Infection and tissue repair of experimental cutaneous candidiasis in diabetic mice

Andréia Carla Eugenio Pupim,¹,²† Tacito Graminha Campois,¹† Eduardo José de Almeida Araújo,² Terezinha Inez Estivalet Svidizinski³ and Ionice Felipe¹,*

Abstract

Purpose. Diabetic patients seem to be predisposed to cutaneous candidiasis. In this study, we evaluated the interference of diabetic conditions in alloxan-induced diabetic mice in relation to the development of C. albicans infection, density of M1 and M2 macrophages, distribution of collagen type I and III and anti-inflammatory cytokines involved in tissue repair.

Methodology. The mice were treated with intravenous alloxan, and all animals with blood glucose levels >250 mg dl⁻¹ were inoculate with C. albicans intradermally in the hind paw and were studied for up to 21 days. Control groups without alloxan were used. The fungal burden was evaluated by periodic acid-Schiff (PAS) and by counting the colony forming units. Total population of macrophages were targeted with antibody to F4/80 antigen and M2 macrophages with anti-arginase antibody. Anti-inflammatory cytokines from popliteal lymph nodes were determined by capture ELISA procedures. Picrosirius red staining allowed quantification of collagen types I and III in the infected skin by using a polarized light microscope.

Results/Key findings. Diabetic mice, versus non-diabetic mice, showed a significant lower density of F4/80 and M2 macrophages, higher fungal burden, deficiency in interleukin (IL)-4 production, and delayed IL-13 responses. The later clearance of C. albicans enhanced tissue injury, leading to a decrease in collagen type I. Moreover, collagen type III was increased by interference of IL-13 and transforming growth factor-β cytokines.

Conclusion. These findings highlight some important changes in diabetic animal responses to C. albicans infection that may be important to the pathophysiological processes underpinning cutaneous candidiasis in diabetic patients.

INTRODUCTION

Diabetes affects hundreds of millions of people worldwide, and these individuals exhibit severe impairment of the healing of acute wounds. Moreover, this population suffers from chronic non-healing diabetic foot ulcers, which are estimated to occur in 15% of all persons with diabetes. These ulcers are a serious complication of diabetes and precede 84% of all diabetes-related lower leg amputations, mainly when coupled with an impaired ability to fight infection [1].

Fungal infections are the most frequent cutaneous manifestations in diabetic patients [2], and candidiasis is a leading occurrence [3]. Diabetic patients seem predisposed to cutaneous candidiasis, as shown in a study by Papini et al. [4], in which onychomycosis was observed in 53.3% and foot skin mycosis in 46.7% of cases. Both fungal infections showed significantly higher prevalence compared to the control group [5]. Given their high complexity in humans, the effector molecules and cells involved in the immune response to cutaneous candidiasis remain to be elucidated.

In adult humans, optimal wound healing involves the following the events: (1) rapid haemostasis; (2) appropriate inflammation; (3) mesenchymal cell differentiation, proliferation and migration to the wound site; (4) suitable angiogenesis; (5) prompt re-epithelialization (re-growth of epithelial tissue over the wound surface); and (6) proper...
synthesis, cross-linking and alignment of collagen to provide strength to the healing tissue [6–8].

In our previous study, we established a model of cutaneous *Candida albicans* infection in BALB/c mice to investigate disease progression, to understand the specific cellular and molecular components involved in immunity to *Candida*, and to determine the balance between pro- and anti-inflammatory cytokines over the course of infection [9]. In this study, we evaluated the interference of diabetic conditions in alloxan-induced diabetic mice in relation to the development of *C. albicans* infection, density of macrophages, distribution of collagen type I and III, the anti-inflammatory cytokines involved in the transition of M1 to M2 macrophages, and tissue repair.

**METHODS**

**Ethical approval**

The experimental protocols were approved by the Animal Research Ethics Committee of the State University of Londrina, Brazil (Approval number 188/12). All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

**Mice and induction of diabetes**

Female BALB/c mice were obtained from the State University of Maringá and housed five animals per cage in a temperature- and humidity-controlled room with a 12 h light/dark cycle. They received sterilized water and food *ad libitum*. BALB/c mice received a single dose of alloxan (Sigma Aldrich, St. Louis, MO, USA) via the tail vein at a dose of 65 mg kg\(^{-1}\) body weight [10]. All animals were 8 weeks of age at the start of the experiments. Mice were considered diabetic if their blood glucose was >250 mg dl\(^{-1}\). Blood glucose measurements were performed with an ACCU-CHEK Active test strip glucometer (Roche Diagnostics, Mannheim, Germany) using blood samples from the tail vein. Control mice were not treated with alloxan.

**C. albicans culture**

*C. albicans* strain 577 was isolated from the skin of a patient with mucocutaneous candidiasis and kindly provided by Dr Luiz Rodolfo Travassos, UNIFESP, São Paulo, Brazil. The pathogenicity of this strain was observed in several investigations by our group. Fungal cells were grown in 1% yeast extract, 2% peptone and 2% dextrose (YPD), at 28°C for 24 h. The cells collected by centrifugation were resuspended in YPD plus 10% foetal bovine serum (FBS) at a concentration of 5×10\(^6\) yeast cells ml\(^{-1}\) and incubated at 37°C for 2 h, to produce pseudohyphae, as described by Kagami *et al.* [11]. Microscopic examination confirmed that 85.7±3.8% of the cells of this strain converted to pseudohyphae.

**Cutaneous *C. albicans* infection and fungal burden**

Two main groups were used, one treated with alloxan and considered diabetics and another one made up of healthy animals (non-diabetic mice). Five mice per diabetic or healthy subgroup were inoculated with *C. albicans* pseudohyphae (5×10\(^6\)/50 µl\(^{-1}\) PBS) in the dermis of the hind paw, and PBS solution was injected into the contralateral paw as a negative control. After 1, 4, 7, 14 and 21 days, the sites of infected skin on the hind paw, on the non-infected hind contralateral paw and the popliteal lymph nodes were collected, weighed and macerated. Homogenates of the hind paw were diluted and 100 µl aliquots were plated on YPD growth medium to allow *Candida* colony growth. The plates were cultured for 24 h at 37°C and the number of c.f.u. per milligram of tissue was determined. Experiments were repeated at least three times. Homogenates of popliteal lymph nodes were then centrifuged, and the clear supernatants were collected and kept in the freezer at −20°C for later determination of cytokines according to a previous study [9].

**Periodic acid-Schiff staining of yeast and filamentous forms of *Candida***

Similar groups of mice as described above were evaluated histopathologically. The collected hind paw tissues were fixed in 10% formalin for 24 h and then subjected to histological processing for paraffin embedding. Serial histological 4 µm sections were stained with periodic acid-Schiff (PAS) staining because *Candida* species stain poorly with haematoxylin and eosiin. PAS staining is a histochemical technique used to stain polysaccharides and chitin present in the fungal cell wall. In this study, PAS staining was used to identify the morphological features of the fungus as well the localization and extension of infection. Small budding yeast or filamentous forms (pseudohyphae, hyphae) show strong cell wall PAS staining as pinkish red.

**Immunohistochemistry**

The protocols of Kagami *et al.* [11] were used for immunohistochemistry. Histological sections, 4 µm thick, of skin from the infected hind paw were deparaffinized in xylene and hydrated in a graded alcohol series. The sections were incubated in 10 mM citric acid (pH 6) at 95°C for 30 min for antigen retrieval, and endogenous peroxidase activity was quenched by treating sections with 3% hydrogen peroxide for 5 min at room temperature. Sections were blocked with 2% BSA, 0.5% Triton X-100 and 10% goat serum in PBS for 60 min at room temperature, followed by incubation with primary rat anti-F4/80 (Santa Cruz, SC-52664, 1:50) or rabbit anti-arginase I (Santa Cruz, SC-20150, 1:50), overnight at 4°C. Samples were washed and incubated at room temperature for 90 min with secondary peroxidase-conjugated goat anti-rat (Vector Laboratories PI-9400, 1:500) or peroxidase-conjugated goat anti-rabbit (Vector Laboratories PI-1000, 1:500), developed using the dianaminobenzidine substrate kit for peroxidase (eBioscience), counterstained with Harris’ haematoxylin and mounted with Permount. From each mouse, 30 microscopic fields of infected hind paw were captured using a high-resolution camera (Moticam 2500, 5.0 M pixel, USB 2.0) attached to a Axioshot Zeiss Axioshot light microscope (×63 immersion objective). Motic Images Plus 2.0 was used for analysis. We counted the total number of macrophages (F4/80\(^+\)) and M2
(arginase I') macrophages in each mouse during the development of the infection. The number of macrophages was expressed per mm².

Quantification of collagen
Histological sections, 4 µm thick, of skin from the infected hind paw were deparaffinized in xylene and hydrated with a graded alcohol series. The sections were stained by Picrosirius red staining for quantification of collagen types I and III using a polarized light microscope. Accordingly, 10 images from each mouse were captured using an AxioCam high-resolution camera (Carl Zeiss, Jena, Germany) attached to an Axioscop Plus light microscope (Carl Zeiss, Jena, Germany; ×40 objective) using the software AxioVisionRel 4.1. The images were evaluated with the software Image-Pro Plus 4.5 to obtain the number of pixels of yellow and red fibres (type I collagen) and green fibres (type III collagen). Results were expressed as percentages (%).

Cytokine analysis
To determine the concentration of anti-inflammatory cytokines (IL-4, IL-10, IL-13 and TGF-β), the supernatants of the homogenates of popliteal lymph nodes of infected and control mice described above were submitted to capture ELISA procedures (eBioscience, San Diego, CA). The cytokine quantification assays were performed in accordance with the manufacturer’s instructions.

Statistical analysis
Initially, all data were analysed to observe the distribution type. As they presented normal distribution, the number of macrophages (F4/80 and M2) and quantification of collagen were compared between diabetic and control groups performing an unpaired sample Student’s t-test. For cytokine analysis, the differences between groups were analysed using an unpaired Student’s t-test, except when the time post-infection (p.i.) was a variable, where one-way ANOVA was applied. P<0.05 was considered statistically significant.

RESULTS
Monitoring of blood glucose during infection
The mice were treated with alloxan to induce diabetes conditions with selective destruction of insulin-producing pancreatic cells in the islets of Langerhans. Samples of blood from the tail vein of the control mouse group (non-diabetic) showed a blood glucose level of 98.2±7.4 mg dl⁻¹, whereas the alloxan-induced diabetic group showed a significantly increased level of 272.8±19.3 mg dl⁻¹ at day one post-treatment. Control mice were considered immunocompetent. The glucose level of each mouse was measured immediately prior to euthanasia, in order to confirm the diabetic conditions over the course of infection.

C. albicans fungal burden
Diabetic mice infected with C. albicans showed a higher fungal burden (Fig. 1b) with predominance of filamentous forms (pseudohyphae and hyphae) compared to non-diabetic mice whose dermis had a predominance of yeast cells 1 day p.i., as observed in Fig. 1(a). From the fourth day p.i., the injured tissue in the dermis of diabetic mice displayed rupture of tissue and disorganizations of extra cellular matrix up to day 14 (Fig. 1d, f and h). These results suggested an impaired ability to fight infection caused by alloxan treatment, making the diabetic mice largely unable to mount an adequate inflammatory response. The elimination of pathogens occurred earlier in non-diabetic mice at 7 days p.i., and tissue organization was little affected (Fig. 1c, e, g and i). C.f.u. were determined to confirm the clearance of C. albicans infection. Fig. 2 shows C. albicans present up to day 14 in skin of diabetic mice, while non-diabetic mice were able to eliminate the pathogen between 4 and 7 days. Candida growth was not observed in the contralateral paw (control), indicating no fungal dissemination from the infected paw.

Population density of F4/80 and M2 macrophages
To consider the possibility of diabetic mice showing disturbances in the functions or number of macrophages, the next step was to study the density of macrophages at the infection site using F4/80 as marker. We observed F4/80 macrophages to be significantly reduced in diabetic mice compared to non-diabetic mice at day four p.i. (Fig. 3a, b and e; P<0.05). In addition, the activation of the alternative pathway to M2 was hindered, since the population of macrophages marked with anti-arginase I was significantly reduced between 14 and 21 days p.i. (Fig. 3c, d and f; P<0.001).

Analysis of anti-inflammatory cytokines during infection
To investigate any deregulation of M1 and M2 macrophage polarization in alloxan-induced diabetes, the profile of anti-inflammatory cytokines was determined. The IL-4 level was significantly reduced in the diabetic group at day 4 p.i., compared with non-diabetic controls (Fig. 4a; P<0.001), whilst IL-13 was produced at a later time point by diabetic mice (Fig. 4b). Similarly, a delayed rise in transforming growth factor-beta 1 (TGF-β1) was evident in diabetic mice versus non-diabetic controls, occurring only in the final phase of infection at day 21 (Fig. 4c). Insignificant IL-10 levels was observed in diabetic groups over the course of infection (Fig. 4d; P<0.001).

Analysis of collagen type I and III in skin from infected mice
Type I collagen was slightly affected in the early phase of infection but there was critical reduction between days 7 and 21 in diabetic mice (Fig. 5a, c and e). In addition, the infected tissue in diabetic mice had more type III collagen between days 7 and 21 (Fig. 5b, d and f). These results are associated with the disorganized tissue observed in diabetic mice, which exhibited a higher quantity of filamentous forms of C. albicans (Fig. 1d, f and h).

DISCUSSION
Previously, our group investigated the disease progression of chronic mucocutaneous candidiasis (CMC), to understand
specific cellular and molecular components involved in immunity to C. albicans during the course of cutaneous infection in immunocompetent BALB/c mice [9]. Several investigations by our group with strain 577 have demonstrated its high virulence when inoculated by the peritoneal route in mice leading to death within 24–48 h of infection [12]. Mice of the same age, when protected with pretreatment with concanavalin-A, survived due to an increase in innate and cellular immunity by an increase in mannose receptor activity and greater TNF-α production, making them better endowed to kill ingested C. albicans. The pathogenicity of strain 577 was also demonstrated in studies of phagocytosis with yeast co-incubated with macrophages in Roswell Park Memorial Institute medium (RPMI) for 2 h, where there was 72.6±2.3 % transition to filamentous forms. The capacity of macrophages to kill C. albicans 577 was only 30 % and therefore 70 % remained viable [13]. Since strain 577 was isolated from the skin of a patient with cutaneous candidiasis and given its high virulence, we considered it important to test it in our model. Therefore, strain 577 was injected into the hind paw of healthy non-diabetic mice and alloxan-induced diabetic mice to study the changes that occurred due to diabetes induced by alloxan, especially in relation to infection with C. albicans and tissue repair.

Although strain 577 was inoculated as pseudohyphae, there was a predominance of yeast cells at day 1 p.i. in the dermis of non-diabetic mice. On the other hand, a higher fungal burden and filamentous forms were observed on day 1 p.i. in diabetic mice (Fig. 1). These results suggest that alloxan-induced diabetes caused impaired migration and activation of macrophages by IFN-γ deficiency as has been reported by van der Graaff et al. [14, 15] in some CMC patients. By comparison between the two groups, it was possible to observe greater injury in infected tissue of diabetic than non-diabetic mice over development of the infection. Our group has observed a significant decrease in IFN-γ and IL-17A production in diabetic mice compared to control mice (data not shown), suggesting that diabetic conditions interfered with this process. Figure 2 confirms delayed clearance of C. albicans by diabetic mice, with implications for the understanding of the nature of the immune response to this infection. According to Kagami et al. [11] mice deficient in IL-17A demonstrated delayed healing after skin infection with C. albicans, compared with wild-type mice.
which corroborates the results of the present study. Such data indicate that prolonged uncontrolled diabetes can facilitate *C. albicans* infection, including in alloxan-induced diabetic rats [16].

To understand the participation of macrophages during the development of *C. albicans* infection we used an antibody against F4/80 antigen, which is present in the cell membrane of macrophages, to evaluate total population of macrophages and anti-arginase antibody as marker for M2 macrophages were used. F4/80 macrophages at 21 days p.i. (arrowhead) (a, b). M2 macrophages at 21 days p.i. (arrowhead) (c, d). absolute number of F4/80 and M2 macrophages during the infection process (e, f). Statistical significance was determined using Student’s t-test, *P*<0.05; **P**<0.01; ***P***<0.001.

As expected, the presence of M2 macrophages in diabetic mice was significantly lower at 14 and 21 days p.i., compared to non-diabetic mice. M2 macrophages are defined by their expression of specific gene products and play an important role in containing inflammation, removing apoptotic cells and repairing damaged tissue [20]. The M2 macrophage phenotype is also characterized by efficient phagocytic activity, including as induced by mannose receptor and macrophage scavenger receptor activation [21], which leads to an increase in TGF-β1 [22]. The decreased

---

**Fig. 3.** Population density of F4/80 and M2 macrophages in non-diabetic and diabetic mice infected with *C. albicans*. Antibody to F4/80 antigen, which is present in the cell membrane of macrophages, to evaluate total population of macrophages and anti-arginase antibody as marker for M2 macrophages were used. F4/80 macrophages at 21 days p.i. (arrowhead) (a, b). M2 macrophages at 21 days p.i. (arrowhead) (c, d). absolute number of F4/80 and M2 macrophages during the infection process (e, f). Statistical significance was determined using Student’s t-test, *P*<0.05; **P**<0.01; ***P***<0.001.
levels of M2 macrophages in diabetic mice indicates a lower restorative capacity.

IL-4 and IL-13 cytokines regulate the transition of M1 to M2 macrophages [23], with IL-4 production significantly decreased in diabetic mice, compared to non-diabetic, in the current study. Meanwhile, IL-13 showed a delay in its production, thereby also hindering tissue repair mechanisms in diabetic mice. Such data are relevant to the frequent presence of ulcers in CMC patients studied by Papini et al. [4]. Secreted aspartyl proteases (SAPs) have been demonstrated in C. albicans in filamentous forms (pseudohyphae or hyphae), mainly SAP6, which enables this fungus to invade tissues [24, 25]. Due to fungus persistence at infection sites being predominantly in filamentous forms, tissue damage may have occurred, at least in part by the participation of SAPs, as well as the consequences of a suboptimal immune response. The proposed functions of these proteinases during infection include the digestion of host proteins for nutrient supply, evasion of host defences by degrading immunoglobulins and complement proteins, as well as their impact via their adherence and degradation of host barriers during invasion [26].

TGF-β1, a cytokine produced by M2 macrophages, has several functions including the proliferation of fibroblasts, which is a cell that is important in collagen production and wound healing [20, 27]. In this work, diabetic mice produced TGF-β1 at lower levels than non-diabetic mice, as would be expected by their reduced density of M2 macrophages (Fig. 3). In addition, IL-10 production was insignificant in diabetic mice (Fig. 4), thereby favouring the destruction of C. albicans-infected tissue, and attenuating tissue repair. Importantly, after elimination of pathogen prior to day 14, TGF-β1 production increased over the following week. Such data indicate that TGF-β1 induces fibroblasts to increase collagen type III, which is essential to re-establishing tissue structure destroyed during infection (Fig. 5). Accord to Liu et al. [28], collagen type III is required for the production of collagen type I, which was also partially destroyed by infection in diabetic mice. Other data indicate an important role for type III collagen in the reconstruction of damaged tissue [29], as demonstrated in this study.

In conclusion, alloxan-induced diabetes leads to alterations in the production of anti-inflammatory cytokines, including suboptimal IL-4, IL-13 and TGF-β1, compared to non-diabetic mice. Thus, the reduced number of F4/80-induced macrophages, especially the M2 macrophage phenotype, hampered pathogen elimination and therefore tissue repair functions. There was a significant loss of collagen type I and
consequently higher production of collagen type III, due to tissue destruction caused by *C. albicans* infection. Such pre-clinical data highlight important alterations in immune responsivity in CMC patients, with consequences for injury and tissue repair in diabetic patients.

**Acknowledgements**

This study was supported by the Fundação Araucaria (298/2012), Paraná, Brazil and Coordination for the Improvement of Higher Education Personnel (CAPES). Dr A. Leyva helped with English editing of the manuscript.

**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**References**


---

**Fig. 5.** Collagen profile of skin of non-diabetic and diabetic mice infected with *C. albicans*. Collagen fibres under an unpolarized light microscope at 21 days p.i. (a, b). Observe the few collagen fibres in diabetic mice. Collagen fibres observed under polarized light microscope at 21 days p.i. (c, d). Observe the few collagen type I fibres stained in red (arrow) and higher number of collagen type III fibres stained in green (arrow). Relative number of collagen type I and III observed during the infection process (e, f). Stain: Picrosirius red. Scale bar, 25 µm. Statistical significance was determined using Student’s t-test. *P*<0.05; **P**<0.01; .


---

*Five reasons to publish your next article with a Microbiology Society journal*

1. The Microbiology Society is a not-for-profit organization.
2. We offer fast and rigorous peer review – average time to first decision is 4–6 weeks.
3. Our journals have a global readership with subscriptions held in research institutions around the world.
4. 80% of our authors rate our submission process as ‘excellent’ or ‘very good’.
5. Your article will be published on an interactive journal platform with advanced metrics.

Find out more and submit your article at microbiologyresearch.org.