Otitis media (OM) (a middle ear infection) is one of the most common pediatric diseases worldwide. It can affect up to 80% of children before the age of 3 years and can lead to permanent hearing loss (1). Up to 70% of cases of acute OM are caused by viral-bacterial coinfections (2). Of particular relevance are coinfections with influenza A virus (IAV) and the bacterium Streptococcus pneumoniae. In clinical cases and experimental models, infection with IAV facilitates the replication of S. pneumoniae in the middle ear (3–8). Using an infant mouse model of OM (designed to mimic the underdeveloped immune system of children), we have previously demonstrated that the development of pneumococcal OM in coinfected mice was due to the inflammation induced by IAV in the middle ear (3, 8). However, the mechanisms by which the host inflammatory response mediates secondary pneumococcal OM remain undefined.

The middle ear has few resident leukocytes, and an infection in the organ results in an influx of neutrophils, macrophages, and lymphocytes (9–11). Neutrophils have traditionally been considered to play a protective role in OM (12, 13). However, recent studies have speculated that neutrophils may contribute to bacterial persistence in the middle ear via the formation of neutrophil extracellular traps (NETs) (14–16). The term NETs refers to the extracellular DNA produced by neutrophils to “trap” bacterial pathogens. This extracellular DNA is studded with histones and antimicrobial compounds to kill the trapped bacteria (17). Interestingly, the pneumococcal capsule and D-alanine residues on pneumococcal lipoteichoic acids can inhibit NET killing (18), potentially enabling the pneumococcus to survive and persist within biofilm-like NET structures in the middle ear.

Pneumococcal OM predominately develops in the absence of preexisting immunity, with incidence peaking between 6 months (when maternal antibodies have waned) and 2 years, when specific immunity develops (19). In these immunologically naive individuals, natural antibodies may represent an important defense mechanism against influenza virus-mediated pneumococcal disease, as is seen in pneumococcal sepsis (20). Conversely, the formation of immune complexes in the middle ear may facilitate, rather than clear, bacterial OM (21), suggesting that organ-specific differences may exist with regard to the role of antibodies during pneumococcal disease. Moreover, the ability of antibodies to interact with neutrophils in the middle ear (19), and the suggestion that neutrophils may facilitate bacterial OM (14, 15), may indicate that the role of antibodies and neutrophils in pneumococcal-influenza virus OM is more complex than simply protecting against disease development.

Here, we use B6.μMT−/− mice (which lack B lymphocytes) (22) to investigate the role of antibodies in pneumococcal-influenza virus OM. Our data suggest that antibodies facilitate the de-
development of secondary bacterial OM by inducing NETs in the middle ear. These NETs, instead of clearing the pneumococci, may then provide scaffolding for bacterial outgrowth. Accordingly, DNase treatment reduced pneumococcal OM. These data provide new mechanistic insight into pneumococcal-IAV coinfections and identify NETs as an important target for treating and preventing pneumococcal OM.

MATERIALS AND METHODS

Viral and bacterial strains. The bioluminescent *S. pneumoniae* strain EF3030lux (type 19F) (23) was used in all experiments. Influenza virus strain A/Udorn/307/72 (H1N2) was used to model infection with IAV. Virus stocks were prepared in embryonated eggs and quantified as described previously (24).

Mice. Animal experiments were approved by the Animal Ethics Committee of the University of Melbourne and were conducted in accordance with the relevant Australian legislation. C57BL/6, B6.μMT−/−, and B6.pIgR−/− mice were bred and housed under specific-pathogen-free (SPF) conditions at the Department of Microbiology and Immunology, the University of Melbourne. B6.μMT−/− mice lack B lymphocytes and antibodies (although these mice can selectively produce some antibodies) (22, 25, 26). In contrast, B6.pIgR−/− mice are deficient in the polymeric Ig receptor (plgR) (27, 28). Accordingly, these mice are unable to secrete polymeric antibodies, and the sera of the mice contain significantly more IgA and IgG than sera from C57BL/6 (B6) mice (27, 28).

Infection of mice. Five-day-old B6 and B6.μMT−/− mice were colonized intranasally (i.n.) with 2 × 10^9 CFU of *S. pneumoniae* EF3030lux or phosphate-buffered saline (PBS) in 3 microliters. At 14 days of age, the mice were infected intranasally with 10^8 PFU of egg-grown IAV in 3 microliters. Six days post-IAV infection, the mice were euthanized, and organs were collected for analysis.

Enumeration of bacterial and viral loads. Tissues used to quantify bacterial and viral loads were collected and processed as described previously (3, 23). The viral load was determined using plaque assays on MDCK cells, as described elsewhere (24).

Histology and immunofluorescence. Middle ears were collected and processed for histological analysis essentially as described previously (3). Antigen retrieval was performed by heating slides in citrate buffer (anti-gen retrieval solution; Dako) in a microwave. The slides were treated with Image-iT FX Signal Enhancer (Invitrogen), as recommended by the manufacturer, and stained with rabbit anti-CRAMP, a marker for mouse NETs (7.75 μg/ml; provided by Richard Gallo, UCSD, San Diego, CA [29]) or the respective isotype controls (rabbit IgG whole molecule; Jackson ImmunoResearch). The slides were subsequently stained with an Alexa 647-conjugated goat anti-rabbit antibody (Invitrogen) and mounted using Prolong Gold with 4',6'-diamidino-2-phenylindole (DAPI) (Invitrogen). For each sample (individual ear), a minimum of three randomly selected images were acquired (Leica DMi6000CS confocal microscope) and used for quantification of NET-producing cells. The data were expressed as percentages of NET-forming cells in relation to the total number of cells. The mean value derived from random images per sample was expressed as percentages of NET-forming cells in relation to the total number of cells. The mean value derived from random images per sample was expressed as percentages of NET-forming cells in relation to the total number of cells. The mean value derived from random images per sample was expressed as percentages of NET-forming cells in relation to the total number of cells. The mean value derived from random images per sample was expressed as percentages of NET-forming cells in relation to the total number of cells. The mean value derived from random images per sample was expressed as percentages of NET-forming cells in relation to the total number of cells. The mean value derived from random images per sample was expressed as percentages of NET-forming cells in relation to the total number of cells.

Antibody transfer. One hundred microliters of sera was transferred to B6.μMT−/− mice (14 to 19 days old) daily via intraperitoneal (i.p.) injection. Sera used for transfer experiments were collected from naive B6.plgR−/− mice (male and female) that were ≥6 weeks of age and pooled.

Transmypanic injection. IgA was enriched from naive B6.plgR−/− sera using ammonium sulfate immunoprecipitation. IgG was then depleted with protein G (Genscript, USA), and IgA was further purified using mouse IgA purification resin (Affiland SA, Belgium) according to the manufacturer’s instructions. IgA purity was confirmed by SDS-PAGE, Western immunoblotting, and enzyme-linked immunosorbent assay (ELISA) (data not shown). Purified IgA (2 μg) or PBS was then injected transtympanically into the middle ears of *S. pneumoniae* EF3030lux-colo-nized B6.μMT mice 4 and 5 days post-IAV infection. Alternatively, 10 μg of DNase (Sigma, USA) or PBS was injected transtympanically into the middle ears of infected B6 mice 4 and 5 days post-IAV infection.

ELISA. Total IgA, IgM, and IgG in serum samples and clarified middle ear homogenates were measured by a standard ELISA (22). Briefly, 96-well MaxiSorp immunoplates (Nunc, USA) were coated with goat anti-mouse IgA, goat anti-mouse IgM, or goat anti-mouse IgG (Sigma). Wells were blocked with bovine serum albumin (BSA) and washed, and then serum or middle ear homogenates were added. To generate a standard curve, serial dilutions of known amounts of purified mouse IgA (BD Pharmingen, USA), mouse IgM (Millipore, USA), or mouse IgG (AbD Serotec, United Kingdom) were included on each plate. Following incubation and washing, goat anti-mouse IgA/IgM/IgG horseradish peroxidase-conjugated antibodies (Southern Biotech, USA) were bound. Bound antibody was visualized using an o-phenylenediamine (OPD) substrate, absorbance was measured at 492 nm in a Multiskan plate reader (Labsystems, Finland), and antibody concentrations were determined using the relevant standard curve.

RESULTS

B6.μMT−/− mice display reduced pneumococcal outgrowth in the middle ear. To assess the role of antibodies in pneumococcal-influenza virus OM, B6.μMT−/− mice and B6 mice were colonized with *S. pneumoniae*. Nine days later, the mice were infected with IAV, and 6 days later, OM was monitored. Six days post-IAV infection was selected as the time point of interest, as we had previously assessed the kinetics of the infection and had shown that day 6 represents the peak of pneumococcal OM in infant B6 mice (3). Consistent with our previous findings (3), B6 mice had high bacterial titers in the middle ear following IAV infection (Fig. 1A), which we had previously shown to result in a 20-dB hearing loss (3). Strikingly, B6.μMT−/− mice displayed significantly lower pneumococcal titers in the middle ear than B6 mice (Fig. 1A). The titers of pneumococci in the middle ears of B6.μMT−/− mice (approximately 10^4 CFU) are consistent with bacterial titers in the middle ears of mice infected with *S. pneumoniae* alone (3, 8), and we have demonstrated that this amount of bacteria in the ears does
not result in hearing loss (3). The difference in bacterial outgrowth between B6 and B6.μMT<sup>−/−</sup> mice was not due to impaired replication of IAV in B6.μMT<sup>−/−</sup> mice or a reduced inflammatory infiltrate in the ears of B6.μMT<sup>−/−</sup> mice (see Fig. S1 in the supplemental material). Importantly, the observed decrease in pneumococcal replication was restricted to the middle ear, as no differences in pneumococcal titers were observed between B6 and B6.μMT<sup>−/−</sup> mice in the nasopharynx (Fig. 1B).

**Antibodies facilitate pneumococcal outgrowth in the middle ear.** B6.μMT<sup>−/−</sup> mice lack B lymphocytes and therefore, for the most part, do not produce antibodies (22). However, these mice can produce at least some level of IgA in response to *Salmonella* (25), as well as IgE/IgG in the lung in response to *Aspergillus fumigatus* (26). Therefore, to determine if antibodies could restore pneumococcal growth, we adoptively transferred serum into B6.μMT<sup>−/−</sup> recipient mice from naive B6.pIgR<sup>−/−</sup> mice (Fig. 2A). B6.pIgR<sup>−/−</sup> mice were used instead of wild-type B6 mice, as they have higher levels of serum IgG and IgA than wild-type mice (27, 28), thereby reducing the number of intraperitoneal injections required. Sera were derived from naive mice, rather than from mice with specific anti-pneumococcal antibodies, as we found that 20-day-old coinfected mice have not yet developed significant levels of specific anti-pneumococcal antibodies in the middle ear (see Fig. S2 in the supplemental material). Therefore, any putative role of antibodies in pneumococcal OM is not likely to be driven by specific anti-pneumococcal antibodies. The transfer of naive serum to coinfected B6.μMT<sup>−/−</sup> mice significantly increased bacterial titers in the middle ear relative to mice treated with PBS (Fig. 2A). Serum transfer also resulted in middle ear antibody titers that were equivalent to those of coinfected B6 mice (Fig. 2B to D). To confirm that antibodies facilitated pneumococcal outgrowth in the middle ear, we injected IgA purified from naive sera directly into the middle ears of coinfected B6.μMT<sup>−/−</sup> mice. Although we were unable to use a large number of mice for these experiments, due to the difficulties associated with administering transtympanic injections to infant mice, the administration of IgA resulted in higher bacterial and IgA titers than the injection of PBS (Fig. 2E and F). No reactivity was observed between the IgA used for these transtympanic injections and pneumococcal or IAV antigens (see Fig. S3A and B in the supplemental material), suggesting that OM can occur in the absence of specific anti-pneumococcal/IAV antibodies.

**NETs facilitate pneumococcal outgrowth in the ears of coinfected B6 mice.** We had previously demonstrated that infection...
with IAV induces an influx of neutrophils into the middle ear cavities of infant mice (3). Moreover, pneumococci in the ear colocalize with this influx of neutrophils (3), and neutrophils are thought to interact with antibodies in the middle ear (19). In light of the suggested role of NETs in OM (14, 15), we reasoned that antibodies in the middle ear may facilitate bacterial outgrowth by inducing NETs. To test this hypothesis, B6 and B6.μMT−/− mice were coinfected with S. pneumoniae, and IAV and NET formation in the middle ear was assessed. Immunofluorescence showed that B6.μMT−/− mice formed significantly fewer NETs in the middle ear than coinfected wild-type B6 mice (Fig. 3A and B). We confirmed with immunofluorescence assays that pneumococci localized to the neutrophilic infiltrate in the middle ears of NET-positive B6 mice (see Fig. S4 in the supplemental material). The production of NETs in the middle ear was independent of S. pneumoniae, as NETs were also detected in the middle ears of B6 mice infected with IAV alone (Fig. 3A and B).

To confirm that reduced NET formation in B6.μMT−/− mice was due to the absence of antibodies in the mice, NET formation was assessed in coinfected B6.μMT−/− mice following transtympanic injection of IgA or PBS. The administration of IgA resulted in a significant increase in NET production in the middle ear compared to injections with PBS (Fig. 3C). PBS-treated B6.μMT−/− mice displayed reduced NET formation compared to B6.μMT−/− mice that were not treated transtympanically (Fig. 3B and C), suggesting that the transtympanic injection itself may limit the detection of NETs by immunofluorescence. Nevertheless, the significant increase in NETs observed in IAV-treated mice relative to PBS-treated mice suggests that antibodies can facilitate NET production in the middle ear.

Due to the sequestered nature of the middle ear, depletion of neutrophils using the 1A8 monoclonal antibody is ineffectual (8). Instead, to investigate whether the antibody-dependent difference in NET production observed between B6 and B6.μMT−/− mice affected bacterial titers in the middle ear, B6 mice were coinfected with S. pneumoniae EF3030lux and IAV. DNase was then injected transtympanically in order to destroy the NETs present in the middle ear. Alternatively, control mice were treated with PBS. Bacterial titers in the middle ear were then determined 6 days post-IAV infection. Treatment with DNase resulted in significantly fewer pneumococci in the middle ear than PBS treatment (Fig. 3D). These data thus suggest that the decreased NET production in B6.μMT−/− mice may help reduce pneumococcal outgrowth in the middle ear.

**DISCUSSION**

OM represents a major health care burden worldwide and can arise from coinfection with IAV and S. pneumoniae. We have previously shown that IAV facilitates pneumococcal outgrowth in the middle ear by triggering middle ear inflammation (3, 8). The present study suggests that the ability of antibodies to trigger NET production in the middle ear may be a key component of the host inflammatory response that facilitates the development of bacterial middle ear disease.

It has previously been suggested that NETs may be an important mechanism by which pneumococci persist in the middle ear (14, 16), as they are able to reside in NETs and resist NET-mediated killing (18, 30). Consistent with this notion, bacterial biofilms entangled with DNA strands from NETs can be found in the middle ear effusions of children with recurrent acute OM (16). Accordingly, it has been suggested that DNases may be a useful adjunct treatment in children with recurrent or chronic otitis media (16). However, the therapeutic benefit of DNase treatment in an animal model of OM has yet to be demonstrated. Here, we show that not only do coinfected mice develop NETs in the middle ear, but the transtympanic administration of a DNase reduces...
pneumococcal outgrowth in the middle ears of IAV-infected mice. DNases have previously been used very successfully to treat patients with cystic fibrosis (31–33). Similarly, a study from the early 1960s showed that a DNase derived from beef pancreas had a therapeutic effect on patients with OM (34). Our data may thus suggest that DNase treatment could be an efficacious treatment option for children with OM. Indeed, the ability of a DNase (dornase alpha) to resolve OM in children is currently the subject of a clinical trial in Australia, where dornase alpha is administered to the middle ears of children undergoing surgery for grommet insertion (35). In the present study, the transtympanic administration of DNase to the middle ear was designed to mimic this surgical administration DNase. Of course, an animal model can never fully recapitulate the complexity of human disease, and it remains possible that DNase treatment would not reduce bacterial titers in children or that this would not result in a subsequent improvement in hearing. Nevertheless, our data suggest that the therapeutic benefits of DNase treatment in OM is an interesting area for further study.

Surprisingly, we identified antibodies as an important trigger for NET formation in the middle ear. Previous studies have suggested that B6.μMT−/− mice can at least produce IgE and IgG upon challenge with a fungal pathogen (26). However, the potential production of IgE/IgG in these mice following microbial challenge (26) does not detract from our findings that antibodies mediate NET formation and pneumococcal OM. We showed that the transfer of purified IgA to B6.μMT−/− mice induced NET production in the middle ear, which clearly indicates that antibodies contribute to disease development. One possible explanation to reconcile our findings with the suggestion that B6.μMT−/− mice still produce some antibodies upon infection (26) may be that a certain threshold level of antibodies is required for the development of secondary pneumococcal OM. Alternatively, it is possible that the CD19+CD9+ IgD+B-1 cells found by Ghosh et al. (26) in the lungs of μMT−/− mice are not present in the middle ear.

A variety of different stimuli have been identified as triggering NET formation either in vivo or in vitro (36). They include interleukin 8 (IL-8), components of the complement cascade, and select bacterial and fungal pathogens (36). It is therefore interesting to consider the ways in which antibodies may trigger NET formation in the middle ear. Antibodies could induce NET formation in an indirect manner by activating the complement cascade (36, 37). The chemotactic complement-derived peptide complement factor 5a (C5a) can then induce NET formation by neutrophils that have been primed with granulocyte-macrophage colony-stimulating factor or interferons (36). While IAV can trigger the expression of interferon-regulated genes in the middle ear (8), a role for complement in middle ear NET production and pneumococcal OM is inconsistent with previous reports that complement protects against the development of pneumococcal OM (38). Moreover, we have found that, in vitro, IgA is able to induce both NET formation and pneumococcal outgrowth in the absence of complement (data not shown). Thus, clearly elucidating the pathway by which antibodies induce NET formation in the middle ear remains an important area for future studies.

In this study, we used purified IgA to confirm the role of antibodies in NETs and pneumococcal outgrowth in the middle ear. However, other antibody isotypes may also be able to induce NET formation in vivo. Indeed, our preliminary data suggest that the presence of IgG (in the absence of IgA) in the middle ears of cointected B6.μMT−/− mice is also sufficient to facilitate pneumococcal outgrowth (data not shown). Thus, the interactions between antibodies and NET-producing neutrophils may not be restricted to one antibody isotype.

The antibodies involved in the development of OM in the present study were unlikely to be specific for S. pneumoniae or IAV, as middle ear disease could be induced in B6.μMT−/− mice by the transfer of IgA, which did not bind to pneumococcal or IAV antigens. Moreover, cointected B6 mice (which develop pneumococcal OM) did not possess pneumococcus-specific antibodies in the middle ear. It currently remains unclear if the “OM-inducing” antibodies observed in this study were natural antibodies (i.e., polyspecific, low-affinity antibodies that can be produced in the absence of apparent antigenic stimuli) (39) or high-affinity antibodies that were produced in response to nonpneumococcal antigens. The possibility also exists that these antibodies actually bind to self-antigens, as autoantibodies are known inducers of NET production (40).

It is important to note that despite the findings of this study, specific anti-pneumococcal antibodies are still likely to protect against pneumococcal OM, as has been observed in clinical trials of anti-pneumococcal conjugate vaccines (41, 42). This is due, in part, to the ability of anti-capsular antibodies to reduce nasopharyngeal colonization with the corresponding serotype strains (43, 44). Colonization is the essential first step in the development of pneumococcal OM, and an increased number of pneumococci in the nose is associated with an increased risk of OM (45, 46). Therefore, regardless of whether anti-pneumococcal antibodies are able to induce NETs in the middle ear, anti-pneumococcal antibodies are still likely to decrease the risk of OM by reducing pneumococcal colonization in the nasopharynx.

Finally, this study found that the role of “OM-inducing antibodies” in pneumococcal outgrowth was at least to some extent tissue specific, as B6.μMT−/− mice did not display reduced pneumococcal titers in the nasal cavity relative to wild-type B6 mice. However, the role of NETs in secondary pneumococcal disease may not be restricted to the middle ear. It has recently been demonstrated that mice cointected with S. pneumoniae and influenza virus display increased pulmonary lesions and NET formation in the lung compared to mice infected with either pathogen alone, and these NETs were unable to kill S. pneumoniae (30). Therefore, determining the role of NETs, and the stimuli required for their production, in the pathogenesis of invasive pneumococcal disease (i.e., pneumococcal pneumonia, sepsis, and meningitis) remains a key area for further study.

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