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# *Candida albicans* up-regulates the Fas-L expression in liver Natural Killer and Natural Killer T cells.

María Sol Renna, Carlos Mauricio Figueredo, María Cecilia Rodríguez-Galán, Paula Alejandra Icely, Hugo Cejas, Roxana Cano, Silvia Graciela Correa, Claudia Elena Sotomayor*

*Inmunología, Departamento de Bioquímica Clínica, Centro de Investigaciones en Bioquímica Clínica e Inmunología CIBICI-CONICET, Facultad de Ciencias Químicas. Universidad Nacional de Córdoba, Córdoba, Argentina*

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ABSTRACT

After *Candida albicans* arrival to the liver, the local production of proinflammatory cytokines and the expanded intrahepatic lymphocytes (IHL) can be either beneficial or detrimental to the host. Herein we explored the balance between protective inflammatory reaction and liver damage, focusing our study on the contribution of TNF-α and Fas–Fas-L pathways in the hepatocellular apoptosis associated to *C. albicans* infection. A robust tissue reaction and a progressive increase of IL-1β, IL-6 and TNF-α were observed in infected animals. Blocking the biological activity of TNF-α did not modify the number of apoptotic cells observed in *C. albicans* infected animals. Fas-L molecule was up regulated on purified hepatic mononuclear cells and its expression progressed with the infection. In the IHL compartment, the absolute number of Fas-L+ NK and NKT cells increased on days 1 and 3 of the infection. *C. albicans* was also able to up regulate Fas-L expression in normal liver NK and NKT cells after *in vitro* contact. The innate receptor TLR2 was involved in this phenomenon. In the interplay between host factors and evasion strategies exploited by pathogens, the mechanism supported here could represent an additional way that allows this fungus to circumvent protective immune responses in the liver.



## 1. Introduction

*Candida albicans* is a commensal organism in humans; however, in immunocompromised individuals this fungus is an opportunistic pathogen which causes localized invasive mucosal mycosis or life-threatening disseminated and deep-seated organ infections (Kim and Sudbery, 2011; Lilic and Haynes, 2007). *C. albicans* infections are increasing due to the expanding use of antibiotics and immunosuppressive agents for cancer management, organ and bone-marrow transplantation, the treatment of autoimmune diseases or in patients with HIV-AIDS (Kim and Sudbery, 2011; Lilic and Haynes, 2007).

The gastrointestinal mucosa is considered the most common endogenous source for hematogenous dissemination of *C. albicans*, while catheter contamination with fungal biofilms represents an exogenous supply of the fungus (Chavada et al., 2011). The liver can be invaded by *Candida* in different circumstances: in disseminated candidiasis (Kim and Sudbery, 2011; Lilic and Haynes, 2007), following immunosuppressive therapy and organ transplants (Kontoyiannis et al., 2010; Hassan et al., 2014), and during ambulatory peritoneal dialysis (Bartoletti et al., 2014). This organ mounts an efficient inflammatory reaction and limits the growth of the fungus through the activation of resident innate immune cells and the recruitment/expansion of polymorphonuclear leukocytes and innate lymphoid subsets (Bogdanos et al., 2013; Crispe, 2009). However, higher levels of inflammatory mediators and immune cell activation required to control infection can also promote pathophysiologic responses that lead to hepatocyte injury (Szabo et al., 2007).

Tumor necrosis factor receptor 1 (TNFR1) and CD95/Fas, members of the TNFR superfamily, can trigger the apoptotic process after binding their specific ligands. These molecules are expressed on the

*Abbreviations:* DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; FC, flow Cytometry; HMC, hepatic mononuclear cells; IHL, intrahepatic lymphocytes; Ca, *C. albicans* infected animals; N, naive non injected animals; PAMPs, pathogen associated molecular patterns; PGN, peptidoglycan; PRR, pattern recognition receptors; TLR2, toll like receptor 2; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling assay; WB, western blot.

* Corresponding author at: Inmunología, Departamento de Bioquímica Clínica-CIBICI-CONICET, Facultad de Ciencias Químicas,Universidad Nacional deCórdoba.Haya de la Torre y Medina Allende, Ciudad Universitaria, 5000 Córdoba, Argentina.

*E-mail address:* csotomay@fcq.unc.edu.ar (C.E. Sotomayor).

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surface of hepatocytes under physiological and pathological conditions (Jörn et al., 2006; Liedtke and Trautwein, 2012). TNF-α is a pleiotropic cytokine that exhibits two opposite effects in the liver: it can elicit apoptosis of hepatocytes in a number of toxic injury models, but also can prime hepatocytes for subsequent cell division stimulated by growth factors in liver regeneration (Cosgrove et al. 2008; Jörn et al., 2006; Liedtke and Trautwein, 2012). Hepatocytes are also greatly sensitive to Fas-induced death promoted by several intrahepatic lymphocytes (IHL) such as activated T lymphocytes and other innate subsets expressing Fas-L (Crispe, 2009; Sharma et al., 2011).

The composition of the IHL subsets is largely shaped by the income–exit dynamic. During inflammatory processes, the frequency of particular intrahepatic subsets is finely modulated and the selective migration of these populations can condition the disease outcome (Bodagno et al., 2013; Szabo et al., 2007 Szabo et al., 2007). IHL subsets are abundant in NK and NKT cells. NK cells respond both to cytokine activation and engagement of activating over inhibitory receptors (Crispe, 2009). Once activated, they show effector functions such as cytokine synthesis and cytotoxicity that are important in innate responses against pathogens but also may contribute to the pathogenesis of liver diseases (Arrunategui-Correa et al., 2004; Swain, 2008). NKT cells are innate T lymphocytes defined by the co-expression of αβ TCR as well as NK1.1, a marker of NK cells (Bogdanos et al., 2013; Protzer et al., 2012), with multiple functions that may fulfill both beneficial (e.g., clearance of virally infected cells) and harmful (e.g., induction of autoimmunity) roles in the setting of liver disease. One of most striking characteristics of both activated NKT and NK cells is their ability to induce Fas-L mediated apoptosis (Dong et al., 2004; Gao et al., 2009), a pathophysiologic mechanism associated to immune activation in several infections.

Working in a well-characterized model of *C. albicans* infection, we reported important biochemical and immunological changes in the liver microenvironment after the settlement of the fungus (Correa et al., 2004; Renna et al., 2006; Renna et al., 2012; Rodriguez-Galán et al., 2001; Rodríguez-Galán et al., 2003; Rodríguez-Galán et al., 2010a,b). A novel contribution to the pathogenesis of this mycosis reported by our group was the *in situ* hepatocellular apoptosis that could be triggered by the TNFR and/or Fas-Fas-L alternative pathways (Renna et al., 2006). Herein we explored the inflammation-injury balance early upon *C. albicans* arrival to the liver. We provide novel evidence about hepatocellular apoptosis during *C. albicans* infection and demonstrate the modulation of Fas-L expression in intrahepatic NK and NKT cells after the contact with the fungus. Additionally, our findings suggest that fungal PAMPs (pathogen associated molecular patterns) sensed by TLR2 could regulate the expression of Fas-L.

## 2. Materials and methods

### 2.1. Ethics statement

All animal experiments were approved and conducted in accordance with guidelines of the Animal Experimentation Ethics Committee, Faculty of Chemical Science, National University of Córdoba (Permit Number 15-03-51021) in strict accordance with the recommendation of the Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care (OLAW Assurance number A5802-01).

### 2.2. Animals

Outbred female 8–10-week-old Wistar rats (body weight, 100–150 g), were collectively housed in the experimental room for at least 7 days before experiments started. Animals were maintained at the Animal Resource Facility of the CIBICI-CONICET (Centro de Investigaciones en Bioquímica Clínica e Inmunología, CONICET) in accordance with the experimental ethics committee guidelines.

### 2.3. Microorganism and infection

The pathogenic *C. albicans* strain N° 387 was from the stock culture collection of the Mycology Division, Department of Clinical Biochemistry, Faculty of Chemical Science, National University of Cordoba. Yeast cells were grown on Sabouraud glucose agar slant at 28 °C, maintained by weekly subculture and periodically checked for assimilation pattern and virulence (Renna et al., 2006; Renna et al., 2012; Rodriguez-Galán et al., 2001). Regularly, we inoculated intraperitoneally (i.p.) $3 \times 10^8$ viable yeasts in normal rats and after 3 days, liver homogenates were plated on Sabouraud agar to isolate the fungus. For each infection, yeast cells were harvested after 48 hours (h) of culture, centrifuged at 1000 × g, washed twice in sterile PBS, counted, and diluted to the desired concentration. The number of viable cells was checked by triplicate on Sabouraud agar after 48 h of incubation at room temperature (RT).

### 2.4. Experimental design

Rats were assigned to two experimental groups: naive non injected animals (N) or infected i.p. on day (D) 0 with 1 ml inoculum of $3 \times 10^8$ viable *C. albicans* yeasts/ml (Ca). On D1–D3 animals were humanely euthanized by cervical dislocation (Renna et al., 2012; Rodríguez-Galán et al., 2010a,b). Livers were removed, placed on individual Petri dishes, weighed and processed for different studies. Fungal burden was determined by the colony-forming assay on Sabouraud agar after 48 h of incubation at RT. The values were expressed as Log of colony-forming units (CFU) per gram of tissue.

For hepatocellular apoptosis experiments, a group of infected rats was injected 6 h before infection with a single i.p. dose of 200 mg $Kg^{-1}$ in 400 μl of $IgG_2$ anti-TNF-α monoclonal antibody (CENTOCOR Discovery Research, Horsham, PA, USA) (Ca-anti TNF-α group) as described (Rodríguez-Galán et al., 2010a).

For histopathological studies, livers were fixed with 10% formalin–PBS for at least 24 h, dehydrated in alcohol, cleared in xylene and embedded in paraffin. Six-micrometer specimens were sectioned, affixed to glass slides and deparaffinized by soaking them in a xylene bath for 15 min, at RT. The tissue slices were hydrated by transferring them through 100% ethanol (for 15 min), 95 and 75% ethanol (for 10 min each) and PBS twice (for 5 min) and then stained with HE, PAS or processed for apoptosis studies.

### 2.5. Determination of DNA nick-end labeling

Apoptotic cells were quantified in liver sections by the terminal deoxynucleotidyl transferase dUTP nick-end labeling assay (TUNEL fluorescent method), which enzymatically labels free 3′-OH ends of DNA with a fluorescently labeled nucleotide (In Situ Cell Death Detection Kit, Fluorescein, Boehringer Mannheim Co., Germany) (Gavrieli et al., 1992). All steps were performed according to the instructions of the manufacturer. Briefly, paraffin-embedded sections were deparaffinized and rehydrated and then permeabilized by incubation with 40 μg $ml^{-1}$ proteinase K (Immunotech SA, Marseille, France) for 15 min at RT. Sections were incubated with the labeling solution containing terminal deoxynucleotidyl transferase in a humidified chamber at 37 °C for 60 min. Next, the slides were rinsed 3 times with PBS, mounted and examined using an AXIOPLAN fluorescence microscope (Böer et al., 2003). The total number of TUNEL + cells was counted in each specimen. Data were

expressed as the number of TUNEL+ cells per 10,000 hepatocytes (Gavrieli et al., 1992).

### 2.6. RT-PCR analysis in liver homogenates

A standard reverse transcription-PCR assay was used in this study. Briefly, mRNA of livers (D1–D3) was extracted with TRIzol reagent (Invitrogen, Life Technologies) according to the manufacturer's protocol. Reverse transcription reactions were performed using two μg of total RNA in a 25 μl of mixture. Total RNA was first incubated with 0.5 μg of oligo(dT) (Biodynamics, Buenos Aires, Argentine) for 5 min at 70 °C and allowed to stand on ice for 5 min. The sample was then incubated for 1 h at 42 °C with 25 U ribonuclease inhibitor (RNasin Promega, Madison, WI, USA), 1.25 mM deoxynucleoside triphosphates (Invitrogen, Life Technologies, USA), 200 U moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega) in M-MLV 53 reaction buffer (Promega). In a total volume of 25 μl PCR buffer (Invitrogen, Life Technologies, Brazil), 2 μl of cDNA were incubated with 1.25 U of Taq DNA polymerase (Invitrogen, Life Technologies), 1.5 mM $MgCl_2$ (Invitrogen, Life Technologies), 0.2 mM deoxynucleotide triphosphates and 0.2 μM sense and anti-sense primers. The cDNA obtained was subjected to PCR amplification in a thermal cycler (MyCycler Termal Cycles, BioRad) using the following rat primers and PCR protocols described previously: β-actin (Dong et al., 2004), TNF-α, CXCL-8, IL-6, IL-1β, CCL-2, and CCL-4 (Porporatto et al., 2004). The conditions were chosen so that none of the RNAs analyzed reached a plateau at the end of the amplification protocol (in most reactions, 35 cycles of amplification were used, but for β-actin and for IL-6 the number of cycles were 25 and 30 respectively). The PCR products were analyzed by 2% agarose gel electrophoresis in the presence of 0.5 mg $ml^{-1}$ ethidium bromide. To semiquantitate and compare cDNA levels, the gels were photographed, and the intensities of the bands were analyzed using Scion Image software. The relative band intensities in each reaction were normalized to the mean intensity of the β-actin band. Results are expressed as arbitrary units corresponding to the ratio of sample to the β-actin band intensities.

### 2.7. Isolation of hepatic mononuclear cells

Hepatic mononuclear cells (HMC) were isolated from the liver of N and Ca groups on D1–D3 of the treatment. Briefly, livers were perfused with 20 ml of PBS-5% Fetal Calf Serum (FCS), pressed through 200-gauge stainless steel mesh, and resuspended in PBS- 5% FCS. After being washed with PBS once and subjected to centrifugation, cell pellets were resuspended in 40% Percoll (GE Healthcare Life Sciences, Latin America) in complete RPMI 1640 medium. Cell suspensions were gently overlayed onto 80% Percoll, centrifuged for 20 min at 1000 × g and HMC were collected from the interface. Cells were washed, resuspended in erythrocyte lysing solution (150 mM $NH_4Cl$, 1 mM $KHCO_3$, 0.1 mM $Na_2EDTA$, pH 7.3) washed twice in PBS, counted and diluted to the desired concentration (Pyz et al., 2006).

### 2.8. Culture assays

N rats were killed and the livers were aseptically removed. HMC were purified as described above and cultured at a concentration of $1 \times 10^6$ cells/well in RPMI 1640-10% FCS-0.1% gentamicin (supplemented media) at 37 °C-5% $CO_2$ in 24 well-plates. Cells were then incubated alone or with viable *C. albicans* yeasts at different yeast:cell ratio according to the assay: 1:10 for immunocytochemistry, 1:1 for Flow Cytometry (FC), and 10:1 for Western blot (WB). To inhibit fungus overgrowth we used 0.5 μg/ml Amphotericin B in 1:1 and 10:1 yeast:cell ratio cultures. The fungal viability at this concentration of Amphotericin B tested by MTT (3-(4,5-dimethythiazol-2-yl) -2,5-diphenyl tetrazolium bromide; Sigma) assay was not modified. After 12 or 24 h of culture, HMC were harvested, washed once with PBS-10 % FCS and processed. The HMC viability was measured by propidium iodide and FC analysis. At concentration of Amphotericin B employed, the HMC viability was not modified.

In another set of experiments, HMC from N animals ($1 \times 10^6$ cells/well) were cultured alone, with *C. albicans* yeasts (10:1 yeast:cell ratio), with *C. albicans* yeasts plus 10 μg $ml^{-1}$ anti-TLR2 blocking monoclonal antibody (30 min at 37 °C, 5% $CO_2$) (Santa Cruz Biotechnology, CA, USA) or with 10 μg $ml^{-1}$ of the TLR2 agonist peptidoglycan (PGN, Sigma–Aldrich, St Louis, MO, USA). After 12 or 24 h of culture, HMC were harvested and processed for WB or FC, respectively.

### 2.8. Western blot

For WB assays, lysates of HMC (28 μg of protein/lane) were size fractionated in 10% SDS polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Matsui et al., 1997). Membranes were blocked for 60 min in PBS-Tween 20–3% milk and incubated overnight with 1/100 diluted rabbit anti-Fas-L or 1/250 diluted goat anti-TLR2 (Santa Cruz Biotechnology, CA, USA). Membranes were washed with PBS-Tween 20 and incubated with a 1/2000 dilution of peroxidase conjugated mouse anti-rabbit IgG or 1/1000 dilution of peroxidase conjugated rabbit anti-goat, respectively (Sigma–Aldrich) (Matsui et al., 1997). Immunodetection was performed using Western Lightning Chemiluminescence Reagent kit (PerkinElmer Life Sciences, Inc., Boston, USA), according to the protocol provided by the manufacturer. After detection of Fas-L or TLR2 proteins, blots were stripped and analyzed for β-actin using an anti β-actin mouse mAb (1/500 final dilution, Santa Cruz Biotechnology) followed by a peroxidase conjugated goat anti-mouse IgG (1/2000 final dilution, Sigma–Aldrich).

### 2.9. Flow cytometry analysis

In different experiments, $1 \times 10^6$ cells were successively stained with PE anti-CD3 mAb, FITC anti-NK1.1 mAb, biotin-conjugated hamster anti-Fas-L mAb (BD Biosciences; USA), and APC-S avidiniistreptavidin (Sigma-Aldrich). The corresponding isotype controls were from Sigma–Aldrich. All the staining steps were performed at 4 °C in a staining buffer (PBS-EDTA-FCS) for 30 min in the dark (Renna et al, 2012). After incubation, cells were washed, fixed in 2% formaldehyde, and resuspended in PBS-EDTA-FCS. The leukocyte population was gated based on forward and side light-scatter parameters. NKT cells were identified as CD3 positive and NK1.1 positive cells in the leukocyte gate (R1). Data were collected using a FACSCanto II flow cytometer (BD Biosciences) and analyzed using WinMDI software (Matsui et al., 1997; Pyz et al., 2006).

Fas-L expression was analyzed in NK and NKT cells and depicted as the % and the absolute number of Fas-L+ NK and NKT cells per gr of liver.

### 2.10. Immunocytochemistry

To study Fas-L expression, 100 μl of HMC ($1 \times 10^6$ cells/mL) was cytocentrifuged at 500 r.p.m. for 5 min using the Shandon Elliot cytospin. Smears were fixed in methanol for 5 min and treated with blocking buffer (3% BSA in PBS–Tween) for 30 min at RT. Then, samples were incubated with a 1/25 dilution of biotin conjugated hamster anti-Fas-L and PE anti-NK 1.1 mAb (BD Biosciences PharMingen) for 30 min at RT. After washing with PBS, the samples were incubated with a 1/400 dilution of FITC- avidin-streptavidin

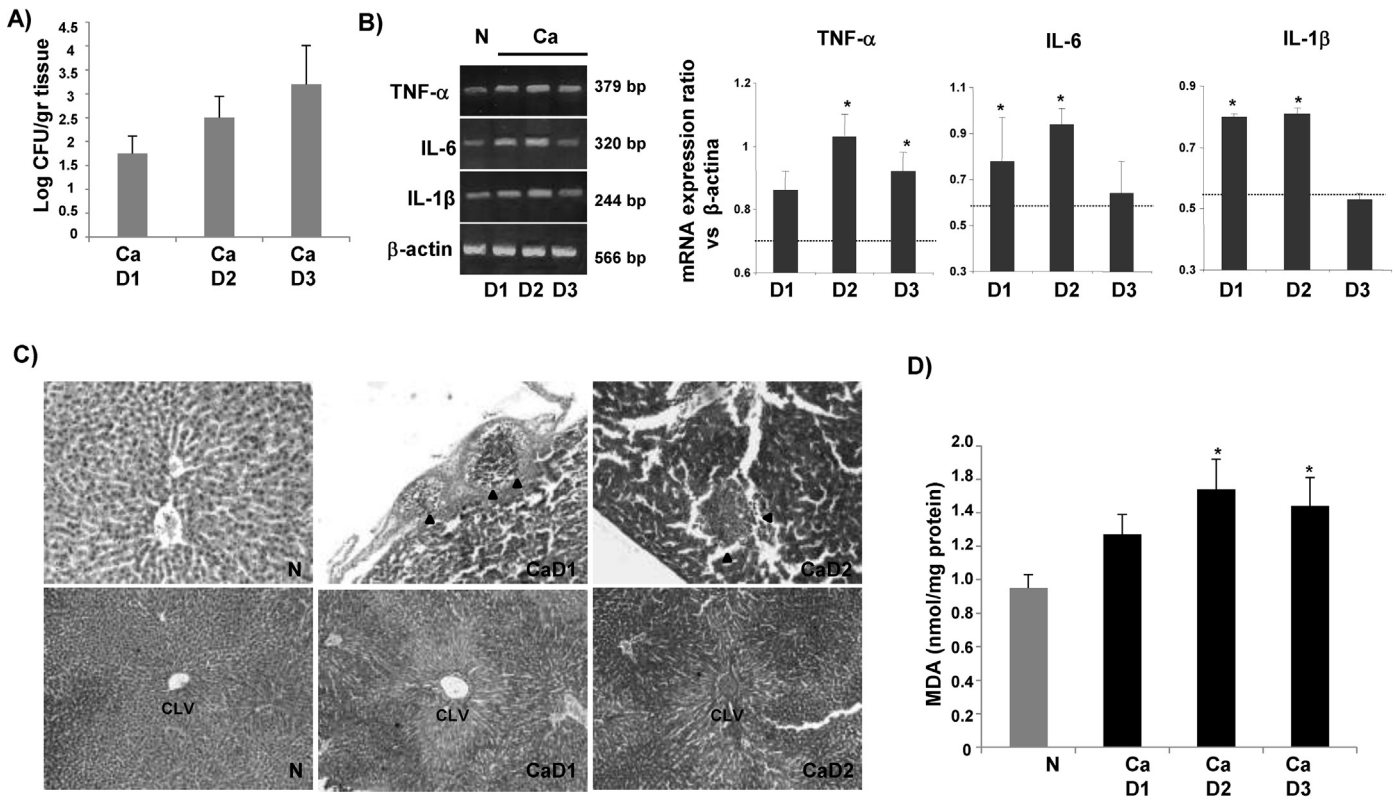


**Fig. 1.** Inflammatory reaction and liver injury during *C. albicans* primary infection.
(A) *C. albicans* liver colonization. Livers from infected animals were removed on D1–D3 of infection. Fungal burden was determined by the colony-forming assay and the values were expressed as log colony-forming units (CFU) per gram of tissue. (B) Transcriptional profile of proinflammatory cytokines. Total RNA was extracted from livers of N and Ca animals on D1–D3 after treatment, and reverse transcription-PCR was performed with the specific primers for TNF-α, IL-6, IL-1β and β-actin. PCR products were electrophoresed on 2% agarose gel and stained with ethidium bromide. A representative kinetic of proinflammatory cytokine mRNAs expression is shown. The intensity of transcripts was normalized with β-actin and expressed as densitometric units. The dotted line corresponds to the constitutive expression. (C) Histopathological findings. Representative microphotograph of H&E and PAS stained sections from N and Ca groups. Upper panels show subcapsular foci of polymorphonuclear leukocytes (CaD1) and parenchymal abscesses (CaD2) present post infection (see arrowheads). Magnification × 10. Lower panels show changes in the hepatic glycogen stores. N animals showed abundant glycogen staining, whereas Ca rats exhibited depletion areas. CLV: central lobulillar vein. Magnification × 10. D) Lipid peroxidation measured as MDA levels, was performed in liver homogenates from N and Ca groups (D1–D3). Data are expressed as means ± SE of two independent experiments (rats per group $N = 5–4$; CaD1 = 6–5; CaD2 = 5–4 and CaD3 = 5–5) (*$p < 0.05$ vs. N).

(Sigma–Aldrich), washed with PBS, mounted and examined using an AXIOPLAN fluorescence microscope.

### 2.11. Statistical analysis

Differences between group means or ratios were assessed using ANOVA followed by Student–Newman–Keuls test for multiple comparisons. A $p$ value <0.05 was considered statistically significant. Data are given as means ± SE.

## 3. Results

### 3.1. Inflammatory reaction and liver damage during C. albicans infection

The proinflammatory cytokines TNF-α, IL-1β and IL-6 are crucial for the organization of tissue response. To assess early signs of liver inflammatory response we analyzed the mRNA levels of TNF-α, IL-1β and IL-6 during the first 3 days of *C. albicans* infection. Fig. 1 shows the liver fungal burden during the time of study (Fig. 1A), the kinetic profile of transcripts and the relative expression referred to β-actin (Fig. 1B). After *C. albicans* arrival to the liver, the expression of IL-6 and IL-1β mRNAs increased on D1 (37% and 45% respectively) and D2 (65% and 50% respectively) returning to constitutive levels on D3 of infection (vs. N p = NS). TNF-α mRNA expression increased on D2 (43%) and D3 (28%) of infection ($p < 0.05$). In order to evaluate the fungal presence and tissue reaction, liver sections were stained with HE and PAS. Fig. 1C (upper panels) shows representative lesions where *C. albicans* can be recognized within the abscesses. Subcapsular foci of polymorphonuclear leukocytes (D1) and parenchymal abscesses (D2) were present post infection. Associated to the inflammatory response we also observed early changes in hepatic glycogen stores as consequence of metabolic imbalance linked to the infection (Rodríguez-Galán et al., 2010a,b). While liver for normal animals showed abundant glycogen staining, infected rats exhibited depleted areas (Fig. 1C lower panels).

In parallel with local inflammatory reaction in response to *C. albicans* arrival, we also detected clear signs of tissue damage during the course of infection. For instance, malondialdehyde, a marker of lipid peroxidation (Wellen and Hotamisligil, 2005), showed increased levels in liver homogenates of infected animals on D2 and D3 of infection ($p < 0.05$) (Fig. 1D). Other markers of liver injury such as serum activities of the enzymes GPT, GOT and gamma-GT have been already reported (Correa et al., 2004; Rodríguez-Galán et al., 2010a,b).

### 3.2. Role of TNF-α in the hepatocelullar apoptosis associated to C. albicans infection

Beyond lipid peroxidation, the increased and sustained levels of TNF-α induced by the fungus, could also be responsible for the generation of more deep tissue damage, as TNF-α is the most relevant

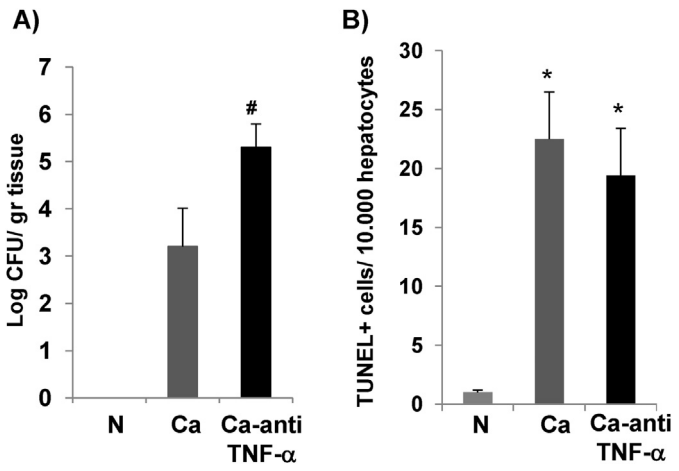


**Fig. 2.** Evaluation of TNF-α contribution in hepatocellular apoptosis associated to *C. albicans* infection.
The assessment of apoptotic cells was performed in liver sections on D3 of experimental design in the following groups: naive non injected animals (N), *C. albicans* infected animals (Ca), and animals injected 6 h before infection with a single i.p dose of 200 mg $Kg^{-1}$ in 400 μl of $IgG_2$ anti-TNF-α monoclonal antibody (Ca- anti TNF-α). A) *C. albicans* liver colonization. Livers from infected groups were removed on day 3 of infection. Fungal burden was determined by the colony-forming assay and the values were expressed as log colony-forming unit (CFU) per gram of tissue. B) Quantification of TUNEL+ in liver sections. Successive counts until 900–1200 cells per sample were made and referred as TUNEL+ cells per 10,000 hepatocytes. $^{*}p < 0.05$ versus N. Data are means ± SE of two independent experiments ($N = 4–4$; Ca = 4–4; Ca-TNF-α = 4–4). # $p < 0.05$, Ca vs. Ca-anti TNF-α.

and well-known inductor of death signals in the liver (Jön et al., 2006; Cosgrove et al. 2008). In order to establish the contribution of this cytokine in the hepatocellular apoptosis observed during *C. albicans* infection (Renna et al., 2006), we used a monoclonal antibody to block any TNF-α released during the 3 days of study, as described previously (Rodríguez-Galán et al., 2010a). After that, we assessed the hepatocellular apoptosis in Ca-anti TNF-α group by TUNEL assay. The effectiveness of the treatment was demonstrated by the significant raise in the number of CFU in the liver ($p < 0.05$) in agreement with the role of TNF-α in the control of fungal growth (Fig. 2A). TUNEL assay showed the presence of apoptotic cells with green fluorescence in both infected groups. The quantification of the phenomenon revealed that the number of TUNEL+ cells in Ca-anti TNF-α group increased significantly compared with N rats ($p < 0.05$) (Fig. 2B), although the level of intrahepatic apoptosis was similar to only infected rats, as previously (Renna et al., 2006). Together, these results suggested a minor contribution of this inflammatory mediator to the intrahepatic apoptosis.

### 3.3. *Assessment of Fas-L expression in intrahepatic immune subpopulations and liver chemokines transcripts after* C. albicans *contact*

Fas/Fas-L interaction contributes to hepatic damage, as established in several animal models of infection (Kuhla et al., 2008; Zhang et al., 2008; Tupin et al., 2007). Among IHL, several subsets can express the Fas-L molecule, as we demonstrated during acute *C. albicans* infection (Renna et al., 2006). We purified total hepatic mononuclear cells (HMC) from N and Ca animals and we analyzed Fas-L expression by WB assays. *C. albicans* infection gradually increased the Fas-L expression on HMC population during the first 3 days of infection (Fig. 3A). Subsequently, we studied the Fas-L surface expression by FC in selected NK (NK1.1 + CD3-) and NKT (NK1.1 + CD3+) subsets from N and *C. albicans* infected animals (Fig. 3B). An initial increase in the frequency of Fas-L+ NKT cells was detected on D1 in Ca group and persisted until D3 post infection. The frequency of Fas-L + NK cells was also increased with the infection (D1), showing a further expansion at D3 (Fig. 3C). The absolute number of Fas-L + NKT cells in Ca animals increased at both times evaluated ($p < 0.05$), while Fas-L + NK cells increased on D1 and after three days duplicated the number (D1 vs. D3 $p < 0.05$) (Fig. 3D).

In agreement with these observations, *C. albicans* triggered the increase of CCL-2, CCL-4 and CXCL-8 mRNA hepatic levels (Fig. 3E), three chemokines involved in the recruitment and mobilization of IHL (Gao et al., 2009) and also relevant for the biology of NK cells (Robertson, 2002). The local transcription of chemoattractants able to recruit and/or enlarge the IHL population suggests the ability of *C. albicans* to induce changes in HMC compartment with apoptotic potential.

To prove that the increment of Fas-L + NKT and NK cells is mediated by *C. albicans,* we co-cultured HMC purified out of N rats with viable *C. albicans* for 12 or 24 h and evaluated Fas-L expression by immunocytochemistry. We found that 12 h after the fungus contact, NK1.1 + cells (include both NK and NKT subsets) up regulated the Fas-L molecule (Fig. 4A, see arrowhead). The expression of Fas-L was also evaluated in each subset by FC analysis; as can be seen, the percentage of Fas-L + NKT and NK cells increased after 24 h of contact with the fungus (Fig. 4B).

### 3.4. *Contribution of TLR2 signaling in the induction of Fas-L*

Using the present model we found that the number of TLR2+ HMC increased during the infection (Renna et al., 2012) and that the in vitro exposure of N-HMC to *C. albicans* increased the number of TLR2+ cells (Renna et al., 2006). On the other hand, previous reports described a relationship between Fas-L and TLR2 expression in mouse models of liver damage associated to gram negative bacterial infection (Hiromatsu et al., 2003; Shimizu et al., 2002). This robust evidence prompted us to explore a possible connection between the fungus, TLR2 and Fas-L in the liver scenario. First, we cultured N-HMC alone (RPMI) or with *C. albicans* in the presence or absence of blocking anti-TLR2 antibody and after 12 h of incubation we examined the Fas-L expression by WB (Fig. 5A left). Fas-L expression increased significantly upon *C. albicans* stimulation and the effect was abolished almost completely upon anti-TLR2 treatment (Fig. 5A right), showing that in the presence of *C. albicans*, signaling through this innate receptor increased the expression of Fas-L. To confirm the involvement of the TLR2 signaling, purified N-HMC were cultured for 24 h with the TLR2 agonist peptidoglycan (PGN) and the Fas-L expression was evaluated by FC (Fig 5B left). The engagement of TLR2 by the agonist PGN triggered a significant increase in the percentage of Fas-L+ HMC ($p < 0.05$) (Fig. 5B right).

## 4. Discussion

Upon arrival to the tissues, pathogens trigger diverse innate effector mechanisms that interfere with their spreading. Although this early response can control the pathogen, the effector immunity can be damaging as a side event associated to the inflammatory response. Previously, we demonstrated liver injury after *C. albicans* infection, including apoptosis of hepatocytes (Renna et al., 2006). It is well known that upon engagement of death receptors by their ligands, Fas-L and TNF-α, intracellular signaling pathways are triggered, but the involvement of these molecules during the local response to the fungus remains to be established. Herein we provide evidence about the simultaneous activation of the inflammatory reaction and effector mechanisms involved in tissue damage. Our results illustrate the ability of *C. albicans* to enlarge NK and NKT innate subsets expressing Fas-L.

The liver immune system consists predominantly of innate elements. This organ produces many innate proteins, contains a large number of macrophages, Kupffer cells (KCs), Dendritic cells (DCs)

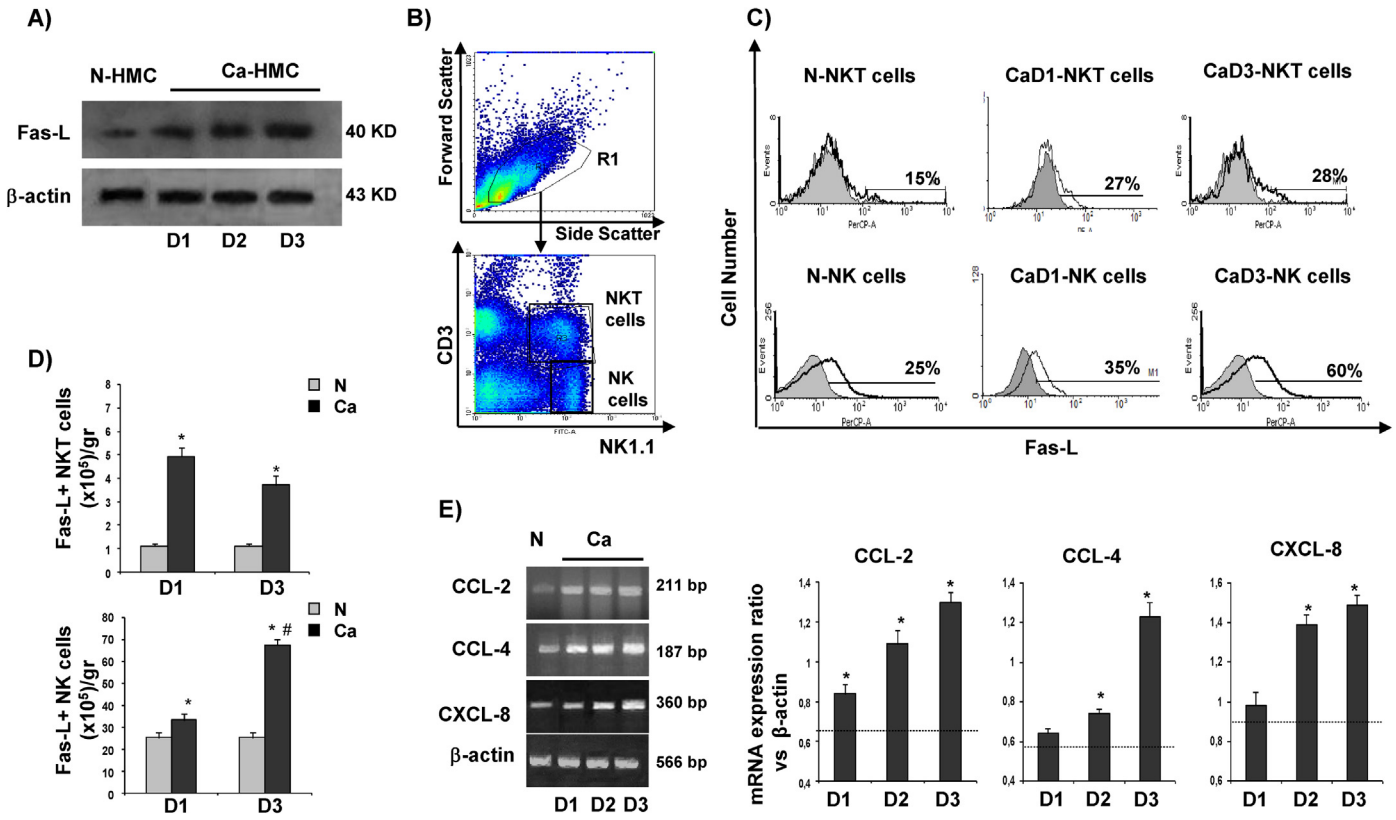


**Fig. 3.** Fas-L expression in HMC, NK, NKT cells and liver chemokine transcriptional profile during C. albicans infection. A) HMC purified from N (N-HMC) and Ca (Ca-HMC) animals (D1–D3) were tested for Fas-L expression by WB assay using anti-Fas-L mAb. The immune reactive protein band is shown. After detection of Fas-L, the membrane was stripped and incubated with anti-β-actin mAb to evaluate equivalent loading of the lanes. B- E) Fas-L expression in NK and NKT subpopulations: N-HMC and Ca-HMC (D1 and D3) were stained with anti-CD3, anti-NK1.1 and anti-Fas-L mAbs and analyzed by FC. B) Representative Forward Scatter vs. Side Scatter density plots showing gate in R1 and CD3 vs. NK density plot showing gate in NK+ cells and in double positive CD3+ NK1.1+ NKT cells for Fas-L expression analysis. C) Representative histograms showing NKT and NK cells stained with isotype control (gray filled) or Fas-L mAb (empty). D) Absolute number of Fas-L+ cells in NKT and NK cell populations after C. albicans infection. Data are expressed as means ± SE of two independent experiments (rats per group $n \geq 4$)(*$p < 0.05$ vs.N; # $p < 0.05$CaD1 vs. Ca D3). E) Total RNA was extracted from livers of N and Ca animals on D1, D2 and D3 after treatment, and reverse transcription-PCR was performed with specific primers for CCL-2, CCL-4, CXCL-8 and β-actin. PCR products were electrophoresed on 2% agarose gel and stained with ethidium bromide. A representative kinetic of chemokine mRNA expression is shown. The intensity of transcripts was normalized with β-actin and expressed as densitometric units. The dotted line corresponds to the constitutive expression. Data are expressed as means ± SE of two independent experiments (rats per group $N = 5–4$; CaD1 = 6–5; CaD2 = 5–4 and CaD3 = 5–5) (*$p < 0.05$ vs. N).

and is selectively enriched in IHL subsets such as NK and NKT cells (Bogdanos et al., 2013; Crispe, 2009; Szabo et al., 2007). Herein, in *C. albicans* infected animals, the transcription of IL-1β, IL-6 and TNF-α genes demonstrated the early activation of innate immune response after pathogen arrival. The progressive increase in the fungal burden was associated to higher levels of mRNA for IL-1β and IL-6 (24-48 h) and an increase in the expression of TNF-α transcript between 48 and 72 h. The mobilization of glycogen stores and the presence of subcapsular and parenchymal abscesses clearly illustrated the changes in the energetic balance (Wellen and Hotamisligil, 2005) and tissue architecture in order to mount an inflammatory reaction (Fig. 1). Previously we demonstrated in this infection model, that KCs and DCs expand significantly and the IHL compartment experiences qualitative and quantitative changes (Renna et al., 2012). The liver CD11b/c+ subpopulation is involved in the activation of NKT cells, and the contact with *C. albicans* further enhances the production of IFN-γ by this subset (Renna et al., 2012), a cytokine involved in the protective mechanisms against the fungus (Lilic and Haynes, 2007). Together these findings demonstrate activation and amplification of innate mechanisms in order to orchestrate an efficient local response against the pathogen.

Early during the infection, TNF-α mediates various physiological processes such as microbicidal activity of macrophages, cell migration and tissue reaction (Crispe, 2009; Szabo et al., 2007). This cytokine also participates in the pathogenesis of acute liver injury triggering apoptosis of hepatocytes *in vivo*, as described in murine models induced by Concanavalin A (Con A), endotoxin/D-galactosamine (Gal/N), alcohol-mediated toxicity and obesity, and some viral infections such as viral hepatitis (VH) C, VHB and Dengue Virus (Sharma et al., 2013; Wang et al., 2013; Nagila et al., 2013). In the present model, when we blocked the biological activity of TNF-α, the fungal burden reached higher levels, in agreement with the well known role of this cytokine in the control of fungal growth (Lilic and Haynes, 2007). Still, in the absence of TNF-α the intrahepatic apoptosis was similar to animals only infected (Fig. 2), suggesting that death signals elicited by this cytokine may not constitute the primary mechanism involved in the hepatocyte apoptosis associated to *C. albicans* infection.

The link between *C. albicans* and apoptosis induction is supported by work performed with phagocytic cells, when the host cell death is triggered following endocytosis of viable fungus (Rostetein et al., 2000), or after *in vitro* exposition of different cells to *C. albicans* virulence factors such as aspartyl proteases (Sap) (Wu et al., 2013) and Lipase (LIP) (Paraje et al., 2008). Beyond the increased fungal burden provoked by TNF-α blocking (about 73%), the number of TUNEL+ cells associated with the infection remained without changes; we believe that the fungus or released virulence factors, are not acting through a direct mechanism to trigger hepatocyte apoptosis. Alternatively, at hepatic level *C. albicans* exhibited the ability to modulate the Fas-L expression in IHL compartment and particularly in NK and NKT subpopulations (Fig. 3 and Fig 4).

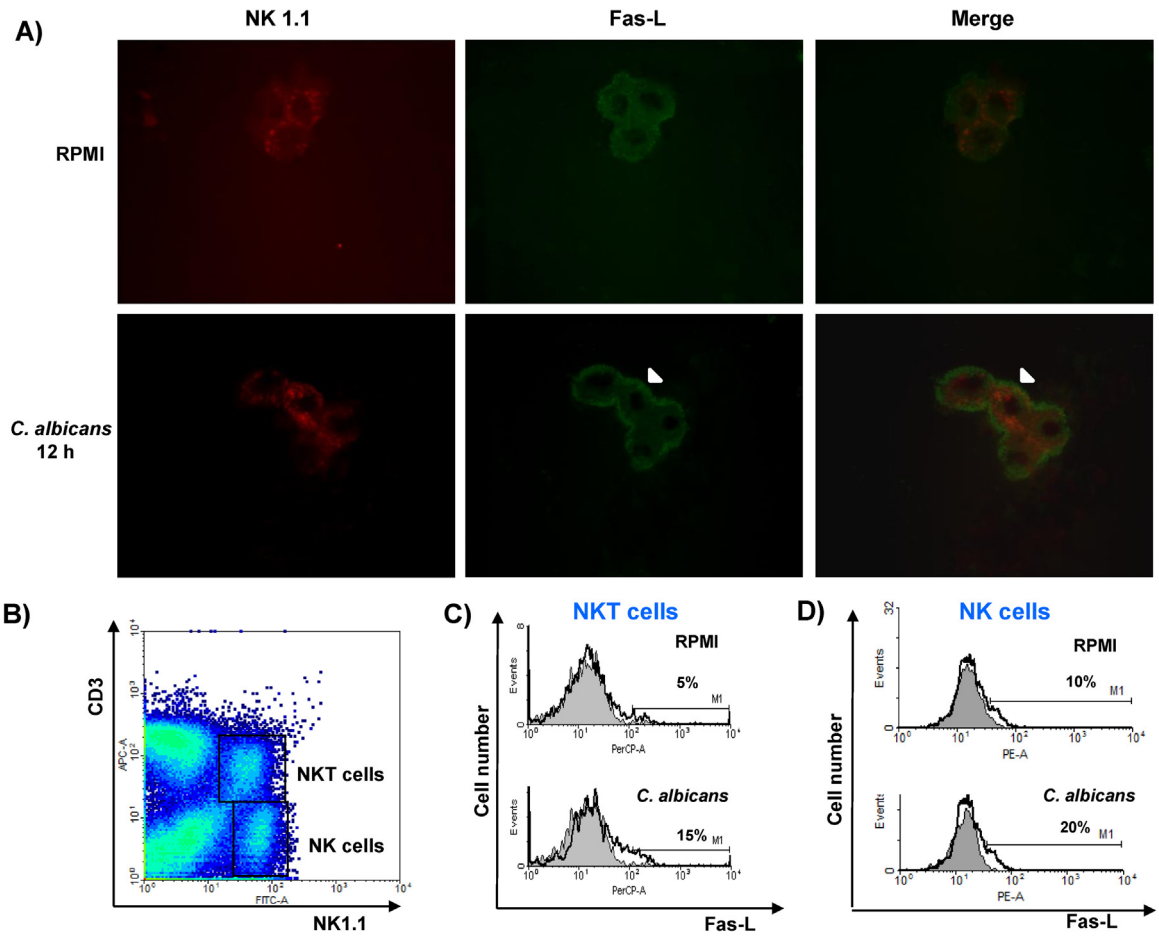


**Fig. 4.** Study of Fas-L expression in NKT and NK cells after *in vitro* contact with *C. albicans*.
HMC purified out of N rats were cultured alone (RPMI) or with viable C. albicans yeasts for 12 h (immunocytochemistry assays) or 24 h (FC assays) at 37 °C and 5% $CO_2$ (see Methods). After incubation, cells were harvested to evaluate Fas-L expression. A) Immunocytochemistry of HMC cytospin smears showing the up-regulation of Fas-L in NK1.1+ cells (NKT and NK cells) after culture (see arrowheads and merge). The slides were stained with PE anti NK1.1 and FITC anti Fas-L mAbs and analyzed using an AXIOPLAN fluorescence microscope. Original magnification × 100. B) Representative CD3 vs. NK density plot showing gate in NK cells and in double positive NKT cells for Fas-L expression analysis. Representative histograms showing up regulation of Fas-L molecule in NKT (C) and NK (D) cells. The histograms are gray filled (isotype control) or empty (Fas-L expression). Experiments were performed by triplicate and repeated three times.

Apoptosis stimulated by Fas-L seems an efficient mechanism to remove transformed hepatocytes in hepatic disorders such as virus infection or malign transformation (Liedtke and Trautwein, 2012; Protzer et al., 2012). Fas-induced apoptosis also occurs in patients with fulminate hepatic failure, in which hepatocyte apoptosis, Fas expression and infiltration with Fas-L expressing cells take place (Wu et al., 2010). Effectors cells in the IHL compartment, such as specific T lymphocytes, NK and NKT subsets express Fas-L. Several studies demonstrate that NK and NKT cells induce Fas-L-dependent apoptosis in the liver (Dong et al., 2004; Hiromatsu et al., 2003; Shimizu et al., 2002; Subleski et al., 2009; Zhang et al., 2008). In animals infected with *C. albicans*, Fas-L expression showed a progressive increment in Ca-HMC in parallel with the enhanced transcription of CCL-2, CCL-4 and CXCL-8 chemokines (Fig. 3). The recruitment and activation of NK and NKT cells in a particular tissue or organ is a critical step during microbe invasion (Robertson, 2002; Tupin et al., 2007). In agreement, already 24 h after infection, we found that the percentage and the absolute number of Fas-L+ NK and NKT cells were considerably enlarged inside the liver and after 72 h of fungus invasion,Fas-L+ NK cell number increased two times compared with uninfected controls. On the other hand, the constitutive levels of Fas in the liver remain without changes during the fungal infection (Renna et al., 2006). These findings demonstrate that early upon fungus arrival, two crucial elements for the promotion of apoptosis meet in the liver, the sustained expression of Fas in hepatocytes and the amplified expression of its corresponding ligand in both NK and NKT cells.

An important observation of the present work is that *C. albicans* up regulated the expression of Fas-L in NK and NKT subsets after *in vitro* contact. In fact, immunocytochemistry studies and FC analysis (Fig. 4) revealed that after 12–24 h of co-culture, the pathogen had the ability, directly or indirectly, to modulate positively a crucial molecule involved in the promotion of the apoptotic phenomenon. Many fungal components recognized by pattern recognition receptors (PRR) can be good candidates to induce death signals. Herein, the expression of Fas-L was notably reduced when the cross-talk between HMC and *C. albicans* was deprived of TLR2 mediated signals (Fig. 5) and a TLR2 agonist up-regulated the expression of Fas-L molecules in N-HMC. In agreement, the relationship between Fas-L and TLR2 expression on a particular subset of IHLs has been reported in liver injury models associated to *Escherichia coli* and *Salmonella* infections (Hiromatsu et al., 2003; Shimizu et al., 2002). In fact, *Salmonella* cell wall components may engage TLR2 on NKT cells, and consequently activate intracellular signals resulting in elevated Fas-L expression on this IHL subpopulation (Shimizu et al., 2002). Interestingly, in this later model the increased number of Fas-L positive IHLs is an important effector mechanism of hepatocyte apoptosis. In the liver, the recognition of microbial molecules through TLRs can trigger inflammatory responses with abundant production of cytokines, chemokines and

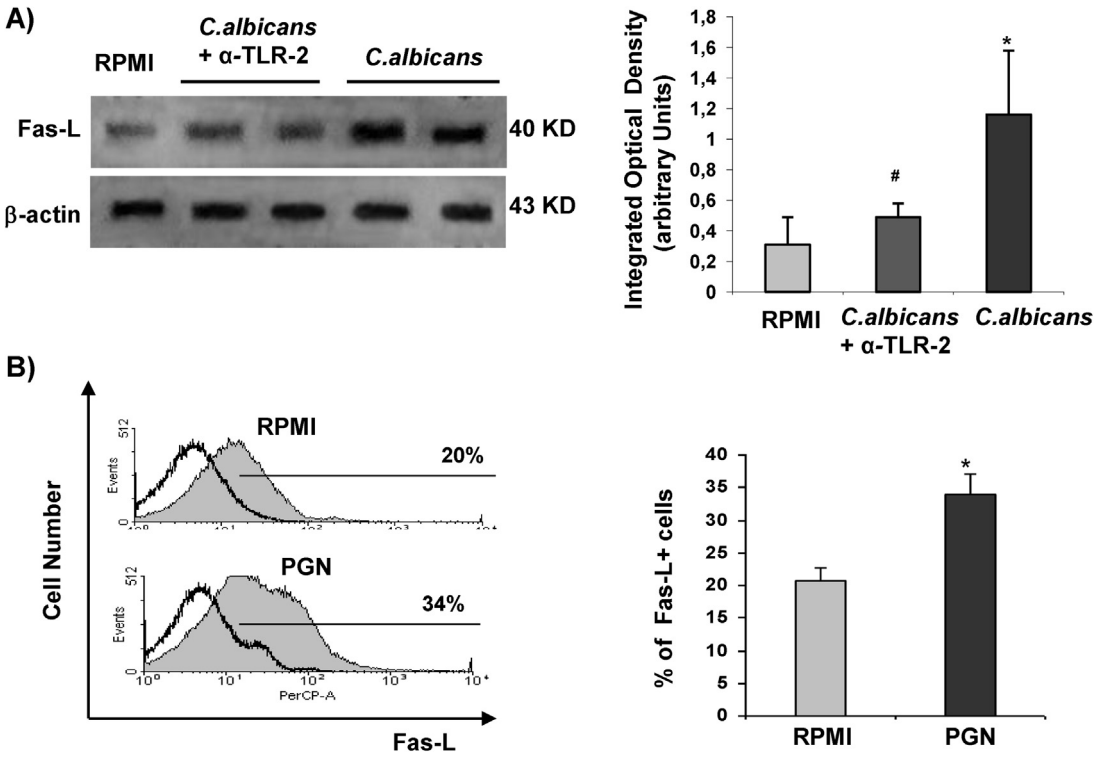


**Fig. 5.** Contribution of TLR2 signaling to the Fas-L expression on HMC.
A) N-HMC were co-cultured alone (RPMI) or with *C. albicans* in the presence or absence of blocking anti-TLR2 mAb. After 12 h of culture, HMC were collected and Fas-L expression was analyzed by WB assay. The immune reactive protein band is shown. After detection of Fas-L, the membrane was stripped and incubated with anti-β-actin mAb to ascertain equivalent loading of the lanes. The relative amount of Fas-L protein is shown. B) HMC purified out of N rats were co-cultured 24 h alone (RPMI) or with the TLR2 agonist PGN, and Fas-L expression was analyzed by FC. Representative histograms showing the frequency of Fas-L+ HMC and the percentage of Fas-L + cells for each experimental condition are shown. The histograms for the isotype control are empty, and for Fas-L expression are gray filled. Data are expressed as means ± SE from three independent experiments where each sample was processed by triplicate (*$p < 0.05$ vs. RPMI; # $p < 0.05$ vs. *C. albicans*).

adhesion molecules and can also participate in liver injury contributing to the pathogenesis of a variety of diseases (Bigorgne and Crispe, 2010). Thus, the activation of particular TLR responses by microorganisms is beneficial to the host but in certain situations might serve as escape mechanism from the defense machinery (Gavrieli et al., 1992; Lilic and Haynes, 2007; Tupin et al., 2007). In the present model signals triggered by TLR2 contributed to mount a robust and protective response against the fungus but also participated in the hepatocyte death mechanism. The proposed TLR2 activation and Fas-L induction on IHLs after *C. albicans* contact could represent a competent strategy of fungal escape that skews the subtle host–pathogen balance promoting the tissue damage and favoring the infection.

From an integrative view, events orchestrated at hepatic level during *C. albicans* infection reveal simultaneous activation of IHL to control infection and expansion of Fas-L+ NK and NKT cells as active players in the hepatocyte apoptosis associated to this mycosis. In the interplay between host factors and evasion strategies of pathogens, the discussed mechanism could represent an additional way exploited by the fungus to circumvent protective immune responses in the liver.

## Disclosures

The authors have no conflict of interest to disclose.

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