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Research paper

Knockout of autophagy gene, ATG5 in mice vaginal cells abrogates cytokine response and pathogen clearance during vaginal infection of *Candida albicans*

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ABSTRACT

The female reproductive tract (FRT) presents a unique challenge to the mucosal immune system as it needs to monitor constantly for the presence of opportunistic pathogens amidst its commensal flora. During infection, autophagy plays a critical role in pathogen clearance, presentation of antigens and production of pro-inflammatory cytokines. However, no information is available that describes the role of autophagy in mouse vaginal infection of *Candida albicans*. The objective of our study is to evaluate the effect of autophagy gene, ATG5 knockout in vaginal cells in response to vaginal *C. albicans* infection. Mice having knockout of ATG5 in the vaginal cells (PR-ATG5-KO mice) were infected intra-vaginally with the yeast form of *Candida albicans*. Vaginal lavages were collected once in a week until the infection was cleared. We detected the expression of autophagy marker genes (LC3, ATG5 and LAMP1) in the vaginal cells. We determined the levels of various cytokines (IL-1α, IL-1β, IL-6, IL-10, IL-17A, IL-22, IL-23p19, TNF-α and G-CSF) involved in anti-candida response. The levels of cytokines in the vaginal lavages were quantified using Aimplex Premixed analyte kit. The vaginal lavages were checked for polymorphonuclear leucocytes (PMNLs) infiltration. The candida clearance rate from the vaginal lumen was determined by Colony Forming Units (CFUs) assay. The results revealed that PR-ATG5-KO mice failed to induce the expression of LC3, ATG5 and LAMP1 indicating an impaired autophagy pathway. The levels of all the cytokines (except IL-10) in *C. albicans* infected PR-ATG5-KO mice were significantly reduced as compared to the wild type infected C57BL/6 mice. The number of PMNLs infiltrated into the vaginal lavages of infected PR-ATG5-KO mice was reduced. The clearance of *C. albicans* from the vaginal lumen was also considerably delayed in PR-ATG5-KO mice. In conclusion, the results revealed that impaired autophagy in vaginal cells influences host response during vaginal infection of *C. albicans* by affecting anti-Candida cytokine levels in the vaginal lavage resulting in reduction of pathogen clearance rate.

1. Introduction

Reproductive tract infections (RTIs) are recognized as a serious global threat to the health of men and women. One of the key reasons for high rate of RTIs, particularly in women, is that vagina acts as a portal for the entry of various microbes. One such organism is *Candida albicans* that acts as a commensal as well as an opportunistic pathogen [1]. This pathogen infects mucocutaneous regions like gastro-intestinal tract (GIT), urogenital tract (UGT) as well as non-mucocutaneous regions such as skin. *Candida* infections of the GIT result in oropharyngeal candidiasis while infections of the UGT lead to Candiduria and Genital candidiasis. Candiduria is more prevalent in immune-compromised individuals such as those suffering from Acquired Immune Deficiency Syndrome (AIDS), elderly hospitalized patients and neonates [2]. Genital candidiasis also causes genital inflammation in both sexes. In men, it culminates into inflammation of glans penis called Candida balanitis and Balanoposthitis [3]. In women, it causes inflammation of the vagina and vulva known as vulvo-vaginal candidiasis (VVC). VVC is the second most common cause of vagina inflammation, clinically known as vaginitis. Among various species of Candida, *Candida albicans* is the most predominant pathogen that causes candidiasis. It is a dimorphic fungus capable of undergoing morphological swapping between yeast form and hyphae form. Yeast form of *C. albicans* is non-infectious while hyphae form is capable of invading into the host. The ability of *C. albicans* to switch from yeast form to hyphae form is a critical feature of its pathogenicity [4].

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Autophagy is gaining importance for its roles in preventing various infections [5–8]. Macroautophagy or autophagy is a conserved process across all eukaryotic cell types and play an important role in pathogen clearance and cellular homeostasis [9]. This process involves sequestration of cytoplasmic material inside a double-membrane vesicle known as autophagosome. The autophagosomes fuse with lysosomes to form autophagolysosomes or autolysosomes that degrade the sequestered cargo using lysosomal proteases [10]. Autophagy occurs under normal physiological conditions for recycling of damaged organelles. It is up-regulated by various factors like nutrient deprivation that is detected by nutrient sensing proteins like mammalian target of rapamycin (mTOR). During infections, autophagy plays a key role in MHC-II mediated antigen presentation, activation of adaptive and innate immune responses, modulation of cytokines/chemokines and clearance of pathogens. Autophagy is known to manifest the activation of innate and adaptive immune responses through the modulation of cytokine and chemokine expression [11]. Recent studies have demonstrated that autophagy plays a vital role in the modulation of cytokine expression (IL-1β, TNF-α and IL-6) by macrophages upon *C. albicans* infection [12,13]. Nicola et al., [14] showed that during lung infection by *C. albicans*, autophagy stimulates the production of various cytokines (IL-4, IL-13, MIP-1 and IP-10). In our recent study, we showed an up-regulation of autophagy marker genes (LC3 and LAMP-1) in human vaginal epithelial cells (VECs) in response to *C. albicans* infection [15]. These *in vitro* studies were extended *in vivo* to understand the role of autophagy process in mice vaginal cells during vaginal infection of *C. albicans*.

Autophagy related gene 5 (ATG5) is involved in the formation of Atg5-Atg12 protein complex, which is critical for the generation of autophagosomes [16]. It is known that knockdown of ATG5 results in the deprivation of Atg5-Atg12 protein complex from the cells, resulting in inhibition of autophagosome formation. Previous studies have demonstrated that conditional knockout of ATG5 affects host immune responses to infections [17,18]. Hence, in the present study, we generated PR-ATG5-KO mice having conditional knockout of ATG5 gene in vaginal cells. These mice were used to decipher the effect of ATG5 knockdown on cellular immune response during *in vivo* vaginal *C. albicans* infection by determining the levels of key immune regulatory molecules such as cytokines.

2. Materials and methods

2.1. Ethics statement

Institutional Animal Ethics Committee of National Institute for Research in Reproductive Health (NIRRH) approved the study (IAEC project no.: 05/08/RB/25/12). All animal procedures were performed as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). All infection experiments were carried out at the animal house facility of Haffkine Institute, Parel, Mumbai after obtaining the necessary permissions from NIRRH Animal Ethics Committee and Haffkine Institute Animal Ethics Committee.

2.2. Procurement of animals

Two pairs of ATG5-LoxP mice (60 days old, 20 ± 2 g body Wt.) were obtained as a kind gift from Prof. Noburu Mizhusima, Japan through RIKEN-BRC. Two pairs of PR-Cre mice (60 days old, 20 ± 2 g body Wt.) and another two pairs of wild type C57/BL6 mice (60 days old, 20 ± 2 g body Wt.) were procured from Jackson Laboratories, USA. All these mice were maintained at 24 ± 2 °C under 12/12 h dark/light cycles at the animal house facility of NIRRH. The mice had access to standard pellet diet and water *ad libitum* throughout the study.

2.3. Generation of mice lacking ATG5 gene in vaginal cells (PR-ATG5-KO mice)

To generate mice lacking ATG5 gene in the vaginal cells, B6.129S-Atg5 < tm1Myok > (ATG5-LoxP) transgenic mice were mated with another transgenic mice, B6.129S (Cg)-Pgr$^{tm1.1\ (cre)\ Shah}$/AndJ (PR-Cre). The ATG5-LoxP transgenic mice were generated by replacing the wild type exon-3 of ATG5 with a mutant exon-3, which consists of LoxP sites on both ends [19]. This mutation allows the deletion of the exon-3 of ATG5 from cells expressing Cre enzyme. PR-Cre mice have the Cre enzyme gene under the regulation of Progesterone Receptor (PR) promoter [20]. PR is known to be expressed by vaginal cells [21,22]. Hence, the progeny obtained by crossing these two mice strains (ATG5-LoxP and PR-Cre) is referred to as PR-ATG5-KO mice and would lack the exon 3 of ATG5 in vaginal cells. Since all these three mice strains have been created using C57BL/6 background, wild-type C57BL/6 mice were used as control.

2.4. Candida albicans procurement and culture

Candida albicans (strain: 3153A) was obtained as a kind gift from Dr. Paul Fidel Jr., Department of Microbiology, Immunology and Parasitology, New Orleans School of Medicine, Los Angeles, USA. The strain was grown on HiChrome Candida agar (Himedia, India). Individual colonies were inoculated in Sabouraud's broth (Himedia, India) and incubated at 30 °C for 16 h in a shaker incubator. Glycerol stocks of candida cultures were stored at −80 °C until experimentation. For vaginal infection, *C. albicans* was seeded in Sabouraud's broth and incubated for 16 h at 30 °C in a shaker incubator. *C. albicans* blastospores were harvested and re-suspended in phosphate buffered saline (PBS). Viable *C. albicans* blastospores were stained with Trypan-blue dye exclusion staining and counted using phase contrast microscope (Olympus, Japan) (Magnification × 40).

2.5. Vaginal infection of mice with Candida albicans and collection of lavages

To induce pseudo-estrous stage, 6–8 week old mice were injected subcutaneously (sc) with of 17β-estradiol (100 µg) 72 h prior to *C. albicans* infection and the injections were repeated once every week to maintain pseudo-estrous stage. These mice were used for vaginal infection of *C. albicans* as per the protocol described previously [23]. Briefly, single dose of *Candida albicans* blastospores (5×10^6 cells/mice) were intra-vaginally administered to pseudo-estrous mice. Control mice received only the vehicle, Phosphate buffered saline (PBS) intravaginally. The infections were carried out for different time points in all four strains of mice (wild-type C57BL/6, ATG5-LoxP mice, PR-Cre mice and PR-ATG5-KO mice) consisting of five mice in each group. Vaginal lavages were collected from infected and control mice at weekly intervals using 50 µl of PBS administered intra-vaginally.

2.6. Total RNA extraction from vaginal cells

Mice vaginal tissue was excised, cleaned from blood stain and damp, washed in PBS, and cut into small pieces (4–5 mm) placed in a sterile multi-well plate containing 0.5 ml/well of Dispase (1.7 U/ml) on a shaking platform overnight at 4 °C. The cells thus obtained were suspended in 10× trypsin-EDTA and incubated at 37 °C for 10 min. Cells were centrifuged, washed with PBS and re-suspended in complete DMEM with 10% FBS. Cells were sheared using syringe with 21-gauge needle, washed with PBS and enumerated by trypan blue dye exclusion. Equal numbers of cells from each group were treated with TRIzol reagent (Invitrogen, USA) to extract total cellular RNA. The extracted RNA was isolated using phenol-chloroform method and treated with DNase I (Sigma-Aldrich, USA). The concentration of the RNA was