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THE ROLE OF INNATE RECEPTOR TLR2 IN NEUTROPHIL RECRUITMENT IN OROPHARYNGEAL CANDIDIASIS (OPC)



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ABSTRACT

Candida albicans (CA) is a dimorphic fungus that commonly colonizes the oral cavity. Overgrowth can result in an infection of the oral cavity, known as oral candidiasis (OPC). Neutrophils play a major role in nonspecific immune defense against fungal infection. Neutrophils then engage in phagocytosis of the *C. albicans* by engagement of membrane innate receptors, called pattern recognition receptors (PRRs) that interact with certain identifying molecules on the cell wall of the infecting fungus, called pathogen-associated molecular patterns (PAMPs). Toll Like Receptors (TLRs) are a type of PPR expressed on neutrophils and are believed to play a major role in its activation and migration.

This study evaluates the role of TLR1, 2, and 6 in neutrophil recruitment in tongues utilizing a murine model of OPC. Tongue sections from C57BL/6, TLR1, TLR2, and TLR6 KO mice were stained with a neutrophil specific NIMP/alex488 antibody. Neutrophil recruitment was quantitatively assessed by comparing the amount of fluorescence in the tongue epithelium.

Our results found that the lowest percent fluorescence was experienced in the tongue sections of the TLR2KO mice meaning that neutrophil recruitment was hindered. We also observed hindered neutrophil recruitment in the TLR1, and TLR6 KO mice, however it was not as severe as in the TLR2 KO mice. These results imply that all three TLR types 1, 2, and 6 play a role in neutrophil recruitment in the OPC model.

INTRODUCTION

The human oral cavity serves as a reservoir for hundreds of commensal microbes that coexist within a delicate balance, between microbes and the host immune system. These microbes live together in complex networks termed biofilms, in which the microbes interact cooperatively to promote survival and growth. Many parameters affect this biofilm such as temperature, pH, oxygen, fluid flow, as well as the host's age. In particular, the tongue offers microbes an extremely fertile environment for biofilm colonization, due to its numerous protected sites¹.

Any disturbance in the oral cavity's normal flora can allow for the overgrowth of normal commensal organisms, such as *Candida albicans*, resulting in a pathogenic infection. The propagation of this fungus can result

in a very painful and in some cases fatal condition known as oropharyngeal candidiasis (OPC)². Candidiasis is an opportunistic pathogen, meaning that it usually does not cause disease in healthy individuals. Hence, hosts who are immunocompromised; typically elderly, those who suffer from AIDS, people that undergo chemotherapy, infants, or transplant patients, are at higher risk of infection. CA is the 4th most common life threatening infection among all bloodstream and nosocomial infections in the United States³.

Candida species are dimorphic fungi capable of existing in both a yeast form and a hyphal form. One of the complicating factors of *Candida* infection is its ability to switch morphogenic states at body temperature. Yeast and hyphae express different surface molecules at different levels making the initiation of effective immune responses to infection more difficult⁴. When invading tissue, *Candida* is most commonly found in the hyphal form, which is capable of destroying surrounding tissue via mechanical processes. The first line of defense against infection involves the physical barrier provided by epithelial cells. Once *Candida* has broken through the barrier of the epithelia, control of infection and prevention of dissemination involves the immediate activity of the innate immune system.

The main cells of the innate immune system that recognize invading pathogens are monocytes and neutrophils. Neutrophils are polymorphonuclear, phagocytic cells that are part of the innate immune system's first responders to a pathogen infection. Upon infection, a variety of chemical mediators, called cytokines and chemokines, are released resulting in the increased surface expression of adhesion molecules involved in neutrophil infiltration and trigger dilation in the blood vessels around the infected area. This in turn causes added permeability of blood vessels and endothelial cells. Neutrophils then migrate from capillaries and pass through the endothelial layer to the site of infection. This migration from the blood to the site of infection utilizes a process known as chemotaxis. Chemotaxis involves the movement of neutrophils from areas of low chemokine concentration, such as blood, to areas of high chemokine concentration, the site of *Candida* infection. There is a multitude of protein and carbohydrate molecules that play a critical role in the migration of neutrophils to sites of infection.

Until recently, little was known about the process by which neutrophils recognize *C. albicans* as a pathogen.

The oversimplified belief that innate immunity was non-specific and employs an 'ingest and destroy' method could not explain the specific and diverse responses of innate immunity. Current research shows that pattern recognition receptors (PRRs) assume the role of recognizing, for the innate immune system, invading pathogens including *C. albicans*. PRRs recognize conserved molecular motifs within pathogens called pathogen-associated molecular patterns (PAMPs). In mammalian molecules, four types of PRRs have been identified as Toll-like receptors (TLRs), C-type lectin-like receptors, Nucleotide-binding oligomerization domain (NOD) like receptors, and retinoic-acid-inducible gene receptors.

TLRs are transmembrane proteins containing an extracellular domain to identify PAMPs and an intracellular domain that activates signaling cascades upon PAMP interaction. Currently, 10 TLRs have been identified in humans that are capable of engaging specific PAMPs. An example is TLR₂, which recognizes phospholipomannan, a phylogenetically unique fungal component. C-type lectin-like receptors mainly constitute membrane bound receptors that recognize polysaccharide structures, such as β -glucans, that are found in the cell wall of *C. albicans*⁵. Nucleotide-binding oligomerization domain (NOD)-like receptors and retinoic-acid-inducible gene receptors are both cytoplasmic receptors, involved in recognizing viral nucleic acids. No studies have documented any involvement of these receptors in the recognition of fungal pathogen components⁶.

Research utilizing fluorescent analysis of neutrophils stained with anti-TLR antibodies has indicated that neutrophils have been shown to express all known TLRs except for TLR₃^{7,8}. These TLRs play a role in the recognition of the components of fungal pathogens, and further activation and recruitment of neutrophils⁹. The aim of this research is to investigate neutrophil recruitment into the epithelial tissue of the tongue in response to oropharyngeal candidiasis (OPC) and its dependence on TLR₂ and its co-receptors TLR₁ and TLR₆. Due to the ability of TLR₂ to recognize fungal PAMPs, we expect to see a much lower neutrophil count in tongue sections of TLR₂KO mice. Doing this will give us a better understanding of the specific roles of TLRs 1, 2, and 6 in the recognition of *C. albicans* for neutrophil recruitment, and further help us understand the mechanisms that propagate the innate immune response.

MATERIALS & METHODS

Animals

C57BL/6 mice were used as the control for this experiment. They were obtained from Jackson Laboratories (Bar Harbor, ME). TLR2KO, TLR6KO, TLR1KO mice were graciously provided by Dr. Shizuo Akira (Osaka University, Osaka, Japan). All animals used in this experiment were housed in filter-covered micro-isolator cages. During breeding and storing they were housed in the Wolstein Animal Facility at Case Western Reserve University. Mice were then transferred to the Wearn Animal Facility at University Hospitals in Cleveland for infection and the duration of the experiment. Case Western Reserve University School of Medicine Institutional Animal Care and Use Committee has approved all protocols and experimen

Fungal preparation

The strain of *C. albicans* (CA) that was utilized in this study was GDH2346, originally isolated from a denture stomatitis patient. The strain was propagated and maintained in Sabourand Dextrose (SD) agar at 4°C. Prior to mouse infection *C. albicans* was scraped off the SD agar and transferred to a yeast-inducing SD broth (Difco, Sparks, MD) and incubated at 37°C with a shaking for a period of 16 – 24 hrs. After incubation, yeast was collected and washed 3x with PBS. Yeast was spun down and pelleted using a centrifuge between each wash step. The CA was then suspended in PBS and cells were counted on a hemacytometer. Yeast was then diluted to the desired concentration of 5x10⁷ yeast/mL OPC infection Model- Five days before infection with *C. albicans*, the mice were fed tetracycline water (2.5g/L), a form of antibiotic, to reduce competing oral flora. The mice were then injected with an anesthesia cocktail (25% Ketamin (Vedco, St Joseph, MO), 7% Acepromazine (Boehringer Ingelheim, Germany)). After the mice had been properly anesthetized, 4-6 evenly spaced incisions were made on the dorsal layer of the tongue making sure not to pass the epithelium and cause bleeding. All incisions were made using a sterile #10 blade scalpel. An autoclaved cotton ball saturated with PBS was then inserted into the mouse's mouth to prevent dryness. After 3 hours the cotton ball was removed and 100 µL of 5.0 x 10⁷ CA suspension was pipetted onto the tongue with a new cotton ball, and the animal was left for approximately 4-6 hours while sedated. After a three day infection period the mice were then euthanized us-

ing a CO₂ asphyxiation chamber. Using sterile autoclaved instruments the tongue was removed and treated for slide preparation. Three different BL6 mouse tongues were obtained as well as two from each of the TLR1KO and TLR6KO mice, and finally only one TLR2KO mouse tongue was obtained.

Immunohistochemistry

After the tongues were removed they were immersed in Embedding Media (Tissue Tek) (EMS Hatfield, PA) and flash frozen using liquid nitrogen. The tongues were then transferred on dry ice to a microtome-cryostat to be sectioned. From each tongue 2 slides containing two 5-micron sections each, and one slide containing two 10-micron sections were prepared. All slides were then stored in a -20°C refrigerator.

The 10-micron slide was stained with Periodic acid-Schiff and hematoxylin (PASH). One of the 5-micron slides was stained with an anti-neutrophil antibody (NIMP supernatant) and incubated for 2 hours. After washing with PBS-triton the slide was then stained and incubated for 45 minutes with the secondary antibody conjugated to the green fluorochrome Alexa-488 at a dilution of 1:400 in 1% normal goat serum, which provided the fluorescent tag that allowed neutrophil detection and imaging with the florescent microscope. After washing again with PBS-triton the slide was then mounted in Vectashield containing the nuclear stain DAPI (Vector Laboratories, Burlingame, CA). The other 5-micron slide was used as the negative control for the neutrophil staining. This was done by using the same method for staining the first 5-micron slide without using the primary antibody (NIMP).

Microcopy and Quantification

The images were taken using a Leica DMI 6000B inverted microscope. For each NIMP slide, a 10x resolution image was taken of the whole tongue at an exposure of 4500 milliseconds. This was accomplished by stitching together a large number of consecutive 10x images of the tongue. In addition to the stitched image a 20x image was taken of the area of high fluorescence both in green fluorescence (Alex488) as well as a combined superimposed image of green and blue fluorescence (Alexa488 & DAPI).

For the PASH stained slides a 10x stitched image of the whole slide was taken as well as 20x images of the area of the epithelium where fungal hyphal infiltration was observed.

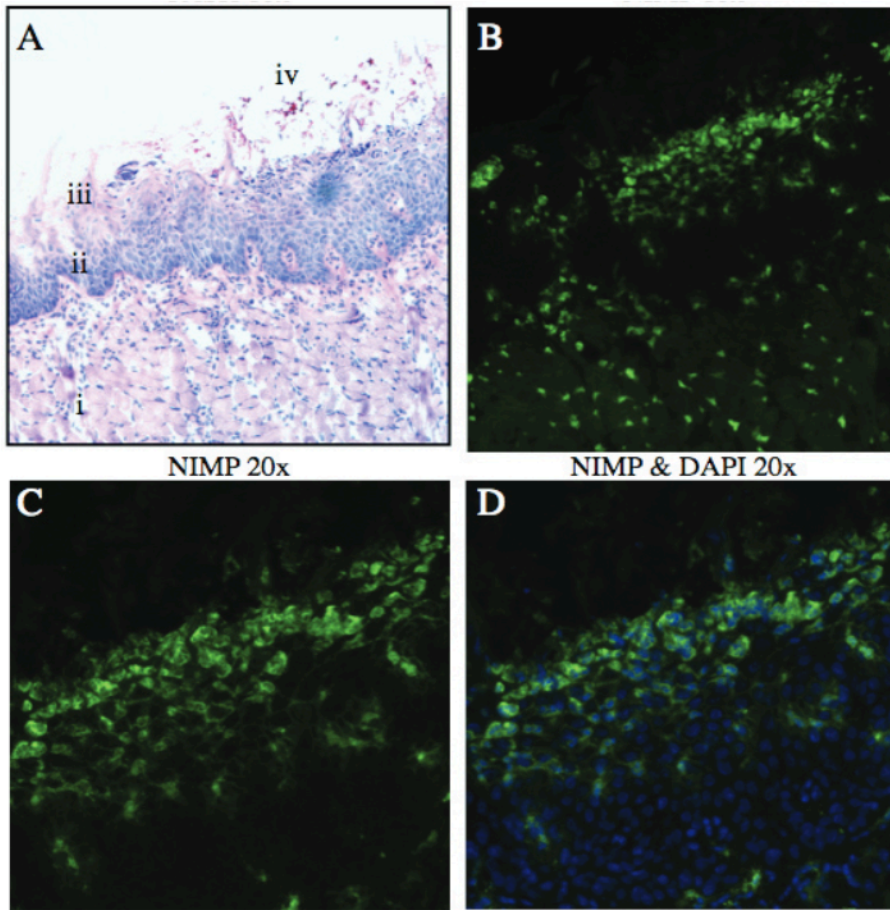


Figure 1: Sections of C57BL/6 OPC infected mouse tongue epithelium tissue, stained with PASH stain to show histology and *C. albicans* growth, and NIMP/Alexa488 and DAPI stain on the neighboring tongue section to show neutrophil migration and nucleus placement. A) PASH stain of tongue epithelium (blue) *C. albicans* is seen on the top of the epithelium (magenta). B) corresponding tongue section with NIMP/Alexa488 (green). Neutrophils fluoresce green and are migrating to the site of *C. albicans* infection. C) 20x image of the same section. D) Same section with NIMP/Alexa488 (green) stain and DAPI (blue) stain.

To quantify the number of neutrophils in each tongue section, the cell counting technique was opted for digital quantifying method using the imaging program Metamorph. For each image taken by the program a number between 1 and 4095 is assigned for each pixel corresponding to its “fluorescing” value. To quantify the results we used the 10x-stitched images of each tongue and using Metamorph the epithelial region of the tongue was selected. In the selected region the total amount of pixels were counted as well as the total amount of pixels that had number values that was considered fluorescing, which was set between 715 and 4095. The percent fluorescence was calculated by dividing the number of fluorescing pixels by the total amount of pixels in the epithelium.

RESULTS & DISCUSSION

Using NIMP/Alexa488 staining, we visualized neutrophil recruitment in the tongues of different OPC infected TL-RKO mice and compared them to the BL6 mouse control. In addition to qualitative assessment done with the fluorescent microscope, we used the analytical program, Metamorph. With Metamorph we quantified our results in order to better analyze the neutrophil recruitment in the tongue epithelium. Figure 1 displays PASH staining and neutrophil staining of a BL6 mouse. Image A displays the PASH stain showing the tongue histology. Part ‘i’ shows the muscle and blood vessel tissue, ‘ii’ shows the superior epithelium of the tongue, ‘iii’ shows the outer most layer of the tongue the stratum corneum that consists of keratinized oral mucosa1, and finally ‘iv’ shows the *C. albicans* infiltration and growth on the surface of the tongue. Images B & C show 10x and 20x images of NIMP/Alexa488 staining of that same location on a serial section of the tongue. The green fluorescence corresponded to neutrophil specific staining. Finally, image D shows a superimposed NIMP/Alexa488 and DAPI staining. DAPI stain is a DNA specific fluorescent stain, hence the blue fluorescence in this image corresponds

to all the nuclei in the image. Looking at the neutrophil nuclei we can visualize the polymorphonuclear properties of these cells confirming that our NIMP/Alexa488 stain corresponded to neutrophils.

Figures 2, 3, 4 and 5 display PASH and NIMP/Alexa488 staining of OPC infected tongues for BL6, TLR1KO, TLR2KO, and TLR6KO mice respectfully. Qualitatively we saw a trend of a much weaker neutrophil recruitment response to the *C. albicans* infection in all of the gene knockout mice compared to the BL6 control. Also we noticed another trend where the *C. albicans* growth and epithelium infiltration were more prominent on the tongues of the TLR1KO, and TLR6KO mice.

We used Metamorph to calculate the presence of fluorescence for each of the tongue sections stained. We then calculated average percent of fluorescence for each

mouse genotype and we graphed the values in Figure 6. Our results are consistent with our qualitative analysis of the staining; we saw a negative trend in neutrophil recruitment with any of the knockout mice. We saw the lowest percent of fluorescence in our TL2KO mouse. The calculated fluorescence value for the TLR2KO mouse was 0.61% compared to the average 1.7% fluorescence of the Bl6 control mice. TLR2 is a cell surface receptor present on neutrophils. It has been implicated in the recognition of the lipopeptide phospholipomannan, a component *C. albicans*. The neutrophils in TLR2KO mouse lack this surface receptor and hence the ability for them to migrate to the site of the *C. albicans* infection becomes severely hindered.

Our TLR1KO, and TLR6KO mice also had hindered neutrophil recruitment, 0.72% and 1.1% respectfully. Both TLR1 and 6 form heterodimers with TLR2 to provide it with ligand specificity. TLR2/TLR1 heterodimers recognize triacyl lipopeptides, and TLR2/TLR6 heterodimers recognize diacyl lipopeptides¹⁰. When comparing the fluorescence values of both genotypes there was less neutrophil recruitment in the TLR1KO mouse, which means that there was a larger number of triacyl lipopeptide ligands involved in the recruitment of neutrophils, however more thorough research is needed.

None of the gene knockout mice displayed zero fluorescence/neutrophil recruitment. This suggests that the signaling pathway for neutrophil recruitment is not solely dependent on either TLR1, 2, or 6 and that neutrophil recruitment is not dependent on one signaling pathway. In a preliminary study, we observed very little to no fluorescence in a NIMP/Alexa488 stained MyD88KO mouse tongue section. This is an indication that MyD88 might be responsible for a signaling pathway that may or may not directly involve TLRs.

In the future, studies will include a larger sample size to give more significant results. Additional studies will further characterize the role of MyD88 and C-type lectin receptor Dectin-1, a receptor believed to collaborate with TLRs¹¹, in neutrophil recruitment to a candidiasis infection.

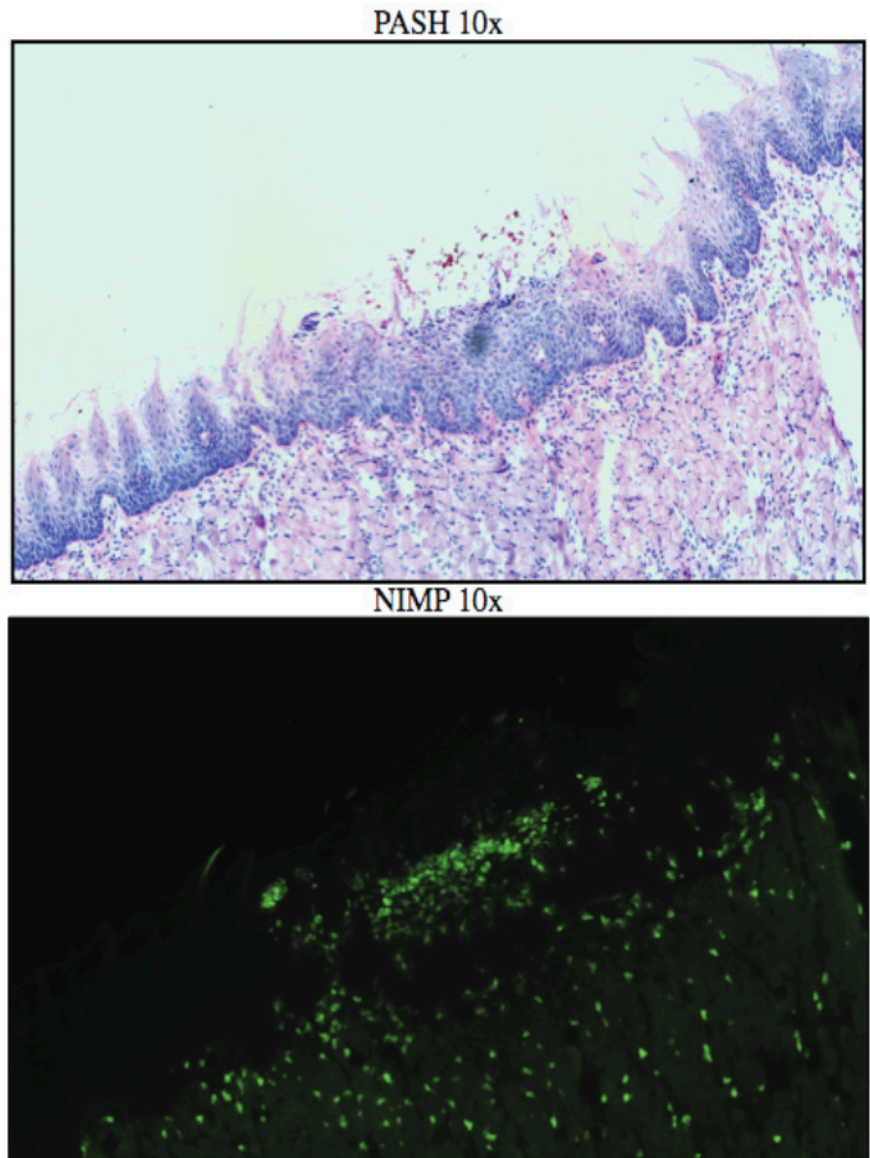
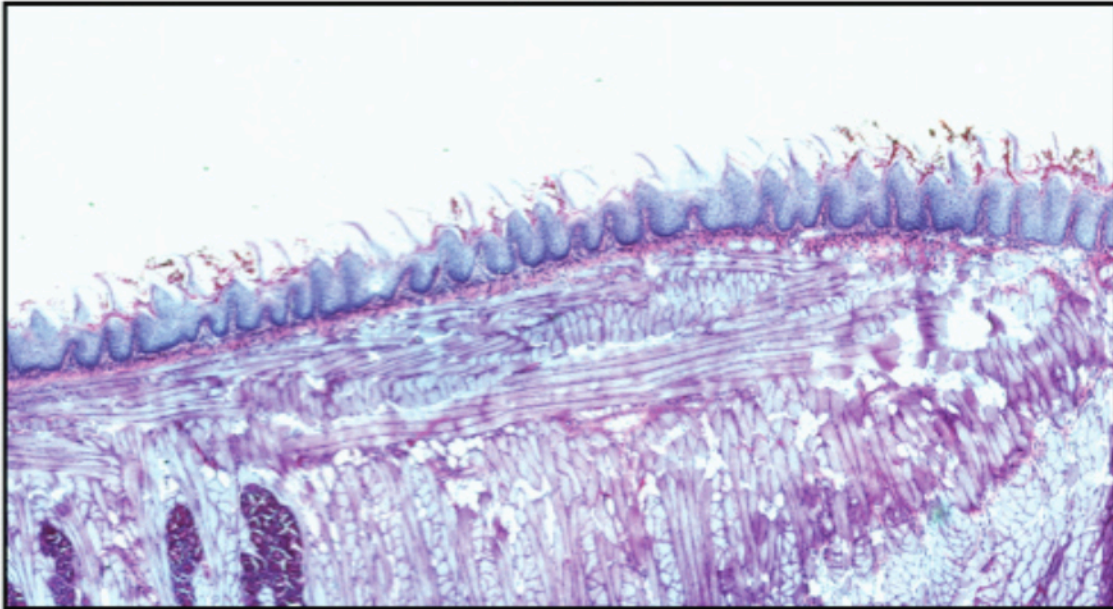


Figure 2: Sections of C57BL/6 OPC infected mouse tongue epithelium tissue, stained with PASH stain to show histology and *C.albicans* growth, and NIMP/Alexa488 stain on the neighboring tongue section to show neutrophil migration.

PASH 10x



NIMP 10x

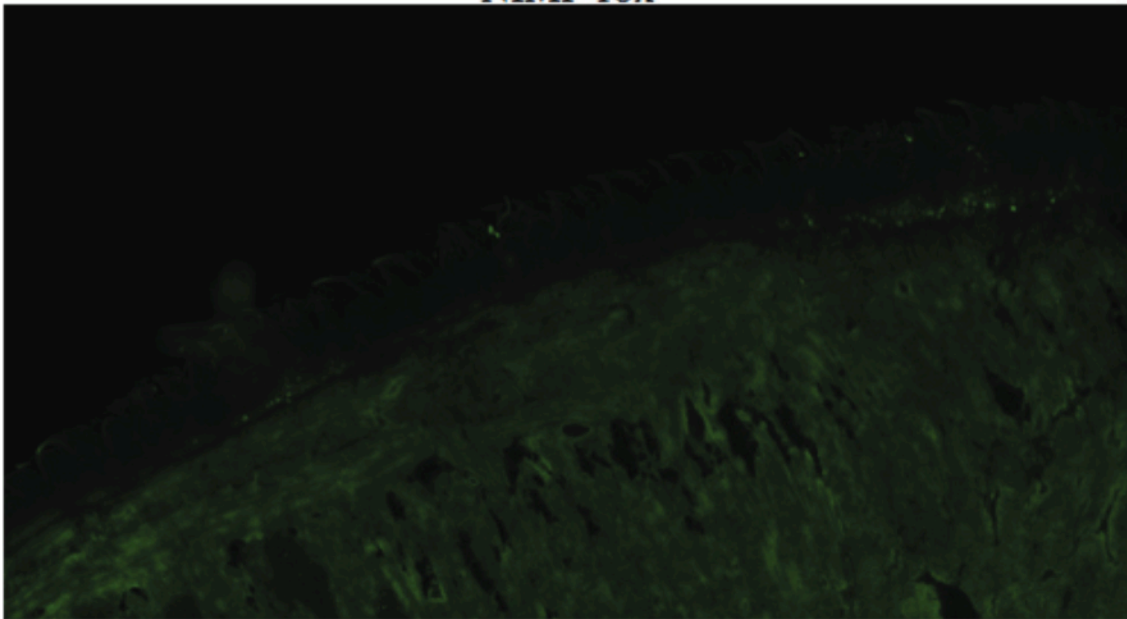


Figure 3: Sections of TLR1KO OPC infected mouse tongue epithelium tissue, stained with PASH stain to show histology and *C. albicans* growth, and NIMP/Alexa488 stain on the neighboring tongue section to show neutrophil migration.

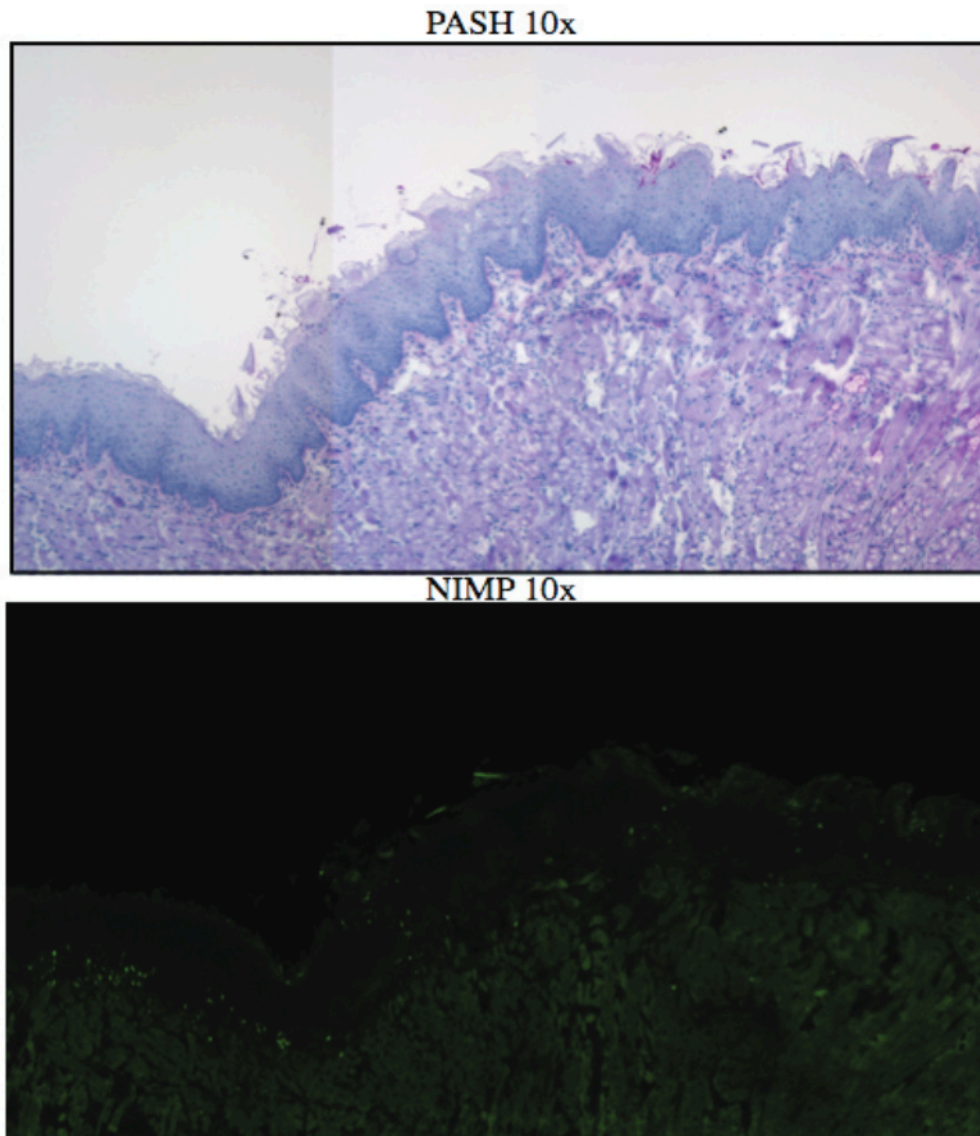


Figure 4: Sections of TLR2KO OPC infected mouse tongue epithelium tissue, stained with PASH stain to show histology and *C.albicans* growth, and NIMP/Alexa488 stain on the neighboring tongue section to show neutrophil migration.

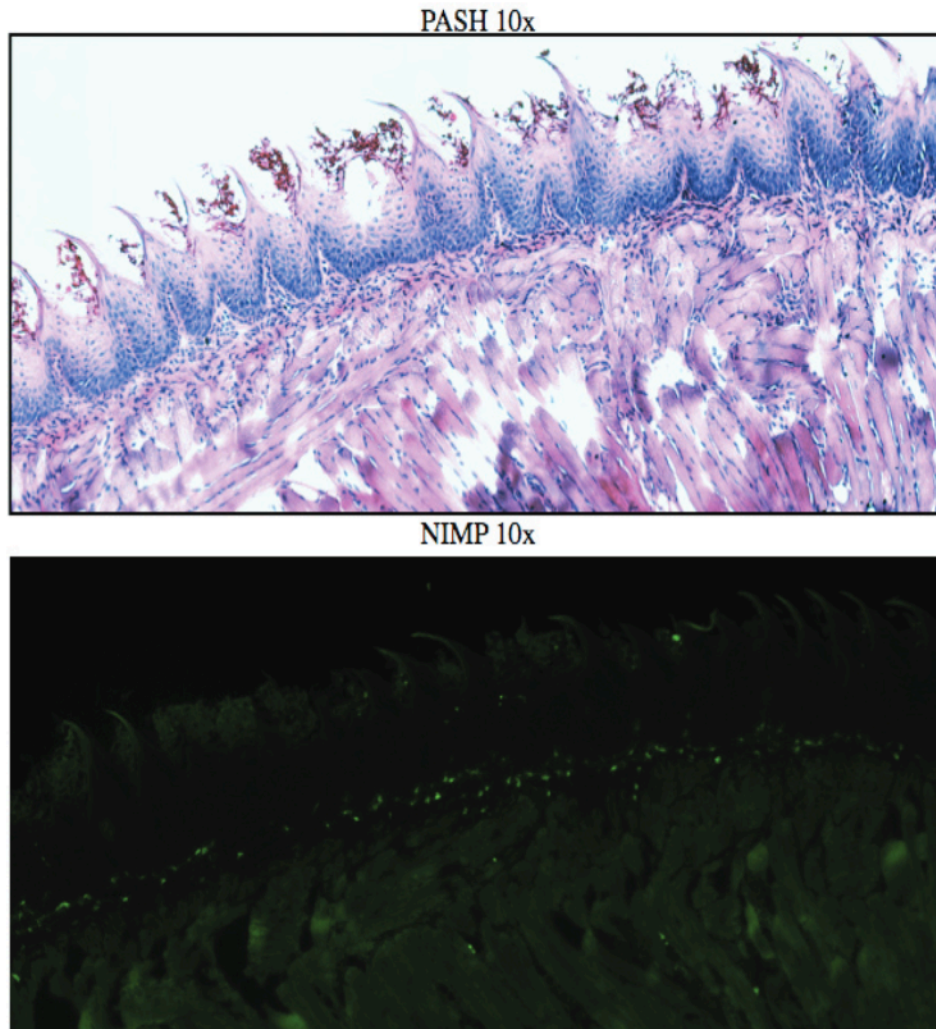


Figure 5: Sections of TLR6KO OPC infected mouse tongue epithelium tissue, stained with PASH stain to show histology and *C.albicans* growth, and NIMP/Alexa488 stain on the neighboring tongue section to show neutrophil migration.

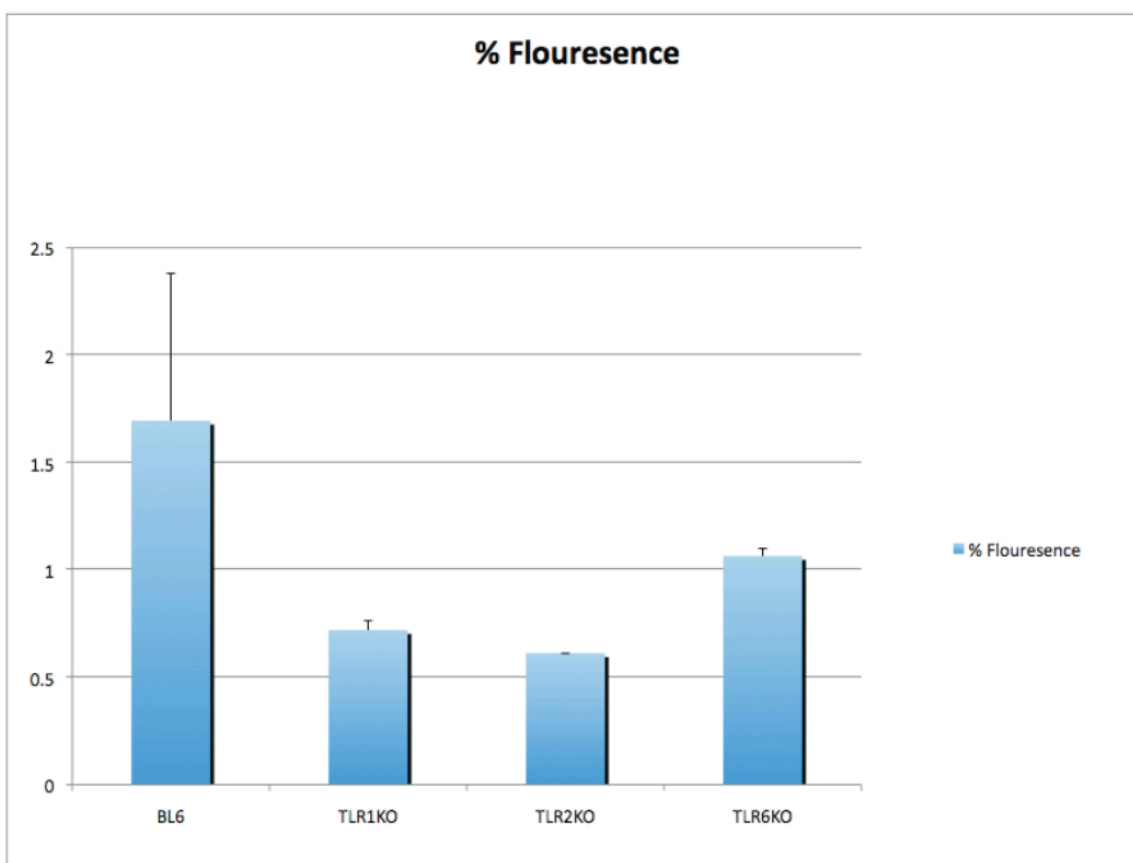


Figure 6: The average percent fluorescence for each of BL6, TLR1KO, TLR2KO, TLR6KO OPC infected mice and their corresponding sample sizes (n). The fluorescing corresponds to the neutrophils in the tongue sections from the NIMP/Alexa488 staining. The percent of fluorescence was calculated by dividing the number of fluorescing pixels by the total number of pixels in the 10x-stitched images in the program Metamorph.

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