Abrogation of spontaneous liver tolerance during immune response to *Candida albicans*: contribution of NKT and hepatic mononuclear cells

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Abstract

Hepatic mononuclear cells (HMC) are a heterogeneous population with innate immune properties involved in the response to several pathogens. Herein, during the primary infection with *Candida albicans*, we observed dynamic changes in CD3⁺, NKT and NKT⁺ intrahepatic lymphoid subsets and a significant increase in the absolute number of antigen-presenting cells (APC). The liver tolerogenic microenvironment sustained by higher levels of IL-10, transforming growth factor-β and IL-4 was severely modified upon the robust IFN-γ production after the fungal colonization. NKT cells purified from infected animals released significant amounts of IFN-γ and the production of this cytokine was exacerbated after a second contact with the fungus. Interestingly, *C. albicans per se* was unable to activate tolerogenic NKT cells from naive animals. In vitro experiments performed with HMC cells depleted of the CD11b/c⁺ population revealed that in the absence of APC, NKT cells are unable to produce IFN-γ in response to *C. albicans*. Our findings constitute the first evidence that this innate lymphocyte population is involved in the pathogenesis of *C. albicans* infection.

Keywords: IFN-γ, TLR2, tolerogenic cytokines

Introduction

The liver is an immune-privileged organ, which occupies a watershed position between gastrointestinal and systemic venous circulations. As a result, it is continuously exposed to potential pathogens, probiotics and food-derived antigens entering via the gut. The liver acts as a complex immune organ, functioning as a site amenable either to mount effective immune responses or to generate tolerance (1, 2). Pathogenic micro-organisms must be efficiently eliminated while a large number of antigens derived from the gastrointestinal tract must be tolerated (1). The liver is selectively enriched in macrophages (Kupffer cells, KCs), NK and NKT cells which are key components of the innate immune system (3). NKT cells represent a subset of mature T lymphocytes defined by the co-expression of αβ TCR as well as NK1.1, a marker of NK cells (4). Because of their capacity to rapidly release large amounts of IFN-γ and IL-4, NKT cells are thought to polarize the local and systemic adaptive immune responses to either a pro-inflammatory type I (IFN-γ and TNF-α) or an anti-inflammatory type II (IL-4, IL-10 and IL-13) profile (3). Given the huge scope of actions that can be mediated by NKT cells, it has become increasingly apparent that these cells 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 (5). NKT cells have been implicated, for better or worse, in a number of different microbial infections including bacteria, virus, protozoan parasites and to a lesser extent fungi (6–9). At the present, the role of NKT cells in candidiasis remains unknown.

*Candida albicans* is a leading cause of infection in hospitalized and immunocompromised hosts. This yeast is a commensal in oral and gastrointestinal epithelia and changes in environmental factors such as competition from other saprophytes, prolonged treatments, immunity alteration and physical perturbations of these niches promote endogenous dissemination (10, 11). Catheter contamination with *Candida* biofilms constitutes an additional font of host infection (12). The liver can be colonized by *Candida* in different circumstances: (i) associated with immunosuppressive therapy and organ transplants (13), (ii) associated with ambulatory peritoneal dialysis (12, 14) and (iii) associated...
Liver immune response during C. albicans infection with disseminated candidiasis (11). The liver constitutes a crucial barrier in the fungal spreading due to its ability to limit the growth of the yeast and to mount an efficient inflammatory reaction. We studied the systemic and hepatic immune response against this fungus in a well-characterized experimental model (15–20). At the local level, we found higher fungal burden associated with hyphal forms and presence of granulomatous reaction. The hepatic inflammatory reaction is compromised with the occurrence of steatosis, depletion of glycogen deposits, increment of functional hepatic enzymes, marked lipid peroxidation and up-regulated activity of inducible nitric oxide synthase and arginase (18). An important contribution reported by our group is the occurrence of in situ hepatocyte apoptosis together with the mobilization of the intrahepatic lymphoid compartment and the up-regulated expression of Fas-L molecule (19). A clear exacerbation of damage parameters and more invasive forms of mycosis is present in immune-compromised hosts (15–18, 20). Together, these findings strongly support the relevance of local immune environment to orchestrate the balance between control/damage.

Here, we explored the role of total hepatic mononuclear cells (HMC) and NKT cells during C. albicans primary infection and their contribution to the balance of inflammatory/anti-inflammatory mediators at local level. Since at the present the function of NKT cells in candidiasis has not been elucidated, we report here new and interesting evidence about this versatile population.

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. 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.

Micro-organism 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 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 (15–17, 20). Regularly, we inoculated intra-peritoneally (i.p.) $3 \times 10^8$ viable yeast 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 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). We used two groups of rats: naive noninjected (N) or rats infected i.p. on day (D) 0 with a 1 ml inoculum ($3 \times 10^8$ viable yeast ml$^{-1}$) assigned to C. albicans-infected (Ca) group. On D1–D3 animals were killed by decapitation (15–20). 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 colony-forming units per gram of tissue. Livers were removed, placed on individual Petri dishes, weighed and processed for different studies. Protocols were approved by the Animal Experimentation Ethics Committee, Faculty of Chemical Science, National University of Cordoba.

Isolation of HMC

Livers were perfused with 20 ml of PBS-5% FCS, pressed through 200-gage stainless steel mesh and resuspended in PBS-5% FCS. Cells were washed with PBS once and 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 NH₄Cl, 1 mM KHCO₃, 0.1 mM Na₂EDTA, pH 7.3), washed twice in PBS and counted and diluted to the desired concentration (19, 21).

Culture assays

HMC were plated and cultured at a concentration of $1 \times 10^6$ cells per well in RPMI 1640-10% FCS-0.1% gentamicin (supplemented medium) at 37°C-5% CO₂ in 24 well plates. Cells were cultured alone, with $10 \mu$g ml$^{-1}$ of peptidoglycan (PGN, Sigma–Aldrich, St Louis, MO, USA), 20 $\mu$g ml$^{-1}$ Zymosan (Sigma–Aldrich) or viable C. albicans yeasts. The 1:1 yeast: cell ratio was chosen after titration studies when the cytokines release was evaluated and the HMC viability was measured by propidium iodide and FACS analysis. To inhibit fungus overgrowth and prevent HMC damage, we used 0.5 $\mu$g ml$^{-1}$ Amphotericin B. Cells incubated with 50 ng ml$^{-1}$ phorbol 12-myristate 13-acetate (PMA, Sigma–Aldrich) and 1 $\mu$g ml$^{-1}$ ionomycin (Io, Sigma–Aldrich) were used as positive control of cytokine production (22). After 24 h of culture, supernatants were collected and stored at −70°C until use. HMC were harvested, washed once with PBS-10% FCS and processed.

In other set of experiments purified NKT ($2 \times 10^9$ cells per well), NKT negative cells ($1 \times 10^6$ cells per well or $2 \times 10^5$ cells per well) or CD11b/c negative cells ($5 \times 10^5$ cells per well) were culture as described above.

Flow cytometry

Rat-specific monoclonal antibodies (mAbs) anti-CD3-PE, anti-CD3-conjugated allophycocyanin (APC), anti-NK1.1-FITC, anti-TLR2-PE and anti-CD11b/c-PE were from BD Biosciences, USA; the corresponding isotype controls were from Sigma–Aldrich. Cells were stained for surface markers in a staining buffer (PBS-EDTA-FCS) on ice for 30 min in the dark (19). 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. The NKT population was 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 (21,22). For each population, the absolute number...
Liver immune response during C. albicans infection: cytokine microenvironment

The hepatic microenvironment is rich in immunosuppressive mediators such as IL-10 and TGF-β, which keep liver immune system in a baseline state (24, 25). To know changes in liver microenvironment after C. albicans arrival, we studied in HMC, the spontaneous or fungus induced release of IL-10 and TGF-β on D1 postinfection. Tolerogenic cytokines IL-10 and TGF-β were not modified neither by the primary C. albicans infection nor after a second contact with the fungus (Fig. 2B and C), with similar levels to N-HMC. On the other hand, when N-HMC were stimulated in vitro with C. albicans, the production of both cytokines remained unchanged. In the same experimental condition, Ca-HMC showed a robust IFN-γ release (P < 0.05), without changes in IL-4 production (Fig. 2D). After restimulation with C. albicans, Ca-HMC released higher levels of IFN-γ (P versus N and Ca < 0.05). Interestingly, N-HMC were able to produce IFN-γ after in vitro interaction with C. albicans (Fig. 2D) (P < 0.05). The production of TNF-α by Ca-HMC yielded a ratio of 2.13 ± 0.12 that was modified after a second contact with the fungus (2.98 ± 0.54) (data not shown). These results demonstrate that the arrival of C. albicans occurred without modifying the expression of cytokines involved in intrahepatic tolerance. Yet, the yeast induced in resident/infiltrating cells, the production of Th1-type cytokines associated with a protective response against the fungus.

Breakdown of liver tolerance by C. albicans infection: cytokine microenvironment

Liver immune response during C. albicans infection

Liver immune response during C. albicans infection

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C. albicans infection up-regulates the TLR2 expression in HMC

In a previous work, we reported that the exposure of N-HMC to the fungus in vitro increases significantly the number of TLR2+ cells in this population (19). Then, we evaluated TLR2 expression during the course of primary infection in total HMC and in each IHL sub-population. The absolute number of HMC-TLR2+ cells augmented on D1 after fungus arrival and returned to basal levels on D3 postinfection (Fig. 3B). Similarly, HMC-TLR4+ cells increased on D1 and recuperated control levels on D3 (data not shown). For NKT, NK and CD3 subsets, no significant changes in the percentage of TLR2+ cells were detected on D1 and D3 postinfection (Fig. 3D). However, as depicted in Fig. 3(E), the absolute number of NKT-TLR2+, NK-TLR2+ and CD3-TLR2+ cells showed a marked enhancement during the liver colonization (versus N < 0.05). No changes in the mean fluorescence intensity (MFI) of TLR2 each TLR2+ populations were observed.

In view of the above results, we evaluated the ability of the TLR2 agonists PGN and Zymozan to induce IFN-γ, IL-4, TGF-β and TNF-α release by HMC purified from N and Ca group on D1 postinfection. As can be seen (Fig. 3F), while PGN stimulated both Ca-HMC and tolerogenic N-HMC, with a significantly higher production of IFN-γ in Ca-HMC cells (P < 0.05), Zymozan do not change the cytokine production in Ca-HMC cultures. On the other hand, PGN and Zymozan induced small increments in IL-4 both in Ca-HMC and tolerogenic N-HMC (Fig. 3F). Basal levels of TGF-β were not modified after TLR2 agonist treatment (data not shown), and the TNF-α production ratio was 3.6 ± 0.3 for Ca-HMC and 3.9 ± 0.2 for N-HMC (data not shown). PMA/Io used as positive control stimulated the release of both IFN-γ and IL-4 in N-HMC and Ca-HMC.

Contribution of NKT cells to the setting of liver microenvironment during C. albicans primary infection

NKT cells respond with vigorous cytokine production within 1–2 h of TCR ligation (3, 5), and these cells go both ways as their activation can polarize the immune response in either Th1 or Th2 direction (26). To assess the contribution of NKT lymphocytes to the Th1 cytokine profile present in the liver during primary C. albicans infection, we worked with purified NKT cells and with NKT-depleted HMC from N and Ca animals. Figures 4A and C show representative regions containing NKT population selected to purify or exclude (top) and populations recovered after sorting (down) in each group. Intrahepatic NKT cells sorted from N animals produced very low levels of IL-4 (~1 pg ml⁻¹) and absence of IFN-γ (Fig. 4B) after in vitro stimulation with the fungus, demonstrating that the direct contact was unable to activate this sub-population. Interestingly, NKT cells from infected livers released higher concentrations of IFN-γ (~100 pg ml⁻¹), which increased even more after in vitro reexposure to the pathogen (~340 pg ml⁻¹). The basal production of IL-4 (~1.2 pg ml⁻¹) was unmodified after re-stimulation.

On the other hand, NKT-depleted N-HMC released spontaneously IFN-γ and low levels of IL-4, but in the presence
of *C. albicans* and PMA/Io, the production of Th1 cytokine was clearly amplified (Fig. 4D); the release of IL-4 remained unmodified (Fig. 4D). As expected, NKT-depleted cells recovered from Ca-HMC produced higher levels of IFN-γ (~490 pg ml⁻¹) in agreement with the *in vivo* priming with the fungus. However, in the absence of NKT cells and in the presence of *C. albicans*, a significant decrease of IFN-γ levels was observed compared with the values obtained without *in vitro* fungus priming (~208 pg ml⁻¹). No changes were detected in IL-4 concentration.

To illustrate the real contribution of NKT subset in the setting of inflammatory liver microenvironment, we compared the IFN-γ production of total HMC- and NKT-depleted HMC cells in the same experimental condition (Supplementary Figure 1 is available at *International Immunology* Online). As can be seen in the absence of NKT cells, the IFN-γ production was strongly diminished with 45.4 and 87.3% of reduction for *ex vivo* and *in vitro* restimulation respectively. Together, these findings demonstrate the true contribution of this innate subset during the liver fungus colonization.

Positive CD11b/c population is necessary to activate NKT cells

As demonstrated above, *C. albicans* was unable to directly activate N-NKT. This population was effectively stimulated to amplify the production of IFN-γ only after *in vivo* priming. As N-HMC produced cytokines after stimulation with TLR2 ligands (Fig. 3D), we wondered if N-NKT cells could be activated with TLR2 ligands. With this purpose, NKT cells were sorted out of N animals and incubated with TLR2 agonists to assess the production of IFN-γ or IL-4. After contact with PGN, N-NKT cells produced low but significant concentrations of both cytokines, while Zymosan only triggered an increase in Th2 type cytokine production. By contrast, when N-NKT cells were stimulated with PMA/Io higher levels of IFN-γ (~120 pg ml⁻¹) were detected with a minor release of IL-4 (~4 pg ml⁻¹) (Fig. 5A). These results show that liver resident NKT cells, in the absence of other cell populations, react poorly to TLR2 agonists maintaining their tolerogenic profile.

The results suggested us that a stronger activation of NKT cells could be dependent on one or more intrahepatic...
populations, as liver resident APC (7, 25, 27). APC could be a good candidate in our model, as the percentage and absolute number of CD11b/c+ cells increased significantly during infection (Fig. 2A). To explore the contribution of this population, we obtained HMC from N (N-HMC) and Ca (Ca-HMC) animals (D1 and D3) were stained with anti-CD3, anti-NK1.1 and anti-TLR2 mAbs and analyzed by FACS. (A) Representative forward scatter versus side scatter density plots showing gate in R1 for further TLR2 analysis. (B) Absolute number of TLR2+ cells in HMC population after C. albicans infection. Data are expressed as mean ± SE (rats per group N = 5–5; CaD1 = 5–6 and CaD3 = 4–6 group, *P < 0.05 versus N). (C) Representative CD3 versus NK density plot showing gate in CD3+ and double positive cells (NKT cells) for TLR2 expression analysis. (D) Representative histograms showing NKT, NK and CD3 cells stained with isotype control (empty) or TLR2 mAb (gray filled). (E) Absolute number of TLR2+ cells in NKT, NK and CD3 cell population after C. albicans infection. Data are expressed as mean ± SE (n ≥ 4 per group, *P < 0.05 versus N). (F) N-HMC and Ca-HMC (D1) (1 × 10⁶ cells per well) were cultured 24 h with medium, TLR2 agonists such as PGN and Zymozan or PMA/Io. Supernatants were collected and IFN-γ or IL-4 production was analyzed by ELISA assay (*P < 0.05 versus basal).

Fig. 3. TLR2 expression in HMC and IHL sub-populations. Contribution of TLR2 ligands in cytokine production after primary infection or in vitro contact with Candida albicans. HMC purified from N (N-HMC) and Ca (Ca-HMC) animals (D1 and D3) were stained with anti-CD3, anti-NK1.1 and anti-TLR2 mAbs and analyzed by FACS. (A) Representative forward scatter versus side scatter density plots showing gate in R1 for further TLR2 analysis. (B) Absolute number of TLR2+ cells in HMC population after C. albicans infection. Data are expressed as mean ± SE (rats per group N = 5–5; CaD1 = 5–6 and CaD3 = 4–6 group, *P < 0.05 versus N). (C) Representative CD3 versus NK density plot showing gate in CD3+ and double positive cells (NKT cells) for TLR2 expression analysis. (D) Representative histograms showing NKT, NK and CD3 cells stained with isotype control (empty) or TLR2 mAb (gray filled). (E) Absolute number of TLR2+ cells in NKT, NK and CD3 cell population after C. albicans infection. Data are expressed as mean ± SE (n ≥ 4 per group, *P < 0.05 versus N). (F) N-HMC and Ca-HMC (D1) (1 × 10⁶ cells per well) were cultured 24 h with medium, TLR2 agonists such as PGN and Zymozan or PMA/Io. Supernatants were collected and IFN-γ or IL-4 production was analyzed by ELISA assay (*P < 0.05 versus basal).
percentage of NKT cells producing IFN-γ in C. albicans infection.

Discussion
In the sterile liver, different cell types, cytokines and components of the innate immunity contribute to a tolerogenic microenvironment that ensures the efficient function of the organ. In the presence of invading pathogens, the balance is broken in order to organize an effective inflammatory response to control the infection (1–3, 25). This paper reports the dynamics of the recruitment of intrahepatic populations and the microenvironment changes that occur during C. albicans liver colonization. We also provide novel evidence about the contribution of intrahepatic NKT cells to the immune response in candidiasis.

In the present study, when C. albicans arrived to the liver, local and progressive transcription of CCL-2, CCL-4 and CXCL-8 mRNA chemokines occurred. Stimulated parenchymal cells and resident APC are involved in this phenomenon (25, 28). In agreement, we demonstrate that the CD11b/c+ population (KC and DCs) significantly expanded in the early stages of the mycosis. In this activated microenvironment, lymphoid cells rise above five times the number of resident cells (19). The analysis of the lymphoid compartment revealed particular kinetics for T, NK and NKT cell subsets with a sustained raise of NKT cells. In this scenario, the activation, recruitment and expansion of intrahepatic populations started to deflect the tolerogenic profile to an inflammatory response in order to control the pathogen infection.

The liver is characterized by the local release of immunosuppressive cytokines such as IL-10 and TGF-β. Cells belonging to HMC population, such as KCs, DCs and Tregs as well as liver sinusoidal endothelial cells (LSECs) would be the main source of these mediators (1, 3, 25). The high

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Fig. 4. Cytokine production by NKT cells and NKT negative population after primary infection or in vitro contact with Candida albicans. NKT cells sorted out of N (N-NKT) and Ca (Ca-NKT) animals on D1 postinfection (2 × 10⁵ cells per well) were cultured 24 h with medium or the fungus. The remaining HMC population (negative cells) was cultured under the same conditions. (A) Representative CD3 versus NK1.1 density plots showing the region containing NKT cell population selected for further purification in each group (top). Representative density plots showing 94–96% purity of NKT cells in both groups (down). Culture supernatants were collected and IFN-γ or IL-4 (B) production was analyzed by ELISA assay. (C) Representative CD3 versus NK1.1 density plots showing the region containing NKT cell population selected for further purification in each group (top). Representative density plots showing 94–96% purity of negative cells (without NKT cells) in both groups (down). Culture supernatants were collected and IFN-γ or IL-4 (D) production was analyzed by ELISA assay. Data from four independent experiments are expressed as mean ± SE in picogram per milliliter. For each experiments, cells sorted out of N (n = 4) and Ca (n = 5) were pooled (*P < 0.05 versus N-NKT cells, #P < 0.05 Ca-NKT and Ca-Neg versus reexposure to C. albicans).
concentration of these cytokines generates a unique micro-
vironment where resident DCs acquire a tolerogenic phe-
notype in order to diminish the activation of immune cells (3). Evidence derived from local infection and liver trans-
plantation models shows that the break of tolerance occurs 
when the tolerogenic cytokine threshold is exceeded by in-
flammatory mediators. During acute viral hepatitis, intrahe-
patic tolerance sustained by IL-4, IL-10 and TGF-
ß produced by KCs, LSECs, NKT cells and Tregs may be dis-
turbed (29). Ma et al. reported that multiple doses of the 
bacterial-DNA mimetic compound, CpG oligodeoxynucleo-
tide (CpG-ODN), elicit acute rejection of the liver allografts 
with significant T-cell infiltration, reduced Tregs and en-
hanced IFN-ß producing cells. These data demonstrate that 
CpG-ODN initiates an inflammatory reaction and abrogates 

spontaneous tolerance in the liver (30). Here, we show that 
C. albicans arrival to the liver did not modify the constitutive 
production of tolerogenic IL-10 and TGF-ß cytokines. The 
in vitro interaction between tolerant N-HMC and the fungus 
also confirmed this fact. Early in the infection, the increment 
of Tn,1 type cytokines, especially IFN-ß, marks the beginning 
of the inflammatory response. A relevant observation was 
that after the second contact with the pathogen, this phenom-
enon was amplified. These results demonstrate the ability of 
C. albicans to prompt sufficiently strong signals to activate 
HMC and reverse the tolerogenic profile that intrinsically 
exists in the liver.

Many studies have been carried out to define the role of 
NKT cells in the response to microbial pathogens. As a po-
tent source of immunoregulatory cytokines such as IL-4,
IFN-γ and TNF-α, these cells are pivotal in the early setting of infection (3, 7, 9). NKT cells have been implicated in the immunity against viruses, bacteria, fungus and parasites. However, their response against different micro-organism can not be predicted and may change depending on each particular NKT-pathogen interaction (26, 31, 32). Regarding the NKT-fungus interaction, only the response against Cryptococcus neoformans has been reported (6, 33, 34). Kawakami et al. demonstrated that NKT cells accumulate in an MCP-1 (CCL-2)-dependent manner in the lungs after C. neoformans infection, playing an important role in the development of Th1 cytokine profile. In the present work, we demonstrate that hepatic NKT cells reacted with vigorous production of IFN-γ during the course of C. albicans infection and the reexposure to the pathogen enhanced the release of cytokines. In the liver microenvironment, others cells can produce significant amounts of IFN-γ in response to C. albicans colonization, as NKT-depleted cells from infected animals spontaneously released IFN-γ as well. Interestingly, in the absence of NKT cells, an opposite behavior was detected and a significant decrease of IFN-γ production was observed, indicating that the abolition of NKT subset has a direct impact in Th1 cytokine profile. In connection with this, other authors demonstrated that NKT cells through their ability to release IFN-γ can modulate and synergistically operate with other innate subsets such as NK cells (35, 36). Taking together, our results constitute the first proof that NKT cells are involved in the pathogenesis of C. albicans infection. Desombere et al. also supplied information about this population (37); however, clear differences between the experimental settings exist: while their work was performed in vitro with human peripheral blood mononuclear cells, commercial antigens of C. albicans and intracellular staining, the ability to evaluate released cytokines upon fungus challenge was not determined. Our work provides evidence in a more complex scenario, testing the ex vivo production of cytokines in a model of primary C. albicans infection.

Activation of NKT cells may involve different pathways. The indirect route depends on cytokines release by activated DCs or other APC and/or the recognition of endogenous glycolipid ligands (25, 32). Only few microbial antigens able to mediate direct activation have been described (25, 32). TLRs are involved in both, the stimulation of APC (25, 32) and the direct activation of NKT cells (38, 39). Two reports demonstrated that TLR2 and TLR4 are abundantly expressed in intrahepatic NKT cells. Working with murine models of Salmonella and Escherichia coli infections, the direct activation of NKT cells through TLR receptors has been shown (38, 39, 40). Even when TLR2 and TLR4 are involved in the host interaction with C. albicans, their specific role during infection has not been established, and conflicting results have been reported (41–44). We previously reported that the expression of TLR2 is up-regulated in N-HCM after in vitro fungus contact (19). Herein, a significant increase in the absolute number of HCM-TLR2+ occurred during the first day of infection that correlated with higher number of NKT-TLR2+, NK-TLR2+ and CD3-TLR2+ cells associated to C. albicans liver colonization. Two classic TLR2 agonists were included in the present study, PGN, a gram-positive bacterial product, and Zymosan, a mimetic compound of fungal cell wall (45). Both ligands can induce inflammatory cytokine production by macrophages and DCs. As PGN binds TLR2/TLR6, whereas Zymosan recognition involves dectin-1 and TLR2, the inflammatory outcome is dependent on both the repertoire of receptors expressed in a particular cell subset and the functional cooperation between the signals downstream the receptor activation (45, 46). The stimulation of Ca-HMC with TLR2 agonists favored the balance toward inflammatory profile linking TLR2 signaling with local protective response. In tolerogenic N-HMC instead, the IFN-γ profile was different dependent on the TLR2 agonist that derived the cell activation. Although in this paper, we did not evaluate the expression of dectin-1 in the HMC, a possible explanation for this observation could be the intracellular signals triggered after activation through this receptor. Further research should be conducted to elucidate this phenomenon.

In this point, a relevant question is if intrahepatic NKT cells are directly activated through TLR2 signaling. We found that tolerogenic resident N-NKT cells produce little amounts of IL-4 in response to TLR2 ligands, and a low release of IFN-γ just after PGN stimulation. Only the vigorous activation driven by PMA/lo was effective to switch the profile. More relevant, the fungus per se was unable to change the secretion profile of tolerogenic NKT cells. These results suggest that the signaling triggered by these innate receptors involved in the pathogen recognition was insufficient to modify the tolerogenic status of liver resident NKT cells.

The current study suggests that N-NKT cells need other cells or soluble factors to become activated in response to C. albicans. As in other models, APC resident in the liver would be the best candidates, as they can sense microbial compounds though TLRs (3, 6, 32). In agreement, infected animals showed up-regulated TLR2 expression in the HMC population and an early expansion of APC subset. When CD11b/c+ cells were negatively sorted, the remaining population mainly formed by CD3, NKT and NK cells was unable to produce IFN-γ against the fungus and the TLR2 triggered a low release of IFN-γ without modifying the lower levels of IL-4. In this system, NKT cells produce IFN-γ only in response to a strong stimulus such as PMA/lo. Together, in the absence of APC, NKT cells were unable to produce IFNγ in response to C. albicans.

In the complex in vivo scenario, the arrival of this pathogenic fungus promoted a coordinated cell interaction leading to NKT activation; in the early stage of infection, the ability of these innate lymphocytes to orchestrate the tone of the ensuing immune response is relevant for the outcome of mycosis. Our work is the first study that addresses the role of NKT cells during the settlement of primary C. albicans infection of the liver. This finding is significant because it provides new evidence about the host-C. albicans interaction and highlights the contribution of NKT cells in the infection. Future studies will delineate the different mechanisms by which NKT cells are activated.
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Supplementary data

Supplementary data are available at International Immunology Online.

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