Institut Pasteur
Microbes and Infection 16 (2014) 678–689
www.elsevier.com/locate/micinf

Original article

Involvement of IL-17A in preventing the development of deep-seated candidiasis from oropharyngeal infection

Paolo Mosci [a,1], Elena Gabrielli [b,1], Eugenio Luciano [b], Stefano Perito [b], Antonio Cassone [b], Eva Pericolini [b], Anna Vecchiarelli [b,*]

[a] *Internal Medicine Section, Department of Veterinary Medicine, University of Perugia, Perugia, Italy*
[b] *Microbiology Section, Department of Experimental Medicine, University of Perugia, Perugia, Italy*

Received 22 January 2014; accepted 16 June 2014
Available online 27 June 2014

Abstract

In this study we show that corticosteroid-treated *Il17a*$^{-/-}$ mice develop invasive candidiasis from oropharyngeal infection whereas WT mice do not. By using an established murine model of oral candidiasis we document the spatial and temporal progression of fungal infection. The histological analysis of tissues in *Il17a*$^{-/-}$ mice showed massive infiltration of the fungus in the stomach and alterations of the gastrointestinal tract segments. Both increased permeability and mucosal ulcerations of the intestinal barrier are seen to favor *Candida albicans* dissemination which was quantified both in kidney and liver where typical candidal abscesses were detected. Neutrophils from *Il17a*$^{-/-}$ were as capable of phagocytosing the fungus comparable to that of WT mice, however, they showed decreased candidacidal ability. Our data implies that IL-17A is crucial for preventing the passage from mucosal to disseminated candidiasis. As such, our model may be suitable to study the mechanisms favoring *C. albicans* translocation to internal organs.

Keywords: OPC; Real-time; Bioluminescence; Imaging; Longitudinal study; *Candida albicans*

1. Introduction

Candida albicans (*C. albicans*), one of the most relevant fungal pathogens, causes different types of superficial infections including oral and vaginal candidiasis, and can disseminate systemically leading to high rate of morbidity and mortality [1]. Compelling evidence shows that the oral cavity can be the site of origin for dissemination of pathogenic microorganisms to distant body sites, particularly in immunocompromised hosts, and *C. albicans* makes no exception [2,3]. Interleukin (IL)-17A is considered a key cytokine for host defense against fungal infections in several experimental models including mucosal and systemic candidiasis [4-6]. In a recent study, allelic variations of genes of the IL-17 pathway have been associated to chronic mucocutaneous candidiasis [7]. It is well established that Th17 as well as IL-17RA receptor are critical in the defense against oropharyngeal candidiasis (OPC), in particular research by Gaffen's group unequivocally demonstrated that Th17 cells secreting IL-17A play a key role in OPC, while Th1 cells and IL-22 seem to play a minor role [8].

More recently, the same research group reported that Th17 cells are also important in conferring long-term adaptive immunity to OPC [9]. Despite all this important information above, the mechanism that leads to impairment of antifungal immunity in animals lacking functional Th17 cells or unable to secrete IL-17A is not fully clarified. Several hypothesis have been advanced including the inhibition of neutrophils recruitment and lack of gene expression of chemokines and GM-CSF [10,11]. Despite the importance of IL-17A in the

* Corresponding author. Microbiology Section, Department of Experimental Medicine, S. Maria della Misericordia Hospital, University of Perugia, Polo Unico Sant' Andrea delle Fratte, 06132 Perugia, Italy. Tel.: +39 075 585 8369.
E-mail address: anna.vecchiarelli@unipg.it (A. Vecchiarelli).
[1] PM and EG contributed equally to the paper.

http://dx.doi.org/10.1016/j.micinf.2014.06.007
1286-4579/

control of the infectious process at local level, no information exists about the role of IL-17A in preventing systemic candidiasis from mucosal infection.

In the present study, based on real-time in vivo imaging technique, we used a recently established model of OPC [12] to evaluate the role of IL-17A in preventing the translocation of *C. albicans* from local to systemic compartment.

2. Materials and methods

2.1. C. albicans strain and culture

C. albicans CA1398 carrying the *ACT1p-gLUC59* fusion (gLUC59) was used [13]. The *gLUC59* luciferase reporter has previously been described [13]. *C. albicans* gLUC59 was cultured in YPD (yeast peptone dextrose) as described by Solis et al. [14].

2.2. Ethics statement

All animal experiments were performed in agreement with the EU Directive 2010/63, the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes, and the National Law 116/92. The protocol was approved by Perugia University Ethics Committee for animal care and use (Comitato Universitario di Bioetica, permit number 149/2009-B). All the animals were housed in the animal facility of the University of Perugia (Authorization number 34/2003A).

2.3. Mouse model of OPC

Female, 6–8 weeks old, inbred C57BL/6J mice (WT) (Harlan Nossan Laboratories, Milan, Italy) and C57BL/6J knock out for IL-17A isoform, homozygote (*Il17a*$^{-/-}$) mice [15,16] (Center for Experimental Medicine and Systems Biology, University of Tokio, Minato-ku, Tokio, Japan) were housed at the Animal Facilities of the University of Perugia. Mice were treated with 225 mg/kg cortisone acetate (Sigma–Aldrich) and infected with 1×10^6/ml *C. albicans* suspension as previously described [14] under anesthesia with a subcutaneous (s.c.) injection of a mixture of Tiletamine/Zolazepam-Xylazine (50 mg/kg-5 mg/kg) [12]. The same infection was also performed in corticosteroid-untreated mice. The oral cavity was swabbed immediately before the infection and streaked on YPD agar plus chloramphenicol (50 μg/ml) (both from Sigma–Aldrich) to verify the absence of *Candida* spp. In selected experiments gut samples obtained before and after infection have been plated onto CHROMagar™ *Candida* medium (CHROMagar, Paris, France), a cromogenich medium which selects colonies of different *Candida* species based on the colony color. The *Il17a*$^{-/-}$ status of the mice was confirmed by using a specific ELISA kit detecting the presence of IL-17A/F heterodimers (eBioscience, Inc.). Moreover, the IL-17B levels by specific ELISA kit was also evaluated (eBioscience, Inc.). No IL-17A/F heterodimers were observed in serum samples of *Il17a*$^{-/-}$ mice pre and post-infection while IL-17B isoform was produced to similar level in both WT and *Il17a*$^{-/-}$ mice.

2.4. Real-time monitoring of OPC

At selected days, starting on day 1 after challenge, 10 μl (0.5 mg/ml in 1:10 methanol:H_2O) of coelenterazine (Synchem, OHM) was added sublingually. Mice were then imaged in the IVIS-200TM Imaging system (Xenogen Inc.) under s.c. anesthesia and then the total photon emission from oral areas within the images (Region Of Interest, ROI) was quantified as previously described [12]. No background luminescence was observed in uninfected mice treated with coelenterazine (data not shown). In selected experiments an ex vivo analysis of pharynx, esophagus and stomach from mice with OPC was performed after 3, 6 and 8 days post-infection as previously described [12]. After 8 days post-infection an ex vivo analysis of liver and kidneys of mice with OPC was performed. Briefly, liver and kidneys were excised from euthanized mice, the latter were dissected and then both soaked with 10 μl (0.5 mg/ml) of coelenterazine (Synchem) to visualize the fungal burden as above described.

2.5. CFU assay

The fungal burden of the tongue, esophagus, stomach, liver, duodenum, ileum and kidneys 3, 6 and 8 days post-infection was evaluated as previously described [12]. In selected experiments gut samples have been also plated onto CHROMagar™ *Candida* medium (CHROMagar, Paris, France). Moreover, to analyze whether *C. albicans* was present in the peritoneal cavity of WT or *Il17a*$^{-/-}$ mice, the peritoneal cavity from both mice was washed with 5 ml RPMI-1640 8 days post-infection. The washes were centrifuged, resuspended with 1 ml of RPMI-1640 and then plated onto CHROMagar™ *Candida* medium (CHROMagar). No *Candida* species were recovered. The disease severity was also evaluated by monitoring individual mice for weight loss.

2.6. Candidacidal assays

After 6 days of OPC infection, peritoneal murine neutrophils from WT or *Il17a*$^{-/-}$ mice were collected 18 h after the intraperitoneal injection of 0.5 ml endotoxin-free 10% thioglycolate solution (Difco). Neutrophils (4×10^6/ml) were incubated in the presence or absence of recombinant mouse IL-17A (rIL-17A) (100 ng/ml, eBioscience) for 30 min at 37 °C plus 5% CO_2 in RPMI-1640, then washed twice and resuspended in RPMI-1640. The oxidative burst of neutrophils was carried out by labeling cells (4×10^6/ml) with 1 μM of 2',7'-dichlorofluoresceindiacetate (DCFH-DA) for 30 min at room temperature. Cells were then incubated with PMA (Sigma–Aldrich) (100 ng/ml) or *C. albicans* gLUC59 cells (2×10^6/ml) into a black 96 wells plates (Nunc) and then the emission of fluorescence was measured in the Tecan plate reader at 25 kinetic cycles and interval time of 5 min.

Killing activity of neutrophils, treated as above described, was determined by CFU inhibition assay. Briefly, neutrophils (10^5 cells) in 0.1 ml suspension/well were incubated in flat-bottom 96-well microtitre tissue culture plates with 10^4 cells *C. albicans* gLUC59 in 0.1 ml RPMI plus 5% FCS and incubated for 2 h at 37 °C plus 5% CO_2. After incubation, plates were vigorously shaken and cells were lysed by adding Triton X-100 (0.1% in distilled water; final concentration in the well 0.01%). Serial dilutions were prepared in distilled water from each well. The samples were then spread on Sabouraud dextrose agar plus chloramphenicol (50 μg/ml) in triplicate and CFU values were evaluated after 24 h of incubation at 37 °C. Control coltures consisted of *C. albicans* gLUC59 incubated in RPMI-1640 plus 5% FCS without effector cells. In selected experiments, 90 μl of freshly collected murine saliva, recovered after injection of 0.2 ml pilocarpine (0.5 mg/kg, s.c. injection) [17], from uninfected or infected mice (day +6), was incubated with 10^4 cells of *C. albicans* gLUC59 for 1 h at 37 °C, plated in triplicate and assayed for colony enumeration [8]. Killing activity was expressed as the percentage of CFU inhibition according to the following formula: % killing activity = 100 − (CFU experimental/CFU control) × 100.

To test the phagocytic capacity of cells, neutrophils (1×10^5/200 μl), after stimulation with *C. albicans* gLUC59 (2×10^5/200 μl), were collected by cytospin (700 g for 7 min) and stained by Hemacolor. The fungal cell internalization was expressed according to the following formula: percentage of internalization = number of cells containing one or more fungal cells/100 cells counted.

To provide information about the proportion of neutrophils in the cellular preparation from the peritoneal cavity of thioglycollate-injected WT and *Il17a*$^{-/-}$ infected mice, cells were fixed with 1.5% formalin, washed, reacted with FITC-conjugated monoclonal antibody (mAb) to Ly-6G (Gr-1) (0.05 μg/test, Rat $IgG_{2b,\ kappa}$, eBioscience, Inc.) for 20 min at room temperature in the dark. After incubation, cells were washed twice with 1X PBS plus 1% FCS and 0.5% NaN_3 2M (fluorescence buffer (FB)), resuspended in 0.5 ml of FB and then analyzed by flow cytometry using FACSCalibur (Becton Dickinson). Data are expressed as percentage of Gr-1 positive cells. Autofluorescence was assessed using untreated cells. Control staining of cells with irrelevant antibody was used to obtain background fluorescence values.

2.7. Histological analysis

Eight days post-infection the animals were sacrificed to analyze gross and histopathologic lesions and tongue, esophagus, stomach, small intestine, liver and kidney tissues were excised. The macroscopic lesions were digitally photographed (Sony Mavica MVC-CD400). Then the tissues were fixed immediately in 10% formalin, then embedded in paraffin. The tongues and stomachs were sectioned longitudinally to verify the extension of the lesions, the esophagus, small intestine, liver and kidneys were sectioned transversally. The 3–5 μm thick sections were stained using the periodic acid-Schiff (PAS) procedure to visualize fungi, and examined by light microscopy (Leica DM2500). The scale bars are in μm.

2.8. Statistical analysis

The data are reported as the mean ± s.e.m. from triplicate samples of three-five experiments. The photon flux emission was compared using Student's t test. CFU counts, weight loss, phagocytic and killing activities of neutrophils were compared using Mann–Whitney U test. A value of $p < 0.05$ was considered significant.

3. Results

3.1. Real-time monitoring of OPC in *Il17a*$^{-/-}$ mice

Firstly, we determined the course of OPC in corticosteroid-treated *Il17a*$^{-/-}$ mice compared with corticosteroid-treated but *Il17a* competent counterpart (WT). To this end, mice were treated subcutaneously with cortisone acetate every two days starting 1 day before infection. We exploited a new in vivo imaging technique that we recently validated [12]. This allows real-time monitoring of the spatial and temporal progression of infection. Briefly, the mice were sublingually infected with blastospores of *C. albicans* and the course of candidiasis was monitored 1, 3, 6 and 8 days after challenge. In Fig. 1 are reported the results of the real-time monitoring of infection. The *Il17a*$^{-/-}$ mice showed a marked increase of susceptibility to infection as compared to the WT animals, as visually evident (Fig. 1A), and as measured by total photon emission (Fig. 1B). Significant increase of luminescence signals, obtained following administration of the luciferase substrate coelenterazine in the oral cavity in *Il17a*$^{-/-}$ mice, was already observed 3 days after challenge. A dramatic increase was manifested in subsequent days (day +6 and day +8). The WT mice developed an appreciable degree of infection only on day +6 to day +8 (Fig. 1). At day +8, all animals were humanely sacrificed.

Macroscopic analysis of tongue and esophagus showed *C. albicans* marked alteration in *Il17a*$^{-/-}$ mice with respect to WT at day +8 post-infection (Fig. 2A).

Fungal load was also assessed by ex vivo bioluminescence emission of explanted esophagus and stomach. No apparent bioluminescence was manifested 3 days after infection in *Il17a*$^{-/-}$ and WT mice counterpart. However, clear and intense signals from both organs of *Il17a*$^{-/-}$ mice were detected after 6 days, and a very strong signal was manifested when the monitoring was performed after 8 days, in keeping with in vivo data (Fig. 2B).

The fungal load in various organs was also monitored by CFU counts in tongue, esophagus, stomach, liver, duodenum, ileum and kidneys. The results reported in Fig. 3A show that the fungal load was significantly increased in tongue, esophagus and stomach of *Il17a*$^{-/-}$ mice as compared to WT mice. This difference was detected 6 days post-infection and reached a maximum 8 days after challenge. As previously observed [12], in WT mice an increase of fungal load was observed 8 days post-infection [12]. Surprisingly, in the duodenum and

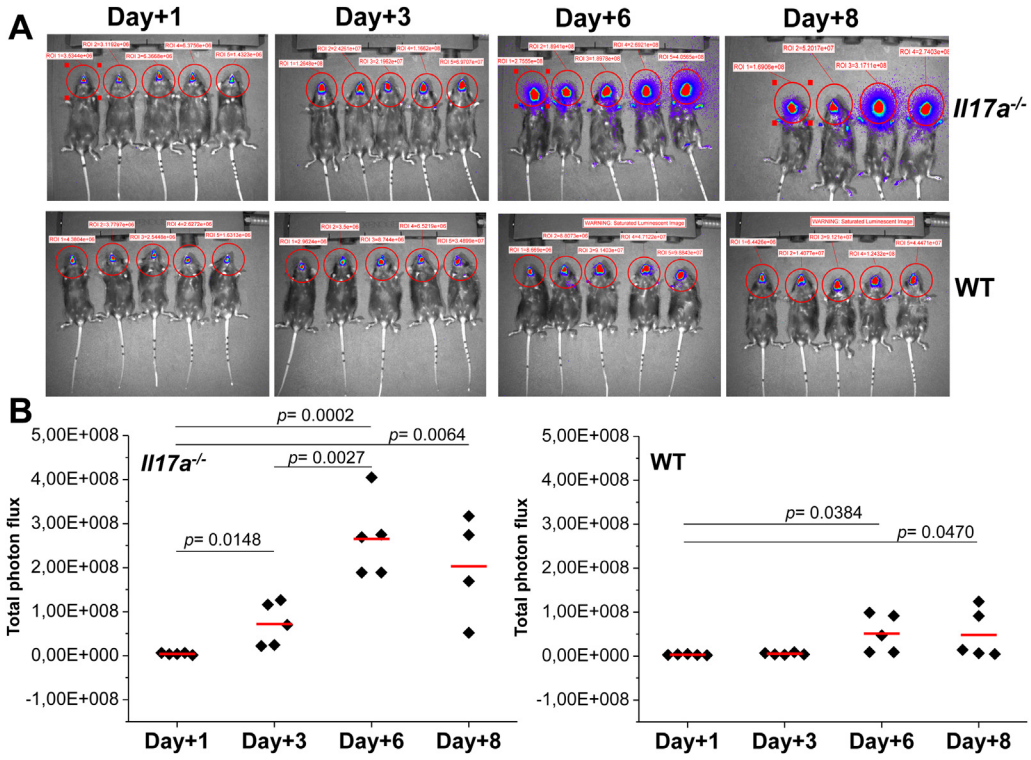

Fig. 1. **In vivo imaging of OPC**. *Il17a*$^{-/-}$ and WT mice were infected with *C. albicans* gLUC59 (1×10^6/ml). 1, 3, 6 and 8 days post-infection anesthetized mice were treated sublingually with 10 µl of coelenterazine (0.5 mg/ml) and imaged in the IVIS-200TM Imaging system. Data are from one of three experiments with similar results. Total photon flux from oral areas in the images (ROI) of each mouse was quantified with Living ImageR software package (A). The statistical significance of Total photon flux from ROI was evaluated with the Student's t test. A value of $p < 0.05$ was considered significant (day +3, +6 and +8 post challenge *vs* day +1; day +6 post challenge *vs* day +3) (B).

ileum CFU recovery after 3, 6 and 8 days post-infection was similar in WT and *Il17a*$^{-/-}$ mice (Fig. 3D). It has been recently reported that the mouse gut contains plenty of fungi, with another *Candida* species [18]. In our experimental system, species of *Candida* other than *albicans* were not detected (data not shown). When the CFU was monitored in the liver (Fig. 3B, left panel) and in the kidneys (Fig. 3C, left panel) significant differences were observed. In particular, a progressive increase of *Candida* load was detected in both organs of *Il17a*$^{-/-}$ mice while no fungus presence was detected in WT mice. The presence of fungal cells in liver and kidneys exclusively in *Il17a*$^{-/-}$ mice was also verified by bioluminescence in an ex vivo analysis (Figs. 3B and C, right panels). Fungal invasion of internal organs in *Il17a*$^{-/-}$ mice was associated with a rapid weight loss starting 6 days after challenge (Fig. 3E). Notably, the WT mice did not show such rather relevant clinical sign, and only at late time (day +8) there was a significant weight loss, though much smaller than the weight loss of *Il17a*$^{-/-}$ mice (Fig. 3E). In our experimental system *Il17a*$^{-/-}$ mice without steroid treatment did not develop systemic candidiasis from local infection (unpublished data).

3.2. Histological analysis

In order to characterize the lesions associated with the OPC, we performed a histological analysis of tongue, esophagus, stomach, small intestine, liver and kidney on day 8 post-infection (Figs. 4 and 5).

The longitudinal sections of the tongue of *Il17a*$^{-/-}$ mice showed a massive fungal colonization of whole dorsal layer of the organ with the formation of pseudomembranous plaques. Cluster of fungi and few inflammatory cells were also present in the epithelium of ventral surface of the tongue. The dorsal papillary architecture appeared to be totally destroyed with loss of the keratinized superficial epithelium (Fig. 4A). Large and extensive erosions, at places terminating in ulcerations, were also evident in the central region of the tongue, in association with massive infiltration of inflammatory cells and presence of some invasive fungal hyphae and blastospores, eventually also penetrating the muscular tissue of the organ.

Differently from *Il17a*$^{-/-}$ mice, the fungal invasion of the tongue of WT mice was much less severe and limited to a portion of the surface with formation of similar pseudomembranes. The mucosa surrounding the fungal burden was intact in all of WT mice. The fungi and inflammatory cells appeared to be confined to the keratinized layer of the tongue with microabscesses formation (Fig. 4A).

In the esophageal sections of the *Il17a*$^{-/-}$ mice the lumen of the organ appeared enlarged and completely obstructed by massive burdens of fungal, inflammatory neutrophils and desquamated epithelial cells. The mucosa showed pseudomembranes, diffuse fungal and inflammatory cells invasion, severe erosion with ulcers deranging the spinous layer, with

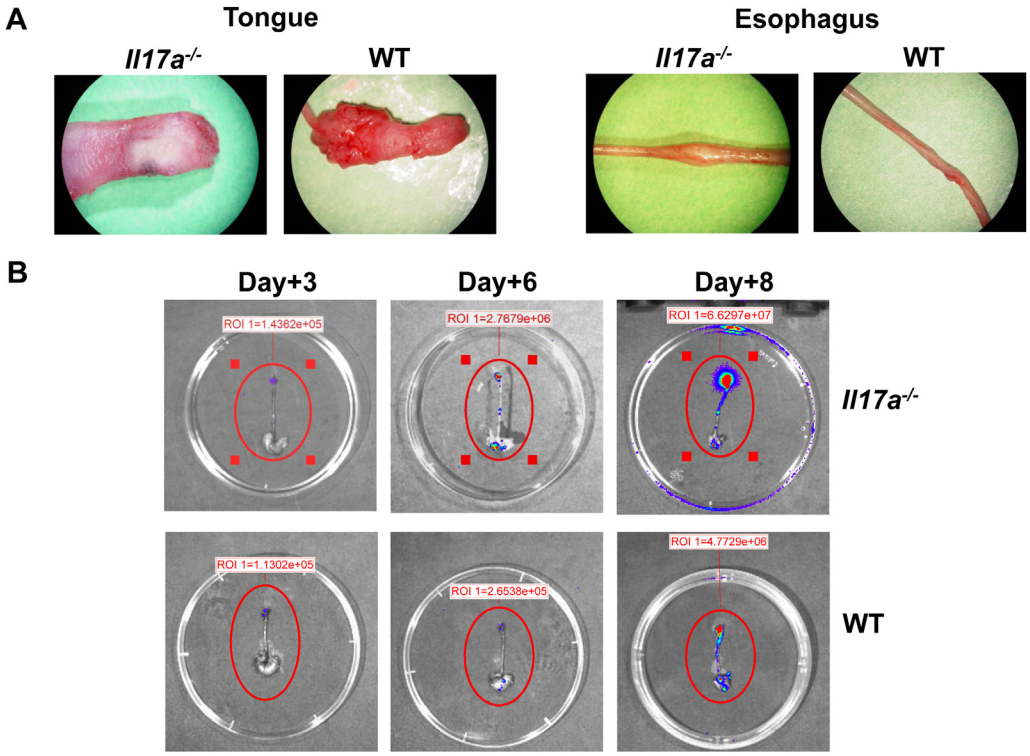

Fig. 2. **Analysis of target organs**. Microscopic analysis of tongue and esophagus recovered after 8 days of infection from *Il17a*$^{-/-}$ and WT mice with OPC was shown (A). Ex vivo analysis of infected pharynx, esophagus and stomach from *Il17a*$^{-/-}$ and WT mice with OPC was shown. 3, 6 and 8 days post-infection, mice were euthanized, gastric tracts were excised and 10 μl of coelenterazine (0.5 mg/ml) were injected through the pharynx into esophagus lumen to visualize the fungal burden and localization by the IVIS-200TM Imaging system. Data are from one of three experiments with similar results. ROI was quantified with Living ImageR software package (B).

muscular edema. At places, the inflammatory infiltrates were seen to surround the invading fungal cells so as to suggest the formation of an inflammatory barrier (Fig. 4B).

In the esophagus of WT mice the few fungal burden and the inflammatory cells appeared confined to a superficial layer of the mucosa without involvement of the spinous layer. The organ doesn't appear enlarged and results moderated obstructed by few hyphae and inflammatory cells (Fig. 4B).

In the stomach section of *Il17a*$^{-/-}$ mice a severe diffuse hyperplasia and hyperkeratosis of the whole forestomach were observed. Moreover, the fungal cells were localized in the forestomach, cardium atrial fold (CAF) and adjacent glandular mucosal surface and in this area an extensive erosion was evident. Hyphae and inflammatory cells, neutrophils and mononucleated cells, penetrated beyond the epithelium deeply within lamina propria. On the contrary, in the forestomach, fungal cells remain localized to the keratinized layer accompanied by a mononuclear cell infiltrate and the reaction doesn't involve the underlying spinous layer (Fig. 4C).

In the stomach of the WT mice a similar hyperplastic reaction was observed while fungal burden or inflammatory reaction were not detected either in the non glandular area or in the glandular one. Mucosal erosion, fungal and inflammatory infiltrate were evident in the portion of stomach cardia (Fig. 4C).

Histological section of the small intestine of *Il17a*$^{-/-}$ mice showed a severe mucosal damage with the loss of the entire intestinal epithelium, vascular congestion and edema, and the intestinal villi appeared almost completely destroyed. No gross damage was observed in WT mice which had totally preserved intestinal villi (Fig. 5A). Finally only in *Il17a*$^{-/-}$ mice an involvement of liver and kidneys was observed. In particular, abscesses characterized by a necrotic central area surrounded by a mass of fungal elements containing both blastospores and hyphae, and an infiltration of neutrophils and mononucleated cells were detected in the liver and kidneys of *Il17a*$^{-/-}$ mice. The colonization of liver and kidneys in these mice evidences the disseminated infection (Fig. 3B and C and Fig. 5B and C). No lesions or/and fungi were found in the liver and kidneys of WT mice (Fig. 5B and C).

3.3. Candidacidal assays

There is rather compelling evidence that neutrophils play a fundamental role in the defense against invasive candidiasis [19]. Since the histological observations reported above showed the presence of neutrophils-containing inflammatory infiltrates at the various sites of the gastrointestinal tract in *Il17a*$^{-/-}$ mice, we wondered about the functional capacity of neutrophils from *Il17a*$^{-/-}$ mice. Thus, we assayed the peritoneal neutrophils candidacidal activity of uninfected and infected mice (day 0 and day +6 after challenge). The percentage of Gr-1 positive cells evaluated in the cellular preparation obtained from both infected groups after thioglycolate treatment was comparable (≥90% in both groups).

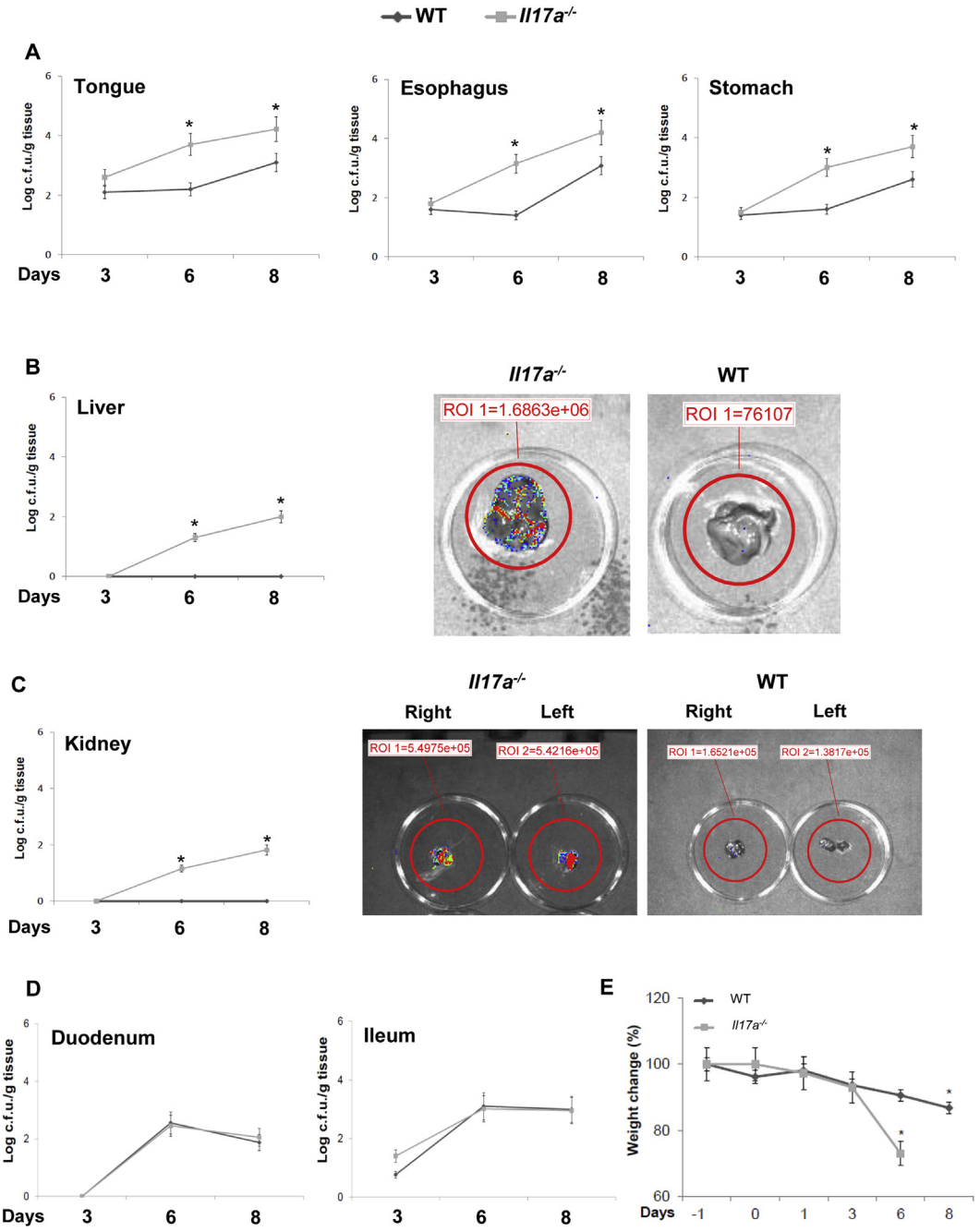

Fig. 3. **Fungal burden in target organs and effect of infection in body weight**. Fungal burden of WT and *Il17a*$^{-/-}$ mice with OPC was evaluated 3, 6 and 8 days post-infection in tongue, esophagus and stomach (A), in liver (B, left panel), kidney (C, left panel), duodenum and ileum (D) *$p < 0.05$ (*Il17a*$^{-/-}$ *vs* WT mice) according to Mann–Whitney U test. Ex vivo analysis of infected liver and kidneys from *Il17a*$^{-/-}$ and WT mice with OPC was shown. After 8 days post-infection mice were euthanized, liver and kidneys were excised, dissected and then soaked with 10 µl of coelenterazine (0.5 mg/ml) to visualize the fungal burden using the IVIS-200TM Imaging system. Data are from one of three experiments with similar results. ROI was quantified with Living ImageR software package (B and C, right panels). Time course of weight change (%) in cortisone acetate treated WT and *Il17a*$^{-/-}$ mice with OPC was shown (E). *$p < 0.05$ (day +6 and day +8 post challenge *vs* day −1) according to Mann–Whitney U test.

As shown in Fig. 6C (left panel), neutrophils from *Il17a*$^{-/-}$ mice expressed similar levels of phagocytic activity as compared to that from WT mice (day 0 and day +6); however, after 6 days the phagocytic activity was increased as compared to day 0. Although both mice showed similar levels of killing activity at day 0, *Il17a*$^{-/-}$ mice displayed a drastic reduction of killing activity at day +6 post-infection unlike the WT in which the killing activity was increased. However, at this time point *Il17a*$^{-/-}$ mice displayed a lower intrinsic oxidative burst (Fig. 6A, left panels), whichever the stimulant. Preincubation of neutrophils from *Il17a*$^{-/-}$ mice with murine rIL-17A did not rescue the reduced antifungal capacity of these neutrophils. No modulation of antifungal activity was also observed in neutrophils from WT mice (Fig. 6A, right panels and Fig. 6C).

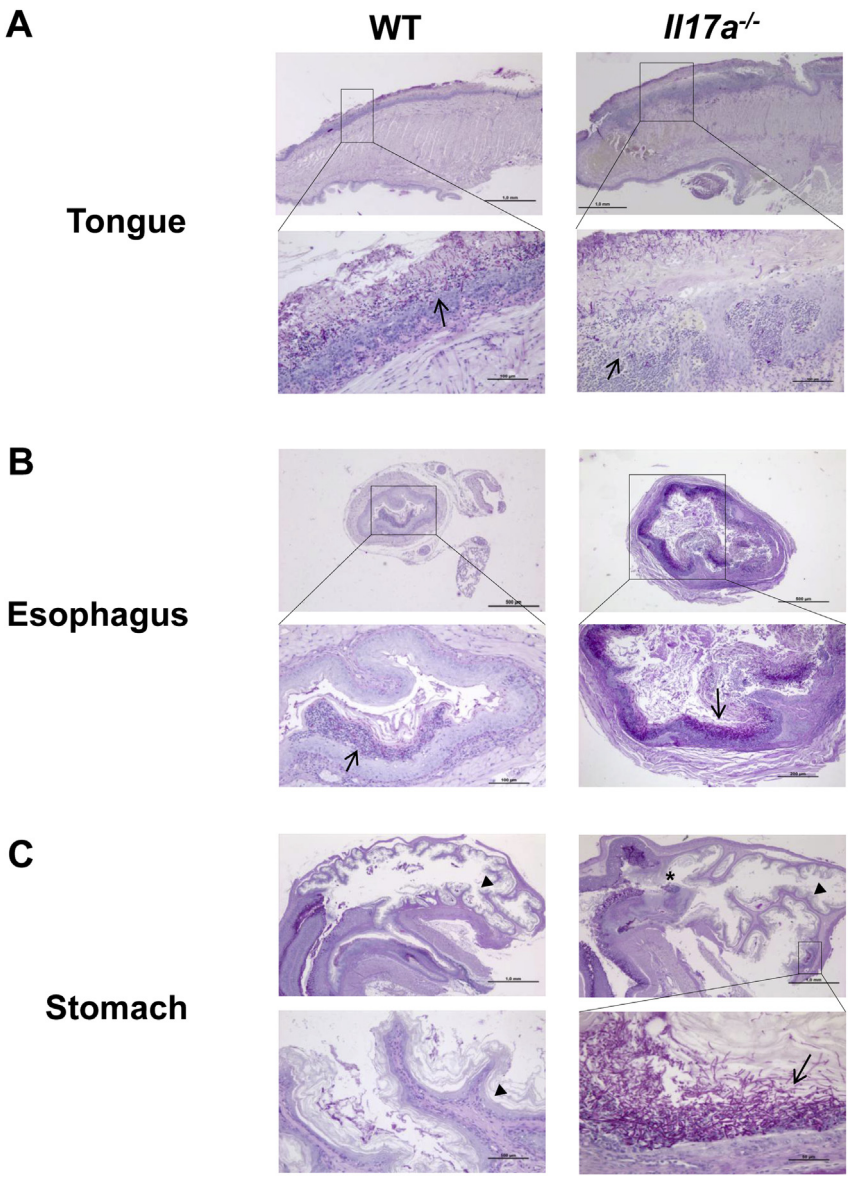

Fig. 4. **Histopathology of tongue, esophagus and stomach**. The tissue sections from *Il17a*$^{-/-}$ and WT infected mice were shown (day +8 post-infection). Tongue sections (A, right panels, scale bar 1.0 mm and enlargement view 100 μm) of *Il17a*$^{-/-}$ infected mice showed fungal and inflammatory cells infiltration penetrating into submucosa up to muscular layers, even throughout the entire tongue (arrow). In the tongue sections (A, left panels, scale bar 1.0 mm and enlargement view 100 μm) of WT infected mice the fungal invasion was much less severe. The fungi and inflammatory cells appeared to be confined to the keratinized layer of the tongue (arrow). In the esophageal sections (B, right panels, scale bar 500 μm and enlargement view 200 μm) of *Il17a*$^{-/-}$ infected mice severe erosions were evident with massive infiltrations of inflammatory cells (arrow). In the esophageal sections (B, left panels, scale bar 500 μm and enlargement view 100 μm) of WT infected mice the fungal burden and the inflammatory cells appeared confined to a superficial layer of the mucosa without involvement of the spinous layer (arrow). In the stomach sections (C, right panels, scale bar 1.0 mm and enlargement view 50 μm) of *Il17a*$^{-/-}$ infected mice a severe diffuse hyperplasia and hyperkeratosis of the whole forestomach were observed (head arrow). Hyphae and inflammatory cells, neutrophils and mononucleated cells, penetrated beyond the epithelium deeply within lamina propria (asterisk). In the forestomach, fungal cells remain localized to the keratinized layer (arrow). In the stomach sections (C, left panels, scale bar 1.0 mm and enlargement view 100 μm) of WT infected mice a similar hyperplastic reaction was observed (head arrow).

In order to get information about candidacidal activity at the site of infection, the killing activity of saliva was analyzed. Saliva was collected, after pilocarpine injection, from WT and *Il17a*$^{-/-}$ mice and incubated with *C. albicans* cells. No statistical differences of the candidacidal activity of saliva taken from WT and *Il17a*$^{-/-}$ mice before challenge were observed (Fig. 6B). A statistically significant increase of candidacidal activity of saliva from WT mice was observed 6 days post-infection compared to the pre-infection level, while the killing activity of saliva from *Il17a*$^{-/-}$ mice resulted completely abolished post-infection (Fig. 6B).

4. Discussion

C. albicans is part of normal microflora of the mucosa and particularly of the reproductive and gastrointestinal tracts [20]. Asymptomatic carriage of oral yeast has been seen to be present in about 60% of normal [21] to reach greater than 80% in HIV-

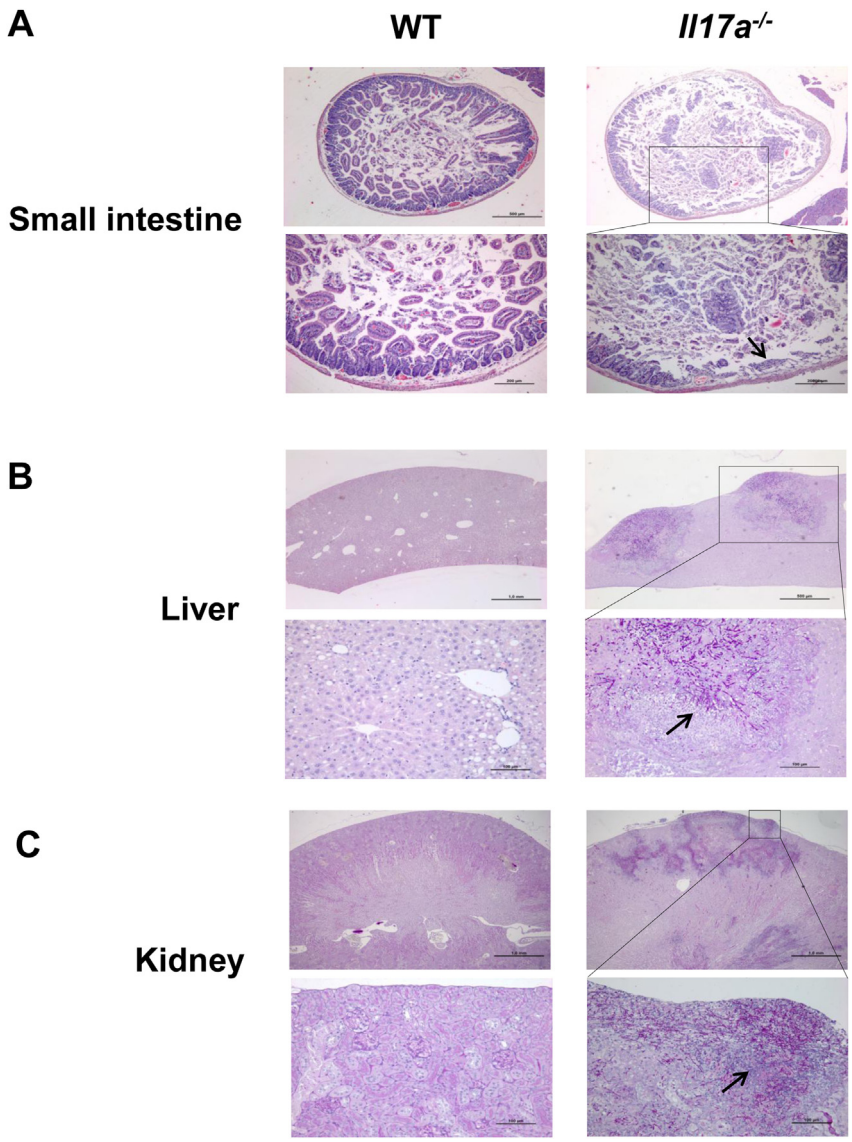

Fig. 5. **Histopathology of small intestine, liver and kidneys**. The small intestine sections (A, right panels, scale bar 500 μm and enlargement view 200 μm) of *Il17a*$^{-/-}$ infected mice showed a severe mucosal damage with the loss of the entire intestinal epithelium, vascular congestion and edema, and the intestinal villi appeared almost completely destroyed (arrow). No such damages were observed in small intestine sections (A, left panels, scale bar 500 μm and enlargement view 200 μm) of WT infected mice. The liver sections (B, right panels, scale bar 500 μm and enlargement view 100 μm) of *Il17a*$^{-/-}$ infected mice showed abscesses characterized by a necrotic central area surrounded by a mass of fungal elements containing both blastospores and hyphae, and an infiltration of neutrophils and mononucleated cells (arrow). No lesions in the liver of WT infected mice were observed (B, left panels, scale bar 1.0 mm and enlargement view 100 μm). The kidneys sections (C, right panels, scale bar 1.0 mm and enlargement view 100 μm) of *Il17a*$^{-/-}$ infected mice showed abscesses and extensive inflammatory reaction that are diffuse in the cortex and medullary area. Large aggregates of yeasts and dense leukocytic infiltration surrounding a necrotic area of cortical zone (arrow). No evidence of inflammatory reaction of fungal cells were observed in the kidneys of WT infected mice (C, left panels, scale bar 1.0 mm and enlargement view 100 μm).

positive subjects [22]. In these latter, OPC [23] represents one of the most common infection, that can spread to esophagus when the CD4^{+} cells fall to less than 100–50/μl and Th17 cells are lost or functionally damaged [24,25]. However, no invasion of internal organs occurs in these subjects, unless other predisposing factors are present, among which numerical or functional impairment of innate immunity cells, particularly the neutrophils are of prime importance [19,26].

Despite the relevant information above, it is unclear how and where *Candida* cells gain access to the systemic compartment from an intact or damaged epithelial compartment, and which determinants of host–fungus interaction play a critical role in dissemination to internal organs from mucosal disease.

In an attempt to gain some insight into this very relevant aspect of candidiasis, we used a previously described experimental model of oropharyngeal candidiasis [12] to examine the course of infection in corticosteroid-treated *Il17a*$^{-/-}$ and WT mice. This model uses a real-time monitoring of the spatial and temporal progression of infection, coupled with standard CFU enumeration. By this approach, here we demonstrate that steroid-treated *Il17a*$^{-/-}$ mice develop systemic infection from

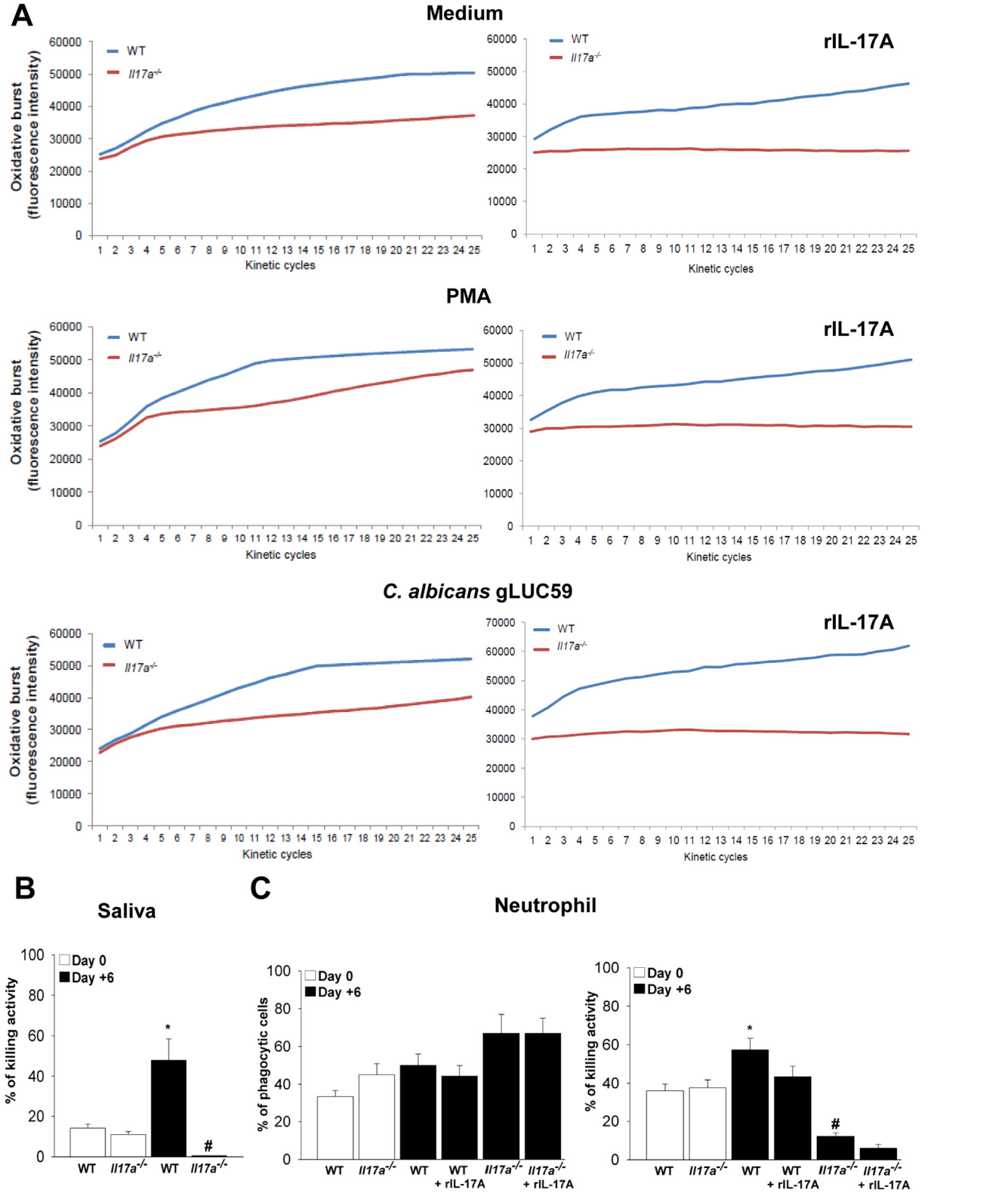

Fig. 6. **Neutrophils activity**. Oxidative burst of peritoneal neutrophils (4×10^6/ml) from *Il17a*$^{-/-}$ and WT mice, incubated in the presence or absence of rIL-17A (100 ng/ml), was evaluated after 6 days of infection in the presence or absence (Medium) of PMA (100 ng/ml) or *C. albicans* gLUC59 (2×10^6/ml). Data are from one of five experiments with similar results (A). At day 0 and +6 post-infection, the percentages of killing activity from saliva (B) and phagocytic and killing capacity (C) of peritoneal neutrophils incubated in the presence or absence of rIL-17A (100 ng/ml), were determined after 1 and 2 h of incubation with *C. albicans* gLUC59 respectively. $*p < 0.05$ was considered significant.

oropharyngeal candidiasis, whereas similarly treated WT mice do not bring about systemic candidiasis from local infection. The presence of IL-17B was detected in *Il17a*$^{-/-}$ mice and the production of this cytokine was similar to that observed in WT mice suggesting that not IL-17B compensatory expression was present in *I17a*$^{-/-}$ mice. Moreover, it has been reported that IL-17A deficiency does not affect the production of other family members [16], suggesting that also IL17F is normally produced. Contrary to what observed by Bishu S. et al. [27] steroid treatment alone, which to some extent favors by itself a late establishment of low-grade OPC, does not bring about systemic candidiasis from local infection in normal mice. On the other hand, in our experimental system *Il17a*$^{-/-}$ mice without steroid treatment did not develop systemic candidiasis from local infection. Thus, the apparent loss of intestinal epithelial barrier caused by IL-17A absence is per se insufficient for *C. albicans* stably infecting the internal organs, suggesting that the presence of another predisposing factor to systemic infection is essential. This model therefore closely mimics the situation of HIV-infected subjects where loss of Th17 cells in advanced stages of mucosal infection does not lead to systemic *Candida* spread in the absence of a predisposing factor or treatment, particularly those affecting number and/or function of neutrophils.

Development of systemic infection in *Il17a*$^{-/-}$ mice most likely occurs via the small intestine, particularly duodenal and ileum tracts. In the intestine of *Il17a*$^{-/-}$ mice the mucosal layer was almost completely destroyed and this could be a critical event considering that this layer is particularly important as defensive wall of the intestinal epithelia, also by preventing the adhesion of *C. albicans* to the epithelial surface. It is of interest that in the non-intestinal tracts, particularly the esophageal one, severe mucosal damage was nonetheless accompanied by remarkable inflammatory infiltrates in the shape of an apparent physical barrier to fungal translocation. This suggests that though grossly damaged, the above tracts were probably less pervious to translocation of fungal cells which might have been substantially retained in these tissues. Immunosuppression with steroids will likely promote infection with resident gut bacteria, therefore we cannot completely exclude that resident bacteria may be involved in the gut damage, however a separate study is necessary to unravel this issue. Previous investigations from Gaffen's research group showed that Th17 and IL-17A are critical for host defense against oral candidiasis. This was related to the inhibition of neutrophils recruitment, reduced expression of β-defensins as well as reduced candidacidal activity of saliva [8]. However, Gaffen's research group detected a large neutrophilic infiltrate in resistant mice after *C. albicans* inoculation, which was greatly diminished in susceptible (IL-17RA^{KO}) mice [8]. Actually, we noticed an equal level of neutrophils infiltrate in both groups of animals. This apparent discrepancy could be due to different strain of mice used. In particular Gaffen et al. used IL-17RA^{KO}, while in our experimental system *Il17a*$^{-/-}$ mice were used. Indeed, we also observed that IL-17B was produced at similar levels in both WT and *Il17a*$^{-/-}$ mice and the presence of this cytokine could account for neutrophils infiltration. Although antimicrobial peptides produced by non-hematopoietic cells at the site of infection may be involved in the observed candidacidal activity, it is likely that in our experimental setting the killing activity observed at the site of infection is also mediated by antimicrobial peptides from recruited neutrophils.

Indeed, genes induced by IL-17 encode antimicrobial proteins such as neutrophils-activating factor [28] and there is a general consensus about the protective role of IL-17A and IL-17A receptor (IL-17RA) in disseminated candidiasis [6,29]. Other recent papers have described different mouse models of gastrointestinal candidiasis [30]. Indeed, intragastric inoculation successfully leads to colonization of the gastrointestinal tract under various conditions such as immunosuppression [31], malnutrition [32] or infant mouse model [33]. In these models it has been shown that a gastrointestinal colonization could be a source of hematogenous candidiasis. A recent interesting paper by Hise et al. [34] showed that Dectin-1, NLRP3 and TLR2 are essential for preventing dissemination from a mucosal source. Here we demonstrate for the first time that IL-17A is critical for preventing systemic dissemination from oropharyngeal candidiasis and that the absence of IL-17A is correlated with severe damage of the gut, loss of fungal retention barrier and access of the fungus to the liver giving rise to classic abscesses of hepatic candidiasis. It has been recently demonstrated that Dectin-1 and NLRP3, that are involved in IL-1β production [35], are essential for preventing *Candida* dissemination from a mucosal source [34]. Our data argue that IL-17A should be added to Dectin-1 and NLRP3 as functionally involved in contrasting the spread of the fungus to the systemic compartment, even in the presence of an immunosuppressant such as the corticosteroid. However, our histological analysis also demonstrate that in the organs of *Il17a*$^{-/-}$ mice, particularly in the esophagus, there is a massive infiltration of inflammatory cells including neutrophils. This indicates that, despite the absence of IL-17A, which is known to be chemotactic for neutrophils, *Il17a*$^{-/-}$ mice are still able to recruit neutrophils in infected organs, and, in some instances (see above) the infiltrating cells assume the apparent shape of a sort of fungus-retaining barrier. Possible replacement of IL-17A functions by other members of IL-17 family and the overproduction of other chemotactic cytokines, such as IL-1β, in *Il17a*$^{-/-}$ mice, could explain the observed recruitment of the neutrophils. Of importance, the neutrophils from *Il17a*$^{-/-}$ mice appear to be partially impaired in their anticandidal activity compared to the neutrophils from WT infected mice. Hence, the presence of neutrophils does not completely mirror their efficacy in terms of antifungal activity. This could explain why in *Il17a*$^{-/-}$ mice fungal cells escape from the local compartment to disseminate elsewhere. Moreover, the neutrophils population was overrun by infiltrating fungus to the point where they can no longer cope with the numbers of encountered fungal cells. While this is consistent with the described influence of IL-17A on the expression of antimicrobial proteins such as β-defensins by innate immune cells [8,36], it is unclear whether the here reported partial impairment of antifungal activity is per se responsible of *C. albicans* passage from mucosal to systemic infection. These aspects, as

well as any possible replacement of IL-17A functions by other cytokines possibly over-expressed in *Il17a*$^{-/-}$ mice deserve further investigations in our model. Conti et al. reported that no evidence of kidney infection was observed in IL-17RA^{KO} mice with oral candidiasis [8]. This apparent discrepancy could be due to different mice used, different inoculum of yeast and different time of CFU determination. In particular Conti et al. used IL-17RA-deficient mice, while we used *Il17a*$^{-/-}$ mice. In addition we used a lower dose of *C. albicans* (1 log less), this allowed to late determination of *C. albicans* load in the kidneys that was specifically performed at day 6 and 8 post-infection. This relatively low dose of infection therefore could closely mimic the human infection.

In humans, hematogenous candidiasis is clinically a typical example of an endogenous infection because of *C. albicans* colonizes the alimentary tract and the translocation occurs particularly in immunocompromised patients due to intestinal barrier damage caused by chemotherapy for cancer or infections [37]. Moreover, it seems that there is an anatomical compartimentalization of candidiasis related to various diseases [38,39].

It has been recently underlined that liver lesions, in high risk patients, after one episode of candidemia within the first 2 weeks, define hepatosplenic candidiasis [39,40]. Indeed, patients with hematologic malignancies in stages of recovery from granulocitopenia have been reported with increasing frequency to suffer from invasive candidiasis localized into the liver [41–43]. Here we report for the first time that the hepatic candidiasis can be secondary to a high-grade OPC. This observation, could be of clinical interest and help timely diagnosis when OPC is associated with liver lesions in patients with hematological malignancies in which some invasive diagnostic procedures are precluded by thrombocytopenia.

In conclusion, our results describe a mouse model that, from oropharyngeal candidiasis, brings to disseminated candidiasis. More importantly, our data point out that the presence of IL-17A is critical for preventing invasive infection, particularly hepatic dissemination. Importantly, the shift to disseminated infection from OPC does not appear to be due to lack of phagocytosis-competent neutrophils recruitment to infected mucosa in *Il17a*$^{-/-}$ mice but rather be favored by some impairment of neutrophils anti-*Candida* functions. This model could be particularly exploited to unravel the biological mechanisms allowing *C. albicans* and possibly other human commensal microorganisms to translocate from intestine to internal organs, hence causing invasive, potentially lethal infections.

Conflict of interest

None.

Acknowledgment

Funding: this work was supported by Fondazione Cassa di Risparmio 2012.0128.021″. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

[1] Naglik JR, Moyes DL, Wachtler B, Hube B. *Candida albicans* interactions with epithelial cells and mucosal immunity. Microbes Infect 2011;13:963–76.
[2] Abbott DJ, Blanchfield JL, Martinson DA, Russell SC, Taslim N, Curtis AD, et al. Neuroantigen-specific, tolerogenic vaccines: GM-CSF is a fusion partner that facilitates tolerance rather than immunity to dominant self-epitopes of myelin in murine models of experimental autoimmune encephalomyelitis (EAE). BMC Immunol 2011;12:72.
[3] Li X, Kolltveit KM, Tronstad L, Olsen I. Systemic diseases caused by oral infection. Clin Microbiol Rev 2000;13:547–58.
[4] Conti HR, Baker O, Freeman AF, Jang WS, Holland SM, Li RA, et al. New mechanism of oral immunity to mucosal candidiasis in hyper-IgE syndrome. Mucosal Immunol 2011;4:448–55.
[5] Conti HR, Gaffen SL. Host responses to *Candida albicans*: Th17 cells and mucosal candidiasis. Microbes Infect 2010;12:518–27.
[6] Huang W, Na L, Fidel PL, Schwarzenberger P. Requirement of interleukin-17A for systemic anti-*Candida albicans* host defense in mice. J Infect Dis 2004;190:624–31.
[7] Huppler AR, Bishu S, Gaffen SL. Mucocutaneous candidiasis: the IL-17 pathway and implications for targeted immunotherapy. Arthritis Res Ther 2012;14:217.
[8] Conti HR, Shen F, Nayyar N, Stocum E, Sun JN, Lindemann MJ, et al. Th17 cells and IL-17 receptor signaling are essential for mucosal host defense against oral candidiasis. J Exp Med 2009;206:299–311.
[9] Hernandez-Santos N, Huppler AR, Peterson AC, Khader SA, McKenna KC, Gaffen SL. Th17 cells confer long-term adaptive immunity to oral mucosal *Candida albicans* infections. Mucosal Immunol 2013;6:900–10.
[10] Iwakura Y, Ishigame H, Saijo S, Nakae S. Functional specialization of interleukin-17 family members. Immunity 2011;34:149–62.
[11] Naglik JR, Moyes D. Epithelial cell innate response to *Candida albicans*. Adv Dent Res 2011;23:50–5.
[12] Mosci P, Pericolini E, Gabrielli E, Kenno S, Perito S, Bistoni F, et al. A novel bioluminescence mouse model for monitoring oropharyngeal candidiasis in mice. Virulence 2013;4:250–4.
[13] Enjalbert B, Rachini A, Vediyappan G, Pietrella D, Spaccapelo R, Vecchiarelli A, et al. A multifunctional, synthetic Gaussia princeps luciferase reporter for live imaging of *Candida albicans* infections. Infect Immun 2009;77:4847–58.
[14] Solis NV, Filler SG. Mouse model of oropharyngeal candidiasis. Nat Protoc 2012;7:637–42.
[15] Nakae S, Komiyama Y, Nambu A, Sudo K, Iwase M, Homma I, et al. Antigen-specific T cell sensitization is impaired in IL-17-deficient mice, causing suppression of allergic cellular and humoral responses. Immunity 2002;17:375–87.
[16] Ishigame H, Kakuta S, Nagai T, Kadoki M, Nambu A, Komiyama Y, et al. Differential roles of interleukin-17A and -17F in host defense against mucoepithelial bacterial infection and allergic responses. Immunity 2009;30:108–19.
[17] Yoshino Y, Nakagawa Y. Salivary 8-OHdG induction by physical exercise training under food restriction. Open Dent J 2011;5:48–51.
[18] Iliev ID, Funari VA, Taylor KD, Nguyen Q, Reyes CN, Strom SP, et al. Interactions between commensal fungi and the C-type lectin receptor Dectin-1 influence colitis. Science 2012;336:1314–7.
[19] Horn DL, Neofytos D, Anaissie EJ, Fishman JA, Steinbach WJ, Olyaei AJ, et al. Epidemiology and outcomes of candidemia in 2019 patients: data from the prospective antifungal therapy alliance registry. Clin Infect Dis 2009;48:1695–703.
[20] Perez JC, Kumamoto CA, Johnson AD. *Candida albicans* commensalism and pathogenicity are intertwined traits directed by a tightly knit transcriptional regulatory circuit. PLoS Biol 2013;11:e1001510.
[21] Glick M, Siegel MA. Viral and fungal infections of the oral cavity in immunocompetent patients. Infect Dis Clin North Am 1999;13:817–31. vi.
[22] Wozniak KL, Leigh JE, Hager S, Swoboda RK, Fidel Jr PL. A comprehensive study of Candida-specific antibodies in the saliva of

human immunodeficiency virus-positive individuals with oropharyngeal candidiasis. J Infect Dis 2002;185:1269–76.
[23] Cassone A, Cauda R. Candida and candidiasis in HIV-infected patients: where commensalism, opportunistic behavior and frank pathogenicity lose their borders. AIDS 2012;26:1457–72.
[24] de Repentigny L, Lewandowski D, Jolicoeur P. Immunopathogenesis of oropharyngeal candidiasis in human immunodeficiency virus infection. Clin Microbiol Rev 2004;17:729–59. table of contents.
[25] Vazquez JA. Optimal management of oropharyngeal and esophageal candidiasis in patients living with HIV infection. Hiv AIDS (Auckl) 2010;2:89–101.
[26] Lionakis MS, Netea MG. Candida and host determinants of susceptibility to invasive candidiasis. PLoS Pathog 2013;9:e1003079.
[27] Bishu S, Hernandez-Santos N, Simpson-Abelson MR, Huppler AR, Conti HR, Ghilardi N, et al. The adaptor CARD9 is required for adaptive but not innate immunity to oral mucosal *Candida albicans* infections. Infect Immun 2014;82:1173–80.
[28] Gaffen SL. Recent advances in the IL-17 cytokine family. Curr Opin Immunol 2011;23:613–9.
[29] Saijo S, Ikeda S, Yamabe K, Kakuta S, Ishigame H, Akitsu A, et al. Dectin-2 recognition of alpha-mannans and induction of Th17 cell differentiation is essential for host defense against *Candida albicans*. Immunity 2010;32:681–91.
[30] Bendel CM, Wiesner SM, Garni RM, Cebelinski E, Wells CL. Cecal colonization and systemic spread of *Candida albicans* in mice treated with antibiotics and dexamethasone. Pediatr Res 2002;51:290–5.
[31] Cole GT, Halawa AA, Anaissie EJ. The role of the gastrointestinal tract in hematogenous candidiasis: from the laboratory to the bedside. Clin Infect Dis 1996;22(Suppl. 2):S73–88.
[32] Takahashi K, Kita E, Konishi M, Yoshimoto E, Mikasa K, Narita N, et al. Translocation model of *Candida albicans* in DBA-2/J mice with protein calorie malnutrition mimics hematogenous candidiasis in humans. Microb Pathog 2003;35:179–87.
[33] de Repentigny L, Phaneuf M, Mathieu LG. Gastrointestinal colonization and systemic dissemination by *Candida albicans* and *Candida tropicalis* in intact and immunocompromised mice. Infect Immun 1992;60:4907–14.
[34] Hise AG, Tomalka J, Ganesan S, Patel K, Hall BA, Brown GD, et al. An essential role for the NLRP3 inflammasome in host defense against the human fungal pathogen *Candida albicans*. Cell Host Microbe 2009;5:487–97.
[35] Pietrella D, Pandey N, Gabrielli E, Pericolini E, Perito S, Kasper L, et al. Secreted aspartic proteases of *Candida albicans* activate the NLRP3 inflammasome. Eur J Immunol 2013;43:679–92.
[36] Pietrella D, Rachini A, Pines M, Pandey N, Mosci P, Bistoni F, et al. Th17 cells and IL-17 in protective immunity to vaginal candidiasis. PLoS One 2011;6:e22770.
[37] MacFie J, O'Boyle C, Mitchell CJ, Buckley PM, Johnstone D, Sudworth P. Gut origin of sepsis: a prospective study investigating associations between bacterial translocation, gastric microflora, and septic morbidity. Gut 1999;45:223–8.
[38] Leigh JE, Barousse M, Swoboda RK, Myers T, Hager S, Wolf NA, et al. Candida-specific systemic cell-mediated immune reactivities in human immunodeficiency virus-positive persons with mucosal candidiasis. J Infect Dis 2001;183:277–85.
[39] Scully C, el-Kabir M, Samaranayake LP. Candida and oral candidosis: a review. Crit Rev Oral Biol Med 1994;5:125–57.
[40] Mikulska M, Calandra T, Sanguinetti M, Poulain D, Viscoli C, Third European Conference on Infections in Leukemia G. The use of mannan antigen and anti-mannan antibodies in the diagnosis of invasive candidiasis: recommendations from the Third European Conference on Infections in Leukemia. Crit Care 2010;14:R222.
[41] Tashjian LS, Abramson JS, Peacock Jr JE. Focal hepatic candidiasis: a distinct clinical variant of candidiasis in immunocompromised patients. Rev Infect Dis 1984;6:689–703.
[42] Meunier F, Gerard M, Richard V, Debusscher L, Bleiberg H, Malengrau A. Hepatic candidosis in a patient with acute leukemia. Mycoses 1989;32:421–6.
[43] Verdeguer A, Fernandez JM, Esquembre C, Ferris J, Ruiz JG, Castel V. Hepatosplenic candidiasis in children with acute leukemia. Cancer 1990;65:874–7.