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Immune Neuroendocrine Interactions during a Fungal Infection in Immunocompetent or Immunosuppressed Hosts

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Abstract
The yeast *Candida albicans* belongs to the microflora of healthy individuals, although it can infect a variety of tissues ensuing changes in the host's immune status. To evaluate the effect of neuroendocrine input on the early immune response during the fungal infection, we use a 3-day paradigm of chronic varied stress in Wistar rats infected with *C. albicans*. We find that stress mediators contribute to the spread of the fungus and downregulate critical functions of phagocytic cells at the infection site. Phenotypic and functional alterations of effector cells account for the decreased resistance to candidiasis and condition the development of the adaptive response. Stressed hosts exhibit a higher fungal burden in kidneys and livers associated with hyphal forms. The hepatic inflammatory reaction is compromised with severe steatosis, increment of functional enzymes, marked lipid peroxidation and hepatocyte apoptosis. Moreover, infection-related sickness symptoms are significantly increased by exposure to stress with anorexia, weight loss, lack of leptin and depletion of glycogen depots. Food deprivation exacerbates the liver injury. Stress mediators perturb the complex immune and metabolic program that operates early during fungal spread and promotes severe tissue damage.

Candida albicans is both a constituent of the normal microbiota and a pathogen of humans that can infect a wide range of body sites. This prototypic opportunistic yeast is able to persistently colonize the host without causing symptoms. The balance between commensalism and pathogenicity is an active process and the impairment of the host immunocompetence is considered one major predisposing factor [1]. *C. albicans* infections increased dramatically in the last decades associated to the use of broad spectrum antibiotics, endocrine disorders, tumors or AIDS highlighting the connection between candidiasis and immunocompetence. To evaluate the effect of the neuroendocrine input on the early immune response during the fungal infection, we developed a model of infection and stress in Wistar rats. For the infection, we injected i.p. 3×10^8 viable *C. albicans* cells and we started the stress exposure immediately after infection (IS group) [2]. The stress paradigm lasted 3 days and

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included cold swimming, restraint and food deprivation [2, 3]. Additionally, uninfected unstressed (N), stressed (S) or infected (I) groups were included as controls. Rats were weighed daily and killed after 3 days of treatment to obtain blood and tissues for different studies. We found a significant increment in the levels of ACTH and corticosterone in the IS group, in agreement with the sustained input of the hypothalamus-pituitary-adrenal axis [2, 4]. We observed increments in liver and spleen weights in both infected (I and IS) groups, although the thymus size remained unchanged. A similar fungal burden was detected in the kidneys and spleens of infected rats, whereas livers were heavily colonized in infected stressed hosts. Stained liver sections showed budding yeasts enclosed inside large and well-developed granulomas in I rats [2], whereas invasive hyphae or pseudohyphae forms predominated in IS hosts exhibiting incomplete granulomatous lesions [2, 5]. As TNF-α is crucial for developing a protective inflammatory reaction and antigens of the fungal cell wall induce TNF-α production [6], we evaluated the production of this cytokine under the different experimental conditions. As expected, we detected the highest serum levels of TNF-α in infected hosts on the first day of the infection. Yet, compared with I rats, the IS group showed a 30% lower concentration of the cytokine [7]. On the other hand, on day 3 we found increments in IL-6 and IL-10 levels in I and S groups, respectively. The analysis of liver sections from infected groups showed fat deposition or steatosis [2, 5]. Interestingly, when compared with I rats, livers of IS rats showed a remarkable higher number of fat vacuoles. Hepatic steatosis is caused by imbalance between the delivery of fat in the liver and its subsequent secretion or metabolism; it is a common feature of many liver diseases with different pathogenic mechanisms. To study the distribution and characteristics of steatosis, we stained sections with the specific lipid dye Sudan Black. Strikingly, in I rats microvesicular steatosis predominated all over hepatic acini while in IS group, we detected mainly lipid accumulation in acinar zone 1, with a mixed pattern of micro- and macrosteatosis. While markers of liver injury such as gamma-glutamyl transpeptidase augmented in all treated groups, the intracellular alanine transferase enzyme increased twice in I and IS rats [4]. As steatosis is frequently associated with oxidative stress, we measured the production of malonaldehyde, an indicator of lipid peroxidation. Interestingly, although this marker increased in all treated groups, the highest levels appeared in IS rats. We also evaluated the presence of apoptotic cells on liver sections after staining with the fluorescent dye DAPI. The microscopic analysis showed clear differences in the frequency and nucleus morphology of hepatocytes from the I and IS groups compared with uninfected controls (N and S), with the strongest alterations on day 3 of the treatment [8]. This phenomenon was quantified by TUNEL assay, where apoptotic cells incorporate specifically FITC-labeled dUTP into DNA strand breaks. Again, a significant number of TUNEL-positive cells was found in IS rats compared with I animals ($p < 0.05$). In an attempt to assess the contribution of fungal factors in the liver injury detected in our experimental condition, we identified a 70-kDa lipase that is released after 24 h incubation in standard culture media. This *C. albicans* lipase exhibited maximal activity at physiological pH and temperature and was able to interact with hepatocytes, to induce cytotoxicity and to promote the deposition of lipid droplets [9], replicating the effect observed in vivo. At the peritoneal cavity, the recruitment of leukocytes that occurred after the fungal injection was severely impaired by stress exposure [7]. Whereas the phagocytosis of peritoneal macrophages was not modified, stress mediators decreased the candidicidal activity, evaluated by flow cytometry and killing assay ($p < 0.001$), as well as the production of nitric oxide (NO) [2, 4]. In consequence, the amount of viable *C. albicans* recovered from the peritoneum was significantly higher in IS rats ($p < 0.05$). Together, this first set of studies enabled us to characterize the early innate response during the *C. albicans* infection in immunosuppressed hosts and to conclude that: (1) at the systemic level these rats exhibited a reduction of TNF-α and IL-6 production, poorly developed granulomas, different type and localization of steatosis and severe liver injury, and (2) at the infection site, they showed lower cell recruitment, reduced candidicidal index and higher fungal load.

During an immune response the host needs to organize and distribute energy resources. For that reason, infection is associated with negative energy balance with reduced food intake, weight loss, increased thermogenesis and fever [10]. Then, we wondered how, in our model, hosts exposed to different experimental conditions could achieve the immunometabolic adjustment. Both infection and stress mediators provoked body weight reductions and anorexia, although with different kinetics. By blocking the active TNF-α (with a specific antibody) or corticosterone (with the receptor antagonist RU 486), we reverted the weight loss observed in the IS and I groups, respectively. Leptin is a central mediator that connects nutrition and immunity and regulates satiety. Compared with controls, leptinemia was significantly reduced in all

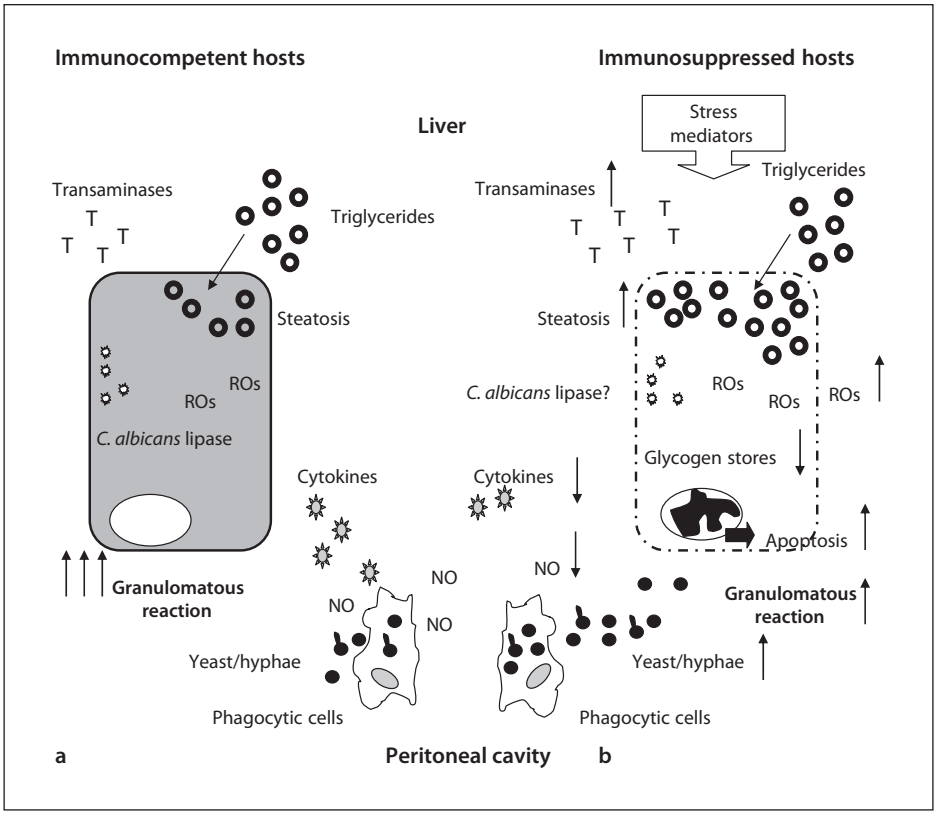

Fig. 1. Outcome of the *C. albicans* infection in immunocompetent or immunosuppressed hosts. Schematic representation of the main biochemical and immune events involved in the early steps of the fungal spreading. The predominant effect of stress mediators on the different parameters evaluated is represented by arrows: ↑ = increment; ↓ = reduction. **a** At the site of the infection, in immunocompetent hosts, the infection activates phagocytic cells that release cytokines such as TNF-α and IL-6 or inflammatory mediators such as NO. The candidicidal mechanisms effectively clear the pathogen significantly reducing spread. In the liver, well-developed granulomas that enclose invasive forms evidence the robust inflammatory reaction. Fungal virulence factors such as *C. albicans* lipase contribute to the fat deposition. Injury markers such as transaminases, lipid peroxidation and microvesicular steatosis are present during the infection although at moderate levels. **b** In the immunosuppressed host, stress mediators severely condition the function of phagocytic cells at the site of the infection. The reduced production of cytokines and NO supports the heavy fungal burden and the higher spreading of invasive hyphal forms. In the liver, the weak inflammatory reaction is evidenced by poorly developed granulomas. Stress mediators could modulate the release of *C. albicans* lipase. Together, in this microenvironment a more severe damage takes place.

treated groups (vs. N, $p < 0.05$) with the stronger reduction in stressed rats. Glycogen stores evaluated in periodic acid-Schiff (PAS)-stained liver sections showed a severe depletion in IS together with a significant reduction in glucose levels ($p < 0.05$). To establish the contribution of food deprivation to the imbalance observed in IS rats, we included two additional experimental groups: (1) rats injected i.p. with 3×10^8 viable *C. albicans* cells as above and exposed to food deprivation on day 2 (IFD group), and (2) rats infected and stressed like the IS group but exposed on day 3 to a 24-hour crowding session instead of food deprivation (ISL group). Clearly, the food deprivation event (IS and IFD) precipitated the complete depletion of glycogen stores and the reduction of glycemia. Steatosis was present in each infected group; however, the highest severity was associated to the stress exposure (IS and ISL). Interestingly, in immunocompetent hosts (IFD), the food deprivation exacerbated the liver injury, with significant increments in lipid peroxidation and transaminase release ($p < 0.05$). We also evaluated the relationship between the ability to clear the fungus and the metabolic imbalance. Compared with immunocompetent hosts, both stressed groups (IS and ISL) showed a higher fungal burden in liver (vs. I, $p < 0.05$) while the single

event of food deprivation on day 2 (IFD) was unable to condition the ability of the host to remove the yeast (vs. I, p = n.s.). On the basis of the extensive data presented here, a model of the mechanism involved in the host-fungus interaction in immunocompetent and immunosuppressed hosts can be proposed (fig. 1). In immunocompetent hosts at the site of the infection (i.e. the peritoneal cavity), resident and recruited phagocytic cells become activated and release inflammatory mediators such as NO and cytokines. Different candidicidal mechanisms effectively clear the pathogen significantly reducing the fungal spread (fig. 1a). In the liver, the robust inflammatory reaction is evidenced by well-developed granulomas surrounding invasive forms. The oxidative environment triggers a mild injury in the organ with lipid peroxidation, microvesicular steatosis and transaminase release. Fungal virulence factors such as *C. albicans* lipase could contribute to the fat deposition. In immunosuppressed hosts instead, stress mediators severely condition the function of phagocytic cells at the site of the infection. The impaired release of cytokines and NO promotes the heavier fungal burden and the higher spreading of invasive hyphal forms. In the liver, the weak inflammatory reaction is evidenced by poorly developed granulomas (fig. 1b). Although we have no evidence yet, the modulatory effect of the stress input on the production of *C. albicans* lipase cannot be discarded. Together, the outcome of a fungal infection as well as the liver damage strongly depend on the metabolic and immune status of the host. As defenses are energetically expensive to develop and to operate, the synchronization of inflammatory and metabolic pathways sustains the redistribution of resources under different conditions. Understanding this complex response may help to explain the progression of systemic infections occurring under different metabolic conditions in immunocompromised hosts.

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