One of the most common causes of community-acquired pneumonia is *Streptococcus pneumoniae*, a commensal organism of upper respiratory tract (1). Pneumonia and other invasive pneumococcal diseases such as bacteremia, meningitis, and sepsis can be caused by *S. pneumoniae*, resulting in 1–2 million infant deaths every year (2). Secondary pulmonary infection by *S. pneumoniae* is commonly associated with higher mortality during major influenza pandemics (2). It is known that *S. pneumoniae*-induced cytotoxicity underlies pulmonary tissue injury during pneumonia and determines the outcome of infection (3). Furthermore, toxicity to alveolar epithelium disintegrates pulmonary architecture as well as weakens the alveolar-blood barrier, facilitating systemic bacterial dissemination. Although we have some understanding of bacterial virulence (4, 5), the underlying molecular processes in mammalian host cells that mediate tissue damage are not fully understood, which limits the development of mitigation strategies.

There are significant data supporting a role for inflammation as a cause for cytotoxicity during infection. *S. pneumoniae* is known to induce a robust inflammatory response at the site of infection that culminates with infiltration and accumulation of inflammatory cells including neutrophils and macrophages (6–8). To defend against infection, activated inflammatory cells produce high levels of genotoxic reactive oxygen and nitrogen species (RONS) including hydroxyl radical, superoxide, peroxides, nitric oxide, and peroxynitrite. RONS-induced DNA lesions such as base damage, single-strand breaks, and double-strand breaks (DSBs) can be cytotoxic and thus damaging to host tissue function (9, 10). DSBs are one of the most toxic forms of DNA damage (11, 12). In response to DSBs, the ataxia telangiectasia mutated (ATM) kinase pathway is activated, leading to Ser-139 phosphorylation of histone H2AX, forming γH2AX. The presence of γH2AX at DSBs recruits downstream DNA repair proteins, including 53BP1 and the Mre11/Rad50/Nbs1 (MRN) complex (13, 14). The major DSB repair pathway in nondividing cells is nonhomologous end-joining (NHEJ) (15). Early in NHEJ, Ku70/Ku80 heterodimer binds the damaged DNA ends. Ku80 plays a vital role in further recruitment and binding of the catalytic DNA-PKcs subunit (16). The DNA strands are then processed by nuclease activity of the MRN complex, and the DNA-PK holoenzyme recruits additional enzymes that complete the repair process (17). Despite the presence of efficient DSB repair, under conditions of excessive RONS, DNA damage can lead to cell death.

Although studies have been done to explore the damaging potential of RONS associated with the host response (18, 19), the possibility that *S. pneumoniae* might directly induce oxidative damage to DNA has not been explored. Studies focused on respiratory, as well as intestinal pathogens (20, 21), call attention to the importance of microbial-induced DNA damage as an important dimension of pathogenicity. For example, *Pseudomonas aeruginosa* has been shown to induce oxidative DNA damage in lung cells accompanied by significant tissue injury (22). This findings...
raise the possibility that *S. pneumoniae* may also induce DNA damage as a means for triggering host cell cytotoxicity. Although induction of cell death is key to pathogenicity, the underlying mechanisms by which *S. pneumoniae* induces apoptosis (23–25) and necrosis (26) in host cells is not yet well understood. Although it is known that certain pneumococcal proteins elicit a potentially cytotoxic inflammatory response (4, 27), here we asked whether *S. pneumoniae* or its secreted factors could directly generate DNA damage responses that could contribute to cell death. One such secreted factor could be hydrogen peroxide (H$_2$O$_2$), produced by action of pyruvate oxidase (encoded by *spxB*) in *S. pneumoniae* (28). H$_2$O$_2$ secreted by *S. pneumoniae* could potentially contribute to pneumococci-induced oxidative stress and elicit DNA damage response during infection. We used in vitro approaches to control H$_2$O$_2$ levels, and we also knocked out the *spxB* gene to reveal the impact of pneumococcal H$_2$O$_2$ in cells and animals. Specifically, we show that *S. pneumoniae* indeed has the potential to induce significant levels of DNA damage via secretion of H$_2$O$_2$ in vitro, that the levels of induced DNA damage contribute significantly to *S. pneumoniae*-induced toxicity, and that the *spxB* gene contributes to genotoxicity associated with disease severity in an animal model. Furthermore, we found that the key NHEJ repair protein Ku80 plays an important role in suppressing *S. pneumoniae*-induced genotoxicity and cytotoxicity. Together, the studies described here show that H$_2$O$_2$ secreted by *S. pneumoniae* is both genotoxic and cytotoxic and call attention to DNA damage and repair as previously unidentified factors in pneumococcal pathogenesis.

### Results

**S. pneumoniae** Induces DNA Damage Responses in Alveolar Epithelial Cells. To learn whether *S. pneumoniae* has the ability to induce DNA damage in host cells, using immunohistochemistry, we measured the frequency of γH2AX foci, which form at sites of DSBs. We also quantified 53BP1 foci, which often colocalize with γH2AX at sites of DSBs. As expected, for untreated human lung epithelial cells, there were very few γH2AX or 53BP1 foci. In contrast, there were abundant foci in the nuclei of cells exposed to bleomycin, a known inducer of DSBs (Fig. 1A) (29). Furthermore, in addition to γH2AX appearing as punctate foci in bleomycin-exposed cells, we also observed nuclei with nearly uniform staining for γH2AX (Fig. 1C1), which is consistent with studies showing that exposure to high levels of a DNA-damaging agent can result in nuclear-wide staining of γH2AX (pan-γH2AX) (30).

To determine if *S. pneumoniae* can induce DNA DSBs, alveolar epithelial cells were cocultured with three virulent serotypes of *S. pneumoniae*, namely, serotype 19F (clinical isolate), serotype 3 (Xen 10 strain), and serotype 4 (TIGR4). Strikingly, the presence of *S. pneumoniae* resulted in a significant increase in the frequency of DSBs, indicated by the presence of both γH2AX and 53BP1 foci, most of which colocalized (Fig. 1A). Interestingly, serotype 4 showed the strongest ability to induce DSBs. Quantification of the percentage of cells harboring a significant increase in DSBs [defined as having ≥5 foci of either γH2AX or 53BP1 (31)] revealed that for serotype 4, ~30% and ~15% of cells have increased γH2AX and 53BP1 foci, respectively (Fig. 1B). In addition to cells having punctate foci, there were also a significant number of cells pan-stained for γH2AX (~45% of the total γH2AX-positive cells) (Fig. S1D), similar to what had been observed.

**Fig. 1.** *S. pneumoniae* induces DNA damage in human alveolar (A549) cells in the form of DSBs. (A) Representative images of alveolar epithelial cells showing DSBs (indicated by γH2AX and 53BP1 foci) after exposure to *S. pneumoniae* serotypes 19F, 3, and 4 for 7 h at MOI 40 (Low MOI). Bleomycin (100 μM) serves as a positive control. (B) γH2AX- and 53BP1-positive cells (≥5 foci per nucleus) were quantified for each condition and expressed as percentage positive. (C-E) Alveolar epithelial cells were exposed to serotypes 19F, 3, and 4 at MOI 200–400 (High MOI). (C) Representative images of alveolar epithelial cells after exposure to *S. pneumoniae* at MOI 300. (D) γH2AX- and 53BP1-positive cells were quantified for each condition. (E) The alveolar epithelial cells were also lysed and analyzed by Western for γH2AX. (F) Alveolar epithelial cells were pretreated with ATM inhibitor KU55933 (20 μM for 2 h) and then exposed to serotype 4 (type 4) at MOI 40 for 7 h. γH2AX-positive cells were quantified for each condition. For B and D, each data point represents mean ± SEM for four experiments. For F, each data point represents mean ± SEM for three experiments. For B, D, and F, *P < 0.05, unpaired Student’s t test.
following exposure to bleomycin. Unlike cells showing clear repair foci, where γH2AX and 53BP1 mostly colocalize, 53BP1 did not stain in γH2AX pan-stained cells, which is consistent with previous observations (30, 32, 33). For serotypes 19F and 3, there was a greater frequency of γH2AX-positive cells compared with uninfected cells, although the ability of these serotypes to induce DSB foci was clearly reduced compared with serotype 4 (Fig. 1B). To determine if observations are specific to the A549 cell type, we performed studies of lung adenoma cells (LA-4). For LA-4 cells, we similarly observed that S. pneumoniae induces DSBs (Fig. S2). These data, as well as analogous results in vivo (see below), indicate that the results of these studies are not specific to one cell line. Finally, we found that induction of DNA damage depends on the multiplicity of infection (MOI). At lower MOI of 3–5, there was no significant increase in γH2AX or 53BP1 foci for any of the strains (Fig. SL4).

To further explore the potential for S. pneumoniae to induce DNA damage, alveolar epithelial cells were exposed to a higher concentration of S. pneumoniae. Given that up to ∼10^9 CFU/mL of S. pneumoniae have been reported to be present in infected human lungs (34), alveolar epithelial cells are likely to be exposed to high levels of S. pneumoniae during acute infections at focal lung regions. Therefore, we used MOI 200–400 for infection of all three serotypes. We found that all three serotypes induced DNA damage in more than 20% of cells, with serotype 4 inducing DNA damage in over 50% of the cells (Fig. 1D). In contrast to the experiments at MOI of 30–50, most cells that were positive for γH2AX were pan-stained (Fig. 1C), suggesting that, at higher MOI, elevated levels of DNA damage were induced (30). As an alternative approach, we also analyzed the levels of H2AX phosphorylation by Western. Consistent with immunofluorescence analysis, we observed significantly increased γH2AX protein levels in lysates of epithelial cells exposed to all three S. pneumoniae serotypes, with the highest levels being associated with serotype 4 (Fig. 1E). To further explore the possibility that H2AX phosphorylation was induced as a response to DSBs, we targeted the canonical DNA damage response pathway centrally regulated by ATM kinase (35). When cells were pretreated with specific ATM kinase inhibitor (KU55933) and then exposed to serotype 4, the frequency of cells with γH2AX staining (both punctate and pan) decreased significantly with respect to mock-treated cells (Fig. 1F), indicating activation of the ATM pathway in response to S. pneumoniae. Taken together, these results demonstrate that S. pneumoniae is able to induce DSBs in human alveolar epithelial cells, that DNA damage responses depend in part on ATM activity, and that the potency of S. pneumoniae-induced DSBs is serotype-dependent.

**S. pneumoniae-Induced DNA Damage Levels Correlate with Levels of Apoptosis and Necrosis.** Host cell death is a key feature of S. pneumoniae pathogenicity (3, 23–26). To reveal the relative cytotoxicity of the three serotypes of S. pneumoniae, we exposed mammalian alveolar epithelial cells to S. pneumoniae. After the epithelial cells had been exposed to bacteria for 7 h in vitro, we analyzed cells for two key events of apoptosis, namely externalization of phospholipids (an early event detected by Annexin V) and late apoptotic fragmentation of DNA (detected by TUNEL). Analysis indicates activation of the ATM pathway in response to S. pneumoniae. Taken together, these results demonstrate that S. pneumoniae is able to induce DSBs in human alveolar epithelial cells, that DNA damage responses depend in part on ATM activity, and that the potency of S. pneumoniae-induced DSBs is serotype-dependent.

**S. pneumoniae-Induced DNA Damage Precedes Apoptosis.** It is well established that DNA DSBs act as a signal to initiate apoptosis, which is then followed by execution of apoptosis, a process that can take many hours (11, 37). In the above experiments, DNA damage levels and apoptosis were evaluated at the same time (7 h after coincubation of alveolar epithelial cells and S. pneumoniae), making it unclear as to whether the observed DSBs could have signaled for apoptosis. To further explore the possibility that S. pneumoniae-induced DNA damage induces apoptosis, we analyzed the levels of both DNA damage and apoptosis at 1, 4, and 7 h postinfection. Analysis shows that all three serotypes cause a significant increase in DNA damage as early as 1 h postinfection (Fig. 3A). The damage levels were then sustained or increased at 4 and 7 h postinfection depending on the serotype,
H2O2, we measured the levels of H2O2 in supernatant from all aerobic metabolism. Given the known genotoxic potential of H2O2, we analyzed the DNA-damaging potential of secreted factors, supernatant was then isolated from log-phase cells, doubling approximately three times in 7 h (Fig. 4A). To explore the possible importance of secreted pneumolysin, we did not detect pneumolysin in the F12-K media contributing to the induction of DNA damage. Interestingly, despite the relatively low level of H2O2 detected in the supernatants of serotypes 19F and 3 (Fig. 5A), these serotypes were nevertheless able to induce DNA damage (Figs. 3A and 4C) (discussed below). Taken together, these results show that H2O2 plays a significant role in pneumococci-induced DNA damage.

H2O2 Secreted by S. pneumoniae Causes DNA Damage and Cytotoxicity. To further explore the biological significance of H2O2, we exploited catalase, an enzyme that neutralizes H2O2 to water. We observed that catalase reduces the frequency of DNA damage-positive cells by 50% or more in cultures of epithelial cells exposed to all three serotypes of S. pneumoniae. During infection of human alveolar epithelial cells, we found that the ability of S. pneumoniae to produce H2O2 was variable among the three serotypes. For serotype 4, within 4 h postinfection of host cells in vitro, the concentration of H2O2 in the bacteria-free supernatant was more than 100 μM, and gradually the concentration increased up to 500 μM over the course of the following 3 h. In contrast, very little H2O2 was detected in the media of serotypes 19F and 3 (Fig. 5A). The difference in H2O2 production among serotypes was not a reflection of bacterial number (Fig. 4A). Moreover, ex situ, we observed that only the supernatant of serotype 4 was able to directly damage exogenous DNA (shown by assaying damage to supercoiled plasmid DNA) (Fig. S6). These results show that S. pneumoniae secretes H2O2 in a serotype-dependent manner and further demonstrate that H2O2 reaches DNA-damaging levels (upward of 500 μM) under coculture conditions.

To learn more about the biological significance of the observed levels of secreted H2O2, we analyzed the DNA-damaging potential of pure H2O2 at a concentration similar to what was observed under coculture conditions. When alveolar epithelial cells were exposed to 100 μM H2O2 in media, we observed that there is a significant induction of DNA damage in 15–20% of the exposed cells (Fig. 5B). Furthermore, H2O2 production for serotype 4 infection at 4 and 7 h parallels our previous observations of serotype 4-induced γH2AX as observed both by immunostaining (Fig. 3A) and Western (Fig. 3S4), consistent with H2O2 contributing to the induction of DNA damage. Interestingly, despite the relatively low level of H2O2 detected in the supernatants of serotypes 19F and 3 (Fig. 5A), these serotypes were nevertheless able to induce DNA damage (Figs. 3A and 4C) (discussed below). Taken together, these results show that H2O2 plays a significant role in pneumococci-induced DNA damage.
serotypes of \textit{S. pneumoniae} (Fig. 6A). The impact of catalase on DNA damage was greatest for serotype 4 infection, which is consistent with its high level of \( \text{H}_2\text{O}_2 \) during infection. We then incubated the bacteria-free supernatant with epithelial cells in the presence or absence of catalase. Consistent with the coculture results, we found that catalase greatly suppresses the DNA-damaging potential of bacteria-free serotype 4 supernatant (Fig. S7A). Importantly, catalase treatment also suppressed the frequency of apoptotic cells as measured by Annexin V assay (Fig. 6B).

To determine the relevance of pneumococcal \( \text{H}_2\text{O}_2 \) in invasion of the blood circulation (Fig. 7B). We found that the presence of WT \( \text{H}_2\text{O}_2 \) significantly increased inflammation-driven collateral damage to pulmonary cell DNA during pneumococcal infection. To determine whether there is a more robust inflammatory response for the mice infected with type 4 WT, we compared the number of macrophages and neutrophils in lung sections. Consistent with our in vitro study, images show a higher frequency of \( \gamma\text{H}2\text{AX} \)-positive cells in lungs of animals infected with serotype 4 WT (Fig. 8A). We found that only \( \sim8\% \) of lung cells were positive for \( \gamma\text{H}2\text{AX} \) on day 1 postinfection for both WT and \( \Delta\text{spxB} \) \textit{S. pneumoniae}. However, on day 2 postinfection, we observed a significant increase in \( \gamma\text{H}2\text{AX} \)-positive cells (>8%) in mice lung infected with type 4 WT, which was statistically significantly higher than that of type 4 \( \Delta\text{spxB} \) (Fig. 8A and B). At day 3 postinfection, results show a similar trend wherein there are more cells harboring DNA damage in mice infected with type 4 WT; however, this result is not statistically significant (Fig. 8A and B).

During pneumococcal pneumonia, there is persistent infiltration of inflammatory immune cells in the lungs that could themselves produce DNA-damaging RONS (6–8). Hence, inflammation-driven collateral damage to pulmonary cell DNA is plausible during \textit{S. pneumoniae} infection. To determine whether there is a more robust inflammatory response for the mice infected with type 4 WT, the total number of macrophages and neutrophils was quantified. We observed that there was a statistically significantly lower number of neutrophils at days 2 and 3 after infection (Fig. 8C).
and fewer macrophages at day 3 for animals infected with type 4 WT compared with type 4 ΔspxB (Fig. 8C and D). If inflammation were to account for the observed DNA damage, one would expect to see reduced DNA damage for type 4 WT infected animals, whereas we observed the opposite. Therefore, the host inflammatory response does not account for the observed type 4 WT-induced DNA damage. Importantly, the level of tumor necrosis factor (TNF-α), a key proinflammatory cytokine, does not vary between type 4 WT and type 4 ΔspxB infection (Fig. 8E), suggesting similar overall levels of inflammation. Together with the results above, the reduced genotoxicity of *S. pneumoniae* type 4 ΔspxB is consistent with a deficiency of H$_2$O$_2$ synthesis, rather than differences in inflammation, thus supporting the significant role of pneumococcal H$_2$O$_2$ in pulmonary genotoxicity and disease severity.

**DNA Repair Deficiency in Host Cells Exacerbates *S. pneumoniae* Infection.** To learn about the potential importance of DNA repair during *S. pneumoniae* infection, we knocked down Ku80, which is indispensable for the NHEJ pathway [knockdown (KD)]

**Fig. 7.** Pneumococcal H$_2$O$_2$ promotes in vivo virulence and invasion. (A) Kaplan–Meier plot for animals infected with *S. pneumoniae* serotype 4 (type 4) and H$_2$O$_2$-deficient ΔspxB mutant (type 4 ΔspxB). BALB/c mice were infected with bacteria at $2 \times 10^7$ cfu per mouse via intratracheal inoculation ($n = 23–24$), and their mortality was monitored. Animals showing symptoms of severe illness (ruffled fur, hunch back, inactive) and ≥20% weight loss were humanely euthanized and considered as fatal cases. (B–D) In this model, pneumococcal cfu were determined in (B) lung homogenate after lavage ($n = 9$), (C) BALF ($n = 9$), and (D) blood of infected animals ($n = 5$). Data are shown in box and whisker plots with median (horizontal line), inner-quartile range (box), and maximum/minimum range (whisker). *P < 0.05, Mann–Whitney test.

**Fig. 8.** Pneumococcal H$_2$O$_2$ is a major genotoxic factor during pathogenesis. (A and B) Lung sections of animals infected with type 4 WT and type 4 ΔspxB were analyzed for γH2AX at days 1, 2, and 3 postinfection ($n = 9$ per group, except for type 4 at day 3 where $n = 5$). (A) Representative images of lung section at days 1, 2, and 3 postinfection showing DAPI (blue), γH2AX (yellow), and costained nuclei (arrows). (Inset) Representative γH2AX-positive nucleus. (B) Frequency of γH2AX-positive cells ($\geq$5 foci). (C and D) Inflammatory responses were evaluated at days 1, 2, and 3 postinfection. BALF from infected animals was analyzed by flow cytometry for (C) neutrophils and (D) macrophages ($n = 9$ per group, except for type 4 at day 3 where $n = 5$). (E) TNF-α concentration was determined in BALF using ELISA. Each data point represents data from one animal, and bars indicate the means. For B–D, results show mean ± SEM. *P < 0.05, unpaired Student’s t test.
Results show that *S. pneumoniae* elicits DNA damage responses in host cells and that serotype 4 is able to secrete high levels of H$_2$O$_2$, giving it the capacity to induce DNA damage and cell death. Moreover, pneumococcal H$_2$O$_2$ alone is able to induce discrete DSBs without bacterial contact. Consistent with a model wherein *S. pneumoniae*-induced DNA damage triggers apoptosis, we observed that cells deficient in DSB repair have increased levels of apoptosis. Finally, results show that *S. pneumoniae*-secreted H$_2$O$_2$ plays a significant role in mediating pulmonary genotoxicity and systemic virulence in an animal model of acute pneumonia. This study underscores the genotoxic potential of pneumococcal H$_2$O$_2$ as well as the potential importance of DNA repair as a defense against *S. pneumoniae*-induced DNA damage and apoptosis.

*S. pneumoniae* is the most common pathogen underlying community-acquired pneumonia. Pneumonia is the major cause of death in children less than 5 y old, accounting for around 19% of childhood deaths worldwide (WHO report), and it also poses a serious threat to the elderly population (43). Moreover, pneumonia from *S. pneumoniae* infection is frequently the cause of fatal secondary infections during influenza pandemics, such as the 1918 influenza pandemic and the recent 2009 pandemic (44, 45). Additionally, *S. pneumoniae* plays a major role in worsening morbidity associated with chronic obstructive pulmonary disease (46). *S. pneumoniae* resistance to mainline therapeutics, such as penicillin and macrolides, has increased strikingly to more than 25% in recent years (47). Given the heavy disease burden and prevailing drug resistance associated with *S. pneumoniae*, alternative approaches are needed for streptococcal disease mitigation.

The plight of the host genome during infection-induced pathogenesis has been largely overlooked. Recently, however, certain pathogenic bacteria have been shown to cause damage to host DNA. For example, *Chlamydia trachomatis* (33) and *H. pylori* (20) have been proposed to elicit dysregulated cell proliferation and mutagenic DNA damage, which in turn are thought to promote bacterial-induced carcinogenesis (33). Interestingly, *H. hepaticus* and *Campylobacter jejuni* have been shown to produce cytolethal distending toxin (CDT), a carcinogenic tripartite protein that shows DNase activity in host nuclei (48–50). Given these examples of pathogens that have evolved mechanisms to induce DNA damage in mammalian cells, it seemed plausible that the host genome might be an intended target of *S. pneumoniae*. Indeed, in the case of *S. pneumoniae*, although it was known that the spxB gene worsens infection (51, 52), our work provides evidence to the ability of the spxB gene to induce DNA damage as the underlying driver of spxB-associated virulence. Furthermore, to date, the link between pathogen-induced DNA damage and disease has been focused primarily on cancer, whereas here we show an example of a pathogen-secreted DNA-damaging factor that has a direct association with pathogen-induced morbidity and mortality during infection.

Severe DNA damage can lead to apoptosis and necrosis (11, 53), which in turn lead to disintegration of lung architecture, promoting pulmonary failure. Here, we observed that *S. pneumoniae* could cause greater permeability of the cell membrane of host cells (observed as PI uptake), indicative of necrosis. H$_2$O$_2$ may contribute to necrosis, as well as exposure to the pneumococcal cell wall, which has previously been reported to be a necrosis-inducing factor (24, 26). With regard to apoptosis, we show that H$_2$O$_2$ secreted by *S. pneumoniae* leads to DNA damage in a contact-independent fashion and that the levels of H$_2$O$_2$ are sufficiently high to induce significant apoptosis (Annexin V and TUNEL-positive cells). These observations are consistent with previous in vitro studies showing that secreted H$_2$O$_2$ can contribute to *S. pneumoniae*-induced cytotoxicity (25).

H$_2$O$_2$-induced DNA damage and resultant cell death is anticipated to enable invasion of *S. pneumoniae* into the blood circulation. Indeed, consistent with a previous report (51), we observed reduced bacterial titers in blood following infection by spxB mutant bacteria compared with type 4 WT, underscoring...
the role of spxB and its H₂O₂ production in development of effective sepsis in our animal model. Previously, Regev-Yochay et al. demonstrated the competitive advantage of having spxB in a nasopharyngeal colonization model (52). It has been reported that spxB inactivation in acapsular pneumococcal serotype 2 strain reduces its adherence to alveolar epithelial cells (28). As this could play a role in virulence, we assayed bacterial adherence (28) and found that inactivation of spxB did not reduce the adherence of the capsular serotype 4 strain (Fig. S8), indicating that a reduction in adherence does not explain the observed reduction in virulence. It is likely that the reported role of spxB in adhesion is specific to acapsular strains (54, 55).

We also report an important and previously unidentified role of the DNA repair protein Ku80 in suppressing S. pneumoniae-induced genotoxicity. This observation is consistent with the known role of Ku80 in protecting alveolar epithelial cells (and other cell types) from gamma-irradiation-induced DSBs (56, 57). Overall, our data suggest a genotoxic model of pneumococcal pathogenesis whereby pneumococcal spxB-derived H₂O₂ induces host DNA damage that overcomes the Ku80-dependent NHEJ repair pathway, leading to cell death. Ultimately, via increased DNA damage, the resultant cell death and tissue damage could enable pneumococci to become more virulent and invasive.

In previous studies of pathogen-induced DNA damage, DSBs have been visualized by immunofluorescence detection of γH2AX (20–22, 33, 58). In our analysis of γH2AX foci, we found that a relatively high MOI produces sufficient H₂O₂ in the media to reach genotoxic levels. At a significantly lower MOI of 3–5, S. pneumoniae was not able to secrete significant amounts of H₂O₂ (Fig. S1B) and hence did not induce any significant increase in DNA damage. However, during pulmonary infection in vivo, S. pneumoniae is known to cause focal pneumonia (59). Consequently, it is anticipated that there could be a significant DNA-damaging effect from S. pneumoniae in vivo at sites of locally high MOI.

Interestingly, during S. pneumoniae infection of alveolar epithelial cells, we observed two patterns of γH2AX staining depending on the MOI used. Low MOI (30–50) yielded foci of γH2AX with 53BP1 in almost half of the total γH2AX-positive population, whereas the other half portrayed pan-γH2AX phosphorylation without any 53BP1 staining. At higher MOI (200–400), only pan-γH2AX staining without 53BP1 was observed. During infection of animals, we observed that pan-γH2AX staining constituted about 30% of the total γH2AX analyzed in the lung sections (Fig S10). The pan-γH2AX staining has been reported to occur in human fibroblast cells subjected to Adeno-associated virus (58, 60), Chlamydia (33), and UV and ionizing radiation (30, 61, 62). Recently, such nuclear-wide γH2AX has been shown to occur in highly DNA-damaged cells and is mediated by ATM kinase (30). Here, we found that most of the pan-γH2AX phosphorylation was dependent on ATM kinase and hence constituted part of the DNA damage response cascade induced by S. pneumoniae.

Although H₂O₂ clearly plays a significant role in the induction of DNA damage and downstream responses to S. pneumoniae, we also found evidence for H₂O₂-independent induction of DSBs. During bacterial infection with alveolar epithelial cells, we observed ~60% γH2AX-positive cells. Interestingly, only 30% of cells showed significant DNA damage when incubated with supernatant alone. It is possible that mammalian cell contact with S. pneumoniae causes DNA damage in host cells, independent of bacterial H₂O₂. One possibility is that direct contact of mammalian cells with S. pneumoniae could activate surface proteins in alveolar epithelial cells and produce signals that impact oxidative status and DNA damage response pathways. Indeed, S. pneumoniae is shown to activate the c-Jun-NH2-terminal kinase (25) pathway, which has the potential to phosphorylate H2AX (63). Direct contact-induced DNA damage is further supported by the observation that deletion of spxB (which is necessary for secretion of H₂O₂) in bacteria does not completely eliminate its ability to induce DNA damage. Thus, although it is clear that pneumococcal H₂O₂ plays an important role in inducing DNA damage, there remain alternative mechanisms by which S. pneumoniae can contribute to DNA damage.

To learn more about possible alternative mechanisms for S. pneumoniae-induced DNA damage, we studied the common pneumococcal toxin, pneumolysin. At high levels (e.g., ~20 µg/mL), pneumolysin has been shown to induce apoptosis in alveolar cells in vitro (24). Although pneumolysin is an intracellular protein without any signal peptide for secretion (39), it is known to be released during bacterial lysis (64), and there are reports of pneumolysin in culture supernatant of certain strains, even without bacterial lysis (40, 41). We therefore tested for the presence of extracellular pneumolysin in culture supernatants. We were unable to detect extracellular pneumolysin, which is consistent with its lack of a signal peptide for secretion. Thus, in the studies presented here, it is unlikely that alveolar cells experience pneumolysin at levels that are sufficient to induce DNA damage. Whether pneumolysin can induce DNA damage when the bacteria undergo lysis, either via autolysis or via action of bactericidal antibiotics, is an interesting question for future studies.

Given the low levels of H₂O₂ produced by serotypes 19F and 3, the observation that catalase could suppress the ability of these strains to induce DNA damage was unexpected. However, it is important to consider the approach that was used to estimate H₂O₂ secretion, namely to sample the media. For strains that have low-level production of H₂O₂, it may be that H₂O₂ is rapidly diluted in the media. However, if S. pneumoniae settle to form a layer above the human cells, the local concentration of H₂O₂ is anticipated to be far greater. Catalase would be anticipated to counteract genotoxicity of H₂O₂ under these conditions. Moreover, the fact that serotypes 19F and 3 are not as cytotoxic as serotype 4 is consistent with H₂O₂ being a dominant mechanism for the induction of DNA damage and apoptosis.

The rise of antibiotic-resistant bacteria calls attention to the need for alternative strategies for mitigating disease. Furthermore, although the capsular polysaccharide-based vaccines have been able to reduce the prevalence of vaccine-targeted invasive serotypes in the past 2 decades (65), the serotypes not covered by these vaccines are still prevalent and invasive especially in patients with cardiopulmonary comorbidities or compromised immunity (66). Developing our understanding of the molecular events that modulate the progression of pneumococcal disease is therefore an important step in advancing alternative treatment approaches for S. pneumoniae infection. Although immune-cell–induced RONS play a role in fighting infections, these inflammatory chemicals can also lead to collateral tissue damage. Furthermore, bacterially secreted H₂O₂ may exacerbate tissue damage caused by inflammation-induced RONS. Importantly, S. pneumoniae strains that secrete H₂O₂ clearly must have mechanisms to tolerate H₂O₂ and these mechanisms may render them resistant to H₂O₂ produced by immune cells. Thus, the use of H₂O₂-neutralizing antioxidants, in concert with antibiotic regime, may be appropriate during severe pneumococcal pneumonia. Indeed, antioxidants have been shown to confer positive outcome during pneumococcal meningitis in a rat model (67). Constant secretion of such oxidants by serotypes colonizing the upper respiratory tract (URT) can potentially damage the URT epithelia, destabilizing its normal barrier function [e.g., ciliary velocity and mucus production (68)] and facilitating carriage of S. pneumoniae to become more invasive. Given that certain strains of S. pneumoniae are resistant to H₂O₂ (69), and that H₂O₂ increases disease pathogenicity, our data suggest that determining the status of the spxB gene in pneumococcal isolates could prove helpful in guiding the use of antioxidants in disease treatment.

In this study, we have shown that S. pneumoniae creates high levels of H₂O₂ and that the levels of H₂O₂ are sufficiently high to induce DNA damage and apoptosis. We have shown that suppressing the levels of pneumococcal H₂O₂ either by treatment
with catalase or by knocking out the gene necessary for H₂O₂ biosynthesis suppresses S. pneumoniae-induced DNA damage and apoptosis. Furthermore, H₂O₂ secreted by S. pneumoniae plays a key role in pathogenesis, as shown by an acute pneumonia animal model. Importantly, human alveolar epithelial cells knocked down for an essential component of the dominant DSB repair pathway are more sensitive to S. pneumoniae-induced DNA damage and apoptosis, highlighting DNA repair as a potentially important susceptibility factor. In conclusion, the results of this study point to a role for S. pneumoniae-induced DNA damage in disease pathogenesis and open doors to new avenues for developing therapeutic strategies that either suppress DNA damage or enhance DNA repair during infection.

Materials and Methods

Mouse Strains and Model. This study was carried out in strict accordance with the National Advisory Committee for Laboratory Animal Research guidelines (Guidelines on the Care and Use of Animals for Scientific Purposes) in facilities licensed by the Agri-Food and Veterinary Authority of Singapore, the regulatory body of the Singapore Animals and Birds Act. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC), National University of Singapore (permit nos. IACUC 117/10 and 54/11). Female BALB/c mice (7–8 wk) were purchased from InVivos Pte Ltd. and housed in an animal vivarium at the National University of Singapore. Mice were induced to pneumonia by a single inoculation of S. pneumoniae at 2 × 10⁵ cfu in 50 µL sterile PBS. BALF was drawn from the right lung using 800 µL sterile PBS, and blood was collected via cardiac puncture before harvesting the lung tissues. BALF and blood was plated onto blood agar on the same day, with appropriate dilution. The left lung was fixed in paraformaldehyde and later embedded in paraffin. The right lung was frozen in liquid nitrogen and later homogenized in 2 mL PBS and plated for cfu count.

Cell Culture and Bacterial Strains. S. pneumoniae serotypes and strains were serotype 3 (Xen 10 A66.1), serotype 4 (TIGR4), and serotype 19F (clinical isolate). Bacteria were cultured in heart brain infusion (BHI) broth (Sigma #S5238), supplemented with 10% (vol/vol) heat-inactivated horse serum at 37 °C. The human lung alveolar carcinoma (type II pneumocyte) A549 cell line was maintained in F12-K medium (Gibco) with 15% (vol/vol) FBS at 37 °C, grown in F12-K medium. After centrifugation, the supernatant was filtered and stored at 20 °C. For culture supernatants, after filtration, supernatants were diluted by heating. Protein concentration was quantified using the BioRad DC protein assay kit according to the manufacturer’s instructions (Biovision #K265-200).

Construction of Ku80 KD AS49. Escherichia coli bacteriophage T4 DNA ligase was used to transform S. pneumoniae, and the λ Hzl E. coli bacteriophage was used to transform S. pneumoniae harboring the λ Hzl E. coli bacteriophage and λ Hzl E. coli bacteriophage DNA ligase. Kuzko shRNA lentiviral constructs were purchased from Sigma (SHCLNG-NM-021141, TRCN 0000295856). Lentiviral constructs were packaged via cotransfection with pMD2.G and psPAX2 into 293T cells using X-tremeGENE9 DNA transfection reagent (Roche) to produce lentiviral particles. A549 cells were transduced with viral particles in the presence of 10 µg/mL Polybrene (Sigma) and were selected in 2 µg/mL puromycin (Sigma) 2 d after transduction. A549 cells were analyzed for Ku80 knockdown by Western analysis of cell lysates with an anti-Ku80 antibody (Santa Cruz).

Flow Cytometry. For flow cytometry of BALF cells, neutrophils were gated at Gr-1 CD11b population, whereas macrophages were gated at F4/80+ CD11b+ population. For apoptosis and necrosis assays, cultured cells were incubated with Annexin V-PE (eBioscience #88-8103-74) and then with PI (2 µg/mL). Formaldehyde-fixed cells were stored in PBS.

Immunofluorescence. After incubation with bacteria, cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min, blocked with 3% (wt/vol) BSA in PBS for 40 min, and washed once with PBS. Primary antibodies against γH2AX (Ser-139) (Millipore #05–636 and 53BP1 (Santa Cruz #sc-22760) were used at 1:100 dilutions in PBS and incubated for 1 h at room temperature with coverslip. For TUNEL staining, the labeling enzyme (Roche #1 8647959 910) was incubated similarly for 1 h at 37 °C. Secondary antibody (Invitrogen) labeled with either Alexa 488 or 564 were used for γH2AX and 53BP1. SlowFade (Invitrogen) mounted slides were stored at −20 °C. For staining of 5-µm lung sections, antigen was retrieved using Dako retrieval buffer, the sections were blocked and incubated with anti-γH2AX antibody overnight and next day was stained with secondary antibody and mounted. All of the stained slides were examined under confocal microscope, and at least 9 images (at 3 × 3 sites) were taken of each well under 60x magnification for cell culture slides, and 20 random images of each lung section was taken under 40x magnification.

DNA Damage Quantification. Olympus FV10 2.0 viewer was used for imaging and counting in the dark room. At least 200 cells were counted. Cells were categorized as those with (i) more than five distinct foci of γH2AX or 53BP1 per nucleus regardless of colocalization, (ii) nuclei with remote foci of γH2AX and 53BP1 counted as “overlap,” and (ii) pan-staining of γH2AX. Cells that showed colocalization of FITC and DAPI were counted as TUNEL-positive cells. For images from lung sections, DAPI was machine-counted using IMARIS software, and Zeiss Zen software was used to examine and count γH2AX-positive cells. All counting was done in a blinded fashion, and nuclei were selected by DAPI alone and subsequently analyzed for γH2AX.

Supernatant Assay. To prepare bacterial supernatant, log-phase bacteria were grown in F12-K medium. After centrifugation, the supernatant was filtered (0.2 µm) and immediately incubated with AS49 cells.

H₂O₂ Assay. To measure H₂O₂ levels, conditioned media was centrifuged, filtered (0.2 µm), and stored at −80 °C for analysis using a hydrogen peroxide assay kit according to the manufacturer’s instructions (Biovision #K265-200).

Western Analysis. Treated cells were washed with PBS and incubated for 10 min with 200 µL of 1× lysis buffer [50 mM Tris-HCl, pH 6.8, 25 mM dithiothreitol, 2% (wt/vol) SDS, 10% (vol/vol) glycerol]. The lysate was centrifuged at 17,000 × g for 10 min at 4 °C and the supernatant was denatured at 100 °C for 10 min and stored at −20 °C. For culture supernatants, after filtration, supernatants were concentrated 10× using Amicon ultrafilter units (0.5 mL, 3 K) and denatured by heating. Protein concentration was quantified using the BioRad DC protein assay kit, and lysates were electrophoresed in 15% SDS/PAGE. Analysis was done using anti-γH2AX (Millipore #05–636) antibody or with anti-pneumolysin antibody and with secondary antibody conjugated with HRP (Dako) and later developed by adding Amersham ECL prime reagent (GE Life Science).

ACKNOWLEDGMENTS. We thank Prof. Andrew Camilli (Tufts University) for the gift of the TIGR4 strain and Dr. Yamada Yoshiyuki and Dr. Orsolya Kiraly for their valuable insights. This publication is made possible by the Singapore National Research Foundation and is administered by Singapore-Massachusetts Institute of Technology (MIT) Alliance for Research and Technology.


