UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Role of the NOD-like Receptor Adaptor RIP2 in Otitis Media

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by

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Professor Emily R. Troemel

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The thesis of Jasmine Lee is approved and it is acceptable in quality and form for publication on microfilm or electronically:

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LIST OF SYMBOLS

AOM	acute otitis media
BHI	brain heart infusion
CARD	caspase activation and recruitment domain
COM	chronic otitis media
DAMP	damage-associated molecular pattern
IL	interleukin
NLR	NOD-like receptor
NTHi	non-typeable *Haemophilus influenzae*
OM	otitis media
PAMP	pattern-associated molecular pattern
TLR	toll-like receptor
TNF	tumor necrosis factor

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ABSTRACT OF THE THESIS

The Role of the NOD-like Receptor Adaptor RIP2 in Otitis Media

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Professor Allen F. Ryan, Chair

The NOD signaling pathway mediates innate immunity through the detection of pathogens in the host system. NOD1 and NOD2 both contain a caspase activation and recruitment domain that interacts with RIP2, leading to the activation of the transcription factor NFkB. However, their roles in otitis media have yet to be examined. We investigated experimental otitis media in wild-type and RIP2-/- mice inoculated in the middle ear with non-typeable *Haemophilus influenzae*. Compared to the wild-type,

RIP2-/- mice showed persistent middle ear infection up to 21 days. This included prolonged mucosal hyperplasia and leukocytic infiltration, which were especially severe in RIP2-deficient mice. Recruitment of neutrophils and macrophages were also substantially delayed. In addition to inflammation, viable bacteria could be cultured in RIP2-/- mice for much longer than in wild-type mice, suggesting that an inability to clear bacterial infection may underlie the persistent nature of otitis media in these mice. These results demonstrate that the NOD-like receptor adaptor RIP2 plays a significant role in the innate immune host response in the middle ear.

I. INTRODUCTION

1. Otitis Media

Otitis media (OM) is the one of the most common health problems seen by pediatricians. It is an infection in the middle ear affecting over 90 percent of children before the age of 2 (Casselbrandt et al., 1993). The regions with the highest prevalence of chronic OM are Asia (China and India) and sub-Saharan Africa. China and India had prevalence rates of 4% and 7.8%, respectively. The prevalence rate in sub-Saharan African nations was 0.4 to 4.2% (WHO, 1996). In the United States and Europe, the prevalence of chronic OM is less than 1%. However, otitis media accounts for a large number of doctors' appointments and antibiotic prescriptions (Klein, 2000). The cost due to diagnosis and treatments associated with OM is believed to be over $5 billion every year in the United States (Elden et al., 1998).

Minority groups in developed countries such as Australia and the United States are disproportionately affected. These include Australian Aboriginals with 12 - 28% prevalence and Native Americans with 4 - 8% prevalence (WHO, 2004). Poverty and its associated living conditions such as inadequate housing, poor hygiene and insufficient nutrition are major risk factors in developing otitis media. There is speculation that the larger diameter and lower resistance of the Eustachian tube in these ethnicities could lead to easier access by fluids containing bacteria from the nasopharynx into the middle ear.

Acute otitis media occurs when pressure builds up behind the eardrum (also known as the tympanic membrane). Fluid in the middle ear becomes trapped within the cavity and negative pressure is produced when drainage cannot occur through the

Eustachian tube (Siebert et al., 2006). This will result in a feeling of pain and a sensation of blockage in the ear. Acute otitis media (AOM) was most accurately predicted after the development of an earache. Signs of fever and sore throat were also significant indicators (Kontiokari et al., 1998). These symptoms follow the physiological changes occurring in children with OM.

This infectious disease usually occurs after a viral upper respiratory infection (Winther et al., 2007). 70% of children were found to have a rhinovirus present in their nasopharynx, with 44% of these coincident with new OM cases. 13% of the cases were attributed to other viruses such as RSV, influenza A virus, adenovirus, coronavirus, and parainfluenza virus (Alper et al., 2009). Due to the viral infection compromising the immune system, a secondary bacterial infection arises. The most common pathogens known to cause otitis media is *Streptococcus pneumoniae*, nontypeable *Haemophilus influenzae* (NTHi), and *Moraxella catarrhalis*. The combination of an increase in bacterial presence in the nasopharynx along with a decrease in activity of cilia in the Eustachian tube can lead to the development of a bacterial infection in the middle ear (Park et al., 1993).

2. Treatment of Otitis Media

Although most children will experience a few cases with AOM, the condition will usually resolve itself on its own without treatment and without any residual effects. Therefore, doctors usually favor the wait-and-see approach in patients who have early symptoms of AOM. However, about 10-20 percent of children will continue to experience recurring incidents over time and develop chronic otitis media (Teele et al.,

1989). Chronic otitis media (COM) is defined as the prolonged infection of the middle ear for at least two weeks. In developing countries, COM is a major cause of hearing impairment and complications from otitis media cause approximately 28,000 deaths a year in children (WHO, 2004). In developed countries, infants may have problems with their behavioral and speech patterns as well as develop impairments in their cognitive and language abilities (Klein, 2000).

There is no general consensus among the scientific community regarding which treatments are the most effective in resolving OM. Antibiotics are typically given to patients who present the symptoms discussed above in order to clear the secondary bacterial infection. However, this treatment may only be helpful to those with severe or prolonged AOM due to its short-term effect (Mandel et al., 2004). If antibiotics are repeatedly given over a period of time, it could lead to the rise of antibiotic-resistant microbes (Pelton, 2002). Therefore, this suggests that the use of antibiotics as a treatment will have limited effect on children with COM (Johnston et al., 2004).

Other treatment options are available that may provide longer-lasting protection against OM. Since bacteria contribute to the infection process, vaccines are one possibility to ward against the occurrence of otitis media. However, there is currently only a vaccine for *S. pneumoniae*. While a vaccine for nontypeable *H. influenzae* is possible, too many barriers such as strain heterogeneity and the need for multiple antigens must be overcome in order for this treatment to become a reality (Barenkamp, 2004). While this may decrease the number of cases caused by this microbe, it may lead to the rise in cases caused by *H. influenzae* and *M. catarrhalis* due to decreased competition and an increase in nutrient availability (Casey et al., 2004). Surgery is

another treatment option offered to patients with COM. A tympanostomy tube is inserted into the eardrum to stop fluid accumulation in the middle ear. Unfortunately, it is a controversial operation that may not result in permanent resolution. Very few children had their COM resolved, even after eight years following the surgery. Morphological changes in the ear such as the closing of the hole at the site of tube insertion and tympanic membrane abnormalities were also still present in more than 50% of children (Daly et al., 2003). Even if the tube insertion was performed on a patient as a child, it may lead to hearing loss during adulthood. It was also observed that multiple tube insertions led to a greater loss of hearing when compared to a single insertion (de Beer et al., 2004).

It is still unclear why most children only develop acute OM while some experience chronic OM. One hypothesis proposed that the immune response of the body towards the infection may play a key role in chronicity. It has been considered that the bacteria could be forming biofilms on the middle ear mucosa in patients with COM and thus the infection could be difficult to resolve (Dohar et al., 2005). Another group thought that genetic mutations could be present in the signaling pathways activated during inflammation. These variations in the sequence may have resulted in a change in cytokine production and increased the susceptibility of OM in children (Emonts et al., 2007). While there is no definitive answer, it can be concluded that immunity may be an important topic to investigate.

3. Importance of Innate Immunity

Innate immunity is an ancient defense strategy that was found in plants and single-cell organisms, helping them to resist infection. It is a defense mechanism that targets and eliminates pathogens from the host. Different from adaptive immunity, this system is immediately ready to detect a variety of antigens using a large population of cells and molecules. However, it only recognizes the pathogens immediately present in the system (Si-Tahar et al., 2009).

Barriers are present throughout the body and contribute to innate immunity by acting as the first line of defense against pathogen entry. These include the skin, eyes, gastrointestinal and respiratory tracts, and nasopharynx. The mucosa present in these areas plays a key role by mechanically and chemically stopping the invasion by foreign organisms (Dwivedy et al., 2011). A tight network of cells and lymphocytes connect to form a physical boundary to prevent pathogens from entering. Antimicrobial peptides are also produced and secreted to kill those that cross this layer of cells (Nochi et al., 2006). However, this obstacle can be overcome as the physical barrier can be penetrated by pathogens, resulting in cell death.

Dying cells attacked by the pathogens that manage to cross these barriers secrete factors known as damage-associated molecular-pattern (DAMP) molecules. This release produces an inflammatory response in the local area through the activation of the innate immune system and the recruitment of cells. These include natural killer cells, phagocytes such as neutrophils and macrophages, myofibroblasts, mast cells, and eosinophils, which prevent the spread of infection and aid in the healing process (Rubartelli et al., 2007).

Phagocytes react to the signals sent out by natural killer cells and are responsible for the engulfment of the detected pathogen. Neutrophils comprise the majority of the phagocytes present and are the first to arrive at the infection site. Although they are similar to macrophages by attacking with respiratory bursts, their size is smaller and lifespan much shorter. Macrophages tend to arrive later and are able to engulf more pathogens for a longer period of time. Cytokines such as interleukins help to regulate macrophage activity and control the inflammatory response (Pluddermann et al., 2011).

Although clearing the pathogens is important in resolving the infection, it is only the first step in the recovery process. Normal tissue structure must be regained through the clearance of inflammatory leukocytes through apoptosis at the site of infection as well as the prevention of more phagocytes from entering the area. Cytokines such as anti-inflammatory and growth factors must be released to start the process of healing (Serhan et al., 2005). Inflammation must be negatively and tightly regulated in order to stop tissue damage. Repair and recovery must also be actively promoted. This process must not only be effective but also restricted in its response. Otherwise, chronic inflammation may occur due to failure in resolving the infection (Lawrence et al., 2007).

To sense the approach of an infection, the host cells are able to recognize molecular structures that are conserved and only present on the pathogen known as pathogen-associated molecular patterns (PAMPs). After these are sensed, signaling pathways involved in innate immunity are activated and ultimately lead to the production of cytokines and chemokines to kill the pathogen and resolve the infection (Kumar et al., 2011). The most notable PAMP receptors include the toll-like receptors and NOD-like receptors.

4. Innate Immune Signaling

The toll-like receptors (TLRs) consists of various pathogen recognition receptors which lead to the production of pro-inflammatory cytokines. The majority of TLRs such as TLR2 and TLR4 are transmembrane receptors located at the cell surface which recognize common structures in bacteria. Each TLR is capable of sensing its own set of bacterial cell wall components from both Gram-positive and Gram-negative organisms (Takeuchi et al., 1999). Other TLRs such as TLR9 are located in the endosome and detect bacterial nucleic acids from host DNA (Hemmi et al., 2000). All TLRs, with the exception of TLR3, recruit the adaptor molecule MyD88 to activate the downstream signaling pathways. This induces the production of cytokines such as interferons, interleukins and tumor necrosis factor alpha (TNFα). The alternate adaptor molecule is TRIF and can be recruited only by TLR3 and TLR4. Similar to MyD88, TRIF can also activate interferons and NFκB (Leichtle et al., 2009).

In an experiment performed on TLR2 and TLR4 knockout mice, it was shown that an innate immune response was not properly functioning as an inflammatory response was not initiated to effectively fight the infection (Leichtle et al., 2009). Another previous study has shown that a deficiency in MyD88 has led to the presence of OM and the inability to rapidly clear bacteria from the infection site in mice for weeks (Hernandez et al., 2008). The exploration of other pathways such as those involved with the NOD-like receptors could provide more detailed information about innate immunity in otitis media.

The NOD-like receptors (NLRs) are another family of innate immune receptors that are known to be involved in the response to bacterial infection. They are cytosolic

molecules that can detect intracellular bacteria. NOD1 and NOD2 are two members of the NLR family that stimulate pro-inflammatory cytokines to fight the infection. They are composed of a C-terminal LRR domain, a central nucleotide binding and oligomerization domain, and an N-terminal caspase activation and recruitment domain (CARD). These structures allow the NOD proteins to detect muropeptides, which are conserved within the bacteria peptidoglycan (Bourhis et al., 2007).

Several inflammatory disorders have already been linked to mutations in the NLRs. NOD1 mutations have been linked to the susceptibility of inflammatory bowel disease (McGovern et al., 2005). Mutations in the NOD2 gene have been associated with Crohn's disease and contribute to a slight defect in the innate immunity of the host. This may be due to a decrease in alpha defensins within the mucosal layer, which could lead to the inflammation of the gastrointestinal tract (Wehkamp et al., 2004). As the NLRs seem to play an important role in inflammatory disorders, further research should be done on the NLRs to provide more insight about innate immunity and the mechanism of host defense.

5. RIP2 Function in NOD signaling pathways

RIP2 is a serine-threonine kinase that has a caspase activation recruitment domain (CARD), and it is also known as RICK or CARDIAK. It is the adaptor molecule for both the NOD1 and NOD2 signaling pathways and associates through CARD-CARD interactions (Figure 1). However, RIP2 also aids these pathways by conferring stability to the receptors and thus is able to sustain the innate immune response. Acting as a downstream signaling mediator, the activation of this adaptor protein will induce pro-

inflammatory cytokines such as NFκB (McCarthy et al., 1998). It has also been shown to play a role in cell death and the activation of the JNK pathway through NOD1 signaling (da Silva Correia et al., 2007). With its ability to initiate inflammatory responses, RIP2 may be an important defense molecule in killing pathogens and resolving infection. Therefore, it can potentially play a crucial role in innate immunity. However, the role of NLRs and RIP2 in OM has yet to be explored in detail.

The present study was designed to assess the role of RIP2 in otitis media induced by NTHi to determine if this pathway can enhance the recovery mechanisms involved in OM. The ultimate goal is to increase our understanding of chronic otitis media and to develop new therapies for patients with COM. Using a RIP2 knockout mouse, pathogenesis of the disease will be studied through histology and bacteria clearance within the middle ear will be assessed.

II. MATERIALS AND METHODS

1. Animals

RIP2 -/- mice on a C57BL/6 background were crossed 6 times to produce the homozygous line. These were generated and provided by Ulevitch and colleagues (Ulevitch et al, 2007). Age-matched C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) and used as the wild-type. Experiments were approved by the Institutional Animal Care and Use Committee of the Veteran Affairs Medical Center (San Diego, CA) and performed according to National Institutes of Health guidelines for the care and use of laboratory animals.

2. Bacteria

Non-typeable *Haemophilus influenzae* strain 3655 (biotype II, NTHi) was originally provided by Dr. Asa Melhus (Lund University) after isolation from the middle ear of a patient who had otitis media. The culture was streaked onto a chocolate agar plate and placed in a 37°C incubator overnight. Two colonies were then selected and grown in 25 ml of brain heart infusion (BHI) media added with 1 ml of Fildes enrichment (BD Diagnostic Systems). The next day, the bacteria were spun down at 7,000 rpm for 10 minutes and the pellet resuspended in fresh BHI media. A final concentration of $10^5 – 10^6$ bacteria/ml was used to induce an inflammatory response in the middle ear (Melhus et al., 2003).

3. Surgery

RIP2 -/- and C57BL/6 mice were divided into groups of 6 mice for each experimental time point (3 each for histopathology and bacterial culture). The mice were deeply anesthetized with an intraperitoneal injection of rodent cocktail (13.3 mg/ml ketamine hydrochloride, 1.3 mg/ml xylazine, 0.25 mg/ml acepromazine). 0.1-0.2 ml of this mixture was given per 25-30 g body weight of the mouse. A midline incision was made on the neck by a ventral approach. Soft tissue dissection was done to bilaterally expose both the middle ear bullae. A hole was carefully drilled with a 20-gauge needle, in which 5 ul of NTHi inoculum (500-5000 cfu) was injected into the cavity of the middle ear. A sterile cotton swab was used to remove excess fluid and the wound was closed through stapling of the skin incision. Lactated Ringer's solution and buprenorphine was given postoperatively to the animals through a subcutaneous injection. The controls used were uninoculated mice (time - 0 day).

4. Histology

The mice used were killed under general anesthesia by intracardiac perfusion. PBS was first injected, followed by 4% paraformaldehyde (PFA). Time points were collected at 0, 6 and 12 hours after inoculation, and 1,2,3,5,7,10,14 and 21 days after inoculation. The 0 hour time point was collected from untreated ears and used as the baseline. Dissection of the middle ear from the rest of the ear was then done, which was then placed into a 4% PFA solution overnight. The next day, these were transferred to 8% EDTA and 4% PFA solution and left to decalcify for 14 days.

The middle ears were embedded into paraffin and cut into 7 um sections. They were then stained with hematoxylin-eosin. The same region from the largest area of the middle ear cavity was then digitally recorded from selected sections. Mucosal thickness was analyzed by computer-averaging the thickness of the epithelium, subepithelium space, and the sum of these two over an area close to 500 um.

Using image analysis software, digital micrographs were then taken of the same standardized region to calculate the percent area of the middle ear lumen occupied by inflammatory cells. The numbers of neutrophils versus macrophages were then counted in 5 randomly chosen sites at 400x magnification for middle ears that contained infiltrates. This was performed independently by 2 observers with similar matching results (Ebmeyer et al., 2005).

5. Bacterial Clearance

The middle ear was opened and a sample from the lumen was obtained using a 1 ul loop. This NTHi culture was then streaked onto a chocolate agar plate. Each loop was streaked onto 3 quadrants for each plate, and then a last quadrant was streaked without touching the previous 3. The plates were then incubated for 24 hours. Verification of NTHi was done through gram-staining the colony forming units and negative cultures that were grown on both blood agar and chocolate agar plates.

A scoring system was used to categorize the degree of colonization in order to analyze the colony forming units obtained from the middle ear lumen cultures. 0 indicated no colony-forming units on the plate, 1 indicated colony-forming units in 1 quadrant, 2 indicated colony-forming units in 2 quadrants, 3 indicated colony-forming

units in 3 quadrants, and 4 indicated colony-forming units in all 4 quadrants on the plate (Hernandez et al., 2008).

6. Statistical Analysis

Using StatView software (version 5.0, JMP-SAS Institute), a 2-tailed t test was done to compare wild type mice with RIP2-/- mice. This was done for each time point on mucosal thickness and middle ear inflammatory cells (neutrophils and macrophages). Differences between the two groups were considered to be significant at $P < 0.05$.

The two ears from each mouse were analyzed separately since they were found to be independent from each other. Descriptive statistics such as means were used to prepare the data obtained from the bacterial load. Semiquantative measures were used to evaluate this parameter.

III. RESULTS

1. Histology of the middle ear during otitis media

The middle ear mucosa consists of the 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 (Figure 2). During bacterial infection with NTHi, the middle ear changes dramatically due to the inflammatory response initiated by innate immunity. For C57BL/6 wild-type mice, the height of this inflammation is usually seen by 2 or 3 days after inoculation. Mucosal thickening is observed for both wild-type and RIP2-/- mice. Cellular infiltrate is widely present in the cavity for the wild-type mice. On the other hand, the RIP2-/- mice showed only a few inflammatory cells (Figure 3). The middle ear returned to normal baseline morphology by day 10 after infection in wild-type mice. The mucosa layer regained its usual characteristic and the inflammatory cells have been cleared from the middle ear cavity. However, mucosal hyperplasia and the presence of leukocytic infiltration in the middle ear lumen were still observed in RIP2-/- mice 10 days after NTHi infection (Figure 4).

2. Prolonging of middle ear mucosa thickening due to deficiency of RIP2

Inflammation in the middle ear can be measured both by the mucosal thickness and the percent of inflammatory cells present within the middle ear cavity. In C57BL/6 mice, the mucosal thickness peaked at day 2 after NTHi infection, held strong until day 7, and returned to baseline levels after day 10. In RIP2-/- mice, the mucosal thickness slowly increased over time until reaching its height at day 10 after infection,

with a dramatic decrease afterwards until remodeling to its baseline at day 21. Mucosal thickness was also higher at day 1 but lower at day 2 for RIP2-/- mice when compared to C57BL/6 mice. Unexpectedly, both the wild-type and RIP2-/- mice experienced a decrease in mucosal thickness at day 5. The levels seem to stay equivalent with the adjacent time points for C57BL/6 mice but was uncharacteristic in the trend for RIP2-/- mice. Overall, mucosal thickness remained greater in RIP2-/- mice from 7 to 14 days (Figure 5).

3. Delay in neutrophil and macrophage recruitment to the site of middle ear infection in the absence of signaling through RIP2

Infiltration of the middle ear cavity began occurring at 6 hours in C57L/6 mice, with the most inflammatory exudate present around 2 and 3 days after NTHi inoculation. However, the lumen was cleared of any leukocytic cells by day 7. In RIP2-/- mice, a drop in the infiltration of inflammatory cells was observed around day 2 and 3 after infection but steadily rose over time by peaking later at day 5 with a minor fluctuation at day 7. However, clearance of the cells did not occur until 21 days after inoculation. Recruitment seemed to be delayed in RIP2-/- mice with the inflammatory exudate exceeding that of C57BL/6 mice from day 5 and lasting throughout the course of the experiment (Figure 6).

Leukocytic infiltration was then further analyzed by looking at the different types of phagocytes, in particular neutrophils and macrophages. Neutrophils comprised the majority of the leukocytes present within the middle ear cavity early on. In wild-type mice, the number of neutrophils peaked at day 1 after NTHi infection and slowly decreased until the cells left the lumen completely by day 10. Neutrophil presence in the

cavity for RIP2-/- mice remained consistent with those observed in wild-type mice until day 5 after inoculation. However, it was day 10 that saw the peak in neutrophil numbers in RIP2-/- mice until clearance of all neutrophils occurred by day 21 (Figure 7).

Macrophage counts entered the middle ear lumen at a later time, reaching its peak in wild-type mice at day 3 after infection. No macrophages were evident after day 10. In RIP2-/- mice, the presence of macrophages began around day 2 and sharply peaked at day 3. A gradual decrease in numbers was observed until complete clearance was seen after 21 days (Figure 8).

4. Requirement of RIP2 for bacterial clearance after NTHi

Bacterial clearance after NTHi infection in middle ears obtained from C57BL/6 mice was fully resolved by day 5 as no cultures could be seen on plates from that time point forward. However, this recovery was prolonged in RIP2-/- mice until 21 days after infection. NTHi bacteria were able to be successfully retrieved and could be cultured throughout the entire time course. The viable bacteria were gradually cleared from the middle ear and infection slowly resolved itself, suggesting a defect in the recovery mechanism due to the deletion in the RIP2 gene (Table 1).

IV. DISCUSSION

The interactive role of the NOD-like receptors in the host response has yet to be deeply explored in infectious disease, namely otitis media. The present study lends support to the hypothesis that RIP2 signaling is important for the timely resolution of otitis media caused by non-typeable *Haemophilus influenzae* (NTHi). This suggests that an abnormality of innate immunity may contribute to otitis media and inflammatory pathways such as the NLRs could potentially become targets in the search for new otitis media treatments.

1. Signaling delay due to absence of RIP2

In the absence of RIP2, resolution of the infection is delayed as acute otitis media seems to develop into a chronic condition. Inflammation in the middle ear persisted for a longer period of time, as mucosal hyperplasia failed to recover until 21 days after NTHi inoculation. Leukocytic cells such as neutrophils and macrophages remained in the cavity as complete bacteria clearance was not seen until day 21. There was also a delay in the recruitment of these inflammatory cells to the middle ear.

There is likely a hindered recognition of the PAMPs when the RIP2 gene is blocked. This will cause a delay in the signaling to downstream effector molecules such as NFκB along with other pro-inflammatory cytokines. Macrophages in RIP2-/- mice saw a reduction in the production of cytokines such as IL-6 and TNFα. These products are normally seen early on in the infection and may hinder the innate immune response (Kobayashi, et al., 2002). This would allow pathogens to remain in the middle ear cavity

for an extended period of time, allowing them to replicate and cause tissue damage at an increased rate.

2. Ability of leukocytes to clear NTHi infection

Both C57BL/6 mice and RIP2-/- mice were able to recruit leukocytes. However, high numbers of neutrophils and macrophages were not seen in the middle ear cavity until 3 days after NTHi inoculation. This indicates that there is a delay in the mechanism of recruitment and migration of leukocytes to the area of infection. Cellular infiltration of neutrophils and macrophages in the middle ear cavity lasted for a prolonged period of time. These cells remained together with the NTHi bacteria until clearance occurred 21 days after infection, which may hint at the inability of some leukocytes to phagocytose or kill the bacteria. One previous study used *Staphylococcus aureus* to determine if peritoneal macrophages were able to kill intracellular pathogens. After incubation of purified macrophages with the *S. aureus* bacteria, gentamycin was used to eliminate any extracellular bacteria that were not engulfed. Macrophages were then lysed and the supernatants analyzed for intracellular bacteria count. It was shown that the number of bacteria retrieved from the peritoneal macrophages originally derived RIP2-/- mice was higher than the number retrieved from wild-type macrophages. Although it seemed that engulfment of the bacteria was not affected by the absence of RIP2, the same macrophages were not as effective at killing intracellular bacteria (McCully et al., 2008). This is consistent with our data that showed impaired bacterial clearance in RIP2-/- mice, and therefore a slow resolution of the infection. It should be noted that a different strain of bacteria (*S. aureus*) was used to conduct the macrophage killing assay from the strain

we used in this study (non-typeable *H. influenzae*). However, this should not change the final outcome of the study in finding that there was a reduction in the strength of the macrophages within RIP2-/- mice to eliminate bacteria within its intracellular compartments.

3. The presence of RIP2-independent mechanisms in innate immunity

Although the NTHi bacteria was completed cleared from the middle ear cavity 21 days after inoculation, the majority of the mice were able to also eliminate the bacteria after 14 days. This suggests that there are other activated pathways that can aid in the inflammatory response besides those involved with the adaptor molecule RIP2. One NLR member that plays a role in innate immunity is the NALP3 (or cryopyrin) protein. Similarly to the NODs, it can interact with the adaptor molecule ASC, another protein that contains a CARD domain found in the intracellular inflammasome. The inflammasome is responsible for the maturation of certain cytokines and the promotion of cell death. It has been associated with inflammatory diseases such as familial cold-induced autoinflammatory syndrome and neonatal-onset multisystem inflammatory disease, which group together to form the cryopyrin-associated periodic syndromes (Walsh, 2009). Both the NODs and NALP3 are able to detect similar PAMPs such as bacterial peptidoglycan and lipoproteins as well as activate certain interleukins such as IL-1β (Eisenbarth et al., 2009). Even though the NALP3 pathway is a distinctly different mechanism, its activation may aid in the inflammatory response and alleviate some of the burden in the innate immune system created by a deficiency in RIP2.

Another set of pathways that may be important in RIP2-/- mice is the TLR family with adaptor molecule MyD88. Similar PAMPs are also recognized by both the NODs and TLR2. With the NOD pathways having their signaling cascade blocked from the RIP2 gene deletion, the TLR2 pathway could also activate innate immunity (Kawai et al., 2011). It was previously shown that NOD2 signaling via its adaptor protein RIP2 operates independently from MyD88 downstream signaling (Suzuki et al., 2011). This demonstrates that effector responses from MyD88 due to TLR activation could indeed affect the inflammatory response seen in RIP2-/- mice during otitis media.

It is important to note that without RIP2 and an intact innate immune system, the strength and responsiveness of the consequent reaction is substantially lowered. Inflammation of the mucosa persists for a longer time and leukocytes could be recruited to clear the bacterial infection but at a slower rate than normally seen. Although other independent mechanisms can compensate for the absence of RIP2, it is not enough to prevent acute OM from developing into chronic OM over time. Further research must be done on the role of other inflammatory pathways involved in innate immunity in order to find new therapies for otitis media.

APPENDIX

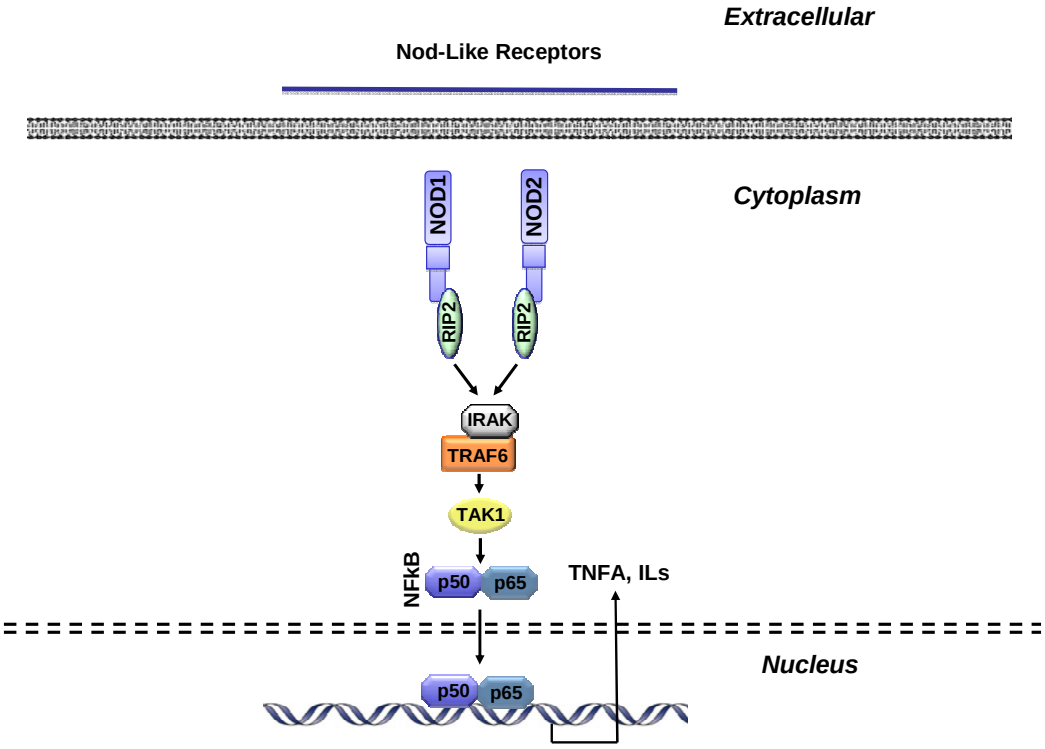
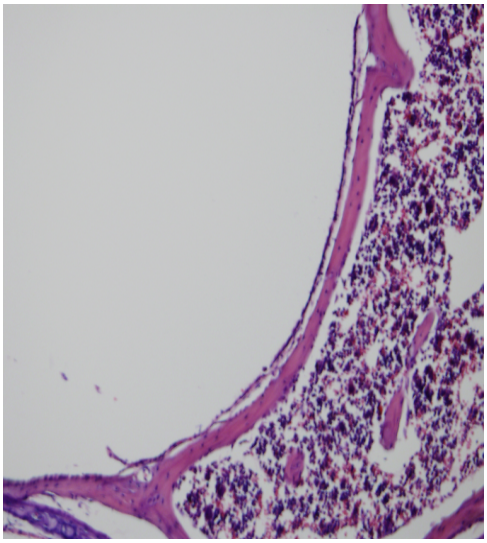
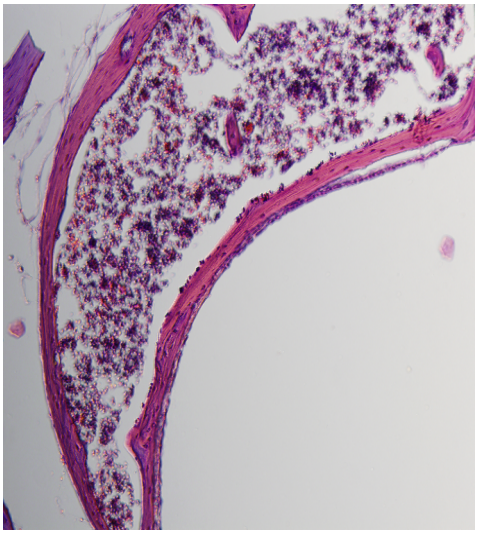


Figure 1. A schematic representation of NOD signaling via the RIP2 adaptor. RIP2 signaling strongly stimulates the production of pro-inflammatory interleukins, primarily via NFκB. NOD, nucleotide-binding oligomerization domain; RIP2, receptor interacting protein kinase 2; IRAK1, interleukin-1 receptor-associated kinase 1; TRAF6, tumor necrosis factor (TNF) receptor associated factor 6; TAK1, mitogen-activated protein kinase kinase kinase 7; NFκB, nuclear factor of kappa light polypeptide gene enhancer in B-cells; p50 NFκB subunit 1; p65, NFκB subunit 3,TNFα, tumor necrosis factor alpha; IL, interleukin.

Day 0 Mucosal Pictures



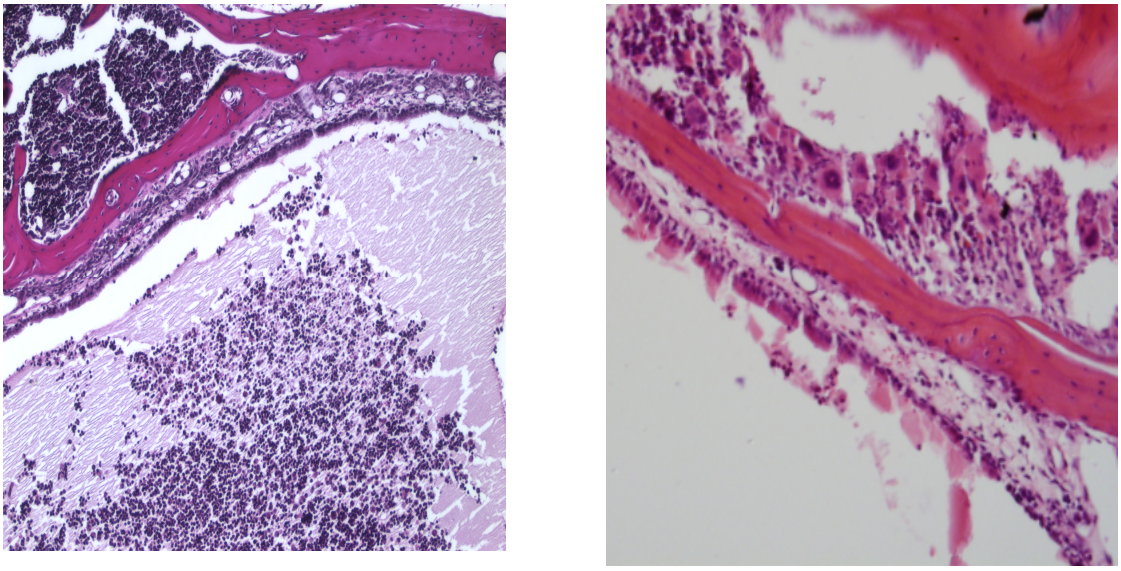
C57BL/6



RIP2

Figure 2. Untreated middle ear response in C57BL/6 mice and RIP2-/- mice as a baseline. The middle ears of C57BL/6 mice before NTHi infection demonstrated a very thin mucosal layer and no cellular infiltrate in the middle ear cavity. In RIP2-/- mice, the morphology at 0 hours (untreated) was similar to that in wild-type mice. Original magnifications, X40.

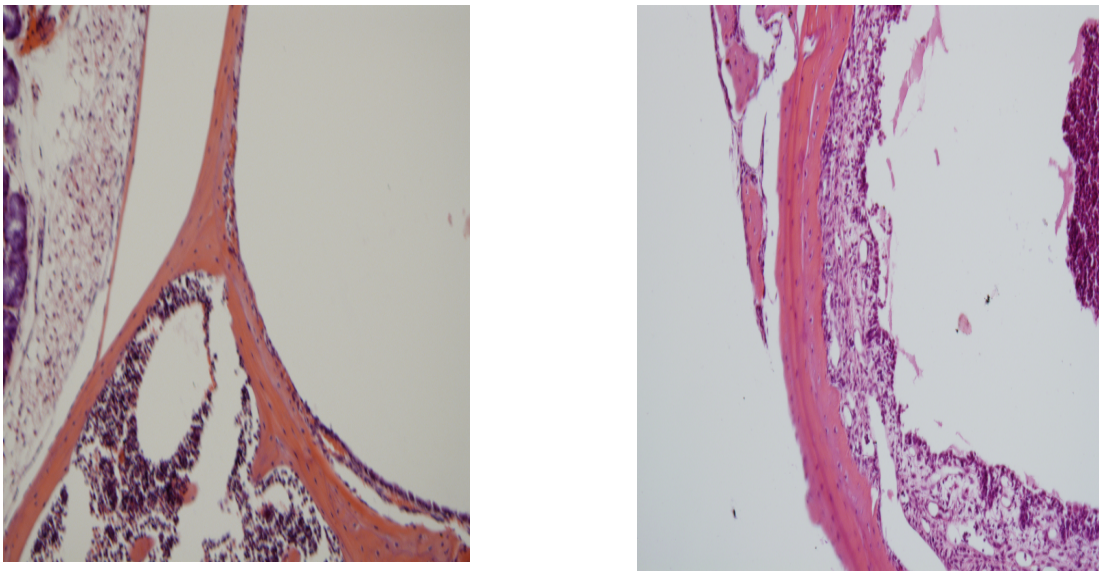
Day 3 Mucosal Pictures



C57BL/6 **RIP2**

Figure 3. Middle ear response to nontypeable *H. influenzae* (NTHi) in C57BL/6 mice and RIP2-/- mice at 3 days after infection. The middle ears cavities of C57BL/6 mice were filled with inflammatory cells and transudated fluid. RIP2-/- mice had fewer inflammatory cells evident in the middle ear cavity. Original magnifications, X40.

Day 10 Mucosal Pictures



C57BL/6 **RIP2**

Figure 4. Middle ear response to nontypeable *H. influenzae* (NTHi) in C57BL/6 mice and RIP2-/- mice at 10 days after infection. No inflammatory cells were evident in the middle ear cavity of C57BL/6 mice and the mucosal layer had remodeled to its baseline appearance. The middle ears of RIP2-/- mice showed a persistent inflammatory cell infiltrate and increased mucosal thickness compared with C57BL/6 mice. Original magnifications, X40.

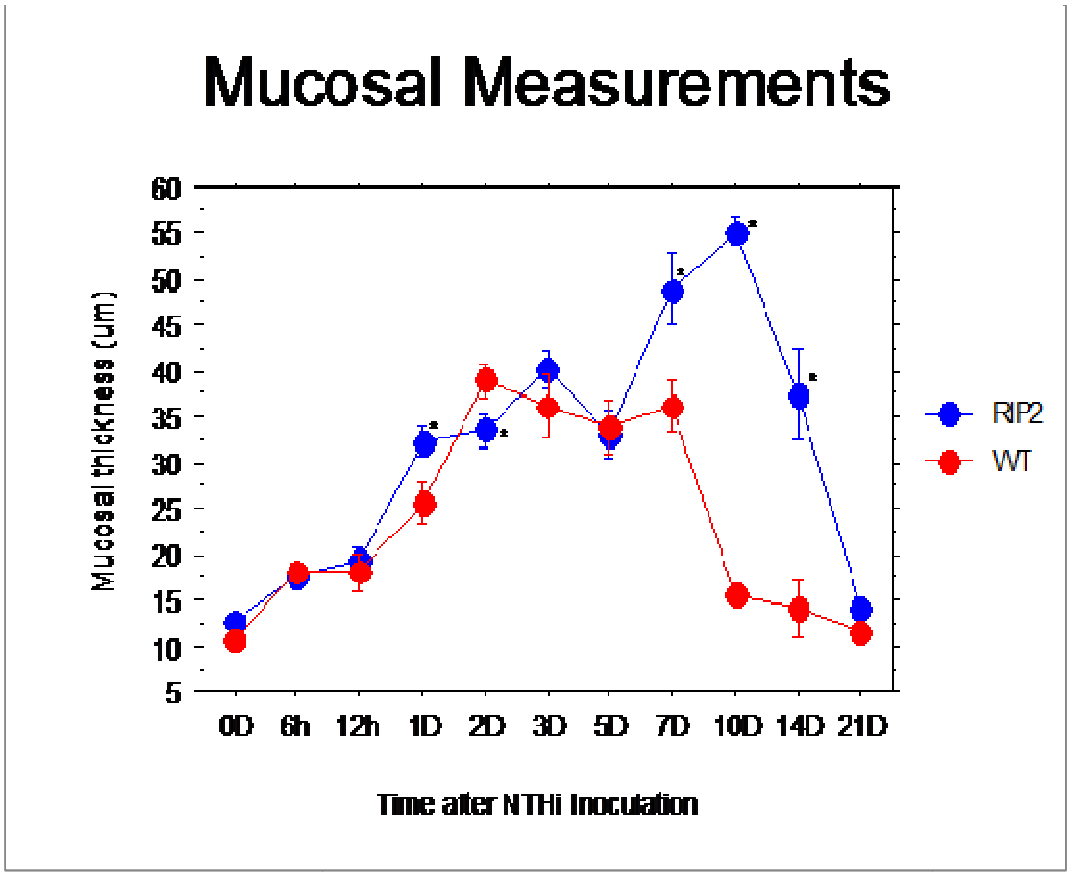


Figure 5. A quantitative evaluation of mucosal thickness of the middle ear cavity throughout the course of otitis media. The middle ears of C57BL/6 mice and RIP2-/- mice showed similar degrees of mucosal thickness through 12 hour after infection with nontypeable *H. influenzae* (NTHi). Thicker mucosal was evident on day 1 for RIP2-/- mice and day 2 for C57BL/6 mice. The degrees of thickness diverged after day 5, when the middle ear mucosa of RIP2-/- mice kept persistently thick while those of C57BL/6 mice returned to near baseline thickness.

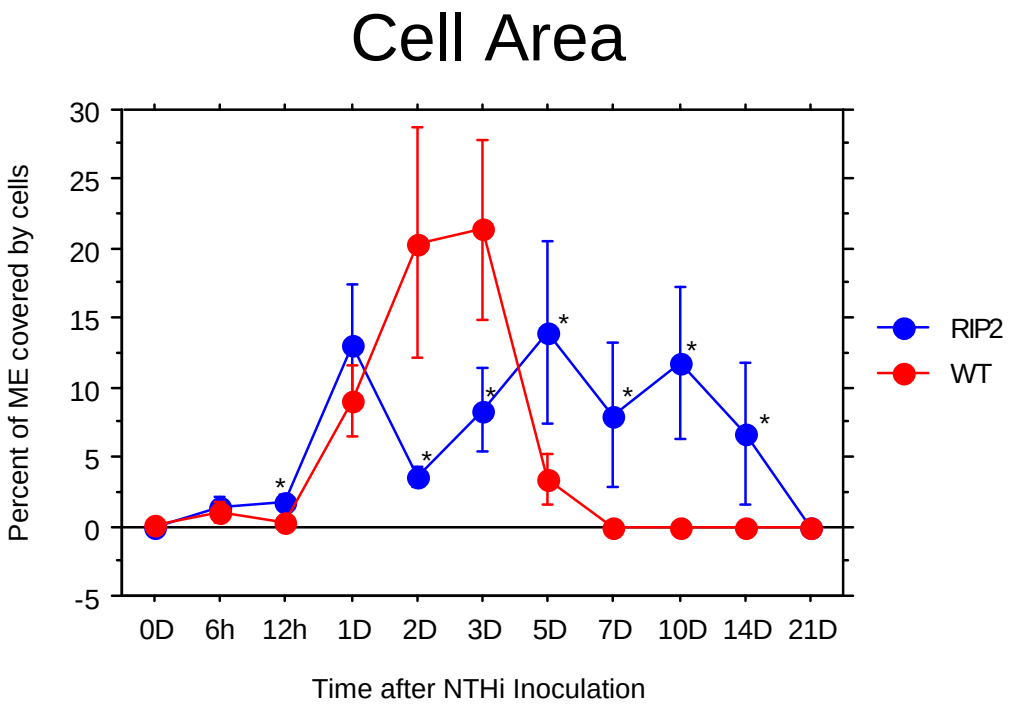


Figure 6. Infiltration of the middle ear cavity by leukocytes after non-typeable *H. influenzae* inoculation. A greater percentage of the middle ear was occupied by inflammatory cells in C57BL/6 mice than in RIP2-/- mice through 3 days after NTHi infection. Leukocyte infiltration is substantially delayed in RIP2 -/- mice, peaking at 5 day after NTHi inoculation, and persisting through day 14. (n=6-8 middle ears per time point; bars represent the mean ±SEM; *P <0.05).

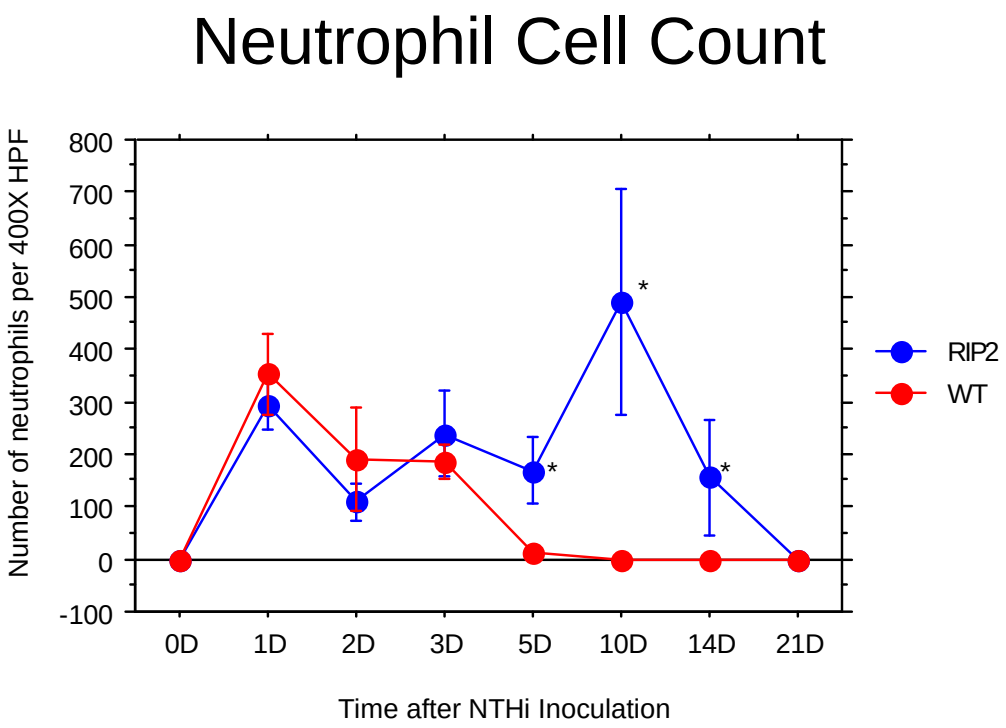


Figure 7. Leukocyte numbers for neutrophils measured in middle ear infiltrates in wild-type and RIP2-/- mice. C57BL/6 mice showed peak neutrophil numbers by day 1 after infection with NTHi with no neutrophils evident by day 10 after infection. Neutrophils showed a striking, late influx after day 5, with the peak at day10 and some neutrophils still present 14 days after infection in RIP2 -/- mice (n=6 middle ears per time point; bars represent ±SEM; *P <0.05).

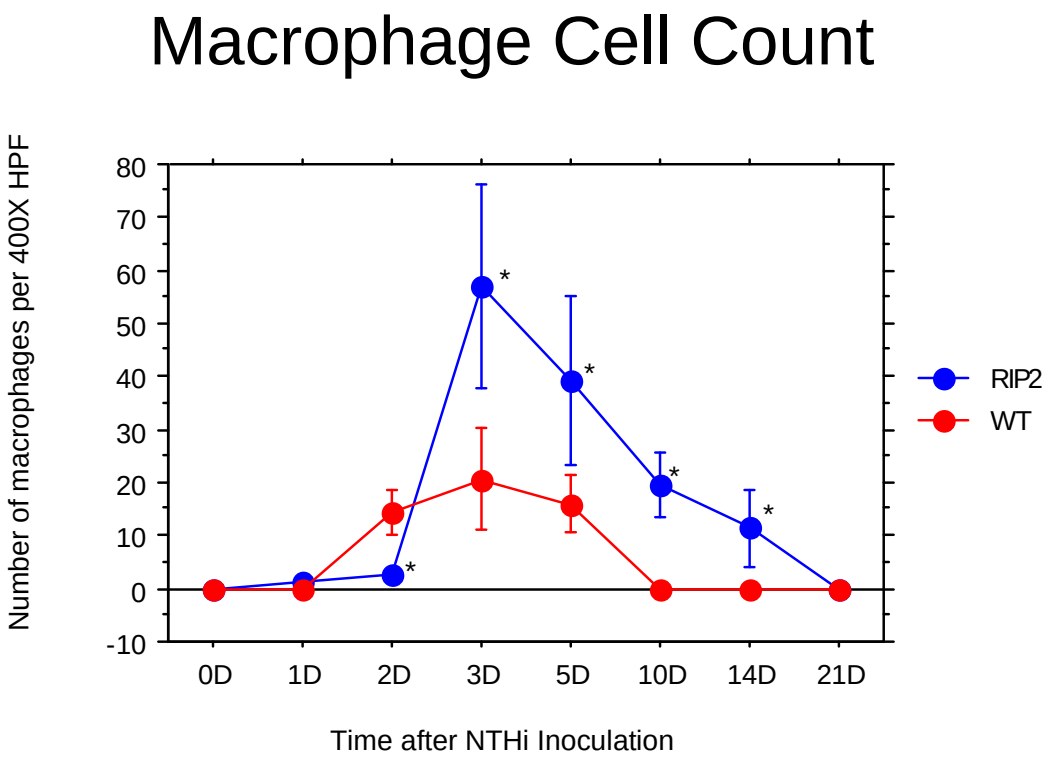


Figure 8. Leukocyte numbers for macrophages measured in middle ear infiltrates in wild-type and RIP2-/- mice. Macrophages were recruited to the middle ear by 2 days after NTHi infection in C57BL/6 mice, with no macrophages noted by day 10 after infection. The RIP2-/- mice had a short delay in macrophage recruitment by day 2 after infection but displayed prolonged macrophage presence in the middle ear until 21 days after non-typeable *H. influenzae* (n=6 middle ears per time point; bars represent ±SEM; *P <0.05).

Table 1. Impaired bacterial clearance of RIP2-/- middle ears. No colony forming units (CFUs) were detected by day 5 after NTHi inoculation in C57BL/6 mice. Bacterial clearance was impaired in RIP2-/- mice until 21 days after inoculation. NTHi was isolated from more than half of the middle ears by 14 days. Mean bacterial colonization of the culture positive plates was evaluated using semi-quantatative analysis of bacterial colonization: 0 indicated no CFUs, 1 indicates one quadrant with CFUs, 2 indicates two quadrants with CFUs, 3 indicates three quadrants with CFUs and 4 indicates four quadrants with CFUs. Data represent positive culture plates out of six.

Bacterial Clearance

Time after NTHi instillation	WT # of culture positive plates	WT Mean bacterial colonization of culture positive plates	Rip2 -/- # of culture positive plates	Rip2 -/- Mean bacterial colonization of culture positive plates
Day 1	4/6	4.00	6/6	3.83
Day 2	6/6	3.00	5/6	4.00
Day 3	3/6	1.00	6/6	3.50
Day 5	0/6	0.00	5/6	3.20
Day 10	0/6	0.00	6/6	3.00
Day 14	0/6	0.00	2/6	2.50
Day 21	0/6	0.00	0/6	0.00

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