Video Article

Th17 Inflammation Model of Oropharyngeal Candidiasis in Immunodeficient Mice

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Abstract

Oropharyngeal Candidiasis (OPC) disease is caused not only due to the lack of host immune resistance, but also the absence of appropriate regulation of infection-induced immunopathology. Although Th17 cells are implicated in antifungal defense, their role in immunopathology is unclear. This study presents a method for establishing oral Th17 immunopathology associated with oral candidal infection in immunodeficient mice. The method is based on reconstituting lymphopenic mice with *in vitro* cultured Th17 cells, followed by oral infection with *Candida albicans* (*C. albicans*). Results show that unrestrained Th17 cells result in inflammation and pathology, and is associated with several measurable read-outs including weight loss, pro-inflammatory cytokine production, tongue histopathology and mortality, showing that this model may be valuable in studying OPC immunopathology. Adoptive transfer of regulatory cells (T_regs) controls and reduces the inflammatory response, showing that this model can be used to test new strategies to counteract oral inflammation. This model may also be applicable in studying oral Th17 immunopathology in general in the context of other oral diseases.

Video Link

The video component of this article can be found at https://www.jove.com/video/52538/

Introduction

Oral infections and inflammation have been related to cancer and cardiovascular diseases, and have dramatic impact on overall human health[2,3]. Opportunistic infections and inflammation caused by *C. albicans* are associated with primary immunodeficiencies (PID)[4,5], inflammatory disorders such as periodontitis [6,7], Sjogren's syndrome, and salivary gland disease[8,9], as well as oral squamous cell carcinoma [10-12]. *C. albicans* is a dimorphic commensal fungus that colonizes the mouths of 60% of healthy humans asymptomatically, yet it is the most common fungal pathogen causing infections when the host defense is weakened[13-15]. It causes recurring and chronic infections and inflammation in patients with AIDS and PID, and also in other immunocompromised individuals. As a commensal, its colonization load is associated with the change in the diversity of the overall oral microbiome[16]. As a pathogen it causes several forms of oropharyngeal candidiasis such as acute pseudomembranous, acute atrophic, chronic atrophic, chronic hypertrophic/hyperplastic, and angular cheilitis.

Protection against *C. albicans* is determined not only by host immune resistance, but also by the ability to appropriately control *Candida*-induced immunopathology. Although commensals such as *C. albicans* contribute to modulation and exacerbation of other oral inflammatory conditions, the mechanisms by which dysbiosis occur during opportunistic infections are unclear. Besides the known role of adaptive Th17 cells in memory response to *C. albicans*[17], their role in initiation and perpetuation of inflammation pathology during chronic infections remain unclear. Furthermore, oral inflammatory diseases such as Sjogren's syndrome and periodontitis are associated with Th17 mediated pathology. Interestingly, these diseases are also strongly associated with frequent OPC. However, the interactions among Th17 cells, oral immunopathology of OPC and other oral inflammatory diseases are unstudied.

Although mouse models of primary and secondary infection of oral candidiasis are available, a mouse model to study Candida infection associated Th17 inflammation, especially in the context of immunodeficiency is unavailable. This study presents a method for establishing oral Th17 inflammation associated with oral *Candida* infection in mice. *Candida* infection in mice is characterized by fungal lesions, inflammation in the tongue, decreased food intake, weight loss and eventually a moribund state. Oral pathology resembles chronic candidal infection lesions, as well as epithelial dysplasia in mouse oral cancer models[12,18].

Protocol

NOTE: The experiments using mice were performed in accordance to the institutional animal welfare committee (IACUC) guidelines.

1. Reconstituting the Rag-1^{-/-} Mice with *In Vitro* Cultured Th17 cells (Three days prior to Infection)

1. For establishing Th17 cells, culture CD4+ CD44low CD62Lhigh CD25- naïve T cells (3×10^4) in U-bottom 96 well plates alone, or co-culture them along with 3×10^4 CD4+CD25+Foxp3+Tregs in the presence of soluble α-CD3 (1 µg/ml), α-CD28 (2 µg/ml) antibodies and antigen presenting cells, under Th17 conditions (polarized using IL-6 (25 ng/ml), TGF-β (2 ng/ml), α-IFN-γ (2 µg/ml) and α-IL-4 (2 µg/ml)) for three to four days.
   NOTE: Naïve cells and T_{regs} were sorted using fluorescence activated cell sorting (FACS) and magnetic cell isolation procedures and cultured as described previously[19-22].
2. On day 4, after resuspending the Th17 cells using the pipette, collect them from cultures, and centrifuge them under sterile conditions.
   1. Take a small aliquot of these cells to examine their viability and for phenotyping. Resuspend them in 500 µl of RPMI medium, add propidium iodide (200 ng/ml) and assess their viability immediately by flow cytometry. Perform phenotyping by flow cytometry assessment of IL-17A production after restimulation (see section 6.3).
3. Centrifuge and wash the main bulk of the cells in sterile PBS at 480 x g for 6 min at 4° C in all the steps unless otherwise specified.
4. Use these cells for adoptive transfer into 6-8 week old CD45.2 Rag1-/- immunodeficient mice.
   1. Resuspend the cells at 1×10^7 cells/ml cell density using cold sterile PBS.
   2. Inject 100 µl of the cells by intraperitoneal injection, using 25 G needles on 1 ml tuberculin syringes, such that one mouse receives 1×10^6 cells. Some mice will receive PBS or Th17 cells only, and other mice will receive Th17 cells that were co-cultured with Treg cells.
   NOTE: Perform all the steps aseptically.

2. Growing *C. albicans* for infection (one day prior to Infection)

1. Before inoculation, disperse five colonies of the CAF2-1 *C. albicans* laboratory strain in 100 µl of sterile PBS suspension, and add the suspension to the sterile broth.
2. Inoculate 5 colonies of the *C. albicans* in 50 ml of the Yeast nitrogen base (YNB without amino acids)/Peptone/Dextrose broth medium and incubate in a shaker incubator at 30 °C for 15–18 hr at 130 rpm.
3. Monitor the broth for cloudiness, as that is an indication for the growth of the fungus.

3. *Candida* Harvesting and Counting Procedure (On the Day of Infection)

1. Collect 10 ml of *Candida* broth in 15 ml tubes. For larger volumes, collect *Candida* in 50 ml tubes.
2. Centrifuge the blastospores at 1900 x g for 5 min at RT in all the steps unless otherwise specified. Pellet the blastospores by centrifugation, followed by the removal of the supernatant.
3. If there is more than one tube, pool the *Candida* blastospores from multiple tubes, adding sterile PBS to the pellets and resuspend them in 10 ml of PBS for counting.
4. Take 20 µl of blastospores in 1.5 ml microcentrifuge tubes, add 20 µl of the 2X paraformaldehyde and incubate at RT for 15-20 min. Count the fixed blastospores using the hemocytometer under the microscope.
   NOTE: Paraformaldehyde is carcinogenic. Open the undiluted solution only in the fume hood.
5. In the meantime, repeat washing the blastospores at least twice, by centrifuging them in PBS, and pelleting the blastospores . Keep the pellets in 15 ml tubes at RT, in the ABSL1 hood, ready for infection.
6. Leave some sterile PBS at RT for resuspending the blastospores for infection.
7. After counting, add sterile PBS to the blastospore pellet to adjust the yeast blastospore cell numbers to 2×10^8 of yeast cells/ml. This is the blastospore suspension that will be used to infect the mice.
   NOTE: Perform all the steps aseptically.

4. Mice Infection (3-5 Days after Cell Transfer)

NOTE: The basic infection procedure is performed as described previously[19,23-25].

1. Weigh the Rag-1-/- mice that received PBS or the cells three days earlier.
2. Calculate the dose of anesthetic agents using their body weight. Anesthetize the mice by administering ketamine/xylazine mixture (16.1 mg/ml and 1.6 mg/ml), using 25 G needles on 1 ml tuberculin syringes, by intraperitoneal injection.
   1. Administer 50 µl per 10 grams of body weight. (*i.e.,* 0.8 mg of ketamine and 0.08 mg of xylazine/10 g of body weight or 80 mg of ketamine and 8 mg of xylazine per kg of body weight respectively). Observe for toe pinch response every 15 min after anesthesia.
   NOTE: This dosage will induce 60-90 min of anesthesia, which is enough for the infection procedure.
   2. Apply the ophthalmic lubricant ointment in the eyes of the mice to prevent the corneas from drying out. As the eye lubricant may dry out, repeat ophthalmic lubrication every 45 min.

3. Inject 1 ml of saline (0.9% NaCl) subcutaneously on the back adjacent to the forelimb, to help rehydration of the mice during anesthesia.
4. Obtain a new, clean cage. Follow the infection procedure one mouse at a time, placing each mouse into the new cage once infected. Perform the PBS/sham infection first,and then proceed to the *Candida* infection groups.
5. Pick up the anesthetized mouse and open the mouth wide to reveal the base of the tongue.
   1. Place a 3 mm diameter cotton ball saturated with 50 µl of PBS or blastospore suspension, sublingually in the oral cavity for 90 min. Flip the mice every 15 min front and back to prevent lung congestion, ensuring that the cotton balls don't move.

6. Set up a timer for every mouse to ensure 90 min of Sham or *Candida* inoculation in the oral cavity.
7. Keep them in a cage under the heat lamp (4 feet away), and each mouse on heat gel pads.
8. Make sure that the tongue is withdrawn inside and away from the teeth, to avoid teeth lacerating the tongue.
9. At the end of 90 min, remove the cotton ball from the mouth of the mouse. Watch for any mouse that may recover from anesthesia sooner than 75 minutes. In such a case, anesthetize them again with ketamine (40 mg/kg) only.

5. Post Procedure Monitoring (during the 90 min Anesthesia)

1. Use heat gel pads during the 90 min anesthesia.
   1. To maintain the body temperature and to prevent suffocation, do not allow mice to recover on the regular corn-cob mouse bedding.

2. After the recovery from anesthesia, administer an additional 1 ml of sterile 0.9% NaCl subcutaneously in two locations on the back.
3. Keep up to five PBS (sham) inoculated mice per cage. House only 1-2 infected mice per cage.
4. Fill out the procedure card with initials every day after the procedure to note down the changes in the body weight, grooming habits and overall health.

6. Assessing the Inflammation

6.1) Weight loss

1. Weigh the mice every day during the infection, starting on the day prior to infection procedure.
   NOTE: Typically, the immunodeficient mice injected with Th17 cells and with no T_{regs} succumb to 20-30% weight loss due to increased fungal burden[19] and exacerbated inflammation.

6.2) Histology scoring of the tongue

1. Sacrifice the mice by CO_2 asphyxiation followed by cervical dislocation.
2. Open the jaws and dissect out the tongue with scissors and forceps. Use blunt forceps to hold the tongue, and using the scissors reach out to the back of the mouth to incise the back end of the tongue.
3. For immunocytochemical hematoxylin and eosin (H&E) staining of the tongue tissues, rinse the tongue tissues with ice cold PBS. Add 5 ml of 10% formalin for 2-3 tongues and fix them O/N in 15 ml tubes.
   1. The next day, remove the formalin and immerse the tissues in 5 ml of 70% ethanol to prevent hyper-fixation. Send them out to commercial service to continue with paraffin embedding, sectioning and staining of paraffin sections.
   NOTE: Commercial histology service is used to perform these steps.

4. Once the slides are back from the commercial histology service, grade the inflammation by observing the tissue sections under a light microscope.
   1. Score from 0 to 5, with 0 being no inflammation, and 5 being the most severe inflammation. Score the inflammation using Table 1.

6.3) Cytokine production by Th17 cells

NOTE: Excessive TNF-α production is one of the readouts for excessive immunopathology. When mice are adoptively transferred with Th17 cells only, it causes immunopathology that is associated with excessive TNF-α production in Th17 cells.

1. Collect a single cell suspension from spleen, cervical lymph nodes, axillary lymphnodes, and tongue, using previously described methods[19,26].
2. Restimulate the cells with phorbol myristate acetate (PMA) (50 ng/ml) and Ionomycin (500 ng/ml) for 4 hr with Brefeldin A (10 µg/ml) added in the last 2 hr. Wash the cells with PBS and fix them using a commercial Fixation/permeabilization kit according to manufacturer's instructions.
3. Perform intra cellular cytokine staining using the fluorochrome conjugated anti-TNF-α, anti-IL-17A and ROR-γt antibodies as described previously[19].
4. Briefly, resuspend the cells in the 1X permeabilization buffer with the cocktail of anti-TNF-α, anti-IL-17A and ROR-γt antibodies, each antibody at 2 - 3 µg/ml final concentration.
5. Incubate the cells for 1 hr at RT.
6. After the incubation, wash the cells with 1X permeabilization buffer.
7. Resuspend the cell pellet in 500 µl of PBS with 0.5% bovine serum albumin for flow cytometry analysis.

Representative Results

In this model, both the *Candida*-infected mice and uninfected mice in Rag-1^{-/-} immunodeficient background were adoptively transferred with Th17 cells 3-5 days prior to the infection. A total of 10-12 mice were used for the experiments, with 2 mice in each Sham infected groups, and 4-5 in each of the *Candida* infected groups. Naive cells were derived from congenic Thy1.1 or CD45.1 mice, so that injected Th17 cells were tracked *in vivo* using Thy1.1 or CD45.1 staining respectively (**Figure 1A**). Co-transferred T_{regs} were derived from CD45.2 mice to distinguish them from CD45.1 Th17 cells. For some experiments, C.B-17 Thy1.2 *scid* recipient mice, Th17 donor cells obtained from Thy1.1 Balb/C mice and T_{regs} obtained from congenic Thy1.2 mice were used. Only *Candida*-infected mice, but not the uninfected mice exhibited the recruitment and expansion of reconstituted CD45.1^{+} Th17 cells in the CLN, the draining lymph nodes. This result demonstrates that the fungus Th17 specific response is initiated in tongue draining lymph nodes (**Figure 1A**). The results represent data from one mouse in each group.

Only in the mice that are co-transferred with T_{regs}, Foxp3+ CD45.1 negative CD4+ cells were detected in the CLN. This shows that the injected T_{regs} were also recruited to the site of infection (**Figure 1B**). The results represent data from one mouse in each group. Whereas infected Rag-1$^{-/-}$ lose weight and recovered after 4 days of infection, cortisone immunosuppressed mice did not recover but further lost weight, as shown previously[27]. Both the groups of mice that received Th17 cells in the presence or absence of T_{regs} lost weight initially. However, the mice that received T_{regs} recovered from weight loss and resolved the infection. 2 mice were used in the Sham infected group, and 4 in each *Candida* infected group. There were 3 mice in cortisone group. The mice that received Th17 cells only, lost weight progressively (**Figure 2**)[19]. Some of the mice in that group had to be euthanized. These results show the average of the weights of all the mice in that group. Although neutrophils are required for the initial clearance of the infection, their continuous presence and recruitment in the tissue is a sign of unresolved inflammation. Therefore neutrophil infiltration was examined in the infected tongue, by determining the expression of a neutrophil marker, Gr-1, at later time points such as day-7 after infection. Mice that received Th17 cells only, had higher inflammatory infiltrates (**Figure 3A**), including Gr-1+ neutrophils even day-4 after infection, compared to the mice injected with T_{regs} (**Figure 3B**). These results showed that in the presence of T_{regs}, mice resolved the inflammation more efficiently and recovered from the infection.

On day 7 after infection, spleen (SPLN), CLN and the tongue tissues were isolated, and single cell suspensions were made for flow cytometry analyses. CLN revealed increased CD45.1 Th17 cells (**Figure 1**) and heightened TNF-α production (**Figure 4A, 4B**) in mice that received Th17 cells only. On the other hand, T_{reg} recipients showed decreased frequency of Th17 cells and reduced TNF-α production by Th17 cells (**Figure 1 & 4A, 4B**). In these experiments, data from CLN of a single mouse, and tongue tissues harvested and pooled from 2 mice are shown. Although at early time-points IL-17A production was higher in T_{reg} recipients[19], at later time points in both the groups, IL-17A production by Th17 cells was minimal (data not shown). Periodic Acid Schiff's staining of the tongue showed increased fungal hyphae in mice that received Th17 cells only, whereas mice that received T_{regs} as well cleared the fungus on day-5 and day-7 after infection (**Figure 5**). Moreover, histopathology scoring of the tongue inflammation also showed reduced inflammation scores in mice that received T_{regs}, compared to mice that received only Th17 cells (**Figure 6**). Scoring the inflammation was based on the tongue histopathological parameters such as, presence of papillae, the intactness of the superficial epithelial layer, visible yeast or hyphae, invading hyphae indicating non-resolving infection, thickening of basal layer (indicative of ongoing tissue repair), and the presence of infiltrating immune cells (neutrophils and Th17 cells). These results demonstrated that injection of Th17 cells did not directly reduce infection but increased the fungal burden and immunopathology caused by primary *C. albicans* infection, whereas co-transfer of T_{regs} ameliorated immunopathology.
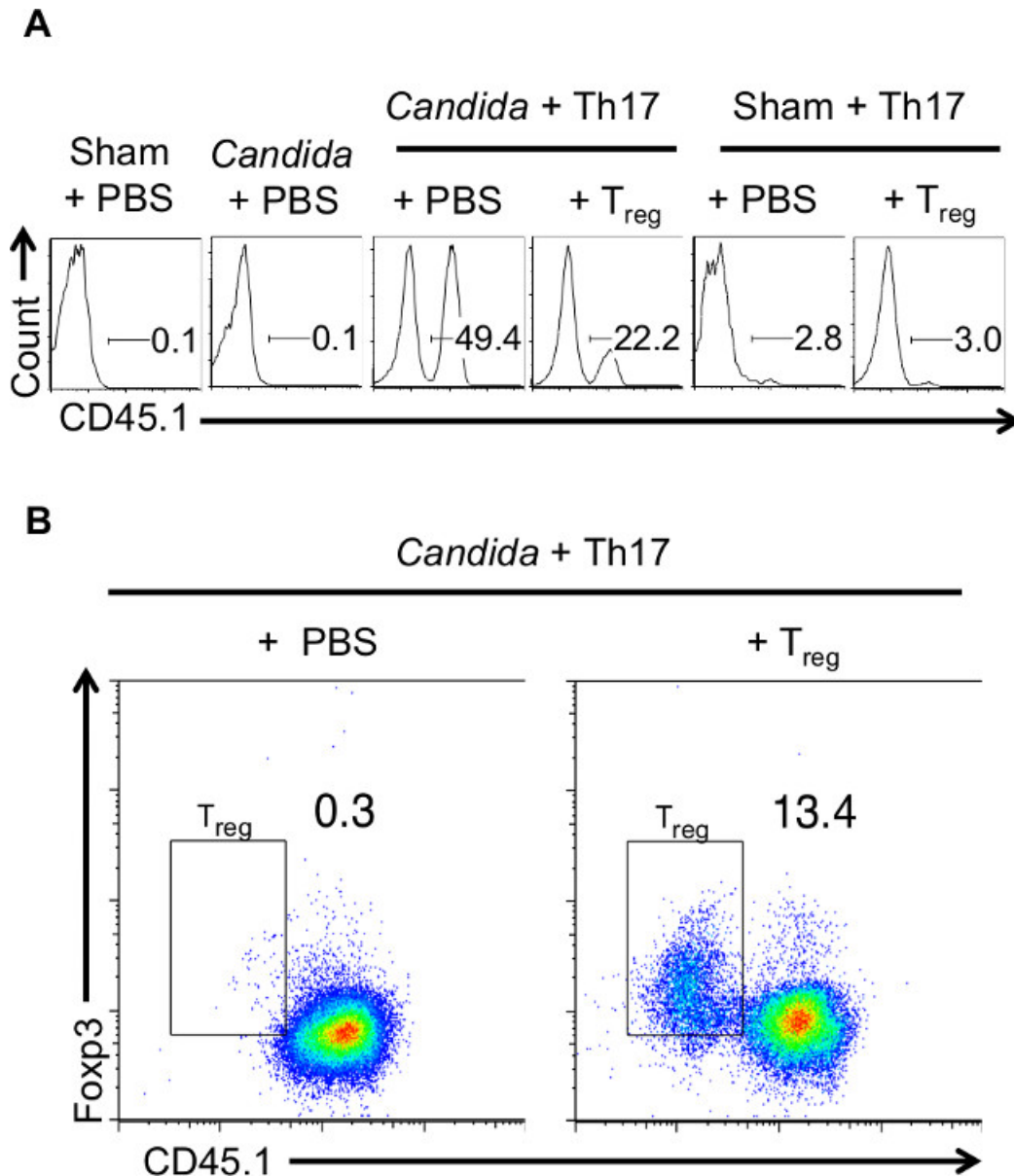
Figure 1. Injected Th17 cells and T_reg cells recruit and expand in the draining lymph nodes (CLN) of the *C. albicans* infected mice.
(A) Rag-1$^{-/-}$ CD45.2 mice were reconstituted with Th17 cells or Th17 + T$_{reg}$ cells that were polarized under Th17 conditions for 5 days. Th17 cells were obtained from CD45.1 congenic mice and T$_{reg}$ cells were obtained from CD45.2 mice. Some mice did not receive any cells (Sham or Candida + PBS). Recipient mice in each group were infected with sham controls or with *C. albicans*. On day 5 after infection, CLN were isolated to make a single cell suspension and track the injected CD45.1 expressing Th17 cells by flow cytometry (gated on all leukocytes in FSC, SSC scatter). (B) Rag-1$^{-/-}$ CD45.2 mice were reconstituted with Th17 cells or Th17 + T$_{reg}$ cells as in "A" and infected with *C. albicans*. CLN were isolated to make a single cell suspension and measure CD45.1 and intracellular Foxp3 expression by flow cytometry (gated on all CD4 cells). The T$_{reg}$ gates in the plots show the CD45.1 negative, Foxp3+ CD4+ cells. Please click here to view a larger version of this figure.
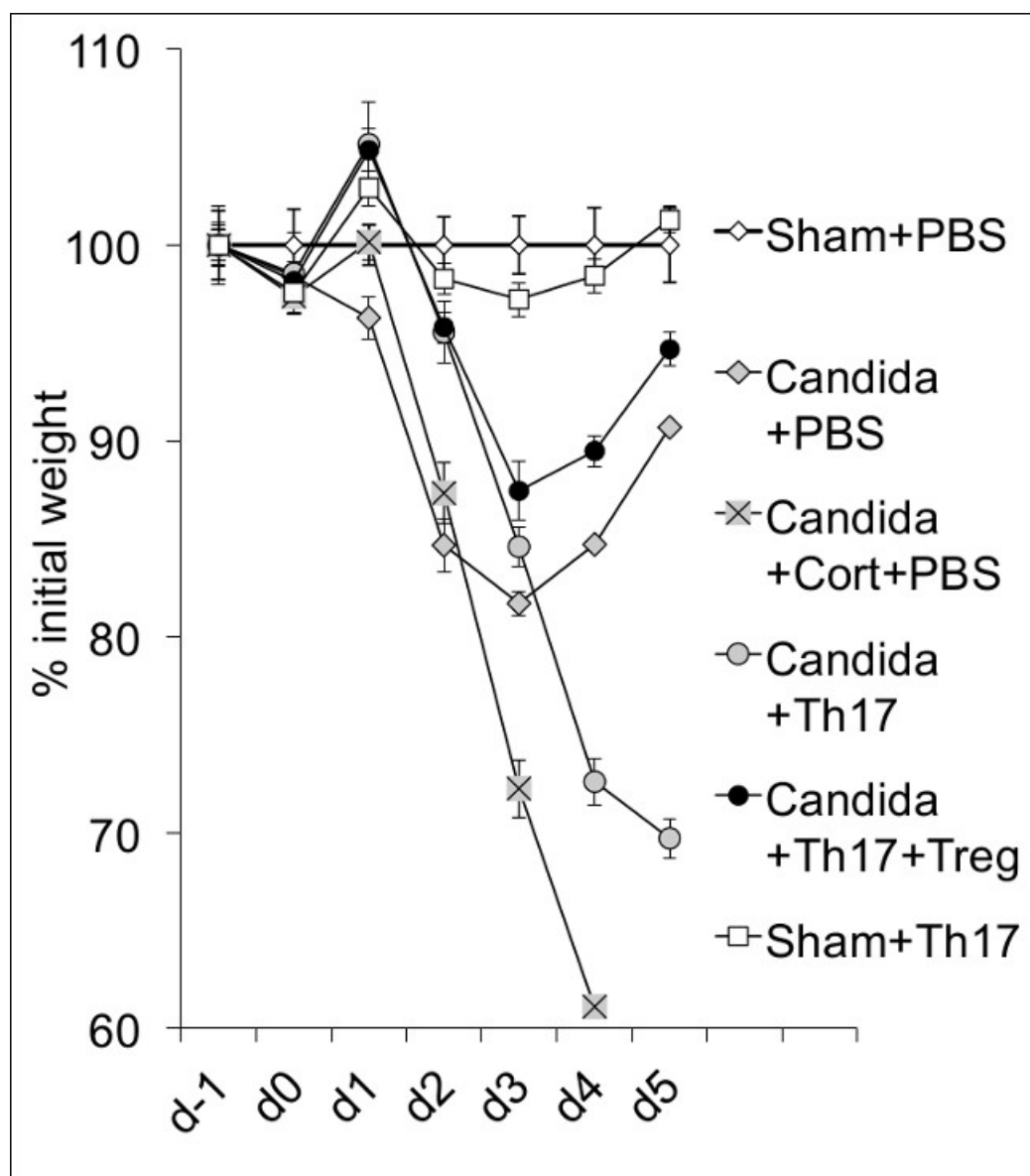
Figure 2. Injected Th17 cells cause weight loss in *C. albicans* infected mice and co-administration of T$_{regs}$ improves recovery from weight loss. SCID CB-17 mice were reconstituted with Th17 cells or Th17 + T$_{reg}$ cells as in **Figure 1A**. 3 days later, the recipient mice were infected with *C. albicans.* Some mice in each group were infected with sham controls. Immunosuppressed mice received cortisone acetate (Cort) injection. The percent weight change in mice reconstituted with indicated cells and infected with *C.albicans,* with respect to d-1 (one day prior to infection) is shown. Data are normalized relative to the weight data from uninfected (Sham + PBS) mice. Please click here to view a larger version of this figure.
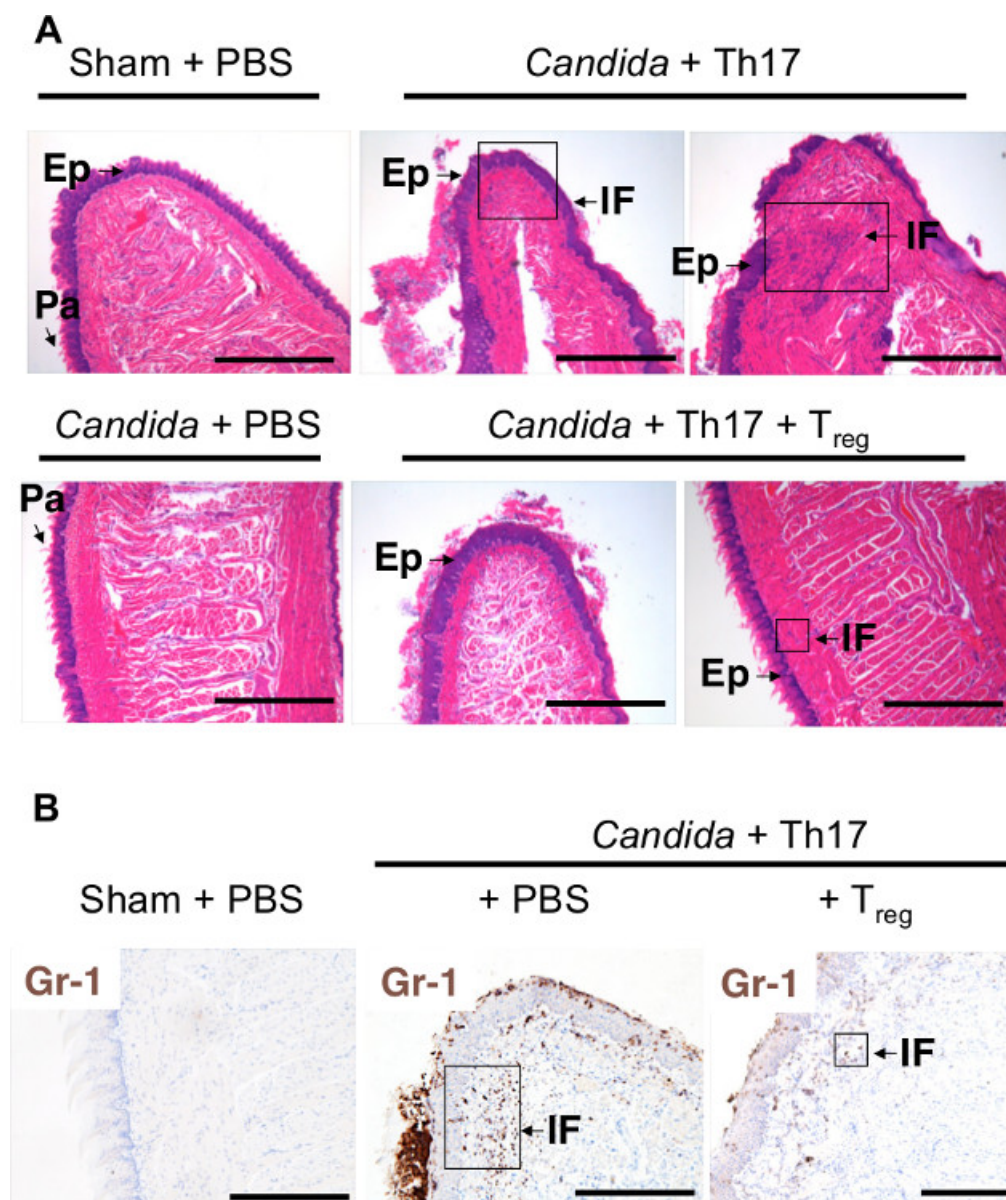
Figure 3. Injected Th17 cells cause tongue immunopathology in *C. albicans* infected mice and co-administration of T_{regs} reduces the immunopathology. Histological evaluation of the tongues of the *C. albicans* infected mice. Mice were reconstituted with indicated cells and infected as in **Figure 1A**. On day 5 after infection, tongues were isolated from mice. **(A)** Sagittal sections of the tongues were stained with H&E to assess inflammation and infiltration (IF) of the immune cells. (Pa) and (Ep) denote papillae and the epithelial layer of the tongue respectively. Microscopic images of the slides viewed at 50X magnification. **(B)** Histological immunostaining for Gr-1 neutrophil marker using anti Gr-1 antibody (Clone: 1A8-Ly6g) in the tongue sections on day 5 after infection (brown, denoted by IF). Microscopic images of the slides viewed at 100X magnification are shown. Please click here to view a larger version of this figure.
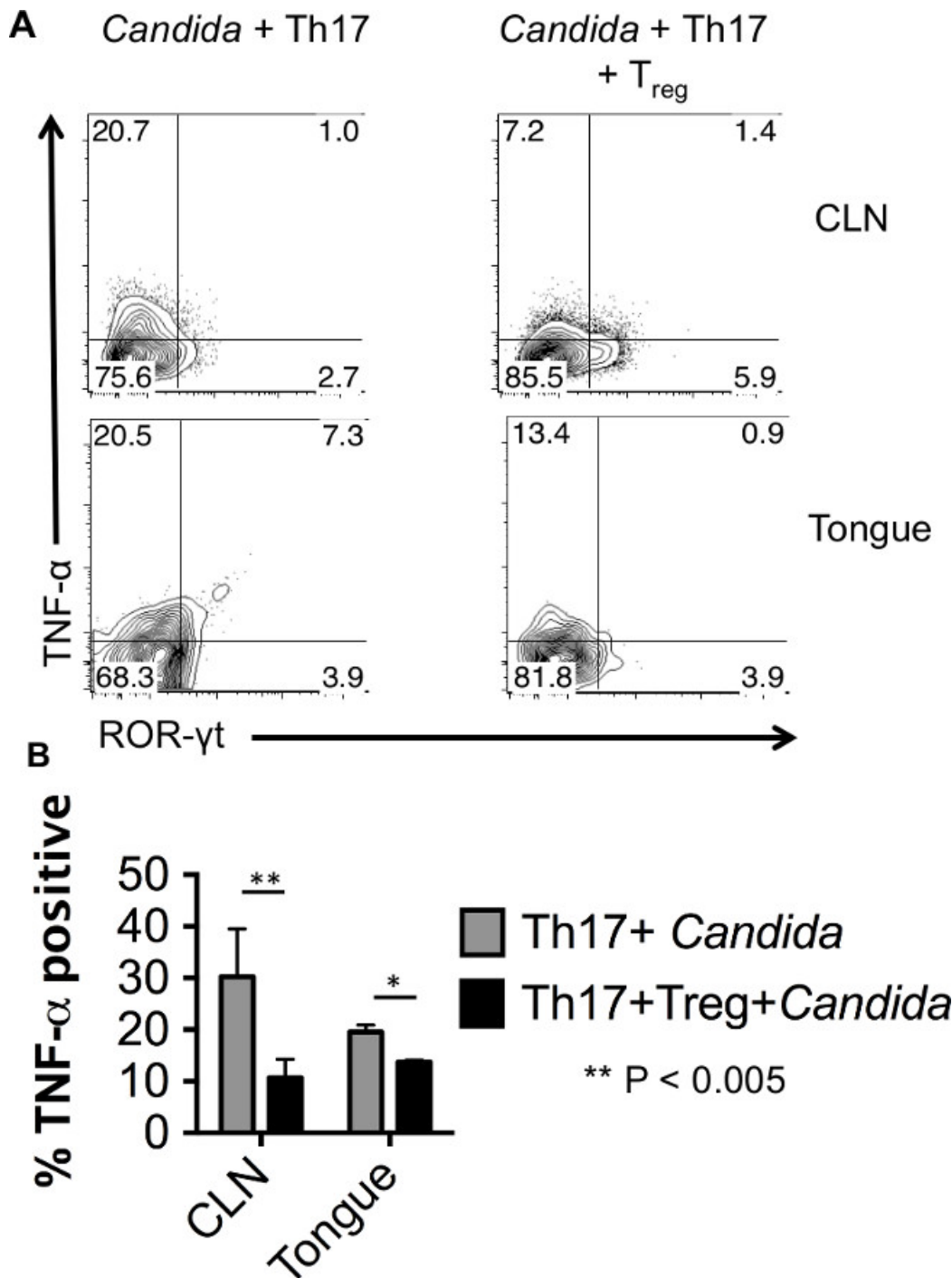
Figure 4. Co-administration of T_regs reduces TNF-α in injected Th17 cells. (A) Rag-1^{-/-} CD45.2 mice were reconstituted with Th17 cells or Th17 + T_reg cells and infected with *C. albicans* as in **Figure 1A**. On day 5 after infection, CLN and tongue tissues were isolated to make a single cell suspension. These cells were restimulated with PMA and Ionomycin before intracellular staining and flow cytometry analyses. Flow cytometric contour plots of intracellular ROR-γt and TNF-α expression of the Th17 cells are shown (gated on CD4+ cells). **(B)** Statistical representation of the percentage of TNFα positive cells from experiments performed as in "A". Data represent 4 mice in uninfected group, and 6 mice in each infected group, and are pooled from two independent experiments. Please click here to view a larger version of this figure.
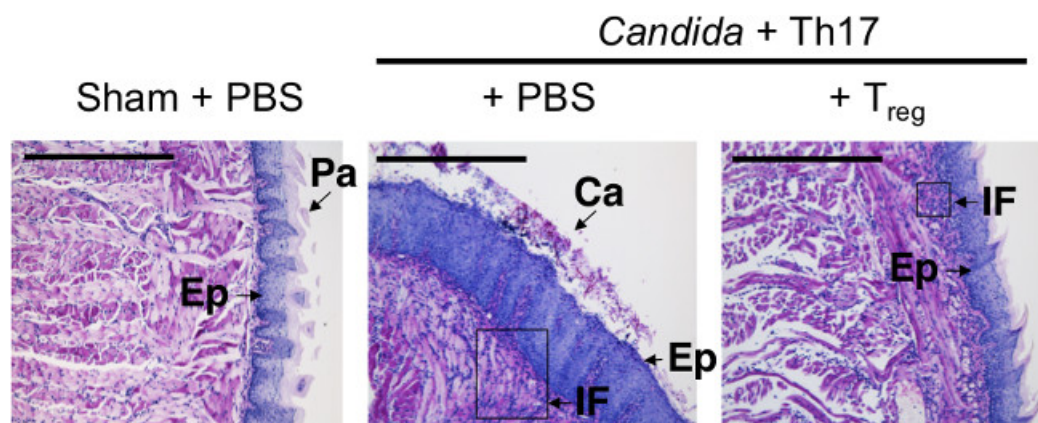
Figure 5. Increased immunopathology is also associated with increased fungal burden in *C. albicans* infected mice. C.B-17 *scid* mice were reconstituted with Balb/C Th17 cells, or Th17 + T_{reg} cells as in **Figure1A**, and were infected with *C. albicans*. Histological evaluation of the tongue from infected mice is shown. On day 7 after infection, tongues were harvested and stained with periodic Acid Schiff's (PAS) to assess fungal lesions, inflammation and infiltration (IF) of cells, and to detect fungus(Ca). The slides were viewed at100X magnification. Please click here to view a larger version of this figure.
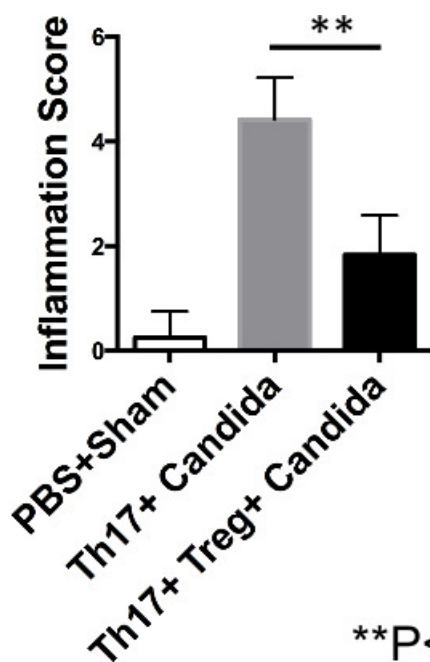

Figure 6. T_{regs} ameliorate immunopathology in *C. albicans* infected mice. C.B-17 *scid* mice were reconstituted with Balb/C Th17 cells, or Th17 + T_{reg} cells as in **Figure 1A**, and were infected with *C. albicans*. Statistical representation of the mean histological scores of the tongues from infected mice assessed as in **Figure 5**. Please click here to view a larger version of this figure.

Score	Characteristics
0	0 – no visible yeast or hyphae, intact superficial epithelium on dorsal surface of tongue, papillae clearly present and undamaged (may or may not be infiltrating mononuclear cells within the tissue), no thickening of basal layer, no infiltrating immune cells.
1	1 - no visible yeast or hyphae, ragged or slightly damaged superficial epithelium, papillae clearly present with minimal damage and infrequent infiltrating immune cells.
2	2 - papillae reduced but present with damage, thickening of basal layer and frequent infiltrating immune cells.
3	3 - occasional clusters of visible yeast and invading hyphae, evidence of damaged papillae, occasional lesions of damaged epithelium, thickening of basal layer and and small clusters of infiltrating immune cells.
4	4 - frequent clusters of visible yeast and invading hyphae, evidence of damaged papillae, frequent lesions of damaged epithelium, minimal infiltration of immune cells, thickening of basal layer and frequent clusters of infiltrating immune cells.
5	5 - widespread evidence of invading hyphae, extensively damaged epithelium across the dorsal surface, large infiltration of immune cells, few or no detectable papillae, thickening of basal layer and highly frequent and large clusters of infiltrating immune cells.

Table 1: Histological Scoring Values

Discussion

This model is based on inducing oral *C. albicans* infection dependent Th17 inflammation. Because of the absence of T_{regs}, the Th17 cell induced inflammation is unrestrained and leads to poorly resolved immunopathology. *In vitro* derived naïve CD4 cells polarized as Th17 cells were used for the adoptive transfer. 40 - 50% of the cultured CD4+ cells show detectable IL-17A expression around day 3 (Th17 cells), and therefore were used for the injection in mice. The major advantage of the model is that Th17 cells cause infection specific immunopathology that can be efficiently ameliorated with T_{reg} injection. Thus the model can be employed to test immunomodulation strategies with T_{reg} injection as a positive control. Inflammation is easily assessed based on the weight loss, histopathological scoring and pro-inflammatory cytokine production by the Th17 cells of the infected mice.

After the adoptive transfer of Th17 cells, they migrated to secondary lymphoid organs and various tissues including the tongue, the primary site of infection. After the infection, other inflammatory cells were also recruited to the tongue to clear the infection. However, without T_{reg} mediated immunomodulation, Th17 cells themselves did not resolve the infection but caused heightened immunopathology. Co-transfer of T_{regs} completely resolved the inflammation. Interestingly, mice with exacerbated inflammation in the absence of T_{regs}, also showed increased fungal burden. Our previous report showed that this was due to reduced IL-17A production in mice, compared to those with T_{regs}. Our current experiments support the idea that it may also be due to increased TNF-α that worsened the inflammation, possibly by increasing epithelial cell apoptosis and increased fungal burden[28]. Considering the immunoprotective effects of IL-17A at the mucosa, we believe that inflammatory pathology that we observe with increasing fungal burden is not due to IL-17A produced by Th17 cells or lack of host resistance. It is caused due to TNF-α and possibly other pro-inflammatory cytokines produced by injected Th17 cells. This can be validated by the observation that Rag-/- mice devoid of Th17 cells show only minimal inflammation and subsequent resolution of infection and only those that received Th17 cells succumbed to severe weight loss (**Figure 1**). Consistent to this data, it has been also previously shown that Rag^{-/-} mice clear the primary OPC infection without much immunopathology[27]. Under these conditions, compensatory mechanisms dependent on IL-17A producing innate immune cells have been shown to clear the infection in Rag-/- mice[17,29,30]. Taken together, although IL-17A produced by Th17 cells and neutrophil recruitment are playing a protective role at initial phases of infection, excessive production of TNF-α and other cytokines by Th17 cells, and the continued presence of neutrophils causes exacerbated immunopathology. The roles of pro-inflammatory cytokines besides TNF-α produced by Th17 cells remain to be investigated in the context of immunopathology.

The major limitation of the model is the question whether it is physiologically relevant to human OPC. Although Th17 cells are shown to be pathological mediators in several oral diseases, whether chronic activation of Th17 cells is pathogenic in OPC remain to be investigated. With recent findings implying the role of *C. albicans* in inflammation and cancer, Th17 immunopathology in the oral cavity is an active field of investigation[31]. Thus the model that is described here can be used to more carefully study specific roles of Th17 cells and how they interact with innate immune cells, giving detailed insights in to oral immunological diseases and oral cancer.

The critical step in the technique is the appropriate recruitment and expansion of the adoptively transferred Th17 cells. This is highly dependent on the viability and IL-17A production of the *in vitro* polarized Th17 cells before the transfer. It is best to test their viability and cytokine production by flow cytometry analyses[19]. Proper execution of the Th17 cell injection and mice infection will lead to infection dependent Th17 immunopathology. This model can be applied to test strategies to thwart Th17 immunopathology and oral inflammation in the context of other infections as well. Also, using this model, one can perform secondary re-infections and study the role of Th17 cells in exacerbating immunopathology in the context of chronic infections. As this model involves immunodeficient mice, it may be very relevant to oral dysbiosis seen in AIDS and PID patients, who suffer from recurring chronic *Candida* infections.

Disclosures

The authors declare that they have no competing financial interests.

Acknowledgements

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