

Letter to the Editor

Nasopharyngeal carriage with *Streptococcus pneumoniae* augments the immunizing effect of pneumolysin toxoid B

To the Editor:

Streptococcus pneumoniae is a major human pathogen responsible for a spectrum of diseases, including pneumonia, meningitis, and sepsis. Morbidity and mortality linked to pneumococcal disease remain high in sub-Saharan Africa, despite the introduction of protein-polysaccharide conjugate vaccines (PCVs), which offer protection against major disease-causing serotypes.¹ PCVs are limited in their affordability and their efficacy in high-risk groups, such as infants.² Furthermore, because the conjugate vaccine is limited in its serotype coverage, the major disease-causing serotypes are being replaced by previously minor serotypes¹ in places where vaccination is widespread. Attempts to overcome the limitations of current vaccination strategies have focused on developing vaccines around conserved pneumococcal proteins that vary minimally between serotypes and are indispensable to bacterial colonization or virulence, such as the cholesterol-binding, pore-forming toxin pneumolysin and its derivatives.³

Pneumolysin is lytic to cholesterol-containing membranes, and lysis of host cells undoubtedly aids bacterial dissemination during infection. At sublytic concentrations, pneumolysin has wide-ranging effects including activation of the immune system,⁴ induction of apoptosis,⁵ and induction of the complement pathway.⁶ Variants of pneumolysin with reduced hemolytic activity have been generated to address the inherent dangers associated with using a potent toxin as an immunogen. Pneumolysin toxoid B (PdB), a pneumolysin derivative with a Trp-433→Phe mutation, has 0.1% of the hemolytic activity of native wild-type pneumolysin and has shown promise in immunization studies in animals.^{7,8}

Here we assess the effect of PdB immunization on pneumococcal carriage. It is debatable, in the context of a protein-based vaccine, whether elimination of carriage would be desirable because low-level exposure to the pneumococcus might serve to strengthen and reinforce immune responses induced by vaccination. Furthermore, in a protein-based approach the problems of serotype replacement that accompany PCV immunization strategies should not exist, and therefore the issue of the nasopharynx acting as a reservoir for further pneumococcal infections should be ameliorated. However, carriage is widely accepted to be a prerequisite for invasive disease, and therefore there is an equally strong argument to be made for eliminating colonization after vaccination. We believed this contentious area warranted further investigation in mouse models that have been used previously with great success to drive forward pneumococcal vaccine development.⁹

Immunization with PdB in alum adjuvant, administered through either the intraperitoneal or subcutaneous routes by using a method based on that of Alexander et al.,⁸ did not affect mouse survival in a subsequent invasive pneumonia infection with the virulent *S. pneumoniae* serotype 2 strain D39 (Table I and see Fig E1, A, in this article's Online Repository at www.jacionline.org), despite inducing a high serum titer of IgG_{2a} anti-pneumolysin antibodies (Table I and see Fig E1, B) that strongly inhibited the ability of wild-type pneumolysin to cause red blood cell lysis *in vitro* (Table I and see Fig E1, C). Intriguingly, although PdB immunization did not protect against invasive

disease and did not reduce bacterial numbers in blood or lungs (Table I), we did observe a significant decrease in pneumococcal numbers colonizing the nasopharynx of PdB-immunized mice after infection with D39 (Table I and see Fig E1, D). To follow up on this observation, we performed PdB immunization and challenged mice 7 days after the final immunization with a dose of pneumococci that establishes stable long-term nasopharyngeal colonization but does not cause pneumonia or invasive disease (Fig 1, A and B). Mice that had received PdB immunization had significantly reduced pneumococcal numbers in the nasopharynx at both 3 and 7 days after infection compared with control mice (Fig 1, A), indicating some protection against long-term carriage in immunized mice. Furthermore, we observed a significant increase in IgG-producing B-cell numbers in the draining cervical lymph nodes of PdB-immunized mice at day 7 after infection compared with that seen in control animals (Fig 1, B). IgA-producing B-cell numbers increased in all colonized mice over the course of infection, but no additional increase was seen in mice that had also received PdB immunization (Fig 1, C).

Because PdB immunization induces strong humoral responses but does not affect lung bacterial numbers or mouse survival and because nasopharyngeal carriage is known to induce some anti-pneumococcal immunity, we assessed the ability of immunization followed by establishment of carriage to affect subsequent susceptibility to invasive pneumococcal disease (Fig 1, D and E). Mice were subcutaneously immunized with PdB/alum or PBS/alum twice 14 days apart. Fourteen days after the second immunization, mice were colonized with D39 or sham infected with PBS. Twenty-one days after colonization, mice were intravenously challenged with D39. Strikingly, 80% of mice that received PdB and were colonized with pneumococci survived subsequent intravenous pneumococcal challenge. This compared with an average of 50% survival of mice that were colonized but did not receive PdB. Mice with the lowest levels of survival included naive mice (30%) and mice that received PdB but were not colonized (30%; $P = .042$, 1-way ANOVA; Fig 1, D). Furthermore, mice that had been colonized or had been PdB immunized and colonized had significantly fewer bacteria in blood at 24 hours after intravenous pneumococcal challenge (Fig 1, E). Importantly, PdB immunization provided added protection against sepsis beyond that seen with colonization alone (Fig 1, E). Because we observed no increase in the titer of inhibition of sera collected at the time of death after invasive infection in PdB-immunized and colonized mice compared with that from mice receiving PdB immunization alone (data not shown), we believe that the additional protection afforded by colonization might be mediated through cellular immunity. This is an area of ongoing investigation.

Collectively, these data suggest that PdB immunization is highly protective against invasive disease in the context of additional, low-level exposure to pneumococcus in the nasopharynx. This protection is greater than both that provided by PdB immunization alone and that provided by previous pneumococcal carriage. This suggests either that PdB immunization induces weak immune responses that are then boosted by subsequent nasopharyngeal exposure to the pneumococcus or more likely that PdB immunization and pneumococcal exposure induce differential immune responses that together form highly effective

TABLE I. Immunization with PdB induces antibody and reduces colonization but does not protect against invasive disease

	Immunization	Survival (%)	Mean survival (h)	Log ₁₀ IgG _{2a} titer (time of death after infection)	Log ₁₀ titer of inhibition	Log ₁₀ CFU/mL blood (24 h after infection)	Log ₁₀ CFU/mg lung tissue (48 h after infection)	Log ₁₀ CFU/mg nasopharynx (48 h after infection)
Control	PBS/alum	9 (2/22)	60.95	0.001 (0-0.001)	0.426 (0.2-0.6)	7.884 (7.7-7.9)	6.429 (6.3-6.5)	3.114 (3.0-3.1)
PdB	PdB/alum	13 (3/23)	56.60	3.044 (2.8-3.2)*	2.308 (2.1-2.5)*	7.903 (7.8-8.1)	6.401 (6.3-6.4)	2.778 (2.7-2.8)*

Data are presented as means $\pm$ SEMs. Mice were immunized with 2 doses of 20 μ g PdB/alum (*PdB*) or PBS/alum (*Control*) 14 days apart and 14 days after final immunization were intranasally infected with 10⁶ colony-forming units (CFU) of *S pneumoniae*.

**P* < .001, 1-way ANOVA.

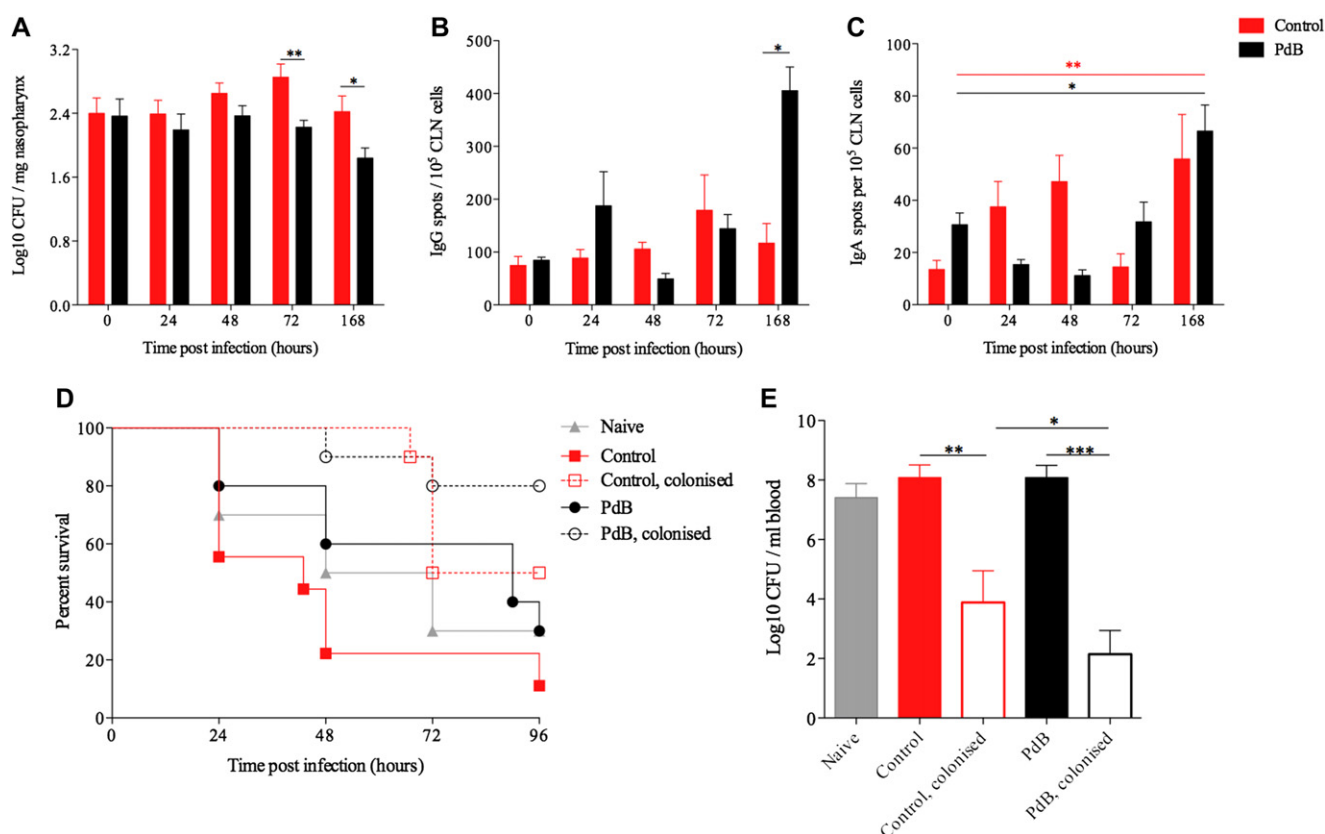


FIG 1. A combination of PdB immunization and colonization with pneumococcus is highly protective against invasive pneumococcal disease. Mice were immunized with 2 doses of 20 μ g PdB/alum (*PdB*) or PBS/alum (*Control*) 14 days apart. Selected groups were then intranasally infected with a colonizing dose (10⁵ colony-forming units [CFU] in 10 μ L) of *S pneumoniae* (colonized). Mice were intravenously challenged with an acute dose of D39. **A**, CFU/mg nasopharynx in colonized mice. **B** and **C**, ELISpot analysis of cervical lymph node (CLN) cells. One spot correlates to 1 IgG-producing (Fig 1, **B**) or IgA-producing (Fig 1, **C**) B cell. **D**, Survival after intravenous challenge. **E**, Sepsis at 24 hours after challenge. Data are representative of 2 experiments (>10 mice per group). Data in Fig 1, **A**, **B**, **C**, and **E**, are means $\pm$ SEMs. **P* < .05, ***P* < .01, and ****P* < .001.

protection against subsequent invasive disease. The evidence presented here confirms that PdB immunization induces strong humoral responses, and therefore perhaps cell-mediated immunity is boosted by carriage. It will be of interest to assess whether the additional protection afforded by carriage after PdB immunization is serotype specific. Previous work has suggested that carriage induces complex immune responses directed at a variety of bacterial protein and polysaccharide antigens,¹⁰ and therefore cross-serotype protection seems likely in this model.

The experiments we describe suggest that immunization with a noncytotoxic pneumolysin derivative is highly protective against invasive disease in the presence of low-level pneumococcal

nasopharyngeal carriage. This serves to reinforce immune responses induced by immunization and hence suggests that total elimination of carriage might not be a desirable outcome for a pneumococcal protein vaccine. If the problem of decreasing protection over time that has been reported for polysaccharide-conjugate vaccines is also evident in pneumococcal protein vaccines, then maintenance of low-level carriage in the nasopharynx after vaccination might serve to reinforce vaccine-induced immunity.

We thank Professor Neil French (University of Liverpool, Liverpool, United Kingdom) for critical reading of the manuscript.

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Supported by a Medical Research Council PhD studentship and additional funding from the Institute of Infection and Global Health, University of Liverpool.

Disclosure of potential conflict of interest: S. Smeaton has been supported by a grant from the Medical Research Council (MRC). A. Kadioglu has been supported by one or more grants from the MRC, internal University of Liverpool funding, and the National Institutes of Health. The rest of the authors declare that they have no relevant conflicts of interest.

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<http://dx.doi.org/10.1016/j.jaci.2012.11.004>

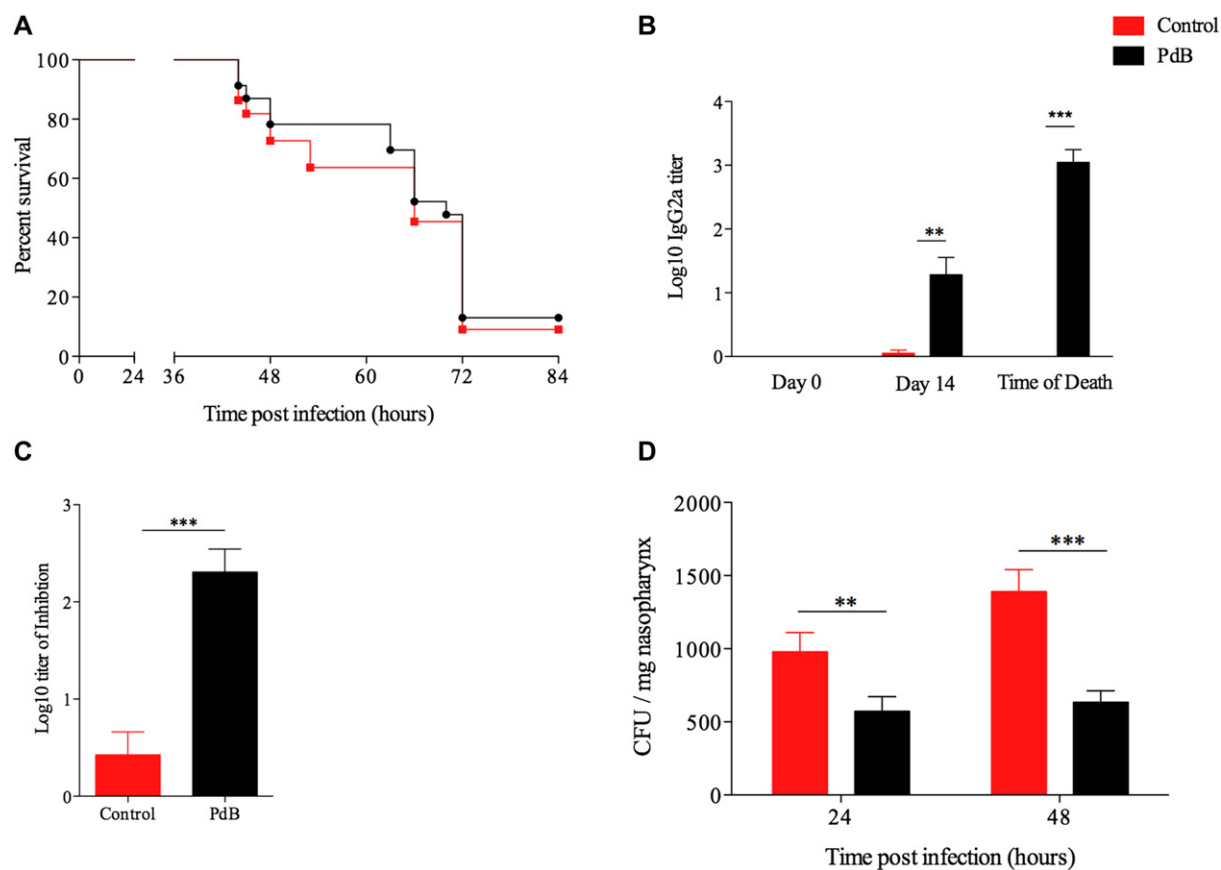


FIG E1. Immunization with PdB induces antibody levels and reduces colonization but does not protect against invasive disease. Mice were immunized with PdB/alum (*PdB*) or PBS/alum (*Control*) and intranasally infected with 10^6 colony-forming units (CFU) of *S pneumoniae*. **A**, Survival. **B**, Serum anti-PdB IgG_{2a} titer after immunizations at day 0 and day 14 and at the study's end point (time of death after infection). **C**, *In vitro* inhibition of red blood cell lysis by sera. **D**, CFU/mg in the nasopharynx. Data are representative of 3 experiments (>10 mice per group) and presented as means $\pm$ SEMs. *Significance in 1-way ANOVA with the Bonferroni posttest. ** $P < .01$ and *** $P < .001$.