UNIVERSITY OF CALIFORNIA SAN DIEGO

Akt Inhibition Effect on Otitis Media Pathogenesis

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

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The thesis of Ye Lin Son is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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(chair)

University of California San Diego

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ABSTRACT OF THE THESIS

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Master of Science in Biology

Professor Allen F. Ryan, Chair

Professor Matthew Daugherty, Co-Chair

The previous studies of the Akt isoforms show that Akt plays a role in cell survivability and growth as well as innate immune system response through macrophage and neutrophil migration and proliferation. However, the role of Akt in Otitis Media pathogenesis is unclear. To mimic otitis media, the middle ears (ME) of Akt1 and Akt2 knockout mice were injected with non-typeable *Haemophilus influenzae*. When looking at ME mucosal thickness, Akt1-/- mice showed a decrease in thickness at 72 hours while Akt2-/- showed a decrease at 48 hours. In

immune system cell count, Akt1-/- mice show an increase in neutrophil count while Akt2-/- mice show a decrease in neutrophil count, both at 48 hours. Finally, the percentage of the ME cavity occupied by inflammatory cells increased at 48 hours in Akt1-/- mice and decrease at 48 hours in Akt2-/- mice. In the immune cell count as well as the percent area covered, Akt1 and Ak2 inhibition leads to a “see-saw” effect where one decreases immune response while the other increases it. This study demonstrates that Akt1 and 2 have a clear role in the ME immune response.

INTRODUCTION

Otitis media

Otitis media (OM) is one of the most common childhood diseases, affecting more than 90% of children under the age of five (Pichichero, 2016). OM is considered to be multifactorial, with many issues contributing to the disease. Upper respiratory infections caused by viruses are often followed a week later by OM (Chonmaitree et al., 2008). This may be mediated by malfunction of the Eustachian tube (Siebert et al., 2006), which then fails to prevent bacteria from entering the middle ear (ME) from the nasopharynx, where a non-pathogenic community of commensal microbes create a microbiological barrier to infection. This community includes *Strep. pneumoniae*, non-typeable *Haemophilus influenzae* (NTHi) and *Moraxella catarrhalis*. Unfortunately, the ME is not adapted to these bacteria, and if they enter the tympanic cavity via the Eustachian tube, active infection typically results. The Eustachian tube also prevents fluid in the ME from draining into the throat. As a result of tubal malfunction, fluid behind the tympanic membrane, or eardrum, builds up and allows the growth of bacteria (Gaddey et al., 2019). Eustachian tube blockage also causes negative pressure and a feeling of blockage. Other risk factors for OM include second hand smoking, family history of chronic ear infections, immune compromisation, and exposure to other children in daycare (Johns Hopkins Medicine).

Although each child can experience OM symptoms differently, children generally experience a combination of the following: irritability, difficulty falling and or staying asleep, tugging at the infected ear due to a feeling of blockage, fever, middle ear fluid, loss of balance, pain, and difficulty hearing (Johns Hopkins Medicine). More severe otitis can promote the buildup of positive ME pressure due to excess fluid, inflammation and puss, even leading to

tympanic membrane perforation (Siebert et al., 2006; Mayo Clinic 2019). This is known as chronic suppurative OM, and may lead to permanent hearing impairment, ultimately causing problems in early childhood development in behavior and language. Other, rarer and serious, complications, such as brain infection (meningitis) and damage to facial nerves can also arise (Eaton, 2020).

The cost of OM in healthcare and associated costs is estimated to be around $6 billion in the U.S. alone, causing more pediatric surgeries, prescriptions, and physician visits than any other condition (Klein, 2000; Ahmed et al., 2014). While OM is a health care and economic burden in developed countries, it is a much more serious disease in the developing world. Chronic OM is highly prevalent in Asia, specifically India (7.8%) and China (4%), and in sub-Saharan African nations (0.4% to 4.2%) (WHO 1996). Undertreated chronic suppurative OM is estimated to cause 28,000 annual deaths due to meningitis, and one half of the world's burden of serious permanent hearing loss (WHO, 2004).

Also according to the WHO, in developed countries, minority groups are affected disproportionately (WHO 2004). When looking at the prevalence of tympanic membrane perforation prevalence in Australian Caucasians ranged from 3-7% while prevalence in Australian Aborigines ranged from 28-43% (WHO 2004). Similar differences have been noted for Native American and Inuit populations. These disparities could be due to ethnic differences in Eustachian tube function or vulnerability, leading to increased bacterial access from the nasopharynx to the ME. Another factor to consider is the socioeconomic status of minority groups. Living conditions associated with poverty, such as poor diet, poor hygiene, and inadequate housing, have been identified as major risk factors in OM development.

OM Treatment

Because most cases of acute OM resolve without treatment, physicians generally favor a "wait-and-see" approach for children over two years of age. For the 10-20% of children who experience chronic OM, defined as prolonged ME infection for more than two weeks or multiple recurrences, or for children with suppurative OM, treatment is recommended (Teele et al., 1989).

Treatment options are limited and may have unfavorable side effects. Currently, treatment involves systemic antibiotics, and ventilation tubes. Antibiotic tend to be most effective for acute OM as it works best under short-term conditions (Mandel et al., 2004). Long-term or repeated usage of antibiotics can lead to antibiotic resistance of bacteria throughout the body (Pelton, 2002) and therefore is not an ideal treatment option for chronic OM. Furthermore, even a single course of antibiotics causes changes in gut microbiome thus leading to increased risk of GI distress or type 1 diabetes (Hu et al., 2017) In a previous study, it was shown that early life antibiotic course (1PAT) exposure in C57/B16 control mice led to a change in gene expression due to a shift in the microbiota in the ileum. In early life, non-obese diabetic (NOD) mice, a single course of PAT altered gut microbiome, reducing diversity without recovery, leading to the development of auto-immunity seen in Type 1 Diabetes, with selection for bacterial taxa involved in high metabolism. Researchers have also found a correlation between repeated antibiotic use and development of Type 2 diabetes (Doheny, 2015). Finally, analysis of ME fluid has shown that approximately 15% of children show no antibiotic in the ME after systemic delivery (Pichichio, 2016).

Another treatment option is insertion of ventilation tubes in a surgical procedure. This option is considered for chronic or recurring OM. Ventilation tubes allow the trapped fluid in the ME cavity to drain, preventing infections and negative pressure. However, it has been shown that

surgery resolved chronic OM for only some children, while more than 50% of children who had tubes inserted showed morphological changes of the ear, such as tympanic membrane scarring, failed closure of the hole in which the ventilation tube was inserted, or a destructive invasion of the middle ear by epidermal keratinocytes known as cholesteatoma (Daly et al., 2003).

Another preventive treatment option is vaccines to provide protection against future infections. Currently, only *Streptococcus pneumoniae* vaccines are available. These vaccines have reduced OM due to several of the most invasive *Strep. pneumoniae* strains. However, their use has lead to an increase in ME infection by other bacteria. (Casey et al., 2010). Since the use of Streptococcus vaccines, gram-negative bacteria, including NTHi infections have become the most common form of OM. High strain heterogeneity and thus the need for vaccines against multiple antigens make it very difficult to create a vaccine for NTHi (Barenkamp, 2004). ME infection by *Moraxella catarrhalis* and uncovered *Strep pneumoniae* strains has also increased (Casey et al., 2010).

A potential area for research on therapies is ME pathogenesis. A better understanding of the relevant genes and eventually the molecular pathways involved in ME hyperplasia, immune responses and OM recovery could be a useful tool in developing novel target therapies for OM.

Role of Innate Immunity in OM

A hypothesis as to why some children have higher susceptibility to OM is that genetic differences in immune responses could cause increased susceptibility to infection (Emonts et al., 2007). Acute OM normally resolves in a few days without treatment. This is too soon for cognate immune antibodies or cellular responses against pathogens to develop, suggesting that

innate immunity is the normal mechanism of OM resolution. For this reason, it is important to understand the role of the innate immune system in OM.

The body's innate immune system begins with the physical barrier: skin, epithelial and mucous membrane surfaces facing the outside world. These act as the body's first line of defense, preventing pathogen entry not only through tight junctions that that form a seal that is difficult to penetrate and separating the outside world from the inside, but also producing antimicrobial substances. The mucus produced at mucosal surfaces also serves a protective function by trapping bacteria.

However, pathogens have evolved to target and break through these physical barriers through elaborated mechanisms involving disruption of the integrity of cells or junctions and migration into deeper tissue (Doran et al., 2013). If pathogens are able to cross the physical barrier, innate immune receptors on tissue cells and resident immune cells such as macrophages and lymphocytes detect pathogens. These receptors do not require sensitization. They have evolved to detect various pathogen molecules including lipopolysaccharides, lipoproteins and pathogen DNA or RNA. The receptors include members of the Toll-like receptor (TLR), Nod-like receptor (NLR) and other receptor families (Mogensen, 2009). Activation of innate immune receptors by pathogens stimulates the production and release of inflammatory mediators, such as cytokines, and activates the complement cascade. This produces inflammation and the recruitment of circulating leukocytes, including neutrophils, lymphocytes and macrophages, via chemoattraction, chemokines, and alterations of vascular permeability. Once at the site of the infection, neutrophils and macrophages attack the pathogen by phagocytosis and respiratory bursts while cytokines control the inflammatory response by regulating macrophage activity (Pluddermann et al., 2011). In a successful inflammatory response, the pathogen is removed and

damaged tissue is repaired. Unfortunately, the ME is a closed space with only the Eustachian tube for clearance, and the ability to remove bacteria, cells, debris and inflammatory mediators is limited. Thus the resolution of ME infection can be difficult.

ME Mucosal Hyperplasia and Cellular Pathways

A major component of the ME inflammatory response is mucosal hyperplasia. The normal ME is lined by a monolayer of simple squamous epithelial cells with a minimal underlying stroma. However, infection and inflammation induce a rapid transformation into a respiratory-type epithelium with multiple layers of epithelial cells, including secretory, goblet and ciliated cells, and a robust stroma (Lim et al., 1971). Activation of stromal cells increases the risk of matrix generation and fibrosis. This can lead to damage in the ME sound conduction apparatus, resulting in permanent hearing loss.

This tissue growth and transformation occurs in response to inflammatory mediators (refs) and the induced expression of growth factors such as HB-EGF (Suzukawa et al., 2014). Growth factors activate receptors that in turn activate downstream signaling cascades to enhance cell division and differentiation. A major such growth-related signaling cascade is the PI3K/AKT pathway. Class I P13Ks (phosphoinositide 3-kinase) activate AKTs, which are a family of serine-threonine kinases (Vanhaesebroeck, 2016). AKTs play a critical role regulating a range of cellular functions including cell proliferation and survival as well as tissue growth. AKTs have also been identified as important regulators of immune cell development and inflammatory responses. Downstream from AKTs are many proteins that can either be activated, including p21 and cFos, or inactivated such as GSK-3 and Bad (Chang et al., 2003).

For example, p21 exists as a complex of four with a cyclin, a proliferating cell nuclear antigen (PCNA), and a cyclin-dependent kinase (Cdk). When p21 is bound to PCNA, DNA pol δ holoenzyme activity is inhibited (Waga et al., 1994; Flores-Rozas et al., 1994). Akt phosphorylates T145 on p21 which causes release of PCNA from the complex, allowing PCNA to form a complex with activator protein-1 (AP-1) and DNA polymerase δ holoenzyme, ultimately inducing DNA synthesis. Activation of p21 through Akt signaling has also been found to increase cell survivability (Lawlor, 2000). AKT activation also increases the activity of c-Fos, a transcriptional regulator of many proliferation and anti-apoptotic genes. AKT inactivates GSK-3, which in its active form, inactivates glycogen synthase, cyclin D, and c-Myc, all proteins involved in cell proliferation. Finally, Bad is a pro-apoptotic protein in the Bcl-2 family. The rheostat theory proposes that the balance of pro-apoptotic, like Bad, and anti-apoptotic proteins determines a cell's fate (Oltvai et al., 1993). Bad is inactivated via phosphorylation by coordination between the P13K/AKT pathway and the Raf/MEK/ERK pathway.

While growth regulation is an important feature of AKT, it is also known to regulate immune responses. In particular, AKT can control the development and function of innate immune cells, including neutrophils macrophages and dendritic cells. In neutrophils and other immune cells, cytokine and NO production in response to bacterial lipopolysaccharide is mediated by AKT signaling (Zhang et al., 2013). Moreover, the apoptosis of normally short-lived neutrophils can be delayed by AKT in response to a variety of inflammatory mediators, thus prolonging their anti-bacterial action at sites of infection (Gardai et al., 2004).

Prior Work on AKT in OM

Our research group previously studied on the effects of P13K/AKT pathway inhibitors on ME mucosal growth *in vitro*. (Lee et al., 2020). In one part of the experiment, ear mucosal lining was explanted and grown *in vitro*. Without treatment with MK2206, an Akt inhibitor, the explants grew steadily for 10 days. When treated with 100nM of MK2206, growth was significantly inhibited. This was true of both NTHi infected and uninfected tissue. Mucosal explants were also treated with PTEN inhibitor bpV. PTEN is a known P13K inhibitor which functions by dephosphorylating PIP3 to PIP2. (Paez et al., 2003). For uninfected explants, the addition of 50 and 500nM bpV significantly inhibited explant growth. In infected explants, the addition of 5, 50 and 500nM bpV inhibited explant growth. The unexpected result of PTEN inhibition suggests that the PI3K/AKT/PTEN pathway is complex (Lee et al., 2020).

One source of complexity may be the different forms of AKT. There are three AKT genes, *akt1*, *akt2* and *akt3*, that encode three distinct isoforms of Akt protein. Although these isoforms are encoded by different genes, they share homologous structure consisting of an N-terminal pleckstrin homology domain, a kinase domain and a C-terminal regulatory domain containing a hydrophobic motif. Different isoforms of signaling molecules can act synergistically or can display distinct functional features and emerging evidence supports distinct functions for Akt isoforms. Whereas *akt1* and -*2* are ubiquitously expressed, *akt3* has been reported to have a more limited tissue distribution (Yang et al., 2003). Differences in the roles of JNK isoforms in OM have been noted (Yao et al., 2014). Moreover, AKT isoforms can differ in their regulation of cell division, differentiation and growth in other tissue systems (Tang et al., 2014). Studies of Akt isoform deficient mice highlight the potentially non-redundant functions of Akt1 and -2. *Akt1* $^{-/-}$ mice are small with significant growth defects, *Akt2* $^{-/-}$ mice have impaired glucose

homeostasis (Cho et al., 2001), and *Akt3*$^{-/-}$ mice display a selective reduction in brain size (Tschopp O et al., 2005).

The purpose of this study was to evaluate the direct roles of AKT isoforms in OM *in vivo*. We hypothesized that selective deletion of Akt isoforms would exhibit different defects on bacterial OM in vivo. To address this hypothesis, we evaluated OM induced by NTHi in mice deficient in Akt1 and Akt 2, the two Akt isoforms that are differentially regulated during OM (Hernandez et al., 2015), and compared the results to those observed in wild-type (WT) mice.

Differences between AKT1 and AKT2 that contribute to differential function

The basis for differences in function between AKT1 and AKT2 are incompletely understood. However, different subcellular localizations and variation in binding partners may determine isoform-specific AKT functions. AKT2 expression is prominent in regions of cell–matrix contact (Arboleda et al., 2003), which may enable interactions with molecules required for cell motility and invasion. Differential localization may reflect distinct protein-binding interactions. Although the AKT isoforms exhibit relatively high sequence homology and similar domain structures, significant variation occurs within the phosphoinositide-binding PH domain. The L-jun NH_2-terminal kinase scaffold proteins POSH interacts only with the PH domain of AKT2, while protein 1 interacts only with the PH domain of AKT1 (Kim et al., 2002; Figueroa et al., 2003). Phosphorylation differences are also a possibility. Girardi et al. (2014) found that a serine residue (Ser129) is directly phosphorylated by protein kinase CK2 in AKT1. However, the homologous residue in AKT2 (Ser131) is not phosphorylated on Ser131 by CK2, which affects the substrate interactions of the two isoforms. CK2-dependent phosphorylation is

therefore an event which, discriminating between AKT1 and AKT2, can account for different substrate specificities.

MATERIALS AND METHODS

Animals

The 60-90 day old AKT1$^{-/-}$ and AKT2$^{-/-}$ knockout mice on a C57BL/6 background (5 times crossed) originally supplied by Jackson Labs were bred and genotyped for this study. Wild type age matched C57BL/6 mice were supplied by Jackson Laboratories (Bar Harbor, ME, USA). This study was performed in accordance to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and approved by the Institutional Animal Care and Use Committee of the Veteran Affairs Medical Center. Both male and female mice were used.

Bacteria

Non-typeable *Haemophilus influenzae* (NTHi) strain 3655 was originally isolated from the ME of a St. Louis, MO OM patient. NTHi was streaked and incubated on a chocolate agar plate at 37°C overnight. Two colonies were added to 25ml of brain heart infusion (BHI) and 1ml of Fildes enrichment to grow overnight. The next day, the mixture was then centrifuged at 7,000 rpm for 10 minutes and the bacterial pellet was resuspended in BHI and diluted to a final concentration of 100-1000 CFUs per μl.

Surgery

The surgeries were performed while mice were under anesthesia using rodent cocktail (2.0 mg/kg xylazine and 40.0 mg/kg ketamine i. m.) via a ventral cervical midline incision. Both ME bullae were exposed and punctured by with a 25g needle. Five μl of NTHi inoculum containing 500-5000 CFU of NTHi were injected into each bulla using a 30g needle (Melhus et

al., 2003). The incision was stapled and the tympanic membranes were visually confirmed to be intact. the mice were subcutaneously injected postoperatively with 0.5 ml of lactated Ringer's solution to maintain hydration and were treated with 0.1 mg/kg of buprenorphine. Control animals (0 hours) were not inoculated with NTHi.

Histology

Once anesthetized by rodent cocktail, the mice were sacrificed by intracardiac perfusion using 5mL PBS followed by 5mL of 4% paraformaldehyde (PFA). MEs were harvested at 0, 2, 3, and 7 days after inoculation with NTHi. The MEs were dissected and decalcified in 8% EDTA and 4% PFA for 21 days. The ears were then embedded in paraffin and sectioned 10 μm. Sections were de-paraffinized, stained with hematoxylin and eosin and imaged at 10X. Using SPOT (Sterling Heights, MI, USA) image analysis software, mucosal thickness was measured at three standardized locations and averaged, from each section.

To assess leukocyte infiltration into the ME, the entire area of the ME was measured and divided into the area occupied by cells. To determine the proportions of neutrophils and macrophages in the infiltrate, an image of the densest rea of infiltrate was imaged at 400X and the number of each cell type was counted.

Finally, an image analysis software was used to take digital micrographs of the same standardized region in order to calculate how much of the middle ear lumen (percent area) was occupied by inflammatory cells.

For each genotype and time point, six WT, AKT1 and AKT2 animals were evaluated.

Statistical analysis

Statistical analyses were performed using statView software. Data are reported as means ± standard errors of the means (SEM). Differences were considered significant at a *P* value of < 0.05. Two-way ANOVA with Bonferroni correction for multiple comparisons was performed on measures of mucosal thickness. ME NTHi colonization rates were evaluated statistically by the Student's t-test. Data normality was evaluated by using the D'Agostino-Pearson omnibus test. The Mann-Whitney U test was employed for data lacking a normal distribution, as for analyses of ME inflammatory cells. Left and right ears in each mouse were considered to be independent of each other, and therefore were analyzed independently.

RESULTS

1. Akt1 and Akt2 are associated with OM.

As previously reported from a gene array study of mouse OM (Lee et al., 2020), the AKT1 gene is upregulated 3-fold at 3 and 6 hours after NTHi inoculation of the ME, and 9-fold at 24 hours before returning to normal at 24 hours. The AKT2 gene is upregulated 3-fold at 24 hours. A single-cell RNA-Seq study in the mouse revealed that AKT1 and AKT2 are expressed by a substantial subpopulation of all cell types present in the normal ME, including epithelial, stromal and monocytic cell types, although somewhat fewer cells expressed Akt2. Akt3 was expressed by substantially fewer cells (Ryan et al., 2020). Six hours after NTHi inoculation, large numbers of neutrophils had entered the ME, but relatively few of them expressed AKT1 or AKT2. Especially AKT1 but also AKT2 expression was observed in a higher proportion of epithelial cells than in the uninfected ME, but not in other cell types. At 24 hours, AKT1 expression remained high in epithelial cells, and was also high in stromal cells, but was low in other cell types. Expression of AKT2 showed a similar pattern, but was expressed at lower levels than AKT1. By 2 days, AKT1 and AKT2 expression had returned to pre-infection levels in all cell types.

2. Effect of Akt deficiency on middle ear inflammation

Middle ear inflammation can be quantified by the thickness of the mucosal lining the ME. The ME mucosa consists of an epithelial and stromal layers. Normal morphology consists of a thin layer of mucosa adjacent to the middle ear bone. The lumen of the middle ear is also clear of any exudate or cells. During bacterial infection with NTHi, the ME morphology changes

dramatically in response to initiated inflammatory innate immune responses. In C57BL/6 wild-type mice, the peak of this inflammatory response is typically seen around 48 to 72 hours after inoculation of NTHi experimentally. The ME returns to normal baseline morphology by day 7 after infection. The mucosal layer usually regains its usual monolayer characteristic structure and the inflammatory cells clear the ME cavity.

In our experiments, mucosal thickening was observed for both wild-type and Akt deficient mice, throughout the course of an acute OM episode induced by NTHi inoculation into the ME. However, there were significant differences between the mutant and WT results. Specifically, we noted that Akt1 deficiency led to a decrease in mucosal thickness at 72 hours but no difference in 48 hours (Figure 1). Meanwhile, Akt2 deficiency led to a decrease in thickness at 48 hours but no difference in 72 hours (Figure 2).

3. Effect of Akt deficiency on neutrophil and macrophage recruitment to middle ear

Inflammation can also be measured by innate immune system response through the percentage of the ME cavity that is occupied by immune cells and the proportions of the different types of innate immune cells.

When looking at the ME cavity area percentage covered by infiltrating leukocytes, Akt1 deficient mice show significantly greater coverage at 48 hours, when compared to WT controls (Figure 5). The percent areas at 72 hours between the AKT1 -/- and WT mice were not significantly different. Akt2 knockout mice show the opposite effect. AKT2 -/- mice showed a much lower percent area coverage by immune cells at 48 hours (Figure 6). Akt2 deficient mice also showed a significant decrease in percent area coverage at 72 hours.

In terms of cell types, Akt1 knockout mice had significantly more neutrophils in their leukocyte clusters at 48 hours than WT controls (Figure 3), but neutrophil counts at 72 hours were similar. Macrophage counts between Akt1 -/- and wildtype mice at 48 and 72 hours were comparable. Akt2 deficient mice showed a significantly lower number of neutrophils at 48 hours compared to the WTs, opposite to the effect of Akt1 deficiency (Figure 4). However, neutrophil counts at 72 hours showed no significant difference, and macrophage counts were similar at both time points.

DISCUSSION

We observed that Akt1 and Akt2 mRNAs were present the ME. They were both regulated during NTHi-induced OM, but with different time courses. Notably, deletion of either Akt1 or Akt2 lead to reduced mucosal hyperplasia at 48-72 hours post ME inoculation with NTHi. In addition, changes in ME cellular infiltration were noted at these times. These results support the hypothesis that Akt isoforms 1 and 2 are important in regulating inflammatory events in the middle ear that are induced by bacterial infection. Mitigating pathogenesis by targeting Akt could be a potential source of new therapies for otitis media.

Akt1 knockout mice showed decreased mucosal hyperplasia at 48 hours, while Akt2 deficiency decreased thickness at 72 hours. This suggests that the two isoforms of Akt regulate cell proliferation, differentiation and survival at different time points in during OM. Currently, there no other studies that have seen a similar link between time of expression of the two Akt isoforms and tissue growth. This may, therefore, be specific to the middle ear.

AKT activation by PI3K occurs downstream from of many growth factor receptors, and has long been associated with cell proliferation and inhibition of apoptosis (Manning et al., 2017). Substrates of PI3K/AKT signaling are known to be involved in OM (Lee et al 2020; Hernandez et al., 2015). A recent study by Lee et al (2020) found that the inhibitor MK2206 reduced ME mucosal growth *in vitro*. MK2206 is a highly selective allosteric inhibitor of Akt kinases, which is in phase I/II clinical trials against cancer (Bimbo et al., 2013). It is clear that cell growth and division are highly involved in the ME mucosal hyperplastic proliferation. It is therefore not surprising that in both Akt knockouts, the ME mucosa showed reduced thickness. However, the difference in timing suggests that AKT2 is more important in early hyperplasia, while AKT1 is more critical later in OM.

Within the middle ear cavity, the volume of cellular infiltrate and the proportions of different leukocyte types in Akt1 versus Ak2 deficiency showed opposite effects during the same time frame. AKT1-/- mice show an increase in neutrophil count and in percent area occupied by leukocytes at 48 hours, consistent with enhanced ME inflammation induced by neutrophils. This implies that AKT1 is a negative regulator of neutrophil infiltration. Since neutrophils showed low levels of AKT1 expression, this seems unlikely to be the results of decreased neutrophil activation. Inhibition of the production of neutrophil-attractant chemokines by cells in the ME seems a more likely alternative. In contrast, AKT2 -/- mice show a decrease in both area of the ME cavity covered by leukocytes and the number of neutrophils in the ME infiltrate. This implies that AKT2 positively regulates neutrophil entry into the ME.

A push and pull effect of the Akt isoforms has been previously noted. In a breast cancer study, the effect of under and overexpression of Akt1 and Akt2 in cell migration and invasion was studied (Riggio et al., 2017). Downregulation was achieved with shRNA constructs of the Akt isoforms, shAKT1 and shAKT2. Human breast cancer cells IBH-6 were placed on a three dimensional basement membrane to model the cells' invasive properties. Akt1 deficiency increased the cells ability to invade into the basement membrane while Akt2 deficiency suppressed this phenotype. Akt isoform functional specificity elucidates the complexity of Akt-regulated cellular signaling and function.

Immune cells exploit the endosome/lysosome system to elicit specific immune responses. The endosome/lysosome compartments play an important role in signal transduction and intercellular communication related to the immune cell functions. For example, antigen-presenting cells prepare antigens via the endosome/lysosome system and macrophages take up

pathogens by endocytosis. The Akt pathway has been shown to regulate the endosome pH and endosome/lysosome-associated mTORC1 activity (Diering et al, 2013; Flinn et al, 2010). Our gene array results (Hernandez et al., 2015) suggest that the Akt/mTORC1 signaling axis is regulated during an NTHi OM infection.

Akt isoforms 1 and 2 have a clear role in the ME immune response. However, the mechanism by which it does so is far from clear. This requires a more complete understanding of the other genes and pathways involved, and the changes exerted by lack of each gene. In this respect, the expression of chemokines in the MEs of each KO would be informative regarding their neutrophil phenotypes. Furthermore, the effects of inhibiting middle ear mucosal hyperplasia and innate immune response on middle ear adaptive immune response must be studied. The response to chronic or re-occurring OM would rely more heavily on adaptive than innate immune responses, and if Akt inhibition were to slow inhibit the adaptive response, Akt pathway manipulation would not be an appropriate alternative for children suffering from chronic OM.

APPENDIX

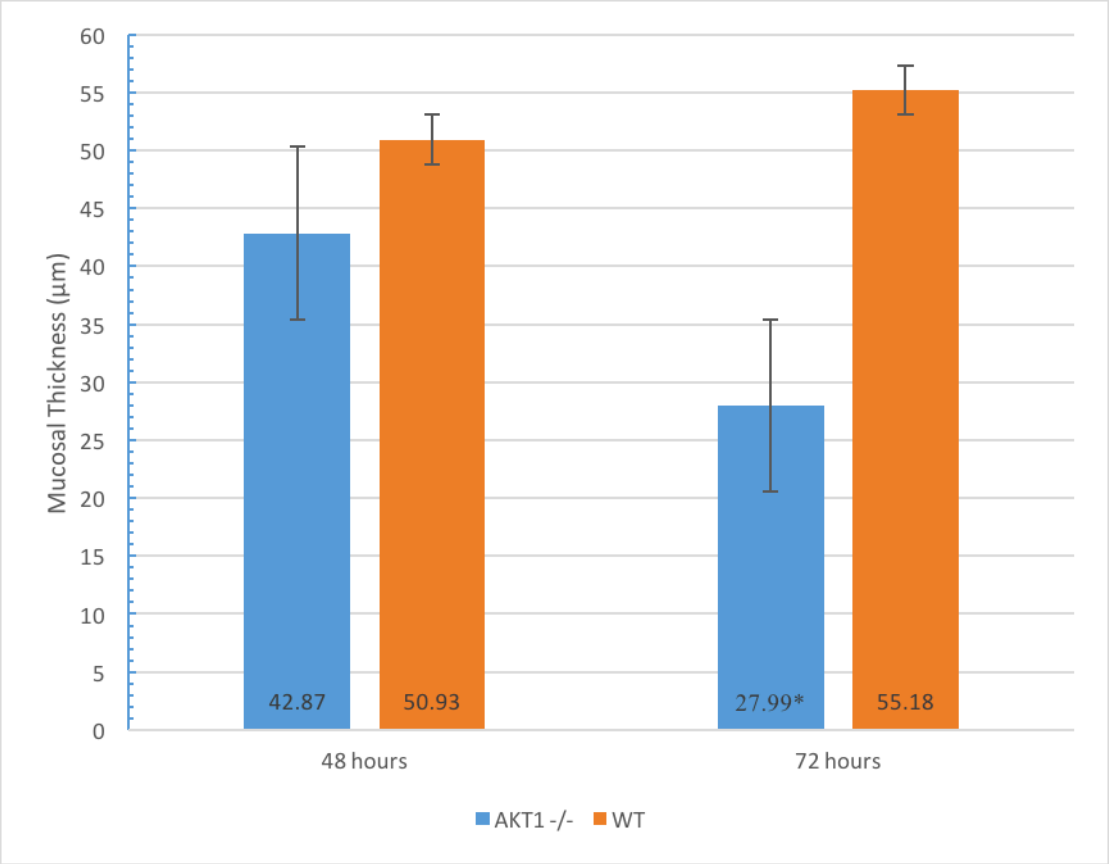

Figure 1. Measurement of the middle ear cavity mucosal thickness at 48 hours and 72 hours in AKT1 -/- and wildtype mice. At 48 hours after infection, the knockout and WT mice show no significant difference in mucosal thickness. At 72 hours, AKT1 -/- mice show a significant decrease in mucosal thickness compared to the wildtype.

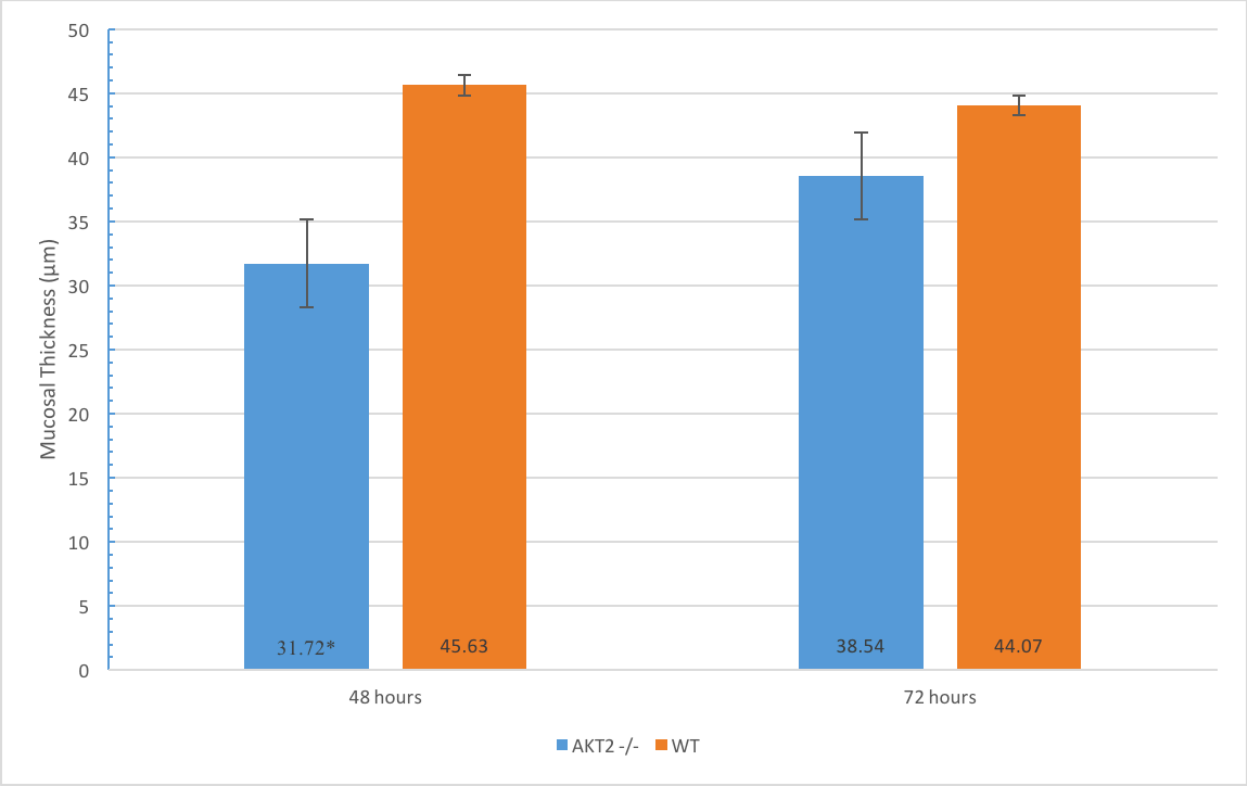

Figure 2. Measurement of the middle ear cavity mucosal thickness at 48 hours and 72 hours in AKT2 -/- and wildtype mice. At 48 hours after infection, AKT2 -/- mice mucosal layer is significantly thinner than that of the WT mice. At 72 hours, the knockout and WT mice show no significant difference in mucosal thickness.

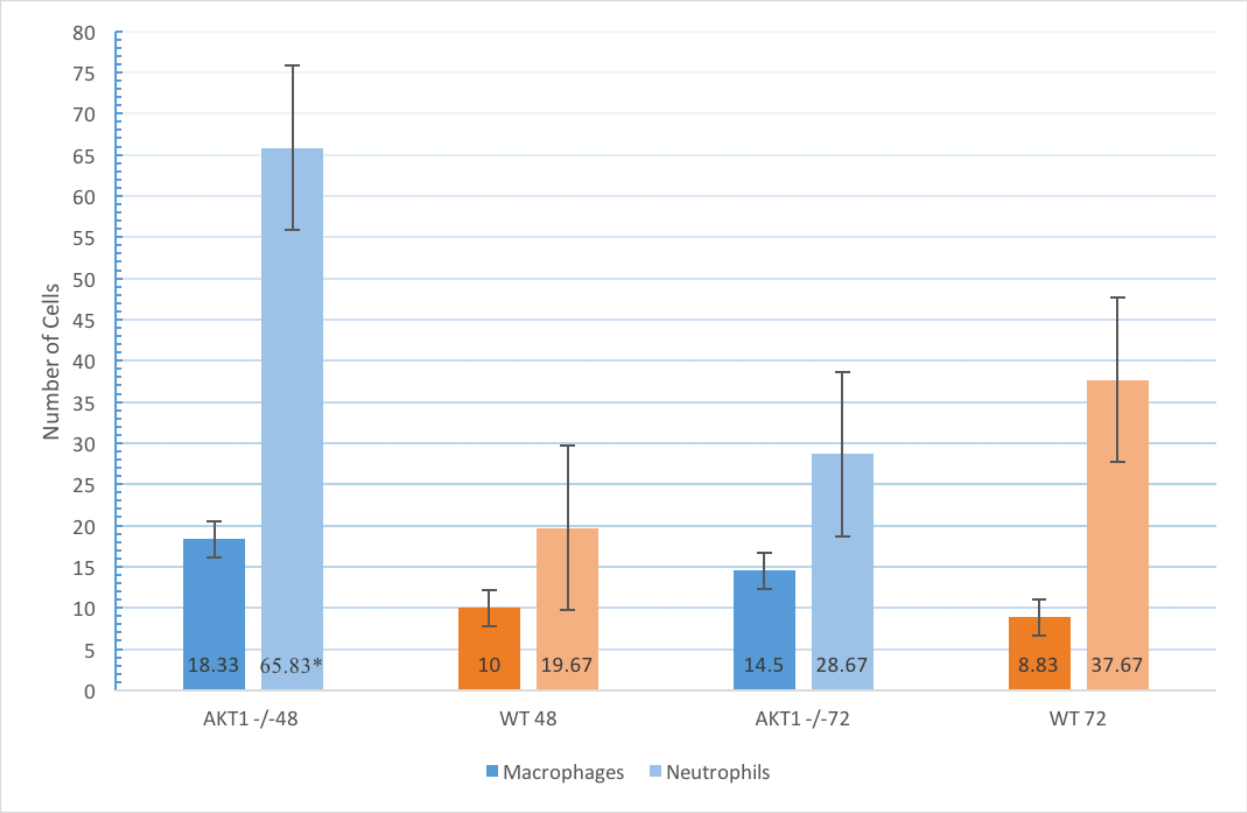

Figure 3. Neutrophils and macrophages counted in middle ear infiltrates for AKT1 -/- and wildtype mice after 48 hours and 72 hours. Compared to the WT mice, AKT1 -/- mice show a statistically significant increase in neutrophil count at 48 hours while macrophage numbers show no significant differences across both time points.

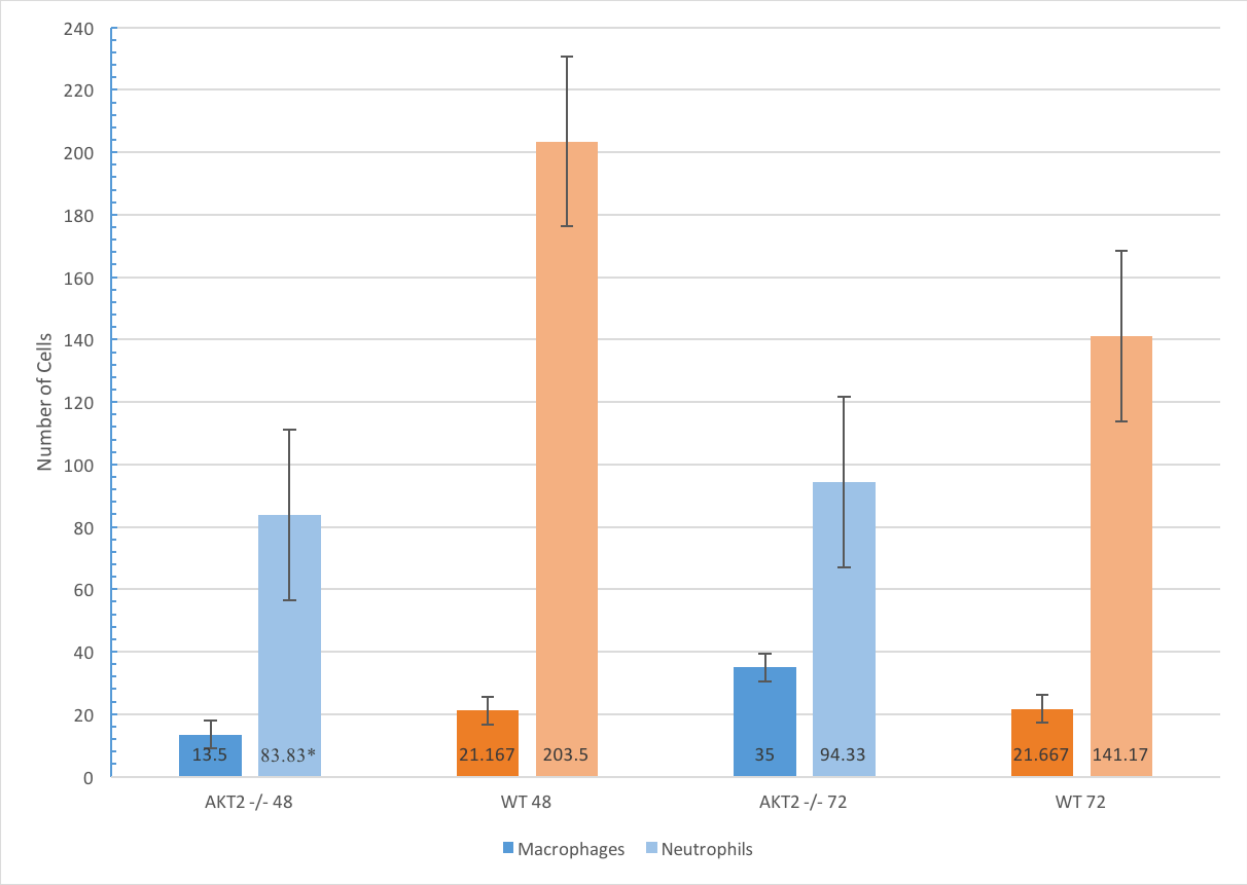

Figure 4. Neutrophils and macrophages counted in middle ear infiltrates for AKT2 -/- and wildtype mice after 48 hours and 72 hours. Compared to the WT mice, AKT2 -/- mice show a statistically significant decrease in neutrophil count at 48 hours while macrophage numbers show no significant differences across both time points.

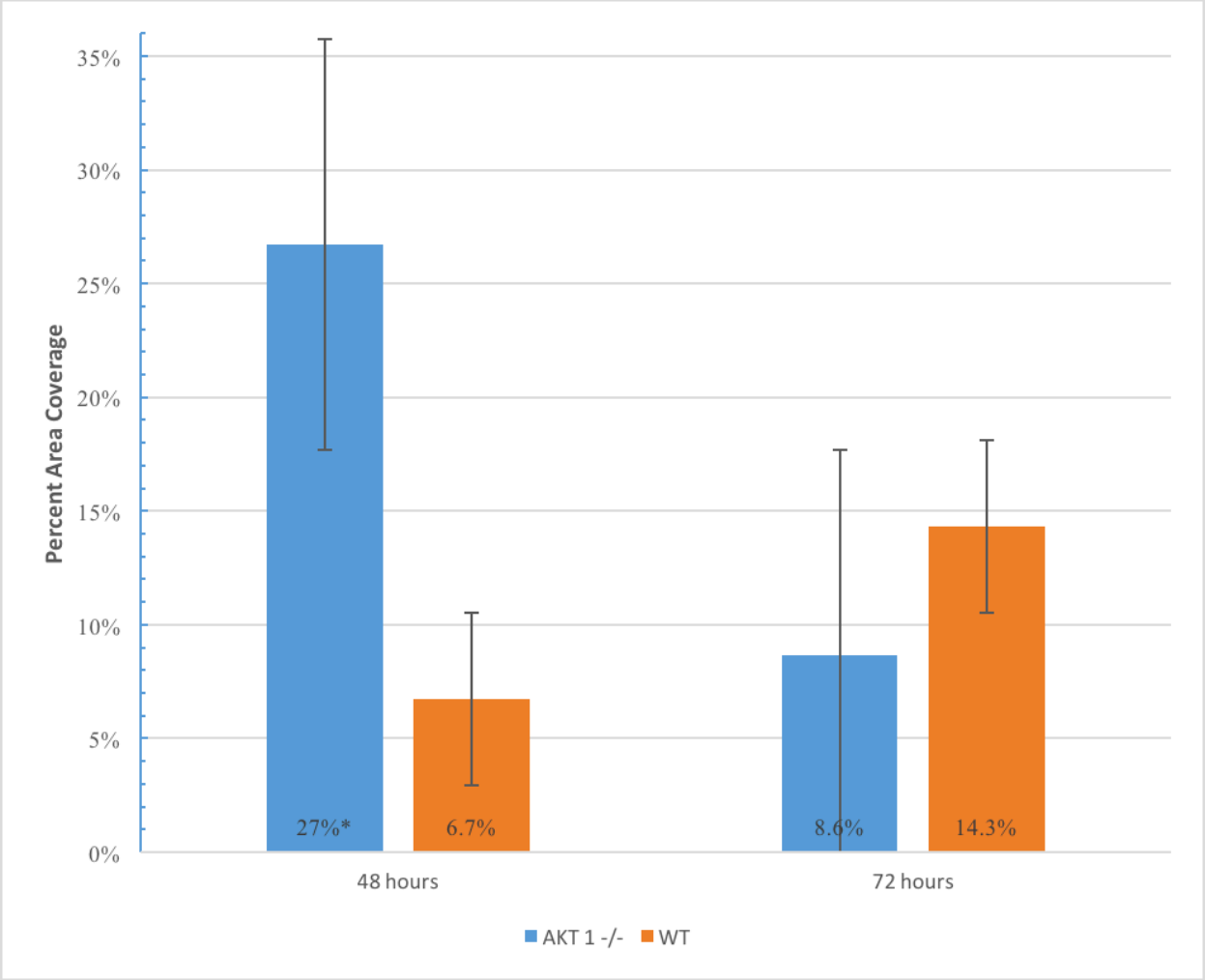

Figure 5. Measurement of the percentage of middle ear cavity occupied by inflammatory cells 48 hours and 72 hours after infection in AKT1 -/- and wildtype mice. At 48 hours, the occupied area percentage of the AKT1 -/- mice is significantly greater than that of the WT mice. At 72 hours, there is no significant difference.

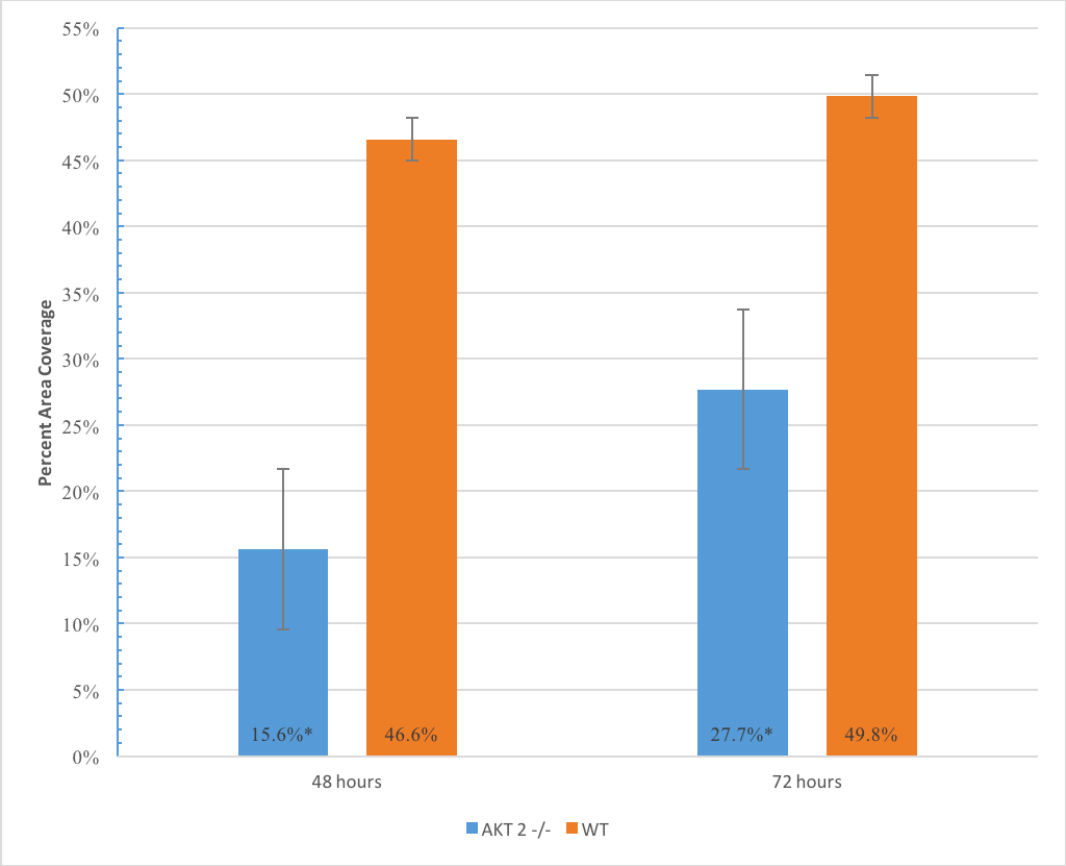

Figure 6. Measurement of the percentage of middle ear cavity occupied by inflammatory cells 48 hours and 72 hours after infection in AKT2 -/- and wildtype mice. At both 48 hours and 72 hours, the occupied area percentage of the AKT2 -/- mice is significantly less than that of the WT mice.

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