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Hepatocellular apoptosis during *Candida albicans* colonization: involvement of TNF-α and infiltrating Fas-L positive lymphocytes

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Abstract

The liver constitutes the first barrier in the control of hematogenous dissemination of *Candida albicans* of intestinal origin. In rats infected with *C. albicans*, this organ limits the growth of the yeast and mounts an efficient inflammatory reaction. However, in rats infected and exposed to chronic varied stress, the hepatic inflammatory reaction is compromised and the outcoming of the infection is more severe. Although in both groups the fungal burden is associated with hepatotoxicity, steatosis, increment of hepatic enzymes and lipid peroxidation, stress-related differences are clearly evident. Herein, we evaluated in infected and infected-stressed hosts the involvement of apoptosis and pro-apoptotic signals in the hepatic injury during the acute step of *C. albicans* infection. We studied *in situ* apoptosis by 4′,6-diamidino-2-phenylindole dihydrochloride and terminal deoxynucleotidyl transferase dUTP nick-end labeling reactions, the levels of local tumor necrosis factor (TNF)-α mRNA by reverse transcription–PCR and the Fas/Fas-L expression by immunohistochemistry and western blot. We also purified intrahepatic lymphocytes (IHLs) to evaluate the dynamic of recruitment following the infection and to characterize the *in vivo* and *in vitro* interaction of *C. albicans* with this subset evaluating the kinetic of Fas-L and Toll-like receptor-2 (TLR-2) expression. This work shows, for the first time, the occurrence of *in situ* apoptosis of hepatocytes as well as the kinetic of IHL recruitment early during the *C. albicans* infection. Moreover, our results demonstrate the ability of the fungus to up-regulate the Fas-L and TLR-2 expression in this subset. In the scenario of early liver injury, the recruited IHLs and the modulated expression of TNF-α, Fas-L and TLR-2 molecules could act coordinately in delivering death signals.

Introduction

Candida albicans is an opportunistic fungal pathogen commonly found in the human gastrointestinal tract (1). It is a unique parasite capable of colonizing, infecting, persisting and stimulating immune responses at mucosal level. Both antibody- and cell-mediated immune responses to *Candida* antigens are evoked in healthy individuals although normally this saprophyte fungus does not cause disease in immunocompetent hosts. It is in the setting of congenital, induced, or disease-related immune dysfunctions that *C. albicans* can cause cutaneous, mucocutaneous and life-threatening systemic diseases (1, 2). A proper interaction between the innate and the adaptive immunity is required for the efficient control of *C. albicans* (2, 3). The regulation of the early fungal burden, the cytokine production, and the expression of co-stimulatory molecules are possible pathways through which the innate immune system may condition the development of the specific response against *C. albicans* (3, 4).

The gastrointestinal mucosa is probably the most common portal of entry of *C. albicans* for hematogenous dissemination. At early stages of the infection, the liver constitutes the first barrier for the control of the fungal spreading (1, 4). The ability of this organ to limit the growth of the yeast and to mount an efficient inflammatory reaction is crucial in determining the outcome of the fungal infection (1, 4). Inflammatory mediators such as proteolytic enzymes, pro-inflammatory cytokines, nitric oxide and oxidative products are released with local and

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systemic effects (5, 6). Considering that liver function depends on the integrated activity of hepatic and non-hepatic resident or immigrating cells, the injury of this organ could result in a disintegrated function (4, 6). In several models of liver damage, the active and passive participation of hepatocytes, the functional status and the modulation of the hepatic activity are considered markers of the different susceptibility to the injury (7, 8).

Tumor necrosis factor receptor 1 (TNFR1) and CD95/Fas/APO-1, members of the TNFR superfamily, trigger the apoptotic process after binding their specific ligands. These molecules are expressed on the surface of hepatocytes under physiological and certain pathological conditions (9). TNF-α is a pleiotropic cytokine that exhibits two opposite effects in the liver depending on the general setting: it can elicit hepatocyte apoptosis in a number of toxic injury models (10–12) but also can prime hepatocytes for subsequent growth factor-stimulated cell division in liver regeneration (11, 13). Hepatocytes are also exquisitely sensitive to Fas-induced death following *in vivo* administration of anti-Fas antibody, where a rapid sequence of pathological changes culminates with massive apoptosis (14). Furthermore, the intrahepatic lymphocyte (IHL) population that includes activated T lymphocytes and many non-T innate immune cells expresses Fas-L (15). The composition of the IHL pool is largely shaped by the income–exit dynamic. During hepatic inflammatory processes, the frequency of particular intrahepatic subsets is finely modulated and the selective migration of these populations can condition the disease outcoming (16).

Several models of alcoholic liver disease (6, 17) as well as parasitic, bacterial and virus infections have been associated with hepatocyte apoptosis (15, 18–21). However, little information is available on the ability of *C. albicans* to induce liver apoptosis. We developed an experimental model of candidiasis to study the contribution of innate effector cells in the pathogenesis of this fungal infection (4, 22–24). Normal immunocompetent hosts or animals exposed to chronic varied stress (CVS) showed marked differences in fungal colonization and inflammatory response in the liver after 3 days of infection. Alterations included lipid accumulation in the cytoplasm of hepatocytes (steatosis), lipid peroxidation and mobilization of hepatic enzymes (4, 22). Interestingly, these hepatic changes were exacerbated by stress mediators (4, 22). Herein, we evaluated the contribution of the apoptotic phenomenon to the liver injury observed in our model of *C. albicans* infection as well as the possible mediators involved in this event. With this purpose, we studied the frequency of liver apoptosis, the TNF-α mRNA levels and the Fas/Fas-L expression. We also evaluated the kinetic of the IHL recruitment in response to *C. albicans* infection, and the ability of this microorganism to modulate *in vivo* and *in vitro* the Fas-L expression in this subset. Moreover, we assessed the induction of the innate receptor molecule, Toll-like receptor-2 (TLR-2), in IHLs stimulated with the fungus.

Methods

Animals

Outbred female Wistar rats (body weight, 100–150 g) were collectively housed in the experimental room for at least 7 days before experiments started. Rats were maintained at 22°C under a 12-h light–dark cycle with continuous access to food and water except when food was removed from the stressed groups as part of the stress procedure (4, 22–24).

Microorganism and infection

The pathogenic *C. albicans* strain no. 387 was from the stock culture collection of the Mycology Division, Department of Clinical Biochemistry, Faculty of Chemical Science, National University of Córdoba. Yeast cells were grown on Sabouraud glucose agar slant at 28°C, maintained by weekly subculture and periodically checked for assimilation pattern and virulence (2, 22–24). Regularly, we inoculated intraperitoneally (i.p.) 3×10^8 viable yeasts in normal rats, and after 3 days, spleen, kidney or liver homogenates were plated on Sabouraud agar to isolate the fungus. For each infection, yeast cells were harvested after 48 h of culture, centrifuged at $1000 \times g$, washed twice in sterile PBS–0.1% gentamicin, 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).

Stress procedure

Animals were exposed to different stressors between 14 and 16 h except for food deprivation, which lasted 24 h. In our model, rats are infected and exposed to a stress paradigm that involves a different stressor each day during 3 days (4, 22–24): day 0, swim (4°C for 5 min); day 1, restraint (for 2 h); and day 2, food deprivation (for 24 h). The Animal Experimentation Ethics Committee, Faculty of Chemical Science, National University of Córdoba approved the protocols.

Experimental design

Rats were assigned to four groups: normal uninfected and unstressed (N), stressed (S), *C. albicans* infected (Ca), and infected and stressed (CaS). Rats were infected i.p. with a 1 ml inoculum (3×10^8 yeasts ml^{-1}) on day 0 and stressed immediately after the infection and during the next 2 days. On days 1, 2 and 3, animals were killed by decapitation and livers were removed, placed on individual Petri dishes, weighed and processed for different studies (4, 22–24).

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 hematoxylin and eosin (H&E) for light microscopic examination, or processed for different studies.

Determination of nuclear apoptotic morphology and DNA nick-end labeling

For apoptosis studies, morphological evaluation of cells was performed by staining with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Molecular Probes, Eugene, OR, USA) as described (25). Briefly, liver sections were stained

with 300 nM DAPI for 5 min in subdued lighting, rinsed three times with PBS and mounted. After that, nuclear morphology was determined using an AXIOPLAN fluorescence microscope with digital camera (dxm 1200, Nikon, Japan). Cells whose nuclei exhibited light blue bright staining and condensed chromatin were designated as apoptotic. For each sample, nuclear staining and morphology were documented photographically and qualitatively evaluated (12).

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) (26). All steps were performed according to the instructions of the manufacturer. Briefly, paraffin-embedded sections were deparaffinized and rehydrated, as described previously, 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 (19). The total number of TUNEL-positive cells was counted in each specimen. Data were expressed as the number of TUNEL-positive cells per 10 000 hepatocytes.

Assessment of Fas and Fas-L in liver sections

For immunohistochemistry, liver sections were treated with blocking buffer (3% BSA in PBS–Tween) for 30 min at RT. The samples were incubated with a 1/25 dilution of biotin-conjugated hamster anti-Fas-L mAb (BD Biosciences PharMingen, San Diego, USA) or with a 1/20 dilution of mouse IgG1 anti-Fas for 30 min at RT. After washing with PBS, the samples were incubated with *Streptomyces avidinii* streptavidin–FITC (Sigma–Aldrich, St Louis, MO, USA) diluted 1/400 or with PE–goat anti-mouse (Santa Cruz Biotechnology, CA, USA) diluted 1/200, for further 30 min. After washing with PBS, the samples were mounted and examined using an AXIOPLAN fluorescence microscope (27). The total number of Fas-L-positive cells per high-power field (×40) was counted in each specimen. Data were expressed as the number of Fas-positive cells per 10 000 hepatocytes.

Reverse transcription–PCR analysis of TNF-α mRNA

Messenger RNA of livers (days 1, 2 and 3) was extracted with TRIzol reagent (Invitrogen, Life Technologies). Two micrograms of total RNA was 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 5× reaction buffer (Promega) in a final volume of 25 μl. 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 (28). Each sample was incubated in a thermal cycler (PTC-100 thermal cycler; M. J. Research) using 1 cycle at 94°C for 5 min followed by 25 cycles for β-actin and 35 cycles for TNF-α. Each cycle consisted of 30 s at 94°C, 1 min at 55°C and 1 min at 72°C. To measure PCR products semiquantitatively, 2 μl of cDNA product was serially diluted 2-fold, and amplified by using 20, 25, 30 and 35 cycles for TNF-α and 20, 22, 24 and 26 cycles for β-actin genes under the same conditions described above. The linear range of amplification for each primer pair was established in independent preliminary studies. The PCR products were analyzed by 2% agarose gel electrophoresis in the presence of 0.5 mg ml^{-1} ethidium bromide. Bands were analyzed with the Scion Image program and expressed as relative densitometric units (28). Results were depicted as the ratio of mRNA levels for TNF-α relative to β-actin mRNA levels.

Isolation and culture of IHLs

IHLs were isolated from the liver of different experimental groups on days 1, 2 and 3 of the treatment as described (18, 29, 30). Briefly, the whole liver was pressed through 200-gauge stainless steel mesh, and suspended in PBS–5% FCS–100 U ml^{-1} of heparin. After washing once with PBS, the cell pellet was resuspended in 50 ml of PBS–5% FCS–100 U ml^{-1} of heparin. Lymphocytes were isolated from parenchymal hepatocytes, hepatocyte nuclei and Kupffer cells by a gradient centrifugation method using Percoll–100 U ml^{-1} heparin (25 min at 2000 r.p.m.). The pellet was resuspended in erythrocyte lysing solution (155 mM NH_4Cl, pH 7.3) and washed two times in PBS. Cells were counted and diluted to the desired concentration (18, 29, 30). The composition of the purified IHLs was assessed by cytocentrifuged slides stained with May-Grundwald Giemsa and flow cytometry (29). In our hands, an increased proportion of lymphoid cells (87%) was obtained in the purified fraction, in agreement with previous reports (18, 29, 30); in our system, CD3, NK and NKT cells represented 70% of the purified IHLs.

IHL cultures were performed as described previously (30). Briefly, 1×10^7 IHLs per well were cultured in RPMI 1640–10% FCS–100 U ml^{-1} heparin–0.1% gentamicin at 37°C and 5% CO_2 in 6-well plates with viable *C. albicans* yeasts at different yeast:cell ratio according to the assay: 10:1 for western blot analysis and 1:10 000 for immunocytochemistry and flow cytometry. After 6, 12, 18 h of culture, IHLs were harvested, washed once with PBS–10% FCS and processed.

Fas-L expression after co-cultures of C. albicans and IHLs

For immunocytochemistry, 100 μl of IHLs (1×10^6 cells ml^{-1}) 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 mAb (BD Biosciences PharMingen) for 30 min at RT. After washing with PBS, the samples were incubated with a 1/400 dilution of *S. avidinii* streptavidin–FITC (Sigma–Aldrich), washed with PBS, mounted and examined using an AXIOPLAN fluorescence microscope.

Western blot

For western blot assays, lysates of liver homogenates (80 μg of protein per lane) or IHLs (28 μg of protein per lane) were size fractionated in 10% SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked for 60 min in PBS–Tween 20–3% milk and incubated overnight with a 1/200 diluted rabbit anti-Fas antibody or 1/100 diluted rabbit anti-Fas-L or 1/250 diluted goat anti-TLR-2 (Santa Cruz Biotechnology). 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) (4). 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 protein, blots were stripped and analyzed for α-tubulin using a monoclonal anti-α-tubulin mouse ascites fluid (1/500 final dilution, Sigma–Aldrich) followed by a peroxidase-conjugated goat anti-mouse IgG (1/2000 final dilution, Sigma–Aldrich).

Flow cytometry analysis

In different experiments, 1.5×10^6 cells were successively stained with biotin-conjugated hamster anti-Fas-L mAb (BD Biosciences PharMingen) and *S. avidinii* streptavidin–FITC (Sigma–Aldrich) or goat anti-TLR-2 (Santa Cruz Biotechnology) and anti-goat IgG–FITC (Sigma–Aldrich). All the staining steps were performed at 4°C in PBS–EDTA–FCS (24). After incubation, cells were washed, fixed in 2% formaldehyde, resuspended and analyzed using a Cytoron Absolute flow cytometer (Ortho Diagnostic System, Raritan, NJ, USA). Isotype controls (Sigma–Aldrich) were run with each sample and matched for fluorochrome. On the basis of forward and side light scatter, lymphocytes were gated in the region 1 (R1). Fluorescence intensity was depicted on a three-decade logarithmic scale and single-parameter analysis as histograms.

Statistical analysis

Differences between group means were assessed using analysis of variance followed by Student–Newman–Keuls test for multiple comparisons. A P value <0.05 was considered statistically significant.

Results

Occurrence of hepatocellular apoptosis during C. albicans infection

Early during the systemic infection with *C. albicans*, rats develop a marked liver injury that is exacerbated by stress mediators (4, 22). To characterize the factors involved in this phenomenon, we explored the occurrence of hepatocellular apoptosis in infected (Ca group) and infected-stressed (CaS group) rats during the first 3 days of the infection. Apoptotic cells were initially visualized by H&E and morphological changes were confirmed after staining with the fluorescent dye DAPI (Fig. 1, panel A). The microscopic analysis of liver sections of Ca and CaS animals revealed remarkable differences in the staining frequency and nucleus morphology compared with uninfected controls (N and S). Changes in nuclear morphology evolved from incipient in the first day of infection, to clearly progressive in the second day. The strongest alterations were observed on day 3 of the kinetic.

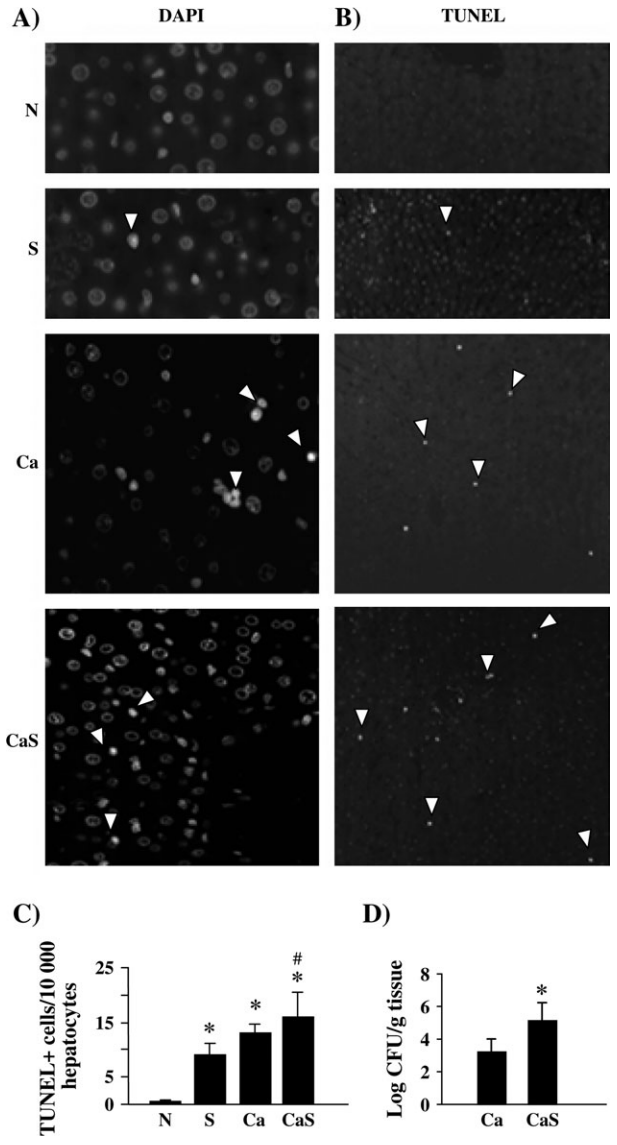

Fig. 1. *In situ* intrahepatic apoptosis after *Candida albicans* infection. The assessment of apoptotic cells was performed in liver sections of animals from all groups (N, S, Ca and CaS) on day 3 of the treatment. (A) For the morphological study, sections were treated with DAPI and evaluated using an AXIOPLAN fluorescence microscope with digital camera (dxm 1200, Nikon). Cells whose nuclei exhibited light blue bright staining and condensed chromatin were considered apoptotic (see arrowheads). Original magnification ×40. (B) The detection of DNA fragmentation was performed using *in situ* TUNEL fluorescent method. Sections were analyzed with an AXIOPLAN fluorescence microscope. TUNEL-positive cells are fluorescent green (see arrowheads). Original magnification ×25. (C) Quantification of TUNEL positivity in liver sections. Successive counts until 900–1200 cells per sample were made and referred as TUNEL-positive cells per 10 000 hepatocytes. *P <0.05, versus N; #P < 0.05, Ca versus CaS. (D) *Candida 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. Data are the mean ± SD ($n \geqslant 8$). *P < 0.05, Ca versus CaS.

Hepatocytes with both bright blue fluorescent chromatin condensation, and more advanced chromatin fragmentation were frequently visualized throughout the parenchyma in the liver of infected animals (Ca and CaS). To quantify this phenomenon, sections were processed for TUNEL assay, where apoptotic cells are evidenced by the specific incorporation of FITC labeled dUTP into DNA strand breaks. As shown in Fig. 1 (panels B and C), the number of TUNEL-positive cells in animals exposed to *C. albicans* infection was significantly increased compared with N animals ($P < 0.05$). The use of this complementary and highly specific technique allowed us to confirm and to demonstrate the occurrence of *in situ* apoptosis early during *C. albicans* spreading. Stress *per se* also triggered the apoptotic death of many hepatocytes (versus N, $P < 0.05$). Remarkably, the highest level of hepatocellular apoptosis was observed in animals exposed to both stress and infection stimuli (Ca versus CaS, $P < 0.05$) that also exhibited the greatest fungal burden (Fig. 1, panel D) ($P < 0.05$). Previously, we demonstrated that CaS rats show a more pronounced liver damage associated with the predominance of the invasive hyphal forms (22). Taken together, these findings suggest that the hepatocyte apoptosis is most prominent in more severe forms of the infection.

Hepatic TNF-α mRNA levels

TNF-α is a pro-inflammatory cytokine released early during the infection that mediates various physiological processes such as cell activation, migration and tissue damage (10, 11). This cytokine also plays a central or additive role in the pathogenesis of acute liver injury triggering apoptosis of hepatocytes *in vivo* (10). Taken into account that the mannoprotein and other components of the *C. albicans* wall induce the production of TNF-α (31), we evaluated the kinetic of expression of TNF-α mRNA in our model. Total RNA was isolated from all experimental groups on days 1, 2 and 3 and the level of TNF-α transcripts was assessed by reverse transcription–PCR (Fig. 2). As can be seen, the transcription of TNF-α gene increased on day 1, peaked significantly on day 2 (versus N, $P < 0.05$) and remained sustained (Ca group) or was abolished (CaS group) on day 3 of the infection. The single exposure to neuroendocrine products released during the CVS also modulated this parameter (S group) (22). Possibly, stress mediators together with the infection down-regulated the TNF-α mRNA expression after 3 days of treatment in CaS group (Ca versus CaS, $P < 0.05$).

Expression of Fas and Fas-L after 3 days of C. albicans infection

Fas-L induces apoptosis in target cells through Fas receptor, a surface glycoprotein that is constitutively expressed on several cells including hepatocytes (6, 32). Using immunohistochemistry and western blot, we demonstrated that the expression of Fas protein remained invariant in liver after 3 days of treatment (Fig. 3, panels A and B), suggesting that neither the fungal infection nor the stress modulated the expression of this molecule involved in death signaling.

By immunohistochemistry, we found that while hepatocytes were negative for Fas-L expression, this molecule was detected on cells infiltrating the liver parenchyma (Fig. 3, panel C). Whereas a reduced number of Fas-L-positive cells was observed in N animals, these cells increased significantly in Ca and CaS groups (Fig. 3, panel D) ($P < 0.05$). These results suggest that Fas-L-positive infiltrating cells could be involved in the apoptotic mechanism triggered during the infection.

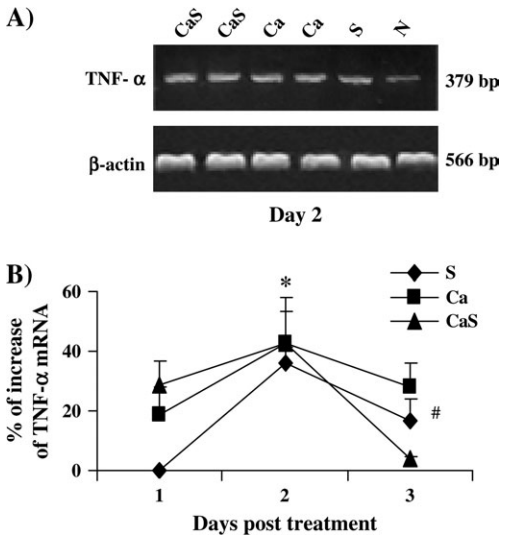

Fig. 2. Kinetic of hepatic TNF-α mRNA expression during *Candida albicans* infection. Total RNA was extracted from livers of different groups (N, S, Ca and CaS) on days 1, 2 and 3 after treatment, and reverse transcription–PCR was performed with the specific primers for TNF-α and β-actin. PCR products were electrophoresed on 2% agarose gel and stained with ethidium bromide. (A) A representative experiment of TNF-α mRNA expression on day 2, including at least two rats per group is shown. (B) The intensity of transcripts was normalized with β-actin and depicted as percentage of increase relative to normal expression. Data corresponding to the kinetic studies are expressed as mean ± SD ($n = 4$–6 per group). *$P < 0.05$, versus N; #$P < 0.05$, Ca versus CaS.

Recruitment of IHLs and assessment of Fas-L expression in vivo and in vitro

In many infections when the liver is the main target where the pathogen is distributed, an increased influx of circulating leukocytes into the hepatic sinus occurs (33). This early recruitment is crucial to circumscribe the primary infection, and also represents the origin of the granuloma formation (16). To evaluate the dynamic of the recruitment of IHLs, we purified IHLs using Percoll gradients on days 1–3 of the treatment (29). Based on the light scatter properties of the purified population, we defined the R1 for further analysis. The absolute number and the percentage (data not shown) of cells in R1 increased significantly on days 1 and 2 after *C. albicans* infection ($P < 0.05$) (Fig. 4, panel A). On day 3, groups showed mainly a diminished number of cells in R1.

Considering that the Fas–Fas-L interaction contributes to hepatic damage in various animal models of infection (34, 35), we analyzed the kinetic of expression of Fas-L in IHLs gated on R1. As a consequence of the infection, Ca and CaS groups increased the percentage of Fas-L-positive cells compared with the N group (data not shown). Moreover, as

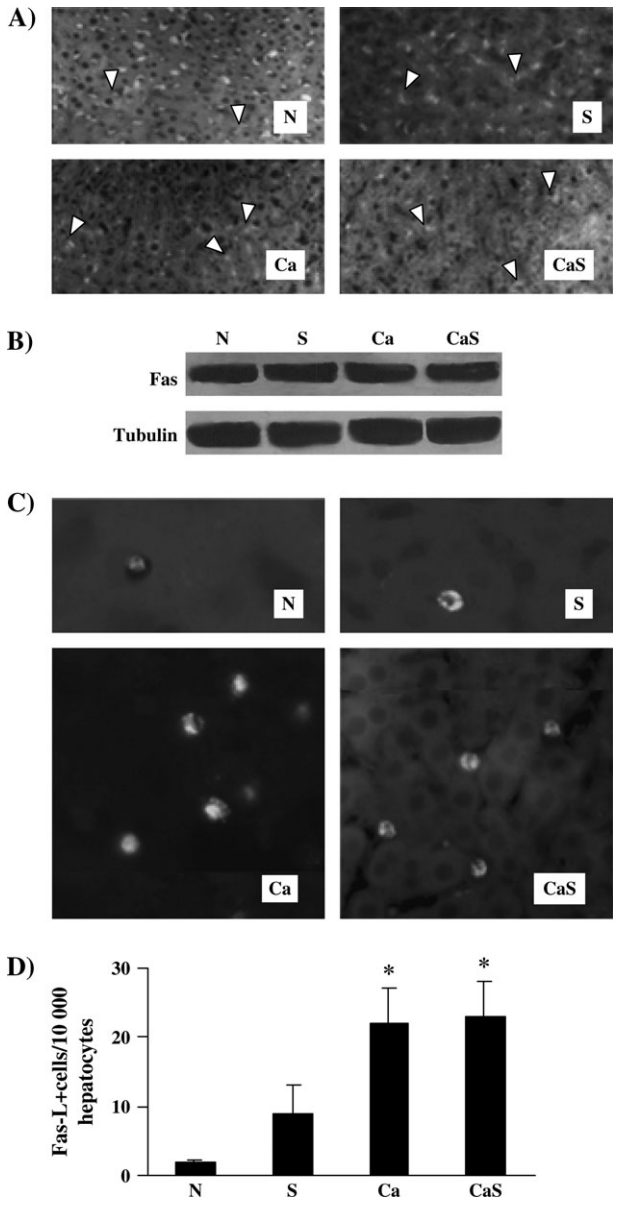

Fig. 3. Intrahepatic expression of Fas and Fas-L after *Candida albicans* infection. The expression of Fas and Fas-L was evaluated on day 3 in rats exposed to different treatments (N, S, Ca and CaS). (A) Fas expression was assessed by immunohistochemistry in liver sections stained with anti-Fas mAb and analyzed using an AXIOPLAN fluorescence microscope. Original magnification ×40. (B) Western blot assay was performed in liver homogenates from different groups using anti-Fas mAb. The immune reactive protein band is shown. After detection of Fas, the membrane was stripped and incubated with anti-tubulin mAb to ascertain equivalent loading of the lanes. (C) Immunohistochemistry for Fas-L was performed in liver sections of all experimental groups. Microphotographs show infiltrating Fas-L-positive cells. Original magnification ×60. (D) Quantification of Fas-L-positive cells in liver sections. Successive counts until 900–1200 cells per sample were made and referred as Fas-L-positive cells per 10 000 hepatocytes. Results are expressed as mean ± SD ($n = 4$–6 per group). *$P < 0.05$, versus N.

shown in Fig. 4B, the absolute number of purified IHLs bearing this molecule involved in death signals increased with the infection (versus N, $P < 0.05$).

Together, by immunohistochemical (Fig. 3, panel C) and flow cytometry (Fig. 4, panel B) studies, we demonstrated that the infection with *C. albicans* occurs together with the increment of Fas-L-positive IHLs *in vivo*. To confirm these findings, we purified IHLs out of normal rats and co-cultured these cells with viable *C. albicans* in different ratios for 6, 12 and 18 h. Following the stimulus *in vitro*, IHLs up-regulated the Fas-L molecule already after 6 h, with a considerable expression after 12 h culture (Fig. 4, panel C; see arrowheads); after 18 h of incubation, the overgrowth of the fungus made difficult this assay. Besides, the western blot assay confirmed the finding with the major expression detected after 18 h of culture.

Induction of TLR-2 expression in IHLs after C. albicans contact

TLRs respond to a wide variety of microorganisms, including fungi, bacteria and viruses. TLRs as well as the TLR adaptor molecule MyD88 have been implicated in the recognition of the molecules located in the cell wall or cell surface of fungal pathogens such as *C. albicans*, *Aspergillus fumigatus*, *Cryptococcus neoformas* and *Pneumocystis carinii* (36). *C. albicans* interacts efficiently with TLR-2 in phagocytes leading to activation of anti-fungal effector functions (2). On the other hand, signaling via TLR-2 on NKT cells up-regulates the surface expression of Fas-L during bacterial infections (15, 19). To determine whether a similar phenomenon operates after *C. albicans* exposition, we analyzed by flow cytometry and western blot the kinetic of expression of TLR-2 in co-cultures of IHLs purified from normal donors and *C. albicans* (Fig. 5, panels A and B). The percentage of IHLs expressing this innate receptor increased approximately three times after 12 h of incubation with a low fungus:cell ratio ($P < 0.05$). Cytometry studies at longer times were technically inconvenient due to the overgrowth of the fungus. For western blot analysis with 10:1 yeast:cell ratio, we observed a kinetic up-regulation of the TLR-2 expression that started already after 6 h of culture (Fig. 5B). To confirm our *in vitro* findings, IHLs were purified from Ca group 3 days after the infection and TLR-2 protein expression was analyzed. As can be seen, the infection up-regulated the TLR-2 expression in the IHL population (Fig. 5, panel C).

Discussion

During the systemic spreading of the opportunistic fungus *C. albicans*, the liver is crucial to regulate the early inflammatory reaction (37). The pathophysiological mechanisms and molecular signals triggered in the liver by the fungus have not been elucidated yet. This work directly demonstrates, for the first time, the occurrence of hepatocyte apoptosis associated to the infection with this opportunistic fungus. Our results also illustrate the timing of IHL recruitment during the early steps of *C. albicans* infection as well as the ability of the fungus to up-regulate the Fas-L expression in this subset. Interestingly, the *in vitro* and *in vivo* contact with the fungus also increases the TLR-2 expression on this population. These findings support the notion that IHLs are involved in the mechanism of liver damage.

Herein, we provide morphological and molecular evidence showing that the apoptosis of host tissue cells occurs during

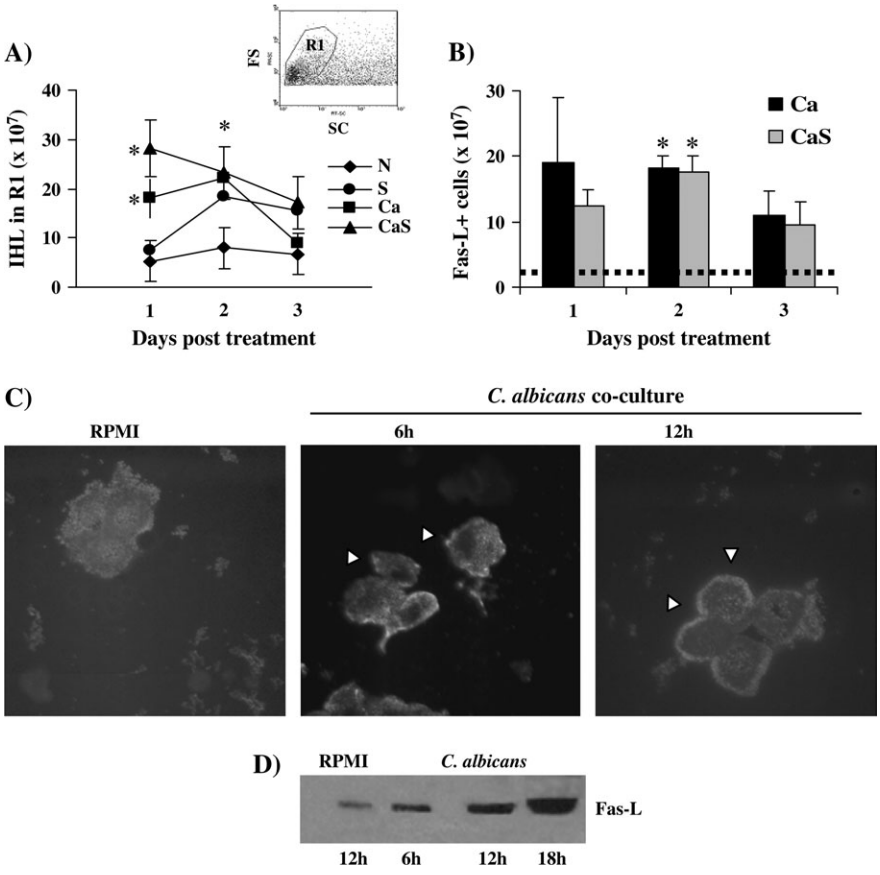

Fig. 4. Recruitment and Fas-L expression on IHLs. IHLs were purified on days 1, 2 and 3 after treatment from different groups by Percoll gradients. The cells were stained with anti-Fas-L mAb and analyzed by flow cytometry. (A) Absolute number of IHLs gated on R1 is shown. Data are expressed as mean ± SD ($n = 4$–6 per group). (B) Kinetic study showing the absolute number of Fas-L-positive cells in IHL population after *Candida albicans* infection. The dotted line represents the mean of Fas-L-positive cells in R1 from the N group. Data are expressed as mean ± SD ($n = 4$–6 per group). *$P < 0.05$, versus N. For the *in vitro* assay, IHLs purified out of normal rats were co-cultured with viable *C. albicans* yeasts for 6, 12 or 18 h at 37°C and 5% CO_2 (see Methods). After incubation, cells were harvested to evaluate the expression of Fas-L. (C) Immunocytochemistry of IHL cytospin smears showing the up-regulation of Fas-L after 6 and 12 h after co-culture (see arrowheads). The slides were stained with anti-Fas-L mAb and analyzed using an AXIOPLAN fluorescence microscope. Original magnification ×100. (D) Kinetic of expression of Fas-L after 6–18 h co-culture with *C. albicans* evaluated by western blot. IHL lysates were resolved on 12.5% polyacrylamide slab gels, transferred to nitrocellulose, and probed with a 1/100 dilution of the anti-Fas-L mAb. The immune reactive protein band is shown.

C. albicans infection. Morphological nuclear changes were observed early in Ca and CaS groups, and alterations were intensified by day 3. With the TUNEL assay, we were able to visualize and quantify a significant number of hepatocytes undergoing DNA fragmentation, as a marker of the *in vivo* process. This finding, in view of the fast mechanisms involved in the homeostatic removal of apoptotic cells, is biologically relevant in the pathogenesis of this infection. In agreement with our previous results showing liver injury (4, 22–24), animals only stressed also exhibited a low number of apoptotic cells, possibly reflecting the liver damage under the activation of the hypothalamo–pituitary–adrenal axis (22). In animals exposed to infection and stress (CaS), the apoptotic phenomenon was higher possibly reflecting an additive effect. In fact, the activation of hepatic sympathetic nerves as well as circulating cathecolamines exacerbate the liver damage by hepatic cell sensitization (38, 39). Interestingly, a major fungal burden was also detected in this group where the immune system was perturbed by stress exposure. That stress has strong suppressive effects on the immune system has been extensively documented. In this particular host–eukaryotic pathogen interaction, the stress-released products could compromise the immune status of host (4, 22–24), favoring the spreading of the pathogen. The major frequency of apoptotic hepatocytes detected in infected stress-immunosuppressed hosts (CaS group) confirms and strengthens the association between fungal susceptibility and liver damage.

What is the biological meaning of the apoptosis observed in the liver during the course of this opportunistic fungal infection? Apoptosis is a finely regulated mechanism of cell death that takes place in several physiological processes but also in many infections favoring pathogen invasion (9, 40). Host cell apoptosis can be induced through direct and indirect mechanisms depending on microbial attributes and the particular host–pathogen interaction (40). At hepatic level, the most relevant and well-known inductors of death signals are TNF-α (9, 10) and the Fas/Fas-L system (9, 14). TNF-α emerges as a key factor in liver disease, not only in the state of fatty liver but also in the transition to more advanced

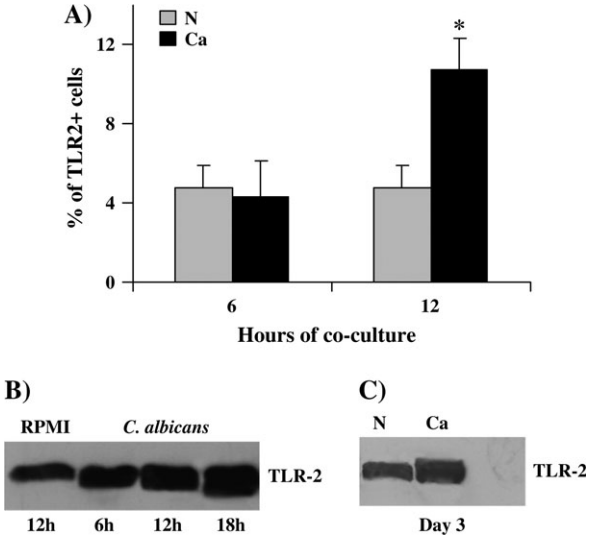

Fig. 5. Induction of TLR-2 expression in IHLs after *Candida albicans* contact. For the *in vitro* assay, IHLs purified out of normal rats were co-cultured with viable *C. albicans* yeasts for 6, 12 or 18 h at 37°C and 5% CO_2 (see Methods). After incubation, cells were harvested to evaluate the expression of TLR-2. (A) Percentage of TLR-2-positive cells gated on R1 assessed by flow cytometry. Data are means ± SD of three similar cultures. $*P < 0.05$, versus medium. (B) Kinetic of expression of TLR-2 evaluated by western blot. IHL lysates were resolved on 10% polyacrylamide slab gels, transferred to nitrocellulose and probed with a 1/250 dilution of the anti-TLR-2 antibody. The immune reactive protein band is shown. For the *in vivo* assay, IHLs were purified from N and Ca rats on day 3 after infection and evaluated for TLR-2 expression. (C) Up-regulation of TLR-2 protein evaluated by western blot. IHL lysates from N and Ca rats were resolved as described above. Data are representative of three separate experiments.

damage. Its early local production triggers the release of other cytokines, and together they promote cell recruitment, hepatocyte death or the healing response (10, 11). In our experimental model, serum levels (4) and liver TNF-α transcripts are detected. However, while infected animals maintain a higher and sustained expression of this pro-inflammatory gene (4, 22), CaS rats exhibit more transient levels of mRNA with a pronounced down-regulation on day 3. Interestingly, this group shows a disorganized tissue reaction and increased liver injury markers (4, 22). The accurate role of cytokines in the liver environment where hepatic and immune cells are exposed to both the pathogen and neuroendocrine products is difficult to dissect. In models of liver injury, such as endotoxin/D-galactosamine (Gal/N), TNF-α/GalN and concanavalin A, TNF-α plays a central role in the damage and induction of apoptosis (10, 41, 42). In alcohol-mediated toxicity and murine obesity models, where steatosis constitutes a typical trait, TNF-α operates as an important hepatocyte sensitization factor acting as a positive control of the apoptotic threshold (6–8, 10, 12). Since the previously reported serum levels of this cytokine (4) and the transcriptional events showed here (Fig. 2) cannot fully explain the pathological alterations observed in our model where steatosis is also present, additional and possibly enhancing mechanisms may also be involved. The sensitization phenomenon and the well-known effect of TNF-α linked to the cell recruitment may offer other possible explanations.

The Fas molecule is constitutively expressed on the surface of hepatocytes and the counter ligand Fas-L appears in many tissues, including immune cells (14, 42). The Fas-Fas-L induced apoptosis seems crucial in regulating the immune response in many infections (15, 18, 21, 40). Eukaryotic pathogens can evade protective host responses to establish and maintain the infection promoting the death of effectors immune cells such as T lymphocytes and macrophages (40). Working with Fas-deficient MRL/lpr mice, Netea *et al.* (42) evaluated the role of Fas–Fas-L interaction during systemic infection with *C. albicans*. These authors demonstrated that mice deficient in functional Fas–Fas-L interaction are more resistant to lethal infection and that their resistance is accompanied by the increment of pro-inflammatory cytokine production (42). Using both immunocompetent (Ca) and stress-inmmunosuppressed hosts (CaS), we show here the presence of Fas-L-positive cells infiltrating the hepatic parenchyma during the acute phase of the fungal infection. This new finding related to the candidiasis pathogenesis suggests that cells which express this phenotype, could trigger the apoptosis program in Fas-bearing hepatocytes, participating in the liver damage.

An increased influx of circulating lymphocytes into the hepatic sinus is observed in many infections when liver is the main target (16, 33). This early recruitment attains utmost importance to circumscribe the primary infection and is the origin of the granuloma formation. Recent studies have demonstrated the existence of a large number of IHLs in inflamed liver as well as the apoptotic phenomenon triggered by activated T or non-T cells bearing the Fas-L molecule in the surface (42, 43). In agreement with these findings, we observed a significant rise in the absolute number of IHLs, mainly Fas-L-positive during the first 2 days of infection. This recruitment is probably related to the fungal liver burden and/or the local production of cytokines, such as TNF-α or INF-γ (2, 3, 5, 22). Moreover, the *in vitro* approach demonstrated that *C. albicans* can modulate *per se* the expression of Fas-L on IHLs. Interestingly, results with models of liver injury associated to gram-negative bacterial infection demonstrated a relationship between Fas-L expression and TLR-2 expression on a particular subset of IHLs (15, 19). In fact, *Salmonella* cell wall components may bind TLR-2 on NKT cells, and consequently activate intracellular signals resulting in elevated Fas-L expression on this IHL population (15). It is important to point here that in these models the increased number of Fas-L-positive IHLs constitutes an important effector mechanism of hepatocyte apoptosis. Herein, we also show that this conserved innate receptor can be up-regulated in IHLs after the fungal contact. We have not explored the complex regulation of Fas-L and TLR-2 yet; however, in view of the previous evidence suggesting a cross-talk between the signaling pathways (15, 19), we can speculate that a similar phenomenon is taking place after *C. albicans*–IHL contact. Studies are in progress to answer this question.

The link between *C. albicans* infection and apoptosis induction is supported by work performed with phagocytic cells, when the host cell death is triggered following endocytosis of fungus (44) or after that *C. albicans* phospholipomanan promotes the dysregulation of the pro-apoptotic molecule transduction pathway (45). Our study provides evidence

about the ability of this fungus to activate, directly or indirectly, at least two surface molecules involved in the promotion of the apoptotic phenomenon. Our findings also enlarge the spectrum of target cells compromised to apoptosis, from immune cells involved in the fungus control to non-immune cells.

In conclusion, in the complex scenario of early liver injury in immunocompetent and immunosuppressed hosts, the recruited IHLs and the modulated expression of TNF-α, Fas-L and TLR-2 molecules could act coordinately in delivering the death signals. The discussed mechanism of Fas-L and TLR-2 induction on IHLs after *C. albicans* contact could represent an ingenious strategy of fungal escape that skews the subtle host–pathogen balance promoting the host damage and favoring the infection.

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Abbreviations

CVS	chronic varied stress
DAPI	4′,6-diamidino-2-phenylindole dihydrochloride
H&E	hematoxylin and eosin
IHLs	intrahepatic lymphocytes
i.p.	intra-peritoneally
M-MLV	moloney murine leukemia virus
R1	region 1
RT	room temperature
TLR-2	Toll-like receptor 2
TNF	tumor necrosis factor
TUNEL	terminal deoxynucleotidyl transferase dUTP nick-end labeling

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