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Original article

Candida albicans increases the aerobic glycolysis and activates MAPK–dependent inflammatory response of liver sinusoidal endothelial cells

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ABSTRACT

The liver, and more specifically, the liver sinusoidal endothelial cells, constitute the beginning of one of the most important responses for the elimination of hematogenously disseminated *Candida albicans*. Therefore, we aimed to study the mechanisms involved in the interaction between these cells and *C. albicans*. Transcriptomics-based analysis showed an increase in the expression of genes related to the immune response (including receptors, cytokines, and adhesion molecules), as well as to aerobic glycolysis. Further in vitro analyses showed that IL-6 production in response to *C. albicans* is controlled by MyD88- and SYK-pathways, suggesting an involvement of Toll-like and C-type lectin receptors and the subsequent activation of the MAP-kinases and c-Fos/AP-1 transcription factor. In addition, liver sinusoidal endothelial cells undergo metabolic reprogramming towards aerobic glycolysis induced by *C. albicans*, as confirmed by the increased Extracellular Acidification Rate and the overexpression of enolase (*Eno2*), hexonikase (*Hk2*) and glucose transporter 1 (*Slc2a1*). In conclusion, these results indicate that the hepatic endothelium responds to *C. albicans* by increasing aerobic glycolysis and promoting an inflammatory environment.

1. Introduction

Candida albicans is an opportunistic human pathogen that has been recently classified as a critical priority fungal pathogen by the World Health Organization (WHO) [1]. Indeed, it causes one of the most important health care-associated infections, being the most prevalent pathogenic fungal infection and occupying tenth place in the ranking of all infectious agents with an incidence of 3.1 % [2]. In some severe cases, *C. albicans* is able to disseminate haematogenously and cause invasive candidiasis, with an estimated mortality rate of 46–75 % [3–5].

Among the organs to which *C. albicans* can disseminate, the liver is the main blood-filtering organ and carries out different functions, including metabolism or the elimination of toxins and cellular debris from blood. More importantly, the liver takes part in microorganism clearance through activation and initiation of immune responses [6,7]. For that, the liver receives blood from two distinct vessels: the portal vein (around 70 % of the blood) and the hepatic artery, which drain into smaller capillaries known as hepatic sinusoids that are mainly formed by liver sinusoidal endothelial cells (LSECs). Since LSECs constitute the first cells of the liver

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that interact with the particles, including microbes, circulating in hepatic sinusoids, they are responsible for different physiological, immunological, and pathophysiological processes [6]. To do that, LSECs exhibit unique characteristics that differentiate them from other endothelial cells (ECs) and make them similar to some immune cells [8]. For example, LSECs have a high endocytic activity mediated by mannose receptor and stabilins, and express adhesion molecules (vascular cell adhesion protein 1 (VCAM1) and intercellular adhesion molecule-1 (ICAM-1)) absent in other ECs [8]. Furthermore, LSECs express several pattern recognition receptors (PRRs), which include Toll-like receptors (TLRs), C-type lectin receptors (CLRs), or NOD-like receptors (NLRs), as well as other specific PRRs (such as liver/lymph node-specific ICAM3-grabbing non-integrin (LSIGN)) [6,8]. These receptors enable the recognition of pathogen associated molecular patterns, leading to the release of cytokines, including chemokines, and the expression of adhesion molecules, which efficiently attract and anchor leukocytes to the infection site to facilitate microbial elimination [6,8].

The study of the interplay between *C. albicans* and other vascular ECs has highlighted the importance of TLRs in recognition of *C. albicans* and subsequent activation of NF-κB and MAPK/p38 pathways leading to the production of cytokines, chemokines, and adhesion molecules [9–11]. However, there are very few studies focused specifically on LSECs and their response to *C. albicans*. Moreover, although these studies describe that the presence of the yeast is able to promote the expression of cytokines and adhesion molecules [12,13], the molecular pathways involved in the process are still unknown.

In view of this, the objective of this study was to analyse the transcriptomic profile of LSECs in the presence of *C. albicans*, and to further study the metabolic and molecular pathways involved in fungal recognition and LSECs activation.

2. Materials and methods

2.1. Microorganisms

C. albicans CECT 13062 strain (Spanish Type Culture Collection) isolated from a patient with systemic candidiasis was used with the approval of the Ethics Committee of the University of the Basque Country (UPV/EHU; M30/2019/114). The yeast was grown in Sabouraud Dextrose Agar (Panreac Química SLU, Castellar del Vallès, Spain) at 37 °C overnight. One day prior to the experiment, 10^5 blastoconidia/ml were inoculated in Sabouraud Dextrose Broth (Panreac Química SLU) and incubated at 37 °C and 120 rpm overnight. The day of the experiment, yeasts were collected by centrifugation and washed twice with Dulbecco's Phosphate Buffer Saline (Corning, VWR, PA, USA). The density of blastoconidia was adjusted for each experiment using a haemocytometer.

2.2. Isolation and culture of primary murine LSECs

Primary cultures of murine LSECs were obtained following a modified protocol of Smedsrød & Pertoft [14], with the approval of the Ethics Committee of the UPV/EHU (UPV/EHU; reference M20/2019/118). Briefly, the LSECs were extracted from C57BL/J6 mice (6–8 week old). After enzymatic perfusion with collagenase type I from *Clostridium histolyticum* (Sigma–Aldrich, St Louis, MO, USA), a cell suspension enriched in LSEC was obtained by isopycnic density Percoll gradient (Thermo Fisher Scientific, MA, USA). Kupffer cells were eliminated from endothelial cell suspension by differential adhesion to a plastic surface. Non-adherent LSECs were collected and seed into 0.05 mg/ml rat collagen type I (Gibco, Thermo Fisher Scientific) coated 48-well plates at 3.5×10^5 cell/cm^2. LSECs were kept in pyrogen free RPMI 1640 (Sigma–Aldrich) supplemented with 1 % fetal bovine serum (Thermo Fisher Scientific), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma–Aldrich), at 37 °C and 5 % CO_2. After 45 min, cells were carefully washed and let to spread for at least 2 h in the absence of serum before further use.

2.3. Culture of immortalized LSEC cell line

Immortalized murine LSECs (Innoprot, Spain) were kept in RPMI 1640 medium with 10 % heat-inactivated fetal bovine serum (Thermo Fisher Scientific), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B (Sigma–Aldrich), in a 5 % CO_2 atmosphere at 37 °C.

2.4. Establishment of LSEC co-cultures with C. albicans

For RNA-seq analysis, primary cultures of LSECs were stimulated at a Multiplicity of Infection (MOI; *C. albicans*:LSEC) of 0.5 with heat-killed *C. albicans* (30 min at 70 °C) for 6 h. Four biological replicates of the experiment were performed.

After the transcriptomic analysis, to study more in depth the effect of *C. albicans* on LSECs, immortalized cells were seeded at 1×10^6 cells/well in 6-well plates for gene expression analysis and, at 5×10^5 cells/well in 12-well plates for cytokine measurement and protein expression analysis. After an overnight incubation, cells were infected for 6 h with live *C. albicans* at a MOI of 5.

2.5. RNA-seq and bioinformatic analysis to study the effect of C. albicans on primary cultures of LSECs

RNA isolation from co-cultures of *C. albicans* and primary LSECs was performed using the Total RNA Isolation Kit (NzyTech, Lisbon, Portugal). The quantity (Qubit 3.0, Thermo Fisher Scientific) and quality (Fragment Analyzer, AATI) of each RNA sample was carried out in the general research services -SGIker- of the UPV/EHU. Then, RNA sequencing and its analysis were performed in the genomics and bioinformatics platform of the *Centro de Investigación Biomédica de la Rioja* (CIBIR). For that, mRNA libraries were prepared using TruSeq Stranded mRNA Kit (Illumina, CA, USA) from 100 ng of total RNA and their concentration was measured using a Qubit 3.0. Libraries were sequenced on a NextSeq platform (Illumina) to generate 75 base single-end reads. Quality control of the raw sequence data was performed using FastQC v.0.11.9 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and MultiQC v.1.9 (http://multiqc.info/), and then, adaptor and low-quality reads (phred <25) were filtered out using TrimGalore v0.5.0 (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) and the rRNAs by SortMeRNA (https://bioinfo.lifl.fr/RNA/sortmerna/).

Clean sequence reads were aligned to the mouse reference genome (GRCm39) using HISAT2 (https://ccb.jhu.edu/software/hisat2/index.shtml). The counts of raw reads per gene were performed using FeatureCounts program (http://subread.sourceforge.net/). The differential gene expression (DEG) in fold change (FC) was then determined using the EdgeR tool using the SARTools package [15,16]. DEGs were assigned above the absolute value of $\log_2$FC of 0.585 (FC > 1.5 (for up-regulated genes) or FC < 0.66 (for down-regulated genes)) and an adjusted p-value of <0.05. The false discovery rate (FDR) was minimized using the Benjamini and Hochberg approach. Gene Ontology (GO) and KEGG pathway enrichment analysis were performed using the clusterProfiler package (https://bioconductor.org/packages/release/bioc/html/clusterProfiler.html), establishing cut-off values for the p-value and the q-value of 0.05 and adjusting the p-value using the Benjamini and Hochberg approach. Further protein interaction networks studies were performed using the STRING tool (v11.5; https://string-db.org/).

2.6. Gene expression by RT-qPCR analysis

For RT-qPCR, LSECs RNA was isolated using the Total RNA Isolation Kit (NzyTech) and measured using the Nanodrop ND 1000 (Thermo Fisher Scientific) before being stored at −80 °C until used. The cDNA was synthesized using the NZY First-Strand cDNA Synthesis Kit (NzyTech) and, then, the RT-qPCR was performed using the NZYSupreme qPCR Green Master Mix (2x) (NzyTech) in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, CA, USA) thermal cycler. The used primers are listed in Supplementary Table 1. To determine the relative concentration of mRNAs in respect to control the threshold cycle (Ct) values were normalized to *Rpl19* housekeeping gene and, then, the ΔΔCt method was used.

2.7. Inhibition of signalling and metabolic pathways

To inhibit different metabolic and signalling pathways, LSECs were treated with inhibitors for 1 h at 37 °C and 5 % CO_2 before *C. albicans* was added. The inhibitors of hexokinase (2-deoxyglucose, 2-DG; 5 mM), GLUT1 (BAY 876; 25 nM), SYK (Piceatannol, 30 μM), MyD88 (TJ-M2010-5, 30 μM), ERK1/2 (PD-0325901; 1 μM), JNK (SP600125, 10 μM), p38a (SB 203580, 10 μM), NF-κB (BAY 11-7082, 2 μM) and c-Fos/AP-1 (T-5224, 10 μM) were obtained from MedChemExpress (NJ, USA).

2.8. Measurement of cytokine release

For cytokine measurement, the supernatants of LSECs were collected and stored at −20 °C until use. Enzyme-linked Immunosorbent Assays (ELISA) to detect TNF, IL-6 and IFN-γ were performed using commercial Uncoated ELISA Kits (Invitrogen, Thermo Fisher Scientific) and IFN gamma Mouse ELISA Development Kit (TMB) (PeproTech® by Invitrogen, Thermo Fisher Scientific).

2.9. Electrophoresis and Western blotting

Protein extraction was performed using RIPA lysis buffer containing 1 mM protease inhibitors (Phenylmethylsulfonyl fluoride, PMSF, Sigma−Aldrich) and incubated on ice for 10 min. After that, each well was scrapped, collected, and incubated on ice another 10 min. Finally, the samples were centrifuged at maximum speed for 5 min and supernatant stored at −20 °C until use.

For Western Blotting (WB), samples were loaded into 10 % SDS-polyacrylamide gels and run at 70 mA, 100 W and 200 V for 45 min in a Miniprotean II (Bio-Rad), using the Page Ruler Plus as protein standard (Thermo Fisher Scientific). Proteins were then transferred to Hybond-P PVDF membranes (GE Healthcare) for 1 h at 400 mA. Membranes were then blocked for 2 h with Tris Buffered Saline Milk (TBSM; 50 mM tris-HCl pH 7.5, 150 mM NaCl, 0.1 % (v/v) Tween 20 and 5 % (w/v) low fat dried milk, both from Sigma−Aldrich, MO, USA). Then rabbit mAbs against c-Fos (9F6), Phospho IκBα (Ser32) (14D4) and the housekeeping protein α-Tubulin (11H10) diluted 1:1000 in TBSM were used as primary antibodies (Cell Signalling Technology, MA, USA) and incubated with the membrane overnight at 4 °C. After three washes of 5 min with TBS, goat anti-rabbit IgG (Invitrogen, Thermo Fisher Scientific) diluted 1:5000 in TBSM was used as secondary antibody and incubated with the membrane for 30 min. All the steps were carried out at room temperature and agitation unless otherwise indicated. Membranes were developed using the NZY Standard ECL (NzyTech) in a G:BOX Chemi system (Syngene, CA, UK). Densitometry analysis of WBs was performed using Image J (Bethesda, MD, USA).

2.10. Extracellular Acidification Rate (ECAR) assay

Glycolytic activity was assessed using the Extracellular Acidification Rate (ECAR) as a proxy, using the Agilent MitoXpress® pH-Xtra kit (Agilent, CA, USA) following manufacturer's instructions.

First, 2.5×10^4 LSECs per well were seeded in black 96-well plates with clear bottoms (Costar, Corning, NY, USA) and incubated overnight. Then, cells were washed twice with 100 μL of pre-warmed respiration buffer adjusted to pH 7.4. After, 90 μL of fresh respiration buffer was added to each well and immediately cells were infected with live *C. albicans* (MOI 5). Finally, 10 μL of pH-Xtra reagent was added to each well and the plates were incubated in the FlexStation 3 CO_2 free machine (Molecular Devices, CA, USA) for 6 h at 37 °C, exciting the sample at 380 nm and measuring the fluorescence emitted by pH-Xtra reagent at 615 nm. In order to perform the statistical analysis, the measured fluorescence was used.

2.11. Statistical analysis

Statistical analysis was performed using IBM SPSS statistical software (version 28.0.1.1; Professional Statistic, IL, USA). All the experiments were performed at least three times and analysed using t-student test. The criterion for significance was set at $p < 0.05$ for all comparisons.

3. Results

3.1. Transcriptional response of primary murine LSECs to C. albicans stimulation

The transcriptional changes with the highest magnitude induced by heat-killed *C. albicans* in primary LSECs were identified through RNA-seq analysis using both adjusted p-value and FC parameters, which showed a total of 79 genes significantly upregulated and 23 genes downregulated in comparison to control LSECs (Fig. 1A). The low number of genes found is likely due to the use of heat-killed yeast, which could cause the loss of some genes associated with the response to the invasive filamentous fungal morphology.

Among the upregulated genes, 36 % (29 out of 79 genes) and 45 of the top 50 Biological Processes (GO-BPs, ordered according to the GeneRatio) were associated with immune response development (Fig. 1B and C). In fact, 16 out of these 45 GO-BPs were classified into "immune system processes" (GO:0002376; 18 genes), nine into "regulation of response to stimulus" (GO:0048583; 24 genes), six into "regulation of cytokine production" (GO:0001817; 16 genes), another six into "regulation of cell adhesion" (GO:0030155; 10 genes), and four into "regulation of cell proliferation" (GO:0042127; 14 genes). All these groups shared genes coding for the cytokines IL-6, IL-10, TNF, and IFN-γ and the Signalling lymphocytic activation molecule 1 (*Slamf1*) (Fig. 1B). With respect to the enriched Molecular Functions (GO-MFs), four processes were upregulated in LSECs stimulated with heat-killed *C. albicans*, which, in accordance with GO-BPs, were all related to immune processes (Fig. 1D). In the same way, KEGG enrichment analysis revealed 17 upregulated pathways, five of which are involved in the inflammatory response to *C. albicans*: cytokine-cytokine receptor interaction (10 genes), the Jak-STAT signalling pathway (five genes), the Toll-like receptor signalling pathway (four genes), and the cytosolic DNA-sensing pathway (three genes) (Fig. 1E).

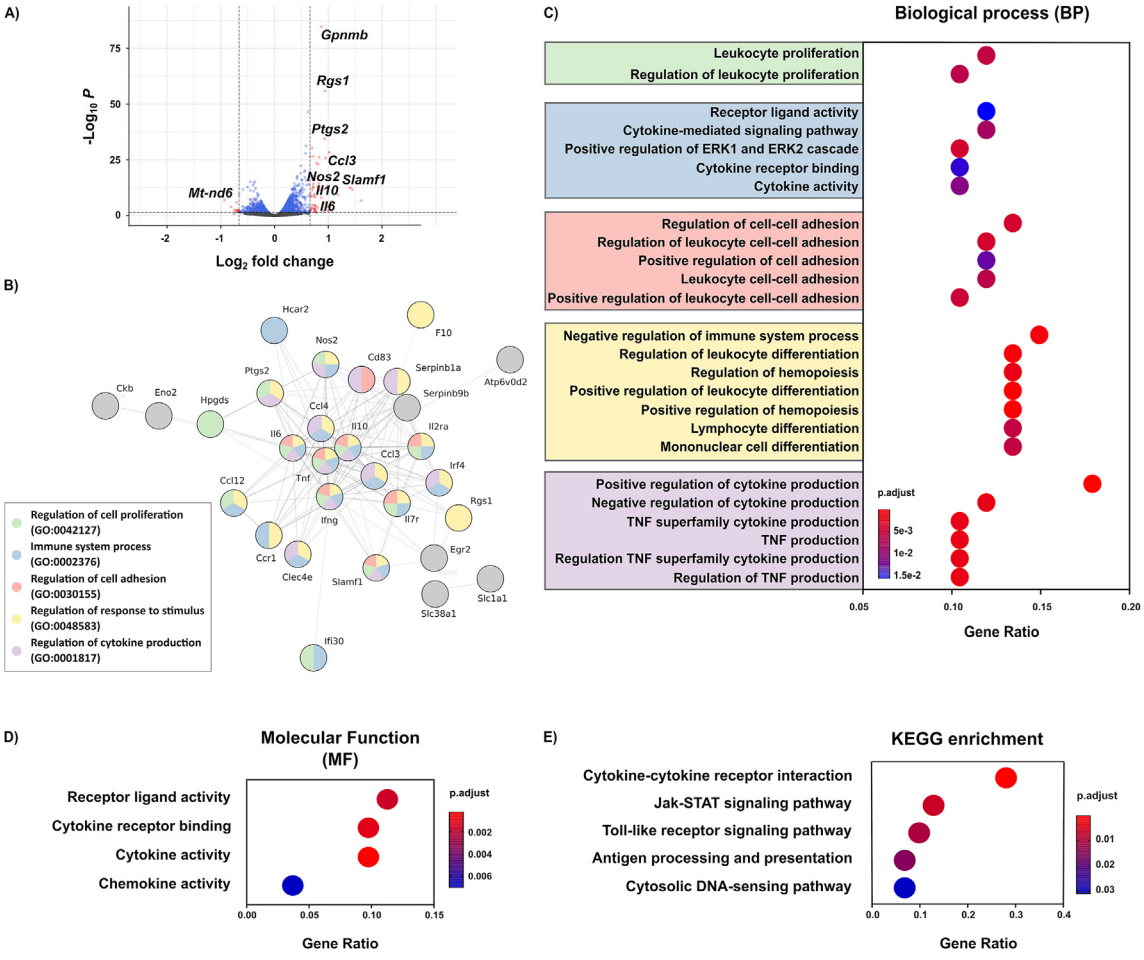

Fig. 1. Analysis of transcriptomic profiles by heat-inactivated *Candida albicans* infection in primary liver endothelial sinusoidal cells (LSECs). A) Volcano plot representing genes differentially regulated by *C. albicans* infection in LSECs. Red dots represent significantly up- or downregulated genes ($|\log_2 FC| > 0.585$; $p_{adj} \leq 0.05$). B) STRING performed with upregulated proteins and their relationship (grey lines) and the Biological Processes (BPs) in which they are implicated (only interrelated genes are shown). C) GO enrichment analysis of the Biological Processes (GO-BP) significantly upregulated *C. albicans* in LSECs. GO-BPs were grouped based on general biological processes. GO enrichment analysis of D) Molecular Functions (GO-MF) and E) KEGG pathways significantly upregulated by *C. albicans* in LSECs.

Having generated evidence of the main pathways altered in LSECs in response to *Candida*, a second analysis was performed, considering only the adjusted p-value as a selecting parameter to investigate thoroughly the differential gene expression induced. Specifically, 548 overexpressed genes were detected and classified using the same five GO-BPs described above (Fig. 2A). Thanks to the less restrictive conditions of this analysis, more genes related to the same processes were detected, such as those coding for PRRs (TLR2, TLR7, Galectin-9, Dectin-2, NLRP3), cytokines (IL-33), chemokines (CXCL1, CXCL2, etc.), and adhesion molecules (VCAM-1, ICAM-1, CADM-1) (Fig. 2B, C, D). Of these upregulated genes, we found that 401 were directly related with GO-BP "regulation of response to stimulus", 325 with "immune system processes", 185 with "regulation of cell population proliferation", 155 with "regulation of cytokine production" and 102 with "regulation of cell adhesion". It should be noted that in addition to *Il6*, *Il10*, *Tnf*, *Ifng* and *Slamf1* genes, in this second analysis all GO-BP groups shared other 26 genes, including the cytokine *Il1a*, the receptors Galectin-9 (*Lgals9*) and CD274 (*Cd274*, also known as PD-L1), the tyrosine kinase SYK (*Syk*), and integrin alpha V (*Itgav*). Furthermore, 55 genes are shared by at least four of the five BP-GO groups. These genes include the cytokine IL-33 (*Il33*), immune response signalling-related genes (*Nos2, Map2k1, Stat3*), and PRRs Galectin-3 (*Lgals3*), TLR2 (*Tlr2*), and NLRP3 (*Nlrp3*). In addition to the genes grouped within these five GO-BP groups, we found other overexpressed genes, such as *Hif1a*, *Hk2* and *Eno2* that were directly related with metabolic reprogramming.

3.2. C. albicans *is recognised by CLRs and TLRs, and induces a MAPK mediated inflammatory response in LSECs*

In order to study in depth the pathways activated by the yeast, live *C. albicans* was used to infect immortalized LSECs. First, to determine the relevance of the two most important groups of outer membrane PRRs detected to be overexpressed in the RNA-seq analysis (CLRs and TLRs), the impact of molecular inhibitors of the key signal transducers SYK and MyD88 was studied after live *C. albicans* infection of LSECs. To do this, the effect of inhibition was evaluated on the secretion of three of the inflammatory cytokines detected in the transcriptomic analysis (TNF, IL-6 and IFN-γ). Surprisingly, TNF and IFN-γ were not detected under any of the conditions tested (data not shown). However, inhibition of both SYK and MyD88 induced a significant decrease in the IL-6 release induced by *C. albicans*, which was even greater when LSECs were exposed to both inhibitors at the same time (Fig. 3A).

To further study the induced signalling cascade, gene expression of the molecules involved in the main inflammatory signalling pathways activated by PRRs (*Nfkb*, *Erk, Jnk* and *P38a*) were studied.

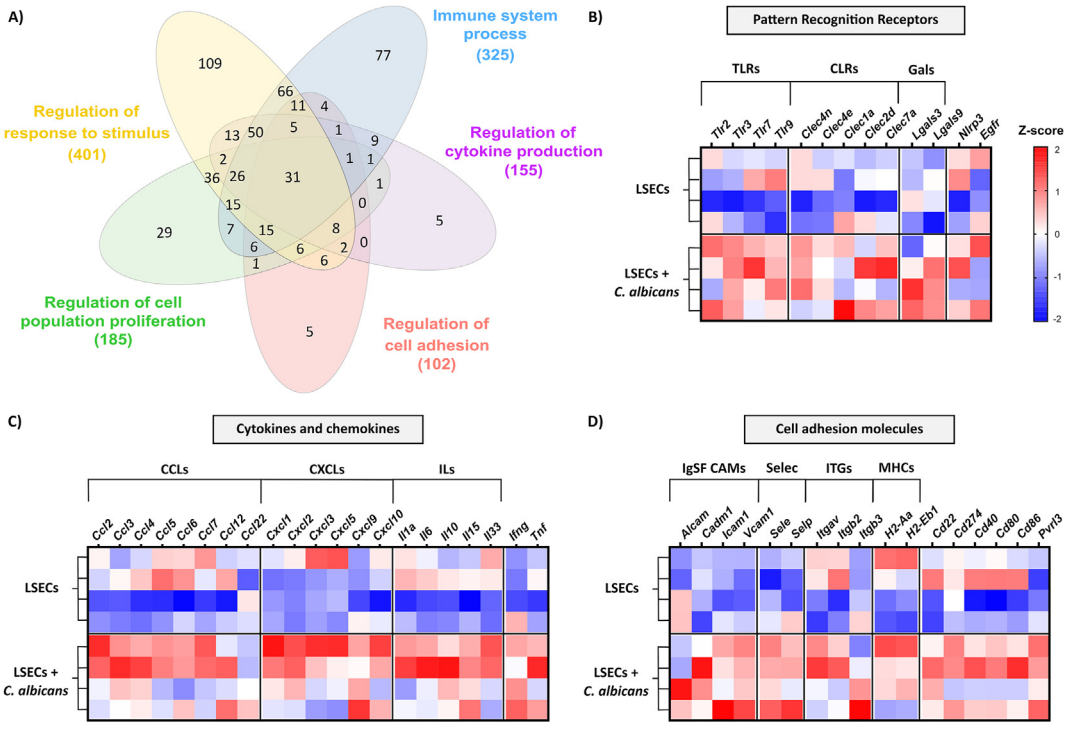

Fig. 2. Pathways regulated by heat-inactivated *Candida albicans* in primary liver endothelial sinusoidal cells (LSECs). A) Venn diagram representing the relations among differentially overexpressed genes (DEGs) ($p_{adj} \leq 0.05$) in the five GO-BPs above-mentioned. Heat-maps showing the expression levels comparison between samples (Z-score) of overexpressed DEGs ($p_{adj} \leq 0.05$) of B) Pattern Recognition Receptors, C) cytokines and chemokines and D) cell adhesion molecules. TLR: Toll-like receptor, CLR: C-type lectin receptor, Gals: Galectins, CCL: CC chemokine ligand, CXCL: CCX chemokine ligand, IL: interleukin, IgSF CAM: Immunoglobulin-like Cell Adhesion Molecules, Sele: Selectin, Itg: Integrin, MHC: Major histocompatibility complex.

As shown in Fig. 3B, none of them were significantly upregulated after *C. albicans* exposure. In view of this, molecular inhibitors for each pathway were also used to study their effect on IL-6 cytokine release (Fig. 3C). The results showed a significant reduction of IL-6 secreted in response to the yeast when inhibiting any of the three MAPK pathways (ERK1/2, JNK and p38), with JNK molecule inhibitor inducing a lesser decrease compared to ERK and p38. On the other hand, the use of the NF-κB inhibitor did not show any effect. These results were further confirmed by WB analysis measuring IκBα phosphorylation, crucial for NF-κB pathway activation, which showed a decreased phosphorylation rate in the presence of the yeast, indicating that this pathway is not involved in the response induced by yeast in these cells (Fig. 3D).

As the three MAPK pathways (ERK1/2, JNK and p38) have been described to converge in the activation of activator protein 1 (AP-1), and more specifically, on the transcription factor c-Fos, the inhibition of the c-Fos/AP-1 complex in the response to *C. albicans* was studied. As expected, a significant decrease in IL-6 release with respect to the fungus alone was shown (Fig. 3E). In view of this result, *Fos* gene (Fig. 3F) and c-Fos protein (Fig. 3G) expression was studied using MAPK pathway inhibitors to try to determine the pathway responsible for its activation. The results confirmed a significant increase in *Fos* gene and total c-Fos protein in *C. albicans*-infected LSECs compared to the control group. The use of inhibitors showed that only the p38 inhibitor significantly decreased expression of *Fos* at 4 h post-infection with *C. albicans,* while at 6 h post-infection this decrease was accentuated with both JNK and p38 inhibitors and, to a lesser extent, with ERK1/2 (Fig. 3F). However, at protein level, the greatest reduction in total c-Fos protein level was observed with the ERK1/2 inhibitor, but the main differences seem to be related to c-Fos phosphorylation since, of the two bands detected in the WB, the intensity of the upper band is reduced (by about 60 %) with the ERK1/2 inhibitor and the lower band is accentuated with respect to the *Candida*-only group (Fig. 3G).

3.3. C. albicans *causes metabolic alterations in LSECs that are primarily characterised by an increase in aerobic glycolysis*

Transcriptomic analysis also showed an upregulation of genes related to HIF-1 (hypoxia-inducible factor 1) signalling (Fig. 4A) and glycolysis/gluconeogenesis (Fig. 4B) in response to *C. albicans*, such as *Eno2* and *Hk2*. Moreover, the *Mt-nd6* gene, encoding a subunit of NADH-ubiquinone oxidoreductase and, thus, associated with oxidative phosphorylation, was downregulated (Fig. 1A). As all these modifications suggested metabolic reprogramming related to the immune response, we decided to analyse it further. To conduct these analyses, infections of immortalized LSECs with live *C. albicans* were performed. In order to confirm the increase in the glycolysis pathway induced by *C. albicans* in LSECs, the expression of three genes was assessed: the class I glucose transporter (GLUT1) – codifying *Slc2a1*, and the glycolytic enzymes *Eno2*, and *Hk2*. In accordance with the transcriptomic analysis results, the three genes showed increased expression following live *C. albicans* infection (Fig. 4C–F). Additionally, the ECAR was measured as a proxy for glycolytic activity to demonstrate that lactate was being produced in excess from pyruvate rather than the latter being derived to the tricarboxylic acid cycle. After 6 h, significant acidification of the media was detected, indicating lactate accumulation in the extracellular media and, therefore, an increase in aerobic glycolysis occurred in the group exposed to *C. albicans* (Fig. 4D).

Finally, the link between the metabolic changes and the inflammatory response induced by *C. albicans* in LSECs was studied. To do that, the impact of glucose uptake and glycolysis inhibition on IL-6 release was studied using molecular inhibitors for the glycolytic enzymes hexokinase and GLUT1 (Fig. 4E). The inhibition of

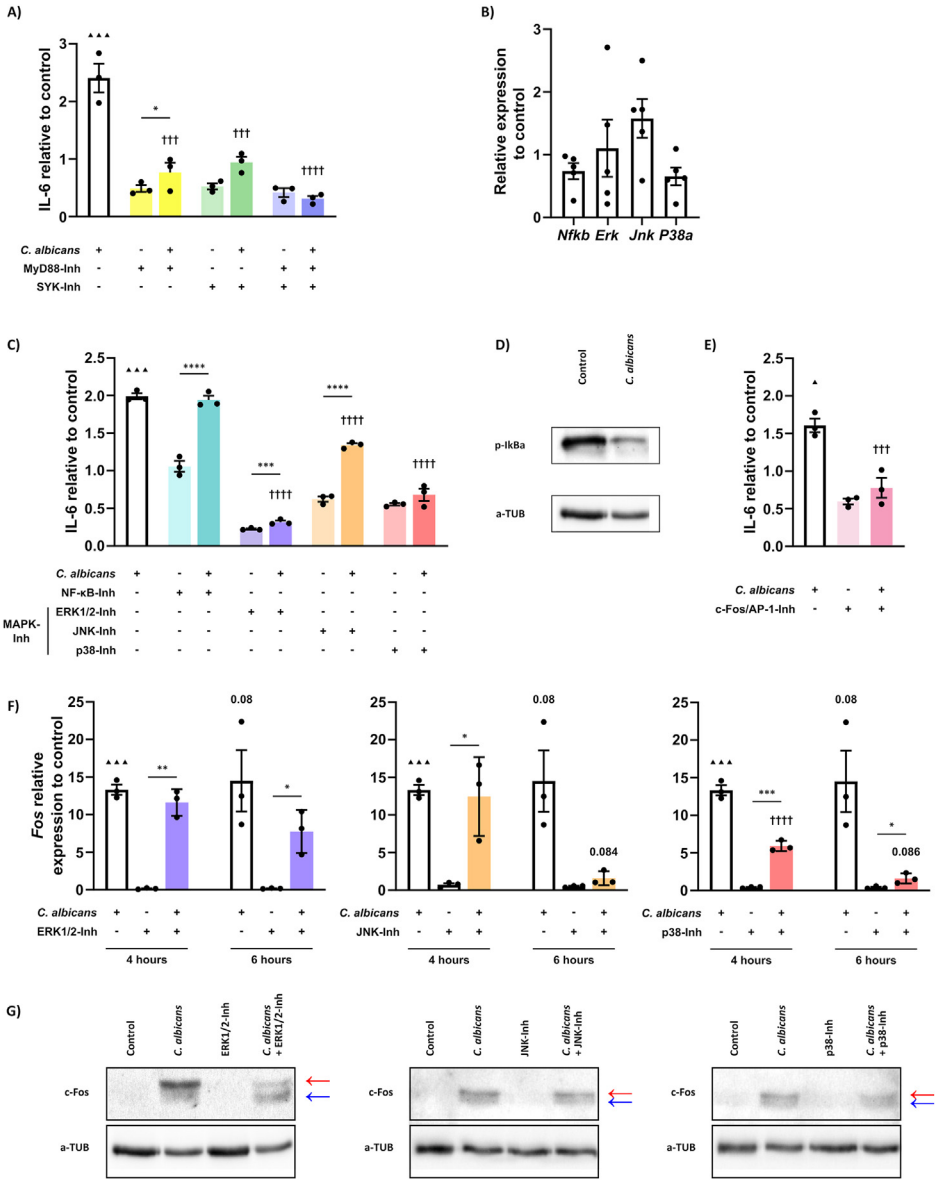

Fig. 3. Live *Candida albicans* recognition and inflammatory pathways induced in immortalized LSECs. A) IL-6 release induced by live *C. albicans* after 6 h in LSECs alone or treated with MyD88 inhibitor (TJ-M2010-5; 30 μM) or SYK inhibitor (Piacetannol; 30 μM). B) RT-qPCR data of the main genes related to inflammatory pathways (*Nfkb*, *Erk*, *Jnk* and *P38a*) after 6 h of infection. C) IL-6 release in controls and after 6 h of live *C. albicans* exposure treated with signalling pathway inhibitors for NF-κB (BAY 11-7082, 2 μM), ERK1/2 (PD-0325901; 1 μM), JNK (SP600125, 10 μM) and p38a (SB 203580, 10 μM). D) WB analysis of IκBα phosphorylation (related with NF-κB activation) after 6 h of exposure. E) IL-6 release in controls and after 6 h of live *C. albicans* exposure treated with c-Fos/AP-1 complex inhibitor (T-5224, 10 μM). F) *Fos* gene and G) c-Fos protein expression in LSECs treated with the three MAPK pathways inhibitors (ERK1/2, JNK and p38a) and infected with *C. albicans* after four and 6 h (gene expression) or only 6 h (protein expression). Individual values and mean ± SEM for each condition are shown. Red arrows and blue arrows represent the upper band and the lower band of c-Fos phosphorylation, respectively. All the experiments were performed in three independent assays. Data were analysed by *t-Student* test: *C. albicans* group compared to controls without *C. albicans* (▲), *C. albicans* group compared to inhibitors with *C. albicans* groups (†) and each inhibitor compared to inhibitor with *C. albicans* (*).

either hexokinase or GLUT1 induced a significant decrease in the pro-inflammatory cytokine IL-6 release in LSECs infected with *C. albicans*. In addition, the role of the three MAPK pathways in *Hk2* expression was also studied (Fig. 4F), and their inhibition reduced the increase in *Hk2* expression when LSECs were infected with *C. albicans*.

4. Discussion

C. albicans remains one of the most commonly isolated infectious agents in health care-associated infections [2]. However, little is known about the response of the specific type of ECs responsible for recognising the pathogen in the liver, the main blood-filtering organ of human beings. To shed light on the mechanisms that underlie this process, an RNA-seq transcriptomic analysis was carried out using primary LSECs, whose analysis indicated that these cells respond to *C. albicans* mainly through the activation of an inflammatory immunological response, including the expression of several PRRs, mainly TLRs (*Tlr2*, *Tlr7*, etc.) and CLRs (*Clec4e*, *Clec7a*, etc.), cytokines (*Il6*, *Tnf*, *Il10*, *Ccl3*, *Ifng*, etc.), chemokines (*Cxcl1*, *Cxcl9*, etc.) and adhesion molecules (*Vcam1*, *Icam1*, *Sele*, etc.).

The role in *C. albicans* recognition of TLRs and CLRs has been widely described in different cell types (primarily epithelial or immune cells), with TLR2, TLR4, Dectin-1 and Mannose Receptor,

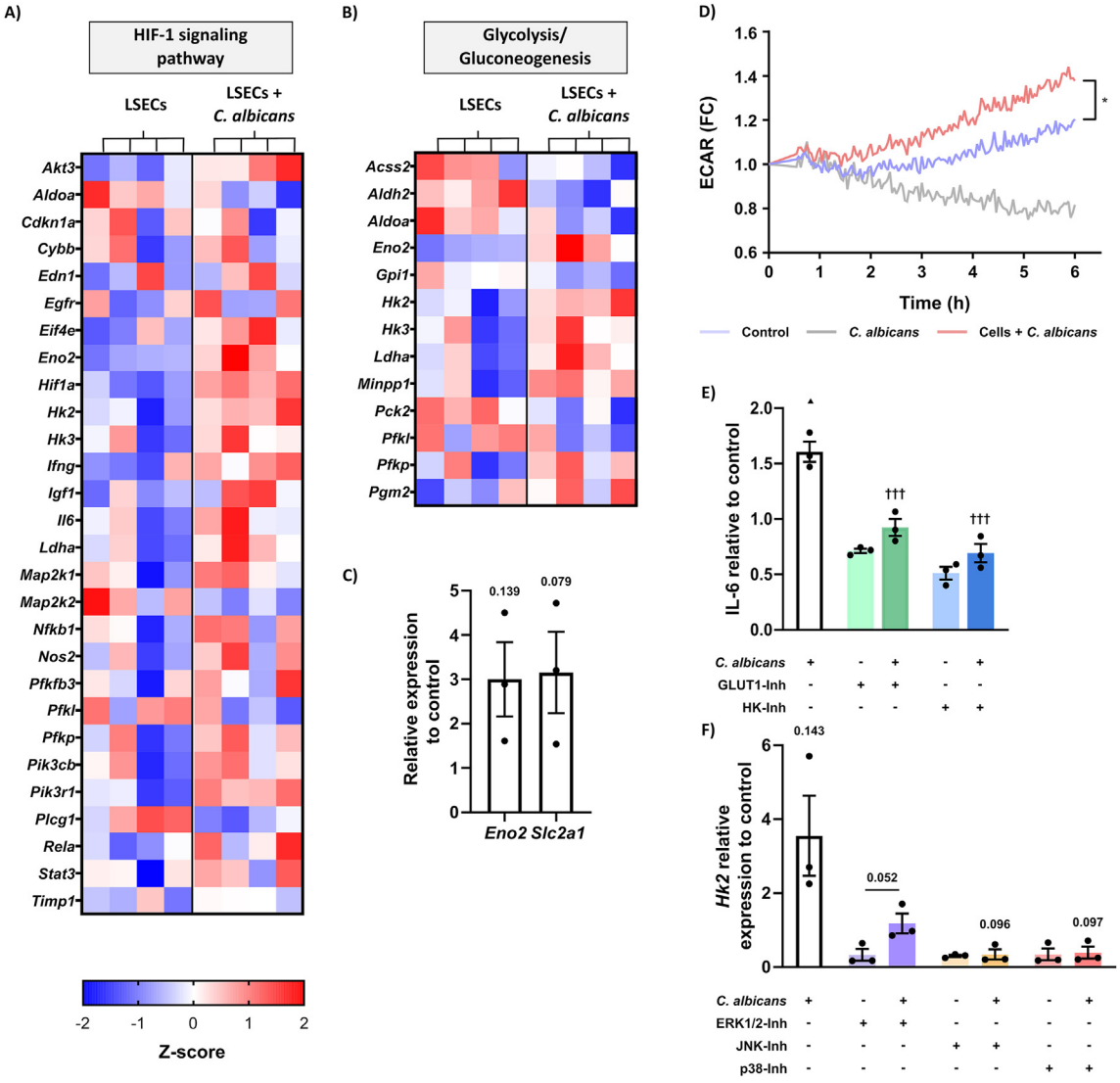

Fig. 4. Live *Candida albicans* promotes metabolic changes in immortalized LSECs. Heat-maps showing the expression levels comparison between samples (Z-score) of over-expressed DEGs ($p_{adj} \leq 0.05$) of A) HIF-1 signalling pathways and B) Glycolysis/Gluconeogenesis. C) Gene expression of aerobic glycolysis related genes (*Slc2a1* and *Eno2*). D) Extracellular acidification rate (ECAR) of LSECs in controls and in contact with live *C. albicans* for 6 h. Data shown as the mean of the fold change of initial fluorescence values in three independent experiments. E) IL-6 release after glucose transporter 1 (GLUT1) (BAY-876; 25 nM) or hexokinase (2-DG; 5 mM) inhibition. F) *Hk2* gene expression in LSECs treated with the three MAPK pathways inhibitors (ERK1/2, JNK and p38a). Individual values and mean ± SEM for each condition are shown. All experiments were performed at least in three independent assays. Data were analysed by *t-Student* test: *C. albicans* group compared to controls without *C. albicans* (▲), *C. albicans* group compared to inhibitors with *C. albicans* groups (†) and each inhibitor compared to inhibitor with *C. albicans* (*).

among others, having particular importance [17]. Concerning ECs, such as HUVECs (Human Umbilical Vein Endothelial Cells), however, the main role of TLR3 has been highlighted [11]. In this regard, our results indicate that both TLRs and CLRs are of great importance for *C. albicans* recognition by LSECs, showing that in different ECs, recognition can occur via different pathways. This result agrees with the fact that LSECs present adhesion molecules and receptors on them that confer unique characteristics and an increased capacity for leukocyte recruitment and microorganism clearance [6]. Moreover, the cytokine and chemokine expression profiles are in line with previous studies performed in other endothelial, epithelial, or immune cells [18–22], where IL-6, TNF, and IFN-γ seem to be of considerable significance. In addition, as shown by gene expression and GO-BPs enrichment analysis, the immunological cascade activated in LSECs promoted the expression of cell adhesion molecules, such as VCAM-1 (*Vcam1*), ICAM-1 (*Icam1*), E-selectin (*Sele*), or P-selectin (*Selplg*). In accordance with our results, studies carried out in HUVECs showed E-selectin expression after *C. albicans* exposure [23].

Given the importance of the inflammatory response to *C. albicans* in our transcriptomic analysis, we analysed the secretion of IL-6, TNF, and IFN-γ by LSECs infected with live yeasts. As expected, *C. albicans* sensing by LSECs induced an increased IL-6 release, but neither TNF nor IFN-γ were detected under any of the conditions studied. This may be due to undetectable quantities produced or even to phenotypical differences between the primary LSECs used for RNA-seq analysis and the LSECs cell line used for the other experiments. Therefore, the measurement of IL-6 release was used from this point on for subsequent studies. To analyse the role of the main PRRs in the production and secretion of IL-6 in response to *Candida*, the inhibition of the principal signal transducers of TLRs and CLRs (MyD88 and SYK, respectively) was performed. The results showed that the inhibition of MyD88 or SYK alone resulted in a significant decrease in IL-6 secretion when LSEC were infected

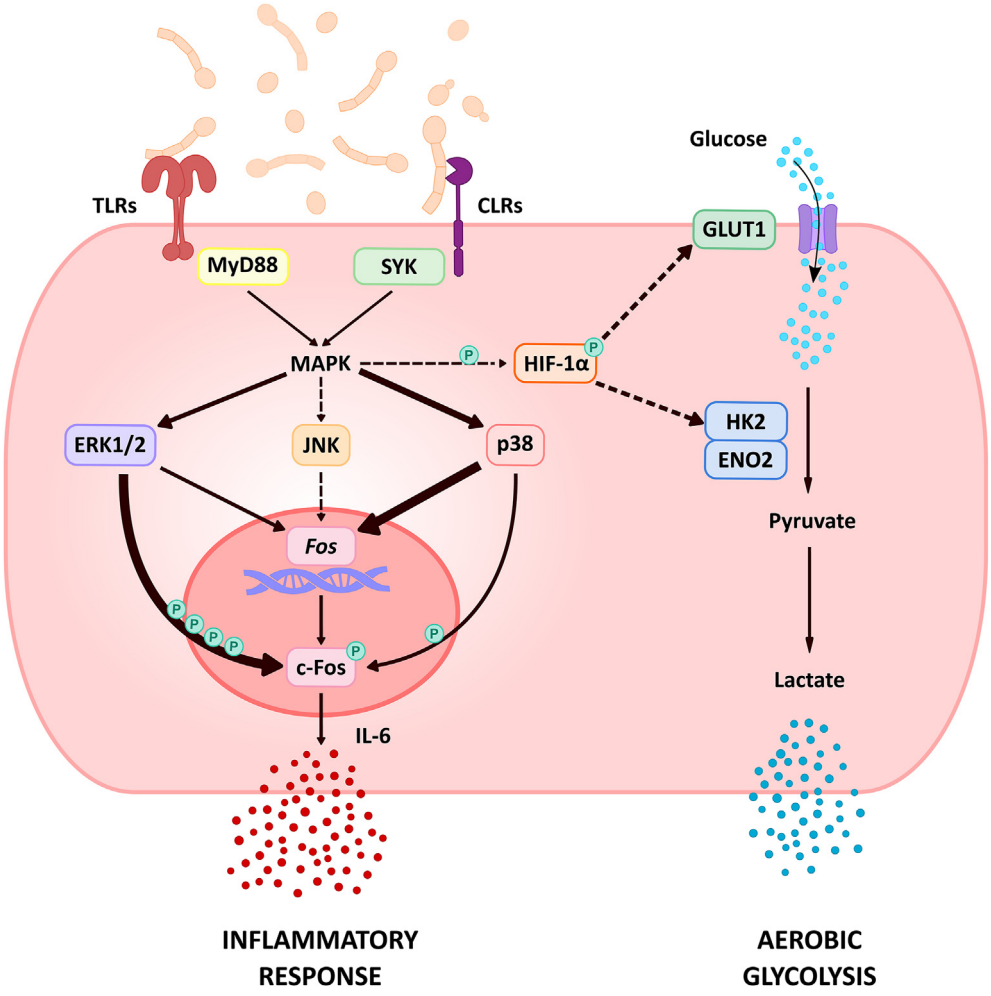

Fig. 5. Graphical representation of the hypothesized LSEC response to *C. albicans*. *C. albicans* is recognized by either TLRs or CLRs present on LSECs surface. Fungal recognition activates the three MAPK pathways (ERK1/2, JNK and p38) leading to an inflammatory response. Activated p38 and ERK1/2 signalling pathways strongly promote *Fos* activation and c-Fos phosphorylation, respectively, eventually inducing IL-6 production. Full arrows (→) represent signalling pathways based on our results and dashed arrows (⇢) hypothesis based on bibliography. Thickness of the arrows represents the strength of pathway activation. Ⓟ indicates phosphorylation.

with the yeast, which was even stronger when both inhibitors were used together. These results suggest that both types of receptors are involved in the response against the fungus, as has been widely described for other cell types [17].

Once the main receptors were identified, we delved into the study of the signalling pathways involved in the release of IL-6. According to previous studies, *C. albicans* induces the activation of several pathways depending on the cell type. Although various hypotheses have been put forward in recent years, it appears that MAPK/p38 and MAPK/ERK1/2 are key in immune responses in oral epithelial cells (OECs) [24,25], while in HUVECs the NF-κB and MAPK/p38 pathways are of great importance [10,26]. In our results, although the expression of *Nfκb*, *Erk*, *Jnk,* and *P38a* genes showed no statistically significant differences between *C. albicans*-exposed and control LSEC cultures, the use of inhibitors for these molecules showed that, except for the NF-κB pathway, the other three pathways, ERK1/2, JNK and p38, have some influence in IL-6 secretion. Of special relevance are ERK1/2 and p38, whose inhibition suppresses IL-6 secretion to baseline levels. The lack of NF-κB pathway activation was confirmed by WB. These results are in line with the hypothesis that the first response of OECs to the yeast-form or low hyphal burdens is mainly NF-κB-mediated, while higher burdens of hyphae make the MAPK-mediated pathways the predominant response [24].

It has previously been shown that c-Fos/AP-1 activation can be mediated by all three MAPK pathways mentioned above [27] and this transcription factor is crucial in different cell types to start an immune response against *C. albicans* [24–26,28]. In common with these previous findings, here we describe that *Fos* expression and its total protein content increased significantly in the presence of *Candida* and that inhibition of c-Fos/AP-1, which specifically inhibits the DNA binding activity of c-Fos/c-Jun, decreased IL-6 secretion induced by the fungus, suggesting its implication in the responses against this yeast. These results agree with previous studies using oral and vaginal epithelial cells, which have already described that c-Fos/AP-1 can be activated via the MAPK/p38 and MAPK/ERK1/2 pathways promoting, among other cytokines, IL-6 release [24,25,28]. However, in our study, the results obtained from gene and protein expression of *Fos* following inhibition of ERK1/2, JNK, and p38 were inconclusive, and we can only hypothesize that the MAPK/p38 and MAPK/JNK pathways might be primarily responsible for Fos expression, with the ERK1/2 pathway possibly having a greater relationship with c-Fos phosphorylation, but further research is required (Fig. 5).

In addition to the inflammatory response, the transcriptomic analysis indicated alterations in metabolic pathways in LSECs in presence of *C. albicans*, mainly in glycolysis/gluconeogenesis and

HIF-1α signalling, the major protein related to hypoxia [29]. It has already been described that this fungus can induce metabolic reprogramming from an oxidative phosphorylation towards aerobic glycolysis in innate immune (e. g., monocytes) and epithelial (e. g., OECs) cells, which allows them to support the increased energy demand due to the activation of immunological responses [22,30–33]. In accordance with these findings, we found increased ECAR activity as a result of lactate production from pyruvate, and increased expression of *Slc2a1*, *Eno2* and *Hk2* in LSECs following live *C. albicans* infection, suggesting a higher glucose consumption and glycolytic activity. Further, our results showed that these metabolic alterations can be due to the action of the three main MAPK pathways, as measured by *Hk2* expression, which might be speculated to be mediated by the HIF-1α signalling pathway. In fact, the above-mentioned MAPK pathways have been widely related with HIF-1 phosphorylation [29] and the activation of glucose transporters and glycolytic enzymes [34].

Finally, we studied the importance of metabolic reprograming to support immune responses in LSECs. The decrease in IL-6 release when hexokinase or GLUT1 were inhibited suggests that the glycolytic pathway is necessary to maintain an inflammatory response. These results are in line with the decreased acidification and release of IL-1β, IL-6, TNF, IFN-γ, and IL-10 observed when hexokinase activity was inhibited in peripheral blood mononuclear cells exposed to *C. albicans* [31]. In the same way, the use of different glycolysis, pentose phosphate pathway, and glucose transporters inhibitors promoted a reduction in c-Fos expression and cytokine production in OECs infected with *C. albicans* [32].

In conclusion, the present study has described for the first time how exposure to *C. albicans* induces both an inflammatory response and a shift in metabolism towards metabolic alterations in hepatic sinusoid endothelial cells. More precisely, we have postulated that the fungus is initially recognised by TLRs and/or CLRs, triggering MAPK/c-Fos pathway activation, particularly through ERK1/2, JNK and p38, promoting an inflammatory environment and metabolic reprogramming to support cellular energy demands.

Declaration of competing interest

The authors have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micinf.2024.105305.

References

[1] World Health Organization. WHO fungal priority pathogens list to guide research, development and public health action. Geneva: Licence: CC BY-NC-SA 3.0 IGO; 2022.
[2] Weiner-Lastinger LM, Abner S, Edwards JR, Kallen AJ, Karlsson M, Magill SS, et al. Antimicrobial-resistant pathogens associated with adult healthcare-associated infections: summary of data reported to the National Healthcare Safety Network, 2015−2017. Infect Control Hosp Epidemiol 2020;41:1−18. https://doi.org/10.1017/ice.2019.296.
[3] Rayens E, Norris KA. Prevalence and healthcare burden of fungal infections in the United States, 2018. Open Forum Infect Dis 2022;9. https://doi.org/10.1093/ofid/ofab593.
[4] Brown GD, Denning DW, Gow NAR, Levitz SM, Netea MG, White TC. Hidden killers: human fungal infections. Sci Transl Med 2012;4:1−10. https://doi.org/10.1126/scitranslmed.3004404.
[5] Lim SJ, Mohamad Ali MS, Sabri S, Muhd Noor ND, Salleh AB, Oslan SN. Opportunistic yeast pathogen *Candida* spp.: secreted and membrane-bound virulence factors. Med Mycol 2021;59:1127−44. https://doi.org/10.1093/mmy/myab053.
[6] Wilkinson AL, Qurashi M, Shetty S. The role of sinusoidal endothelial cells in the axis of inflammation and cancer within the liver. Front Physiol 2020;11:1−25. https://doi.org/10.3389/fphys.2020.00990.
[7] Kubes P, Jenne C, Snyder J. Immune responses in the liver. Annu Rev Immunol 2018;44. https://doi.org/10.1146/annurev-immunol.
[8] Shetty S, Lalor PF, Adams DH. Liver sinusoidal endothelial cells — gatekeepers of hepatic immunity. Nat Rev Gastroenterol Hepatol 2018;15:555−67. https://doi.org/10.1038/s41575-018-0020-y.
[9] Orozco AS, Zhou X, Filler SG. Mechanisms of the proinflammatory response of endothelial cells to *Candida albicans* infection. Infect Immun 2000;68:1134−41. https://doi.org/10.1128/IAI.68.3.1134-1141.2000.
[10] Müller V, Viemann D, Schmidt M, Endres N, Ludwig S, Leverkus M, et al. *Candida albicans* triggers activation of distinct signaling pathways to establish a proinflammatory gene expression program in primary human endothelial cells. J Immunol 2007;179:8435−45. https://doi.org/10.4049/jimmunol.179.12.8435.
[11] Swidergall M. *Candida albicans* at host barrier sites: pattern recognition receptors and beyond. Pathogens 2019;8. https://doi.org/10.3390/pathogens8010040.
[12] Ramirez-Garcia A, Gallot N, Abad A, Mendoza L, Rementeria A, Hernando FL. Molecular fractionation and characterization of a *Candida albicans* fraction that increases tumor cell adhesion to hepatic endothelium. Appl Microbiol Biotechnol 2011;92:133−45. https://doi.org/10.1007/s00253-011-3540-8.
[13] Ramirez-Garcia A, Arteta B, Abad-Diaz-de-Cerio A, Pellon A, Antoran A, Marquez J, et al. *Candida albicans* increases tumor cell adhesion to endothelial cells in vitro: intraspecific differences and importance of the Mannose Receptor. PLoS One 2013;8:1−10. https://doi.org/10.1371/journal.pone.0053584.
[14] Smedsrod B, Pertoft H. Preparation of pure hepatocytes and reticuloendothelial cells in high yield from a single rat liver by means of Percoll centrifugation and selective adherence. J Leukoc Biol 1985;38:213−30. https://doi.org/10.1002/jlb.38.2.213.
[15] Varet H, Brillet-Guéguen L, Coppée JY, Dillies MA. SARTools: a DESeq2- and edgeR-based R pipeline for comprehensive differential analysis of RNA-Seq data. PLoS One 2016;11. https://doi.org/10.1371/journal.pone.0157022.
[16] Robinson MD, McCarthy DJ, Smyth GK, edgeR. A Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 2009;26:139−40. https://doi.org/10.1093/bioinformatics/btp616.
[17] Bojang E, Ghuman H, Kumwenda P, Hall RA. Immune sensing of *Candida albicans*. Journal of Fungi 2021;7. https://doi.org/10.3390/jof7020119.
[18] Swidergall M, Khalaji M, Solis NV, Moyes DL, Drummond RA, Hube B, et al. Candidalysin is required for neutrophil recruitment and virulence during systemic *Candida albicans* Infection. JID (J Infect Dis) 2019;220:1477−88. https://doi.org/10.1093/infdis/jiz322.
[19] Rai MN, Parsania C, Rai R. Mapping the mutual transcriptional responses during *Candida albicans* and human macrophage interactions by dual RNA-sequencing. Microb Pathog 2022:173. https://doi.org/10.1016/j.micpath.2022.105864.
[20] Wang Y, Zou Y, Chen X, Li H, Yin Z, Zhang B, et al. Innate immune responses against the fungal pathogen *Candida auris*. Nat Commun 2022;13. https://doi.org/10.1038/s41467-022-31201-x.
[21] Bruno M, Kersten S, Bain JM, Jaeger M, Rosati D, Kruppa MD, et al. Transcriptional and functional insights into the host immune response against the emerging fungal pathogen *Candida auris*. Nat Microbiol 2020;5:1516−31. https://doi.org/10.1038/s41564-020-0780-3.
[22] Horváth M, Nagy G, Zsindely N, Bodai L, Horváth P, Vágvölgyi C, et al. Oral epithelial cells distinguish between *Candida* species with high or low pathogenic potential through MicroRNA regulation. mSystems 2021;6. https://doi.org/10.1128/msystems.00163-21.
[23] Barker KS, Park H, Phan QT, Xu L, Homayouni R, Rogers PD, et al. Transcriptome profile of the vascular endothelial cell response to *Candida albicans*. JID (J Infect Dis) 2008;198:193−202. https://doi.org/10.1086/589516.
[24] Moyes DL, Runglall M, Murciano C, Shen C, Nayar D, Thavaraj S, et al. A biphasic innate immune MAPK response discriminates between the yeast and hyphal forms of *Candida albicans* in epithelial cells. Cell Host Microbe 2010;8:225−35. https://doi.org/10.1016/j.chom.2010.08.002.
[25] Nikou SA, Zhou C, Griffiths JS, Kotowicz NK, Coleman BM, Green MJ, et al. The *Candida albicans* toxin Candidalysin mediates distinct epithelial inflammatory responses through p38 and EGFR-ERK pathways. Sci Signal 2022;15. https://doi.org/10.1126/SCISIGNAL.ABJ6915.
[26] Yang J, Feng W, Zhang J, Xi Z, Ma Y, Wang Y, et al. *Candida albicans* triggers the expression of inflammatory genes in human umbilical vein endothelial cells. Exp Ther Med 2016;12:1490−4. https://doi.org/10.3892/etm.2016.3464.

[27] Eferl R, Wagner EF. AP-1: a double-edged sword in tumorigenesis. Nat Rev Cancer 2003;3:859–68. https://doi.org/10.1038/nrc1209.
[28] Zhang J, Peng J, Li D, Mei H, Yu Y, Li X, et al. Divergent EGFR/MAPK-mediated immune responses to clinical *Candida* pathogens in vulvovaginal candidiasis. Front Immunol 2022;13. https://doi.org/10.3389/fimmu.2022.894069.
[29] Korbecki J, Simińska D, Gąssowska-Dobrowolska M, Listos J, Gutowska I, Chlubek D, et al. Chronic and cycling hypoxia: drivers of cancer chronic inflammation through Hif-1 and Nf-κb activation. A review of the molecular mechanisms. Int J Mol Sci 2021:22. https://doi.org/10.3390/ijms221910701.
[30] Tucey TM, Verma J, Harrison PF, Snelgrove SL, Lo TL, Scherer AK, et al. Glucose homeostasis is important for immune cell viability during *Candida* challenge and host survival of systemic fungal infection. Cell Metabol 2018;27:988–1006.e7. https://doi.org/10.1016/j.cmet.2018.03.019.
[31] Domínguez-Andrés J, Arts RJW, ter Horst R, Gresnigt MS, Smeekens SP, Ratter JM, et al. Rewiring monocyte glucose metabolism via C-type lectin signaling protects against disseminated candidiasis. PLoS Pathog 2017;13:1–23. https://doi.org/10.1371/journal.ppat.1006632.
[32] Pellon A, Nasab SDS, Bidkhori G, Vaga S, Begum N, Blagojevic M, et al. Fungal infection drives metabolic reprogramming in epithelial cells via aerobic glycolysis and an alternative TCA cycle shunt. ResearchSquare Pre-Print 2022. https://doi.org/10.21203/rs.3.rs-2159406/v1.
[33] Naik S, Mohammed A. Consensus gene network analysis identifies the key similarities and differences in endothelial and epithelial cell dynamics after *Candida albicans* infection. Int J Mol Sci 2023:24. https://doi.org/10.3390/ijms241411748.
[34] Lee SH, Golinska M, Griffiths JR. Hif-1-independent mechanisms regulating metabolic adaptation in hypoxic cancer cells. Cells 2021;10. https://doi.org/10.3390/cells10092371.