioned above, plasmodia of isolate IT4 were used, which were enriched on HBEC-5i cells over several rounds, with adhesion taking place via CSA, thus presenting IT4var04 (var2CSA) on the surface of the iRBCs (Dorpinghaus et al., 2020). A Venn diagram was constructed to illustrate the differential expression (true vs. false) of the three parameters niRBC, iRBC, and febrile (Figure 1). Specifically, genes exhibiting a differential expression of a fold change of ≥ 2.0, a false discovery rate (FDR) of ≤ 0.05, and a minimum transcript per million (TPM) threshold of ≥ 2.0 were included in this analysis.

Our findings reveal distinct regulatory effects: 60 genes were solely modulated by the presence of niRBCs, while elevating the temperature from 37°C to 40°C alone resulted in altered expression patterns in 98 genes. Similarly, the presence of iRBCs alone influenced the expression of 318 genes. Notably, 285 genes exhibited regulation in response to either niRBCs or iRBCs, with 80 genes showing such regulation specifically in the presence of $iRBCs^{37°C}$ and $iRBCs^{40°C}$. Additionally, ten genes were differentially expressed either by the febrile stimulus or presences of niRBCs. Seventy-nine genes were found that overlap in all three comparisons (Figure 1, Supplementary Table 1).

Of the 59 genes that are significantly differentially expressed by the presence of niRBCs, 38 are up-regulated and 21 are down-regulated. With minor exceptions, the majority of these genes demonstrate a 2- to 3-fold regulation. Notably, the most prominently up-regulated gene, BOLA2, exhibits a striking 79-fold increase (from TPM 2.5 to 198.3) in response to niRBCs presence (Figure 1, Supplementary Table 1).

Of the 98 genes that are significantly differentially expressed by the temperature elevation from 37°C to 40°C, 50 display up-regulation, while 48 undergo down-regulation. Once again, most of these genes

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show a 2- to 3-fold regulation. The increase in temperature upregulates the expression of 10 genes associated with the Gene Ontology Biological Process (GO-BP) term "protein folding" (FDR 4.82e-16) (Figure 1, Figure 2A, Supplementary Table 1).

Analysing the 318 genes significantly differentially expressed by the presence of iRBCs, 121 are up-regulated, while 197 are down-regulated (Supplementary Table 1). Approximately 90% of these genes undergo a 2- to 3-fold regulation, while the remaining 10% (22 genes) exhibit a 3- to 6-fold regulation. String analysis reveals the up-regulation of GO-BP terms such as "defense response to virus" (FDR 7.75e-09), "nucleosome assembly" (FDR 0.00013), "NF-κB complex" (FDR 0.0018), and "ribosome biogenesis" (FDR 0.00086) (Figure 1, Figure 2B, Supplementary Table 1).

Within the intersection of genes derived from the analyses of niRBC true vs. false and iRBCs true vs. false, a total of 285 genes exhibit regulation. Noteworthy among these are 13 genes associated with "cellular response to cytokine stimulus" (FDR 4.82e-10), 9 genes linked to "nucleosome assembly" (FDR 2.56e-15), 11 genes involved in "I-κB kinase/NF-κB signaling" (FDR 4.32e-06), 5 genes contributing to "negative regulation of I-κB kinase/NF-κB signaling" (FDR 0.0017), and 3 genes associated with "defense response to virus" (FDR 3.38e-05) GO-BP terms. Remarkably, all these pathways demonstrate up-regulation (Figure 1, Figure 2C, Supplementary Table 1).

Within the intersection encompassing genes regulated in both the iRBC true vs. false and febrile true vs. false analyses, a cohort of 80 genes is observed. Among this group, 3 genes are associated with the "vascular endothelial growth factor signaling pathway" GO-BP term (FDR 0.00025), while 4 genes are linked to the "response to unfolded protein" term (FDR 2.50e-05), all demonstrating up-regulation (Figure 1, Figure 2D, Supplementary Table 1).

In the intersection of all three comparative analyses (niRBC true vs. false; febrile true vs. false; iRBC true vs. false), a set of 80 regulated genes is identified. Notably, the pathways significantly up-regulated include the "chemokine-mediated signaling pathway" (FDR 2.26e-12), comprising 13 genes, and the "protein folding" GO-BP term (FDR 3.29e-10), comprising 7 genes (Figure 1, Figure 2E, Supplementary Table 1).

Focusing solely on the "iRBCs true vs. false" analysis, comprising a total of 762 genes, a comprehensive string analysis reveals 7 distinct clusters housing 6 to 24 of the regulated genes. These clusters correspond to the GO-BP terms "response to cytokine" (FDR 2.01e-23; 24 genes), "response to unfolded protein" (FDR 1.67e-30; 17 genes), "positive regulation of RNA metabolic process" (FDR 7.04e-10; 15 genes), "nucleosome organization" (FDR 3.83e-11; 9 genes), "defense response to virus" (FDR 7.04e-16; 12 genes), and "I-κB kinase/NF-κB signaling" (FDR 3.24e-08; 6 genes). Remarkably, all these clusters exhibit up-regulation. Conversely, the sole down-regulated cluster pertains to "nucleosome assembly" (FDR 3.64e-08; 7 genes) (Figure 1, Figure 3A, Supplementary Table 1).

Using ShinyGO analysis, the proteins encoded by the identified genes were categorized according to their biological functions. Among the 10 most significantly regulated functions are the TNF, IL-17 and NF-κB signaling pathways and cytokine-cytokine receptor interaction, as well as the response to viral (herpes simplex virus 1 infection) and bacterial (legionellosis) infections (Figure 3B).

3.2 Specific response of HBEC-5i cells to cytoadhesion of iRBCs$^{37°C}$ and iRBCs$^{40°C}$

It is important to emphasize that in the above comparisons, although the expression of genes within different intersections is altered compared to the respective controls, the magnitude of these changes in expression levels is not considered in the analyses. Hence, a more detailed investigation is warranted to delineate the specific impact of iRBC cytoadhesion on HBEC-5i cells and the role of the cultivation temperature (37°C vs. 40°C). In contrast to the previous analysis, this subsequent investigation only includes the mean values of normalized reads from three biologically independent transcriptomes. However, the focus remains exclusively on genes identified as significantly regulated within the analysis above (Supplementary Table 1).

In the first step, we identified those genes whose expression was up- or down-regulated (fold change ≥ 2.0, FDR ≤ 0.05, TPM threshold ≥ 2.0) in HBEC-5i cells in response to iRBCs compared to the presence of niRBCs at 37°C ($iRBCs^{37°C}$ vs $niRBCs^{37°C}$). Cytoadhesion of $iRBCs^{37°C}$ up-regulates the expression of 162 genes and down-regulates the expression of 219 genes compared to the presence of $niRBCs^{37°C}$ (Supplementary Table 1).

To get an idea of which metabolic pathways the proteins encoded by the 162 up-regulated genes are involved, a g:Profiler analysis was performed. The GO Molecular Function (GO-MF) terms "structural component of chromatin" and "chemokine activity" had the highest significance. For the GO-BP terms, these were "cellular response to organic substance" and "response to cytokine". The KEGG pathway analysis showed the highest significance for "TNF signaling pathway" and in the Reactome analysis these were "interleukin-10 signaling" and "cytokine signaling immune system" (Figure 4A). This is also reflected in the ShinyGO analysis. Here, as described in the previous paragraph, different signaling pathways of the immune system and the response to viral and bacterial infections were mainly regulated ("TNF, IL-17 and NF-κB signaling pathways", "measles", "legionellosis") (Figure 4B). The use of a string analysis yielded very similar results. Here, among the 162 up-regulated genes, the KEGG pathways exhibiting the most stringent regulation include the "TNF signaling pathway" (FDR 6.70e-09), "viral protein interaction with cytokine and cytokine receptor" (FDR 1.03e-07), "IL-17 signaling pathway" (FDR 4.42e-07), "cytokine-cytokine receptor interaction" (FDR 7.64e-07), and "NF-κB signaling pathway" (FDR 8.31e-07). In total, 8 genes are up-regulated more than 10-fold, while another 16 genes are up-regulated more than 5-fold. Notable among these are the chemokines CCL31, CCL3, CCL20, and IL-6 (Supplementary Table 1).

Furthermore, the up-regulated genes can be classified in the clusters of the following GO-BP terms: "response to cytokine" (FDR 5.50e-18, 19 genes), "nucleosome assembly" (FDR 1.99e-18, 12 genes), "defense response to virus" (FDR 6.28e-13, 10 genes), "cellular response to hormone stimulus" (FDR 0.00030, 5 genes). (Figure 5A, Supplementary Table 1). The 219 genes whose expression is down-regulated code for proteins that fall under the GO-MF term "DNA-binding transcription factor activity" (FDR 2.008×10^{-20}) and the GO-BP term "regulation of nucleobase-containing compound metabolic process" (FDR 3.664×10^{-18}) according to the g:Profiler analysis (Supplementary Table 1).

The next question was to investgate the effect of increasing the temperature from 37°C to 40°C ($iRBCs^{40°C}$), simulating fever as an additional factor besides cytoadhesion of $iRBCs^{37°C}$. Therefore, the expression profile of HBEC-5i cells following cytoadhesion $iRBCs^{40°C}$ was compared to HBEC-5i cells cultured in the presence of $niRBCs^{37°C}$. This analysis revealed 381 genes up-regulated and 213 genes down-regulated in expression (fold change ≥ 2.0, FDR ≤ 0.05, TPM threshold ≥ 2.0) (Supplementary Table 1). G:Profiler and ShinyGO analyses group of the 381 genes up-regulated at 40°C were also carried out here. By and large, the same pathways were identified. However, for many of the pathways, the significance was more than doubled, e.g. for the TNF signaling pathway (Figures 4C, 4D). A string analysis of this comparison also shows great agreement with the analyses mentioned above. The same KEGG pathways are regulated at 40°C, that were already addressed at 37°C but with one exception with higher significance ("TNF signaling pathway" (FDR 1. 54e-17), "IL-17 signaling pathway" (FDR 2.06e-12), "cytokine-cytokine receptor interaction" (8.20e-11) and "NF-κB signaling pathway" (FDR 8.00e-11), "viral protein interaction with cytokine and cytokine receptor" (FDR 6.70e-07)). GO-BP terms identified as significant in the $iRBC^{37°C}$ with $niRBCs^{37°C}$ comparison, such as "defence reaction to viruses" and "cellular reaction to hormone stimuli", are no longer found in the $iRBC^{40°C}$ with $niRBCs^{37°C}$ comparison. Instead of 12 genes, the GO term "nucleosome assembly" (FDR 8.01e-12) still shows 9 genes as significantly up-regulated in their expression. The significance for the GO-BP term "response to unfolded protein" increases from FDR 1.09e-09 (6 genes) to FDR 1.67e-30 (17 genes). This is also true for the GO-BP term "cytokine response" (FDR 3.62e-24), to which 23 genes can be assigned. Furthermore, the GO-BP term "I-κB kinase/NF-κB signaling" (FDR 2.36e-06, 5 genes) was identified as regulated (Figure 4B, Supplementary Table 1). In addition, significant up-

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regulation of gene expression was detected, with a notable subset showing ≥10-fold increase (32 genes including *CCL26, CCL20, CCL3L1, CXCL3, CXCL8* and *Il6*) and a further 55 genes showing 5- to <10-fold increase when comparing niRBC$^{37°C}$ vs. iRBC$^{37°C}$ (Supplementary Table 1).

The 213 genes whose expression is down-regulated encode for proteins that fall under the GO-MF term "DNA-binding transcription factor activity" (FDR 1.805×10^{-12}) and the GO-BP term "regulation of transcription by RNA polymerase II" (FDR 4.185×10^{-9}) according to the g:Profiler analysis (Supplementary Table 1).

The next stage was to examine the genes whose expression was regulated in the presence of iRBCs at 40°C compared to those at 37°C (fold change ≥ 2.0, FDR ≤ 0.05, TPM threshold ≥ 2.0). 205 genes (18 genes fold change >10-, 25 genes fold change 5-10) were identified for which cytoadhesion at 40°C leads to a further increase in expression compared to cytoadhesion at 37°C. Down-regulation was observed for 38 genes (Supplementary Table 1). For some genes, a continuous increase in expression can be observed, starting from cultivation in the presence of niRBCs$^{37°C}$ via iRBCs$^{37°C}$ to iRBCs$^{40°C}$. For instance, this trend is evident in the case of CXCL8, where the expression rises from 11.7 TPM (no niRBCs) to 82 (niRBCs$^{37°C}$), further to 392.6 (iRBCs$^{37°C}$), and peaks at 1932 (iRBCs$^{40°C}$) (Figure 6, Supplementary Table 1). For other genes, such as HSPA1A, the expression with and without niRBCs is comparable, and an increase in expression is seen in the presence of iRBCs at 37°C and increases further at 40°C (TPM: 177 (without niRBCs); 160 (niRBCs); 537 (iRBCs$^{37°C}$), 23001 (iRBCs$^{40°C}$) (Supplementary Table 1).

The g:Profiler and ShinyGO analyses of the 205 genes whose expression was upregulated in the presence of iRBCs$^{40°C}$ compared to iRBCs$^{37°C}$, which were also carried out here, show that immunological signaling pathways are again positively influenced by the combination of iRBCs and elevated temperature (Figure 4F, G). Analysis of the 38 down-regulated genes reveals no significantly regulated pathways.

The genes whose expression is further increased in a temperature-dependent manner can be assigned to the KEGG pathways "TNF signaling pathway" (FDR 2.96e-12), "IL-17 signaling pathway" (FDR 2.76e-11), "NF-κB signaling pathway (FDR 1.09e-09) and "cytokine-cytokine receptor interaction" (FDR 2.98e-09). In particular for genes belonging to the GO-BP terms "response to cytokine" (17 genes, FDR 5.96e-19) and "protein folding" (13 genes, FDR 6.55e-23), an increase in expression can be observed with an increase in temperature from 37°C to 40°C (Figure 5C; Supplementary Table 1).

3.3 Influence of iRBCs on the expression of genes encoding proteins involved in the immune response, nucleosome assembly, angiogenesis, and interferon-alpha/beta signaling pathway

The comparative analyses described above show that cytoadhesion of iRBCs leads to an increase in the expression of genes whose encoded proteins are involved in the host's immune response. For some of these genes, the higher temperature leads to a further increase in expression, although temperature alone has little or no effect on the expression level of these genes. The encoded cytokines can be divided into four groups. 1) Genes that show an increase in expression when co-cultured with iRBCs$^{37°C}$, but maintain a similar level of expression when the temperature is raised to 40°C (*CXCL10, CXCL11, CCL5, CCL20, IL12A*); 2) Genes whose expression is up-regulated at 37°C and whose expression is further up-regulated when the temperature is raised to 40°C (*CXCL2, CXCL3, CXCL6, CXCL8, CCL3, CCL3L1, CCL26, IL1a, IL6*); 3) Genes whose expression increases only during co-cultivation at 40°C (*CXCL1, CXCL5, IL1b, IL32*); 4) Genes that show similar levels of expression under all conditions (*CXCL12, CXCL14, CXCL16, CCL2*) (Figure 6A, Supplementary Table 1).

Six genes whose encoded proteins can assume functions in angiogenesis are also differentially expressed. These include three genes (*MMP1*, *MMP2*, *MMP3*) encoding metalloproteinases as well as the genes (*NR4A1, NR4A2, NR4A3*) encoding the three members of the NR4A nuclear hormone receptor superfamily. For MMPs, the increased expression was only observed at elevated temperature in the presence of iRBCs, whereas for NR4A1, NR4A2, NR4A3, the expression increased in the

380 presence of iRBCs and a further increase was observed after raising the temperature from 37°C to 40°C
381 (Figure 6B, Supplementary Table 1).
382 In addition, twelve genes coding for histones or histone linker proteins were significantly up-regulated
383 in the presence of iRBCs. However, raising the temperature to 40°C did not lead to a further increase
384 in expression, but on the contrary, the expression level for four genes has the expression level
385 significantly decreased in the presence of niRBCs (Figure 6C, Supplementary Table 1).
386 Interestingly, additional 14 genes were identified coding for proteins of the antiviral immune response
387 or the interferon-alpha/beta signaling pathway (Reactome: FDR 2.22E-15) (Figures 6D, 6E,
388 Supplementary Table 1). The presence of iRBCs alone leads to a significant up-regulation in
389 expression. With the exception of *IL6*, there is no further increase in expression at elevated
390 temperatures. On the contrary, for four genes (*IFI6, ISG15, MX1, MX2*) the expression level falls back
391 to the baseline level of niRBCs (Figures 6D, 6E, Supplementary Table 1).
392

393 **4 Discussion**

394

395 *P. falciparum* malaria elicits a robust host immune response that contributes significantly to the
396 pathogenesis of the disease through increased systemic and local inflammation. Various studies have
397 shown that the cytoadhesion of iRBCs strongly influences the gene expression of the ECs. Among
398 other things, it has been shown that binding to HBEMCs mainly influences the signaling pathways
399 involved in the inflammatory response, apoptosis, cell-cell signaling, signal transduction, and the NF-
400 kB activation cascade (Tripathi et al., 2009; Othman et al., 2023). However, not only does cytoadhesion
401 seem to influence the expression, but it also seems to depend on which *Pf*EMP1 is presented on the
402 surface of the iRBCs (Othman et al., 2023). Similar effects have also been demonstrated for HUVECs.
403 However, some studies show that the stimulation of HUVECs by cytoadhesion of iRBCs is only
404 possible in the presence of low levels of TNF (Viebig et al., 2005; Tripathi et al., 2006; Chakravorty
405 et al., 2007).
406

407 In the present study, we analysed the influence of iRBC cytoadhesion on HBEC-5i cells. For this
408 purpose, an iRBC population enriched for binding to HBEC-5i cells was used. This resulted in a
409 homogeneous cell population presenting IT4var04 (var2CSA) on their surface, as CSA is the dominant
410 antigen on HBEC-5i cells (Dorpinghaus et al., 2020). This model was employed to investigate whether
411 stimulation of HBEC-5i cells by binding of iRBCs to CSA is possible and how cytoadhesion affects
412 the expression profile also at elevated temperatures, simulating fever.
413

414 First, the influence of the presence of niRBCs (niRBC true vs. false), iRBCs (iRBC true vs. false), and
415 fever (febrile true vs. false) on the expression profile of HBEC-5i cells was analysed. For these true vs.
416 false analyses, only one stimulus is considered. For example, in the case of iRBC true vs. false, all
417 HBEC-5i transcriptomes in which iRBCs were co-incubated with HBEC-5i cells were analysed,
418 regardless of the temperature used, and in the case of febrile true vs. false, all transcriptomes in which
419 HBEC-5i cells were cultured at 40°C were analysed, regardless of the presence or absence of niRBCs
420 or iRBCs. In this way, all three comparisons were analysed in a similar way.
421 The analysis showed that the presence of niRBCs alone has a strong influence on the expression profile
422 of HBEC-5i cells. 59 genes are differentially expressed only in the presence of niRBCs. Since the
423 presence of niRBCs is an organic component of EC homeostasis, the overlap of the two DEG analyses
424 between niRBC (true vs. false) and iRBC (true vs. false) allows the isolation of genes that are affected
425 by the presence of the parasite compared to those of niRBCs. However, there is a large overlap of 364
426 genes that are also differentially expressed in the presence of iRBCs. It is important to note that this

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427 analysis does not provide any information about the magnitude of expression level changes.
428 Particularly interesting are, for example those genes showing up-regulated expression in the presence
429 of niRBCs and further up-regulation in the presence of iRBCs. Some examples of genes showing this
430 behavior are *JUNB* which encodes a subunit of the AP-1 transcription factor (TPM: 33_ no RBCs;
431 72_niRBCs; 227_iRBCs), *IL6* (TPM: 3_no RBCs; 13_niRBcs; 70_iRBCs), and *CXCL8* (TPM: 11_no
432 RBCs; 82_niRBcs; 392_iRBCs). As expected, increasing the temperature from 37°C to 40°C also has
433 a significant effect on the expression profile of HBEC-5i cells, mainly affecting genes encoding heat
434 shock proteins involved in protein folding and stress response.
435 *BOLA2* is the most up-regulated gene in the comparison of niRBCs true vs. false, increasing from TPM
436 2.5 to 198 (almost 80-fold). So far, little is known concerning the function of BOLA2. However, it was
437 described that it could interact with glutaredoxin-3 and act as a redox-regulated regulator of Fe-S
438 cluster biogenesis or as a transcriptional regulator (Banci et al., 2015).
439
440 Looking at the iRBC true vs. false analysis, a total of 762 genes are differentially regulated. In
441 particular, many genes involved in immunological signaling pathways, such as the TNF signaling
442 pathway, the IL-17 signaling pathway, the NF-kB signaling pathway, and the cytokine-cytokine
443 receptor signaling pathway are up-regulated. Interestingly, also genes coding for proteins involved in
444 the defense response to viruses and in nucleosome organization or assembly are expressed at higher
445 levels.
446 A direct comparison of the influence of iRBCs compared to niRBCs at 37°C and 40°C shows that the
447 positively regulated pathways are by and large the same as found in the iRBC true vs. false analysis.
448 Some of these signaling pathways have already been identified as regulated in the study by Tripathi
449 and colleagues (Tripathi et al., 2009). In addition to the most strongly affected signaling pathways
450 "cytokine-cytokine receptor interaction" and "cell surface receptor-linked signal transduction", many
451 genes of the NFkB activation cascade were also found to be positively regulated (Tripathi et al., 2009).
452 On the other hand, some of the signaling pathways identified as regulated in the present study were not
453 identified as regulated in Tripathi *et al.*, including nucleosome assembly and the defense response to
454 viruses (Tripathi et al., 2009).
455 It is known that the complex immune response elicited by *P. falciparum* infection involves the release
456 of numerous cytokines, of which more than 30 have been detected at elevated levels in the serum or
457 plasma of malaria patients compared to healthy individuals. These cytokines include CXCL2, CXCL4,
458 CXCL8, CXCL9, CXCL10, CXCL11, CCL2, CCL3, CCL4, CCL5, CCL20, CCL28, IL-1b, IL-6, IL-
459 9, IL-10, IL-12, IL-13, IL-15, IL-17A, IL-31, IL-33, IL1-RA, TNF-a, interferon-gamma (INFg),
460 VEGF, and G-CSF (Lyke et al., 2004; Prakash et al., 2006; Armah et al., 2007; Jain et al., 2008;
461 Ayimba et al., 2011; Wilson et al., 2011; Che et al., 2015; Colborn et al., 2015; Herbert et al., 2015;
462 Bujarbaruah et al., 2017). Certain cytokines (such as CXCL4, CXCL8, CXCL10, CCL2, CCL3, and
463 CCL4) show a direct correlation with the severity of malaria infection (Armah et al., 2007; Ayimba et
464 al., 2011; Wilson et al., 2011) and it has been shown that patients with cerebral malaria have
465 significantly increased levels of CCL2, CCL4, CXCL4, CXCL8, CXCL10, IL-1RA, IL-6, TNF and G-
466 CSF (Jain et al., 2008; John et al., 2008a; John et al., 2008b; Wilson et al., 2011). Our study, as well
467 as previous studies, show that cytoadhesion of iRBCs increases the expression of many genes encoding
468 cytokines (for review (Dunst et al., 2017)) even at 37°C. For nine of these 14 genes, in addition to
469 cytoadhesion, raising the temperature from 37°C to 40°C (simulation of fever) leads to a further
470 increase in expression of > 2-fold. The latter group includes genes encoding the inflammatory
471 chemokines CXCL2, CXCL3, CXCL6, and CXCL8, which attract neutrophils, CCL26, which attracts
472 eosinophil granulocytes, and CCL3 and CCL3L1, which attract macrophages and other immune cells.
473 For genes encoding the chemokines CXCL10, CXCL11, CCL5, and CCL20, there is a significant
474 increase in expression due to cytoadhesion, but this is not further increased by the rise in temperature.
475 These chemokines attract various immune cells, including T-cells, monocytes, and dendritic cells (for

476 review (Hughes and Nibbs, 2018). *CXCL1* and *CXCL5* are the only chemokines encoding genes whose
477 expression only increases by the temperature shift to 40°C in the presence of iRBCs. These chemokines
478 play a role in the attraction of neutrophil granulocytes. Recently, stimulation of ECs by plasma from
479 malaria patients was shown to significantly increase the expression of *CXCL1* and *CXCL5* compared
480 to healthy controls (Raacke et al., 2021). Interestingly, the increase in expression observed here for
481 *CXCL6* and *CCL26* has not yet been described to our knowledge. *CXCL6* (granulocyte chemotactic
482 protein-2 (GCP-2)) is one of the genes whose expression increases in the presence of iRBCs and is
483 further increased when the temperature is raised to 40°C. As described above, CXCL6 attracts
484 neutrophils and is a ligand of the chemokine receptors CXCR1 and CXCR2 (Fan et al., 2007). Since
485 neutrophils are important for the development of cerebral malaria, the expression of the gene was
486 analysed in the malaria mouse model *Plasmodium berghei* ANKA (Chen et al., 2000). Still, infection
487 of both C57BL/6 and Balb/c mice did not lead to an increase in expression in the brains of mice
488 compared to non-infected mice. However, this was consistent with the observation that no neutrophils
489 accumulated in the brains of mice with cerebral malaria (Van den Steen et al., 2008). To our knowledge,
490 there is only one study that has compared the plasma concentration of CCL26 in malaria patients with
491 that of non-infected controls. It showed that the plasma concentrations of CCL26 in the malaria-
492 exposed group were significantly lower than in the unexposed group. This observation correlates very
493 well with our results. The TMP increases only slightly from 1 to 3 in the presence of iRBCs, only by
494 increasing the temperature it does increase to TPM 26 (Mancebo-Perez et al., 2022). It can therefore
495 be postulated that CCL26 is only important during the fever attacks of malaria.
496 The expression of genes encoding various interleukins is also up-regulated by the presence of iRBCs.
497 These include the pro-inflammatory cytokines IL-1β, IL-1α, IL-6, IL-12A, and IL-32, whereby for *IL-*
498 *1a and IL6* a more than 2-fold increase in expression is seen from co-incubation of niRBCs to
499 iRBC37°C to iRBC40°C, while for *IL1β* and *IL32* an increase is only observed after co-incubation
500 with iRBCs$^{40°C}$. A recent meta-analysis showed that serum IL1β levels are higher in patients with
501 severe malaria than in patients with uncomplicated malaria. However, the level in patients with
502 uncomplicated malaria was very similar to that of healthy controls (Mahittikorn et al., 2022). This
503 observation fits very well with the results presented here. Only when the temperature increases from
504 37°C to 40°C, does the expression of *IL1β* increases by about 2.5 times. IL-32 shows the same
505 expression profile as IL1β, i.e. an increase in expression only occurs in the presence of iRBCs$^{40°C}$. IL-
506 32 is expressed in different isoforms in immune and non-immune cells, including ECs, and its functions
507 include the induction of the production of several pro-inflammatory cytokines (TNF-α, IL-1β, IL-6,
508 and CXCL8). The function of IL-32 in *P. falciparum* infection is not yet known. However, IL-32 has
509 been described as a master regulator of intracellular bacterial, viral, and eukaryotic infections (for
510 review (Dos Santos et al., 2018). In this context, it has been shown that in *Mycobacterium tuberculosis*
511 (Mtb) infection, endogenous IL-32 induces the production of TNFα, IL-1β, and CXCL8, as well as the
512 production of antimicrobial peptides and apoptosis, so that it can be assumed that IL-32 is associated
513 with protection against Mtb infections (Bai et al., 2010; Montoya et al., 2014; Bai et al., 2015). As
514 shown for MtB infection, the production of various cytokines such as TNFα, CXCL8, IL-1R, IFNγ,
515 and IL-10 as well as antimicrobial peptides were induced in *Leishmania* infection (Dos Santos et al.,
516 2017; Dos Santos et al., 2018).
517
518 Our results shown here are very much in line with the findings of Tripathi and colleagues, who
519 investigated the response of HBMECs to the presence of iRBCs. Significantly increased expression
520 was detected for CXCL1, CXCL2, CXCL3, CXCL6, CXCL8, CCL20, and IL-6 (Tripathi et al., 2009).
521 However, we were able to identify eleven further cytokines whose expression is up-regulated in the
522 presence of iRBCs$^{37°C}$ and/or iRBCs$^{40°C}$. Furthermore, a number of genes encoding proteins involved
523 in the regulation of inflammation and immune response are also increased in their expression by the
524 cytoadhesion iRBCs compared to the control. These proteins include among others PTGS2 (COX-2)

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525 (Martin-Vazquez et al., 2023) and TXNIP (thioredoxin interacting protein) (Mohamed et al., 2021).
526 Other genes that encode proteins involved in inflammation and immune reactions were expressed more
527 strongly at elevated temperatures. The corresponding encoded proteins include, among others,
528 TNFAIP3 (A20) (Wu et al., 2020), LGALS4 (galectin-4) (Cao and Guo, 2016), ATF3 (activating
529 transcription factor 3) (Hai et al., 2010), CD74 (cluster of differentiation 74) (Beswick and Reyes,
530 2009), CH25H (cholesterol 25-hydroxylase) (Cho et al., 2023), and CD274 (Huang et al., 2013).
531 The three members of the NR4A nuclear hormone receptor superfamily (NR4A1 (Nur77), NR4A2
532 (Nur-related factor-1), NR4A3 (Neuron-Derived Orphan Receptor 1)) are also increasingly expressed
533 in the presence of iRBCs, and a further increase was observed after elevating the temperature. In
534 inflammatory diseases, NR4A family members are rapidly expressed and control the differentiation
535 and activity of innate and adaptive immune cells. In addition, these proteins mediate cytokine signaling
536 to control angiogenesis (for review (Hamers et al., 2013; Murphy and Crean, 2022)). Furthermore,
537 three genes encoding members of matrix metalloproteinases (MMP1, MMP2, MMP3) are specifically
538 up-regulated only in the presence of iRBCs at elevated temperatures. Among other things, MMPs are
539 involved in the breakdown of extracellular matrix proteins and regulate functions related to
540 inflammation, such as the activity of inflammatory cytokines and chemokines (for review (Lee and
541 Kim, 2022)). MMP1 cleaves collagen type I and is involved in the modulation and remodeling of the
542 extracellular matrix. MMP2 cleaves collagen type IV and is involved in angiogenesis by degrading the
543 basement membrane of blood vessels and thus promoting the migration of endothelial cells. MMP3
544 can degrade various types of collagens as well as proteoglycans and fibronectin and is involved in
545 tissue remodeling and inflammatory reactions, among other things (for review (Lee and Kim, 2022).
546 Therefore, it can be speculated that the increase in the expression of MMP and NR4A encoding genes
547 could be a reason for the endothelial dysfunction observed in malaria.

548 Strikingly, 14 genes coding for proteins involved in the antiviral immune response or interferon-
549 alpha/beta signaling pathway are upregulated by cytoadhesive iRBCs. However, with the exception of
550 *IL6*, there is no further increase in the expression at elevated temperatures. On the contrary, for four
551 genes (*IFI6*, *ISG15*, *MX1*, and *MX2*) the expression level drops back to that in the presence of niRBCs.
552 In summary, the genes up-regulated in their expression encode the pro-inflammatory cytokine IL-6,
553 which can be induced by the presence of viruses, MX1 (MX dynamin-like GTPase 1), MX2 (MX
554 dynamin-like GTPase 2), IFI6 (interferon alpha inducible protein 6) and ISG15 (interferon-stimulated
555 gene 15), which inhibit viral replication, OAS2 (2'-5'-oligoadenylate synthetase 2) and OAS3 (2'-5'-
556 oligoadenylate synthetase 3), which synthesize secondary messengers that serve to activate RNase L,
557 which degrades viral and cellular RNA, and ISG20 (interferon stimulated exonuclease gene 20 kDa),
558 which is an endoribonuclease that supports the degradation of viral RNA (Tanaka et al., 2014), (Sun et
559 al., 2012; Haller et al., 2015; Zhu et al., 2015; Sajid et al., 2021; Deymier et al., 2022). In addition, the
560 genes *BST2* (bone marrow stroma cell antigen 2), IFI27 (interferon alpha-inducible protein 27), *DDIT4*
561 (DNA-damage-inducible transcript 4), *GBP4* (guanylate binding protein 4), *DDX60* (DEAD-box
562 helicase 60), which are also involved in the antiviral immune response, are up-regulated (Miyashita et
563 al., 2011; Tretina et al., 2019; Busse et al., 2020; Ullah et al., 2021; Zhao et al., 2022). It was shown
564 that a type I interferon (IFN) response occurs during *P. berghei* replication in the liver. A *P. berghei*
565 RNA was identified as a pathogen-associated molecular pattern (PAMP) that can activate a type I IFN
566 response. Thus, liver cells have sensor mechanisms that mediate a type I IFN-driven anti-parasite
567 response (Liehl et al., 2014). However, in the case of iRBCs, it is unclear how such an response is
568 induced in HBEC-5i cells and whether cytoadhesion is the trigger for this response or whether other
569 factors, such as extracellular vesicles secreted by iRBCs and containing various microRNAs, might be
570 involved (Wu et al., 2023).

571 In conclusion, the presence of iRBCs, mediated by cytoadhesion or other mechanisms such as cell-cell
572 communication via extracellular vesicles, stimulates brain endothelial cells. In particular, as described
573 in other studies, several immunological pathways are activated, and the stimulation of antiviral (-
574 parasitic) mechanisms and angiogenesis was also identified in the present study. In particular, the
575 expression of genes encoding cytokines, MMPs, and NR4As is further increased under fever
576 conditions.

577

578 **Data availability statement**

579

580 Raw data available as expression browser in Supplementary Table 1

581

582 **Author contributions**

583 JA: Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing –
584 original draft, Writing – review & editing. MB: Conceptualization, Formal analysis, Investigation,
585 Methodology, Writing – review & editing. HT: Writing – review & editing. MPMT: Writing –
586 review & editing. NGM: Methodology, Writing – review & editing. TR: Writing – review & editing.
587 TG: Funding acquisition, Writing – review & editing. IB: Conceptualization, Data curation, Formal
588 analysis, Funding acquisition, Project administration, Supervision, Visualization, Writing – original
589 draft, Writing – review & editing.

590 **Funding**

591 This work was supported from the Deutsche Forschungsgemeinschaft (BR 1744/20-1 and GU 568/9-
592 1), Leibniz Center Infection. and Jürgen Manchot Stiftung.

593 **Acknowledgments**

594 The authors would like to thank Susann Ofori for excellent technical assistance.

595

596 **Conflict of Interest**

597 The authors declare that the research was conducted in the absence of any commercial or financial
598 relationships that could be construed as a potential conflict of interest.

599

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877

878 **Figures**

879 **Figure 1**

880 Venn diagram showing the number of overlapping and differentially expressed genes in HBEC-5i cells
881 of the groups niRBC true vs. false (yellow circle), febrile true vs. false (red circle) and iRBC true vs.
882 false (blue circle). The niRBC true vs. false group contains the genes for which the presence of niRBC
883 affects the expression of HBEC-5i cells. The febrile true vs. false group contains genes for which
884 increasing the culture temperature from 37°C to 40°C has an effect on the expression of HBEC-5i cells.
885 The group iRBC true vs. false contains the genes for which the presence of iRBCs changes the
886 expression of HBEC-5i cells. Each circle represents one of the groups, with overlaps including genes
887 present in more than one group.

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889 **Figure 2**

890 Protein-protein interaction network analysis. A STRING analysis was performed to identify protein-
891 protein interaction networks that are regulated in HBEC-5i cells in response to the presence of niRBCs,
892 febrile temperature and/or iRBCs. The nodes represent proteins and the connecting lines between the
893 nodes indicate known or predicted interactions. All interaction networks that are shown are up-
894 regulated compared to the respective controls. The thickness of the connecting lines indicates the
895 confidence level of the interaction. Nodes marked with an asterisk are proteins whose encoding genes
896 were identified in the respective analyses. A selection of significant networks from the GO-BP analysis
897 is shown. (**A**) Comparison febrile true vs. false, contains 98 group-specific significantly regulated
898 genes. (**B**) Comparison iRBC true vs. false, contains 318 group-specific significantly regulated genes.
899 (**C**) Intersection of the groups niRBC true vs. false and iRBC true vs. false. (**D**) Intersection of the
900 groups iRBC true vs. false and febrile true vs. false. (**E**) Intersection of the groups niRBC true vs. false,
901 iRBC true vs. false, and febrile true vs. false. Genes exhibiting significant differential expression,
902 meeting stringent criteria including a fold change of ≥ 2.0, a FDR of ≤ 0.05, and a minimum threshold
903 of TPM of ≥ 2.0, were considered for inclusion in the analysis.

904

905 **Figure 3**

906 Identification of the pathways regulated by the presence of iRBCs in HBEC-5i cells. (**A**) A STRING
907 analysis was performed to identify protein-protein interaction networks regulated in HBEC-5i cells in
908 response to the presence of iRBCs (iRBC true vs. false including 762 genes). The nodes represent
909 proteins and the connecting lines between the nodes indicate known or predicted interactions. The
910 thickness of the connecting lines indicates the confidence level of the interaction. Nodes marked with
911 an asterisk are proteins whose encoding genes were identified in the analyses. A selection of significant
912 networks from the GO-BP analysis is shown. Genes exhibiting significant differential expression,
913 meeting stringent criteria including a fold change of ≥ 2.0, a FDR of ≤ 0.05, and a minimum threshold
914 of TPM of ≥ 2.0, were considered for inclusion in the analysis. (**B**) ShinyGO analysis to categorize the
915 proteins encoded by the identified genes according to their biological functions. The 10 most
916 significant pathways are shown.

917

918 **Figure 4**

919 Pathway analysis of the specific response of HBEC-5i cells due to the cytoadhesion of iRBCs$^{37°C}$ and
920 iRBCs$^{40°C}$ co-cultivation. g:Profiler ((**A**), (**C**), (**E**)) and ShinyGO ((**B**), (**D**), (**F**)) analysis of the
921 significantly up-regulated genes (fold change ≥ 2.0, FDR ≤ 0.05, and minimum threshold of TPM of ≥
922 2.0) of the comparisons (**A**), (**B**): niRBC$^{37°C}$ vs. iRBC$^{37°C}$; (**C**), (**D**): niRBC$^{37°C}$ vs. iRBC$^{40°C}$; (**E**), (**F**):
923 iRBC$^{37°C}$ vs. iRBC$^{40°C}$.

924

925 **Figure 5**

926 Identification of the pathways regulated by the presence of iRBCs in HBEC-5i cells at 37°C and 40°C
927 using a STRING analysis. Nodes represent proteins and the connecting lines between the nodes

928 indicate known or predicted interactions. The thickness of the connecting lines indicates the confidence
929 level of the interaction. Nodes marked with an asterisk are proteins whose encoding genes were
930 identified in the analyses. A selection of significant networks from the GO-BP analysis is shown. Genes
931 exhibiting significant differential expression, meeting stringent criteria including a fold change of ≥
932 2.0, a FDR of ≤ 0.05, and a minimum threshold of TPM of ≥ 2.0, were considered for inclusion in the
933 analysis. STRING analysis was performed to identify protein-protein interaction networks regulated in
934 HBEC-5i cells after co-cultivation of (**A**) iRBCs$^{37°C}$ in comparison to niRBCs$^{37°C}$, (**B**) iRBCs$^{40°C}$ in
935 comparison to niRBCs$^{40°C}$, and **C** iRBCs$^{37°C}$ in comparison to iRBCs$^{40°C}$.

936

937 **Figure 6**

938 Compilation of genes whose expression is increased by the presence of iRBCs and/or an elevated
939 temperature compared to the control. (**A**) Cytokine encoding genes. (**B**) Genes encoding
940 metalloproteinases and NR4A nuclear hormone receptor proteins. (**C**) Genes encoding histones and
941 histone-linker proteins, (**D**) Genes encoding proteins of the interferon-alpha/beta signaling pathway.
942 The expression level is shown as TMP. Blue: Expression level in the presence of niRBCs$^{37°C}$; Red:
943 Significant increase in expression (>2-fold) due to the presence of iRBCs$^{37°C}$; Red-brown: Significant
944 increase in expression (>2-fold) due to the presence of iRBCs$^{40°C}$ in comparison to iRBCs$^{37°C}$. (**E**)
945 Reactome analyses of the genes listed in D. The pathways to which the genes can be assigned are
946 shown in yellow.

947

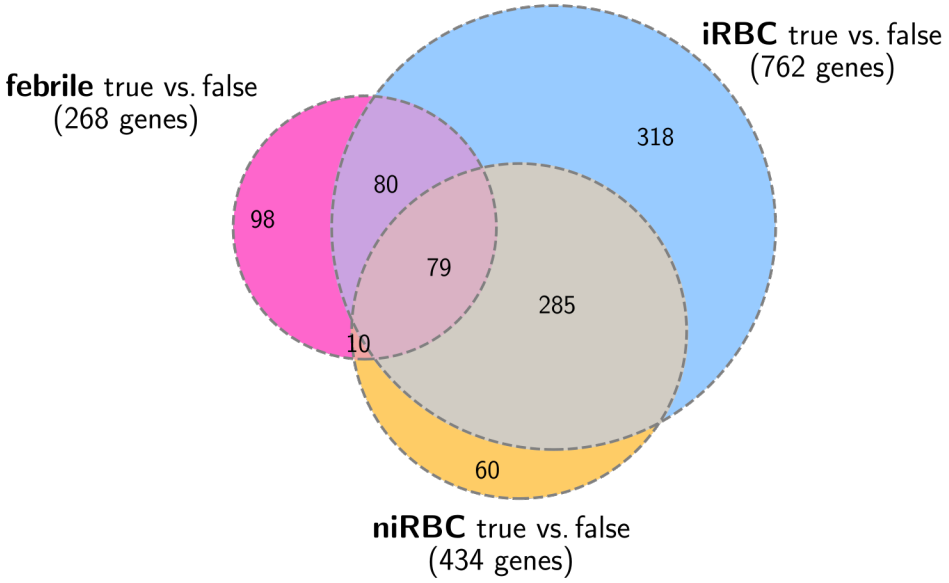
948 **Supplementary Table 1**

949

950

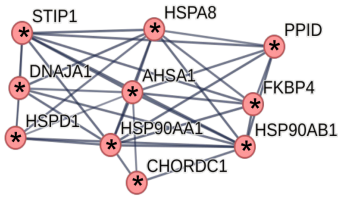
951

This is a provisional file, not the final typeset article


febrile true vs. false
(268 genes)
iRBC true vs. false
(762 genes)
niRBC true vs. false
(434 genes)
98
80
318
79
285
10
60

A

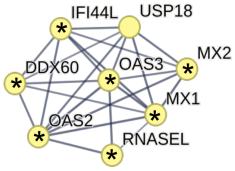
Febrile true vs. false (98 genes)



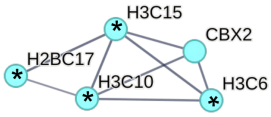
Protein folding
FDR 4.82e-16

B

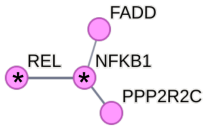
iRBC true vs. false (318 genes)



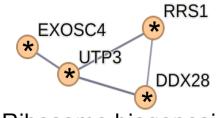
Defense response to virus
FDR 7.75e-09



Nucleosome assembly
FDR 0.00013



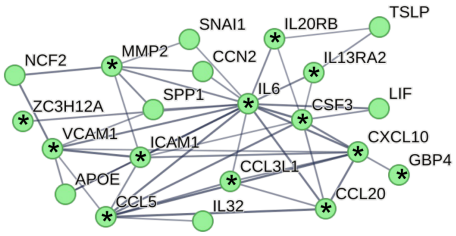
NF-kappaB complex
FDR 0.0018



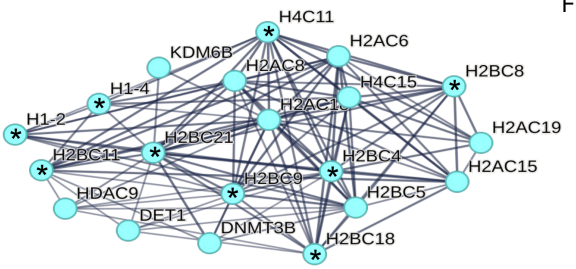
Ribosome biogenesis
FDR 0.00086

C

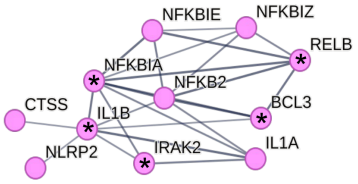
Intersection niRBC true vs. false and iRBC true vs. false (285 genes)



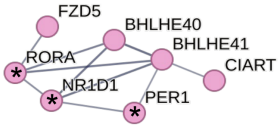
Cellular response to cytokine stimulus
FDR 4.82e-10



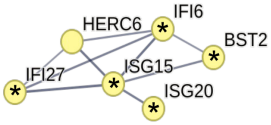
Nucleosome assembly
FDR 2.56e-15



I-kappaB kinase/NF-kappaB signaling
FDR 4.32e-06



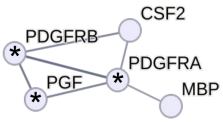
Negative regulation of I-kappaB kinase/
NF-kappaB signaling; FDR 0.0017



Defense response to virus
FDR 3.38e-05

D

Intersection iRBC true vs. false or febrile true vs. false (80 genes)

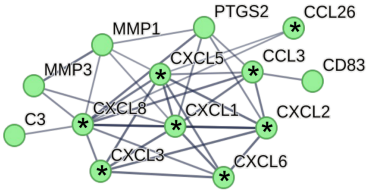


Vascular endothelial growth factor
signaling pathway; FDR 0.00025

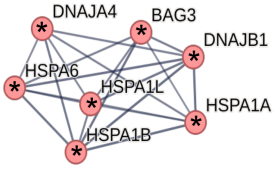
Response to unfolded protein
FDR 2.50e-05

E

Intersection iRBC true vs. false and febrile true vs. false and niRBC true v.s false (79 genes)



Chemokine-mediated signaling pathway
FDR 2.26e-12

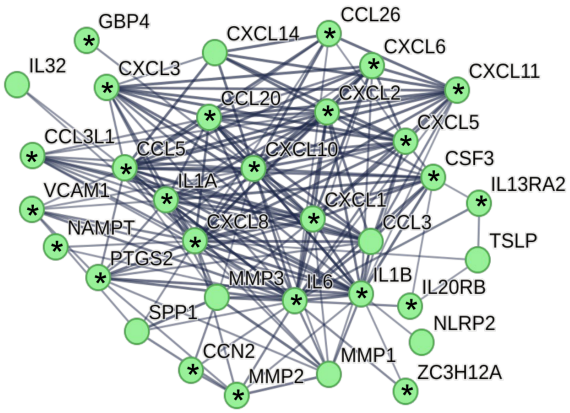


Protein folding
FDR 3.29e-10

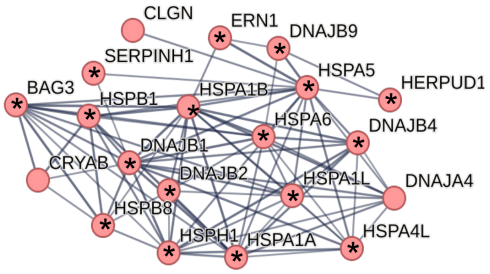
A

iRBC true vs. false (762 Genes)

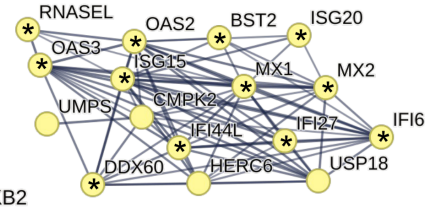
Up-regulated



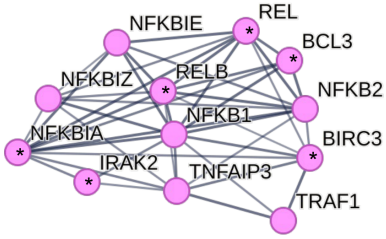
Response to cytokine
FDR 2.01e-23



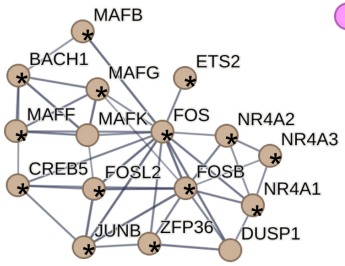
Response to unfolded protein
FDR 1.67e-30



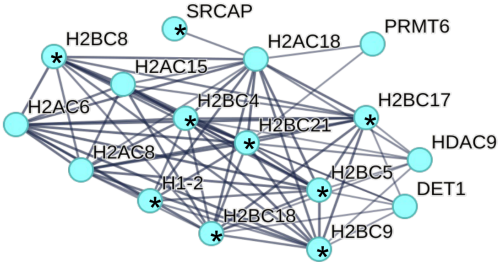
Defense response to virus
FDR 7.04e-16



I-kappaB kinase/NF-kappaB signaling
FDR 3.24e-08

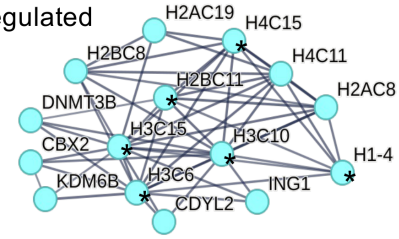


Positive regulation of RNA metabolic process
FDR 7.04e-10



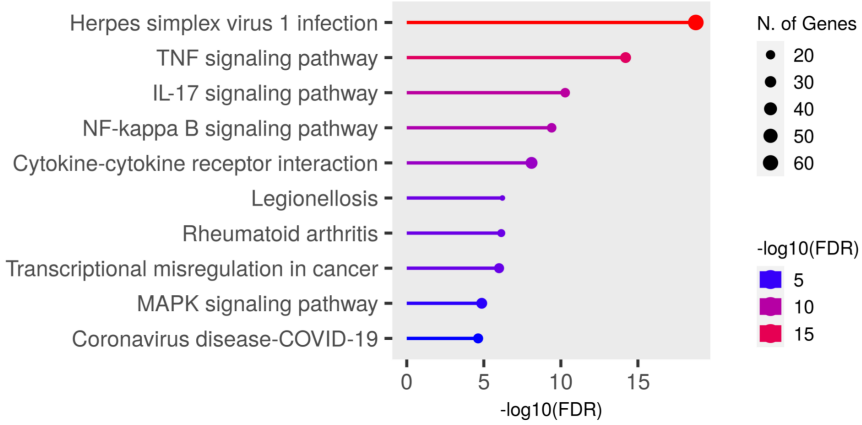
Nucleosome organization
FDR 3.83e-11

Down-regulated



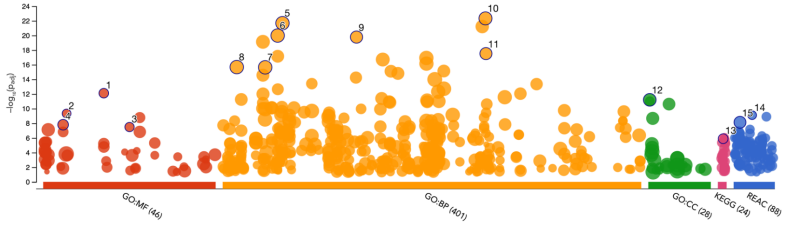
Nucleosome assembly
FDR 3.64e-08

B



A

niRBC$^{37°C}$ vs. iRBC$^{37°C}$



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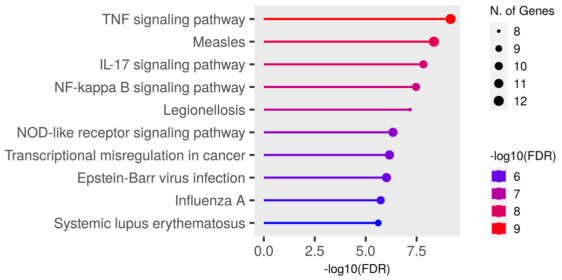
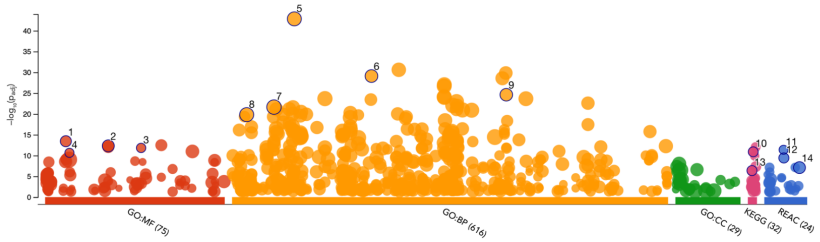
2	GO:MF	GO:0008009	chemokine activity	4.630×10^{-10}
3	GO:MF	GO:0042379	chemokine receptor binding	3.244×10^{-8}
4	GO:MF	GO:0005125	cytokine activity	1.574×10^{-8}
5	GO:BP	GO:0010033	response to organic substance	2.110×10^{-22}
6	GO:BP	GO:0009605	response to external stimulus	1.031×10^{-20}
7	GO:BP	GO:0007166	cell surface receptor signaling pathway	2.257×10^{-16}
8	GO:BP	GO:0002376	immune system process	2.104×10^{-16}
9	GO:BP	GO:0034097	response to cytokine	1.669×10^{-20}
10	GO:BP	GO:0071310	cellular response to organic substance	4.668×10^{-23}
11	GO:BP	GO:0071345	cellular response to cytokine stimulus	3.012×10^{-18}
12	GO:CC	GO:0000785	chromatin	6.251×10^{-12}
13	KEGG	KEGG:04668	TNF signaling pathway	1.281×10^{-8}
14	REAC	REAC:R-HSA-6...	Interleukin-10 signaling	7.789×10^{-10}
15	REAC	REAC:R-HSA-1...	Cytokine Signaling in Immune system	7.306×10^{-9}

B



C

niRBC$^{37°C}$ vs. iRBC$^{40°C}$



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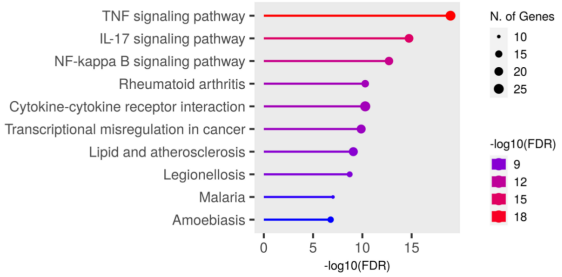
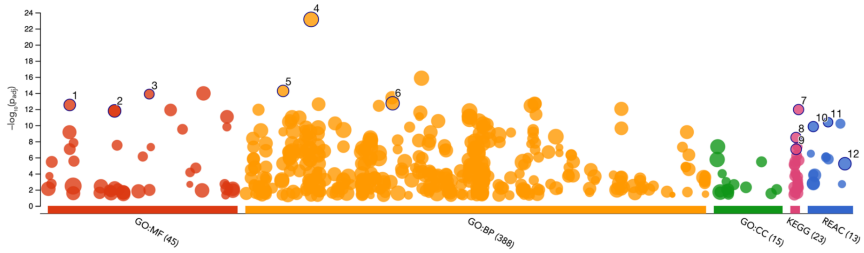
3	GO:MF	GO:0044183	protein folding chaperone	1.510×10^{-12}
4	GO:MF	GO:0008009	chemokine activity	2.258×10^{-11}
5	GO:BP	GO:0010033	response to organic substance	1.417×10^{-43}
6	GO:BP	GO:0034097	response to cytokine	7.933×10^{-30}
7	GO:BP	GO:0006950	response to stress	2.283×10^{-22}
8	GO:BP	GO:0002376	immune system process	1.511×10^{-20}
9	GO:BP	GO:0071345	cellular response to cytokine stimulus	2.461×10^{-25}
10	KEGG	KEGG:04657	IL-17 signaling pathway	1.296×10^{-11}
11	REAC	REAC:R-HSA-6...	Interleukin-10 signaling	4.220×10^{-12}
12	REAC	REAC:R-HSA-6...	Interleukin-4 and Interleukin-13 signaling	3.856×10^{-10}
13	KEGG	KEGG:04064	NF-kappa B signaling pathway	4.205×10^{-7}
14	REAC	REAC:R-HSA-4...	Signaling by Interleukins	8.137×10^{-8}

D



E

iRBC$^{37°C}$ vs. iRBC$^{40°C}$



ID	Source	Term ID	Term Name	p_{adj} (query_1)
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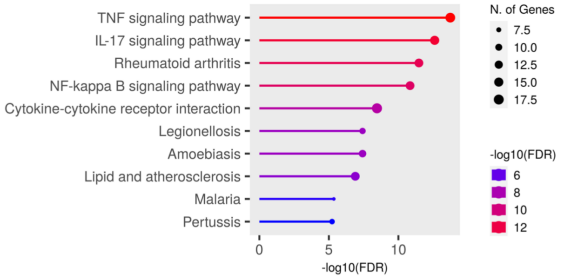
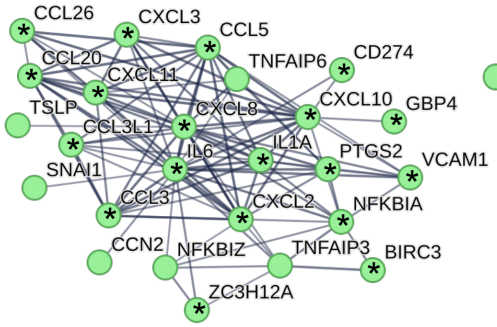
2	GO:MF	GO:0030546	signaling receptor activator activity	1.627×10^{-12}
3	GO:MF	GO:0044183	protein folding chaperone	1.326×10^{-14}
4	GO:BP	GO:0010033	response to organic substance	7.069×10^{-24}
5	GO:BP	GO:0006457	protein folding	5.384×10^{-15}
6	GO:BP	GO:0034097	response to cytokine	1.840×10^{-13}
7	KEGG	KEGG:05323	Rheumatoid arthritis	1.105×10^{-12}
8	KEGG	KEGG:04657	IL-17 signaling pathway	3.238×10^{-9}
9	KEGG	KEGG:04668	TNF signaling pathway	9.388×10^{-8}
10	REAC	REAC:R-HSA-3...	Cellular response to heat stress	1.436×10^{-10}
11	REAC	REAC:R-HSA-6...	Interleukin-10 signaling	4.139×10^{-11}
12	REAC	REAC:R-HSA-4...	Signaling by Interleukins	5.864×10^{-6}

F

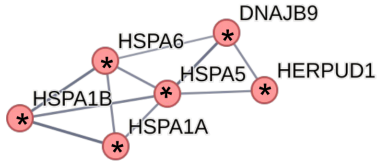


A

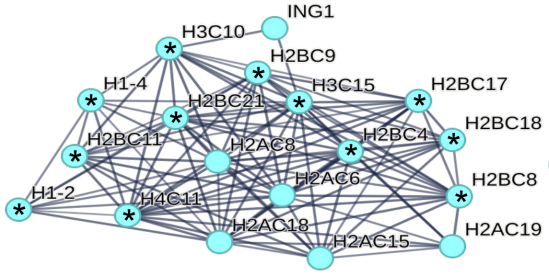
niRBC$^{37°C}$ vs. iRBC$^{37°C}$



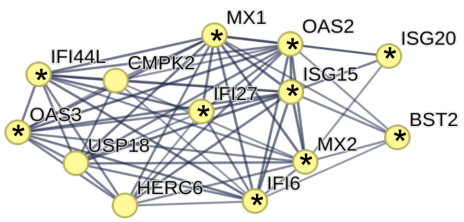
Response to cytokine
FDR 5.50e-18



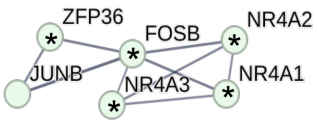
Response to unfolded protein
FDR 1.05e-09



Nucleosome assembly
FDR 1.99e-18



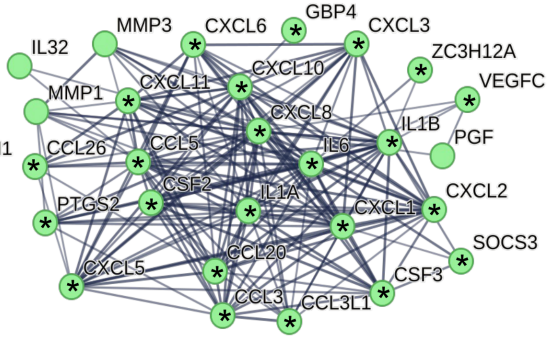
Defense response to virus
FDR 6.28e-13



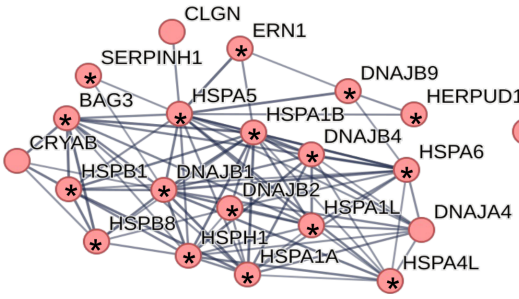
Cellular response to hormone stimulus
FDR 0.00030

B

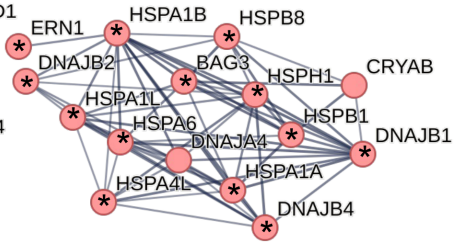
niRBC$^{37°C}$ vs. iRBC$^{40°C}$



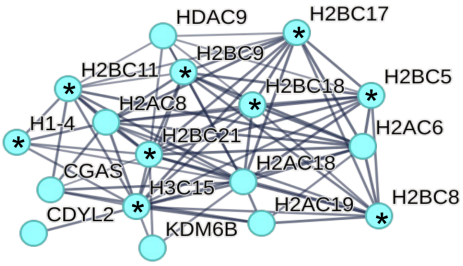
Response to cytokine
FDR 3.62e-24



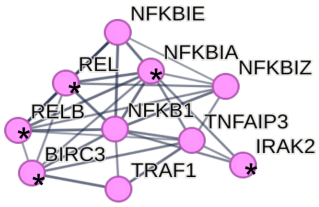
Response to unfolded protein
FDR 1.67e-30



Response to unfolded protein
FDR 6.55e-23



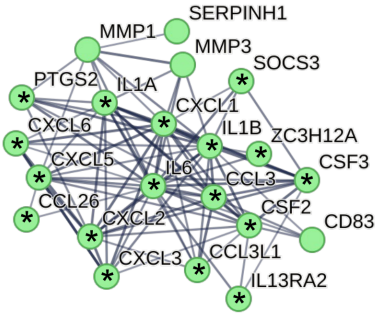
Nucleosome assembly
FDR 8.01e-12



I-kappaB kinase/NF-kappaB signaling
FDR 2.36e-06

C

iRBC$^{37°C}$ vs. iRBC$^{40°C}$



Response to cytokine
FDR 2.32e-16

A

	niRBC$^{37°C}$	iRBC$^{37°C}$	iRBC$^{40°C}$
CXCL1	101	84	448
CXCL2	25	55	173
CXCL3	11	33	150
CXCL5	5	6	28
CXCL6	23	44	156
CXCL8	82	392	1932
CXCL10	1.3	4.2	4.9
CXCL11	1	3.4	2.8
CCL3	0.4	4.7	31
CCL3L1	0.4	10	25
CCL5	34	91	74
CCL20	0.2	3.7	6
CCL26	1	3	26
IL1A	3.4	10.7	30.5
IL1B	332	305	788
IL6	13	70	193
IL12A	10	32	53
IL32	38	33	91

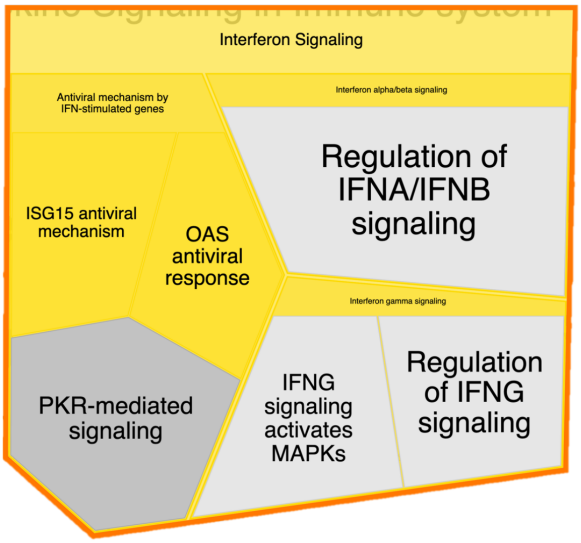
B

	niRBC$^{37°C}$	iRBC$^{37°C}$	iRBC$^{40°C}$
MMP1	6	3	27
MMP2	115	173	317
MMP3	6	6	52
NR4A1	10	70	215
NR4A2	2	57	77
NR4A3	1.5	9	29

C

	niRBC$^{37°C}$	iRBC$^{37°C}$	iRBC$^{40°C}$
H1-2	73	150	116
H1-4	0.5	4.8	2.3
H2BC4	7	14	7
H2BC8	2	9	9
H2BC9	5	16	16
H2BC11	18	101	55
H2BC17	4.5	9	11
H2BC18	0.8	4.3	4.1
H2BC21	10	96	30
H3C10	3.7	11	7
H3C15	2	7	9
H4C11	3.6	12	2.5

E

	niRBC$^{37°C}$	iRBC$^{37°C}$	iRBC$^{40°C}$
BST2	43	116	74
DDIT4	202	838	806
DDX60	6	12	14
GBP4	2	9	9
IL6	13	70	193
IFI6	591	1282	814
IFI27	22	49	96
IFI44L	11	46	23
ISG15	509	1079	733
ISG20	9	20	17
MX1	78	199	95
MX2	11	37	19
OAS2	20	73	40
OAS3	32	87	62

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